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Calcium Signalling and Disease

Molecular Pathology of Calcium

Edited by

Ernesto Carafoli
and Marisa Brini

Springer

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Subcellular Biochemistry
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University of Padova, Italy

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LIST OF CONTRIBUTORS

M.J. Hayes, R.E. Longbottom, M.A. Evans and S.E. Moss
Div. of Cell Biology, University College London Institute of Ophthalmology, 11–43 Bath Street, London EC1V 9EL, UK

I. Bertipaglia and E. Carafoli
Department of Biochemistry, University of Padova, and Venetian Institute of Molecular Medicine, Padova, Italy

L. Spinardi
Direzione Scientifica, IRCCS Fondazione Ospedale Policlinico, Mangiagalli e Regina Elena, Via Francesco Sforza 28, 20122 Milano, Italy

W. Witke
EMBL Monterotondo, Via Ramarini 32, 00096 Monterotondo, Rome, Italy

W. Baehr
Department of Ophthalmology and Visual Sciences, University of Utah, Salt Lake City, Utah 84112-5339, USA

K. Palczewski
Department of Pharmacology, Case School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106-4965, USA

C.W. Heizmann, G.E. Ackermann and A. Galichet
Division of Clinical Chemistry and Biochemistry, Department of Pediatrics, University of Zurich, Switzerland

E.M. Brown
Division of Endocrinology, Diabetes and Hypertension, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, 221 Longwood Avenue, 02115 Boston, MA, USA

J. Colomer and A.R. Means
Department of Pharmacology and Cancer Biology, Duke University Medical Center

P.J. Adams
Michael Smith Laboratorie, University of British Columbia, Vancouver, BC, Canada V6T1Z4

T.P. Snutch
Neuromed Pharmaceuticals Inc., 2389 Health Sciences Mall Vancouver, BC, Canada V6T1Z4

S.E. Jordt and B.E. Ehrlich
Department of Pharmacology, Yale University School of Medicine, Department of Pharmacology, 333 Cedar Street, New Haven, CT 06520-8066

W.J. Durham, S. Sood and S.L. Hamilton
Department of Molecular Physiology and Biophysics

X.H.T. Wehrens
Department of Molecular Physiology and Biophysics, Department of Medicine (Division of Cardiology), Baylor College of Medicine, Houston, TX 77030

I. Bezprozvanny
Department of Physiology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390, USA

A. Hovnanian
INSERM, U 563, Toulouse, F-31300 France; Université Toulouse III Paul-Sabatier, UMR-S 563, Toulouse, F-31400 France

B.L Tempel and D.J. Shilling
The Department of Otolaryngology-Head and Neck Surgery, the Department of Pharmacology, and the V.M. Bloedel Hearing Research Center; University of Washington School of Medicine, Seattle WA 98195-7923, USA

J. Vanoevelen, L. Dode, L. Raeymaekers, F. Wuytack and L. Missiaen
Laboratory of Physiology, KULeuven Campus Gasthuisberg O&N1, Herestraat 49 bus 802, B-3000 Leuven, Belgium

T. Capiod, R. Skryma and N. Prevarskaya
INSERM U800, Laboratoire de Physiologie Cellulaire, Université des Sciences et Technologies Lille 1, 59655 Villeneuve d'Ascq Cedex, France

Y. Shuba
Bogomoletz Institute of Physiology, NASU, Bogomoletz Str, 4, 01024 Kiev-24, Ukraine

F.W. Hopf
Ernest Gallo Clinic and Research Center, University of California, San Francisco, 5858 Horton St., Suite 200, Emeryville, CA 94608

P.R. Turner
Panomics, 6519 Dumbarton Circle, Fremont, CA, 94555

R.A. Steinhardt
Department of Molecular and Cell biology, University of California, Berkeley, Berkeley, CA 94720-3200

A. Verkhratsky
Faculty of Life Sciences, The University of Manchester, 1.124 Stopford Building, Oxford Road,Manchester M13 9PT, UK

P. Bernardi and A. Rasola
Department of Biomedical Sciences, University of Padova, Viale Giuseppe Colombo 3, 35121 Padova, Italy

K.N. Green, I.F. Smith and F.M. LaFerla
Department of Neurobiology and Behavior, University of California, Irvine CA 92697-4545

E.G. Kranias
Department of Pharmacology and Cell Biophysics, College of Medicine, University of Cincinnati, Cincinnati, OH 45267

D.M. Bers
Department of Physiology, Loyola University Chicago, Stritch School of Medicine, Maywood, IL 60153

H.C. Blair, P.H. Schlesinger, Christopher L.-H. Huang and M. Zaidi
Departments of Pathology and of Cell Biology and Physiology, University of Pittsburgh and Veterans' Affairs Health System, Pittsburgh, PA 15261, Department of Physiology and Cell Biology, Washington University School of Medicine, St. Louis, MO 63110, Physiological Laboratory, University of Cambridge, Cambridge, CB2 3EG, United Kingdom, and The Bone Program, Mount Sinai School of Medicine, New York, New York 10029

PROLOGUE

M. BRINI[1] AND E. CARAFOLI[1,2]

[1]*Department of Biochemistry and Department of Experimental Veterinary Sciences, University of Padova, Viale G. Colombo, 3,35121, Padova, Italy*

[2]*Venetian Institute of Molecular Medicine, Via G. Orus 2, 35129 Padova, Italy*

About 120 years have elapsed since the days of the landmark experiment by Ringer that opened the Ca^{2+} signalling area (Ringer, 1883). The experiment was followed by an unusually long period of incubation, after which interest and knowledge in the topic started growing exponentially, eventually establishing Ca^{2+} as a universal carrier of messages to a constantly expanding number of cell function. As knowledge advanced, it gradually became clear that Ca^{2+} has properties that set it apart from all other carriers of biological information. Among them one could quote the auto regulatory properties of the signal, and the ability of Ca^{2+} to act as both a first and a second messenger (Carafoli et al., 2001). But another distinctive property gradually became evident: the ambivalence of the signal. Although it is now clear that cells would not function properly without Ca^{2+} messages, it is also clear that the messages must be delivered to cells, and decoded by them, in a carefully controlled way. Should cells become somehow unable to control the free concentration of Ca^{2+}, letting it increase persistently in their interior above the optimal 100–200 nM level, all Ca^{2+} controlled activities would become permanently activated, including those (e.g., proteases) that are potentially harmful to cells. Various degrees of damage, up to cell death, would inevitably ensue. Cells may activate rescue attempts: as a rule, however, they only buy time, i.e., they enable cells to survive until the emergency disappears. If it doesn't, however, the cells are doomed.

The massive dysregulation of Ca^{2+} signalling that culminates in the death of cells is a dramatic example of the ambivalent nature of the Ca^{2+} message. The literature describing the numerous ways in which the homeostasis of Ca^{2+} may become so dramatically altered, and the molecular mechanisms by which they terminate cell life has now become impressively large. However, a number of less dramatic conditions have become known more recently, in which the Ca^{2+} signal is not altered globally and persistently to the extent necessary to rapidly precipitate cell

death. It is instead altered in more specific ways that only affect individual actors in the control of Ca^{2+} signalling pathways. This is a field that is now expanding very rapidly thanks especially to the rapid advances in the area of genetics. It will be covered in this book, which has attempted to collect essentially all what is known today on the dysfunctions, mostly genetic, of Ca^{2+} controlling systems and of Ca^{2+} signalling pathways. The Chapters of the book will describe and discuss critically diseases linked to the dysfunction of membrane transporters, including channels, that control the fluxes of Ca^{2+} in and out of the cell cytoplasm, as well as diseases caused by the malfunctioning of Ca^{2+}-sensor proteins that are modulated by Ca^{2+} and/or process its signal. No comprehensive book on this important and rapidly expanding topic has so far appeared. It is hoped that the discussion in the chapters collected in the book will expand interest, and help suggesting new work, in this very stimulating field.

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CHAPTER 1

ANNEXINOPATHIES

M.J. HAYES, R.E. LONGBOTTOM, M.A. EVANS AND S.E. MOSS

Div. of Cell Biology, University College London Institute of Ophthalmology, 11–43 Bath Street, London EC1V 9EL, UK

Abstract: Annexins comprise a conserved family of proteins characterised by their ability to bind and order charged phospholipids in membranes, often in response to elevated intracellular calcium. The family members (there are at least 12 in humans) have become specialised over evolutionary time and are involved in a diverse range of cellular functions both inside the cell and extracellularly

Although a mutation in an annexin has never been categorically proven to be the cause of a disease state, they have been implicated in pathologies as diverse as autoimmunity, infection, heart disease, diabetes and cancer. 'Annexinopathies' were first described by Jacob H. Rand to describe the pathological sequelae in two disease states, the over-expression of annexin 2 in a patients with a haemorrhagic form of acute promyelocytic leukaemia, and the under-expression of annexin 5 on placental trophoblasts in the antiphospholipid syndrome. In this chapter we will outline some of the more recent observations in regard to these conditions, and describe the involvement of annexins in some other major causes of human morbidity

Keywords: Annexin, phospholipid, fibrinolysis, endosome, cancer, glucocorticoid, inflammation

1. INTRODUCTION

"The view [of causation] that we adopt has consequences which reach beyond informal discussion during coffee breaks." –(Olsen 1993)

Observers of 'annexinology' may be astonished at the involvement of annexins in almost every aspect of human morbidity. There is probably no corner of medicine into which annexins have not insinuated themselves, and there is an abundance of studies that support their involvement in disease. Pathogenesis however can be defined as the origin and development of morbidity and to date it remains the case that an annexin has never been identified as the 'origin' of a disease state. The rigorous application of epidemiology and of Koch's postulates has never been

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successfully applied to an annexin. However, they repeatedly emerge as 'accessories after the fact'; they always seem to turn up in the wrong place at the wrong time.

The annexins form a large family of mostly abundant proteins which are expressed in almost all tissues of multicellular organisms and in many unicellular organisms. Any individual cell-type may be described as having an 'annexin fingerprint' in that it will express a number of annexins, some at high levels. The annexin family of proteins (there are at least 12 of them in humans) is characterised by the presence of 4 so-called 'annexin repeats' (annexin 6 which occurred as a result of gene fusion has 8). These are 70 amino acid sub-domains each of which is composed of 5 alpha helices joined by short loops. The protein adopts a remarkably well conserved, tightly folded, compact, slightly curved structure which looks like a rhombus when viewed from the convex 'top' of the protein. This upper surface possesses class II calcium binding sites and it is this surface that fulfils the major defining biochemical characteristic of annexins, their ability to associate with anionic charged phospholipid-containing membranes. The N-terminal 'interacting domain' of the protein varies considerably in length and sequence between the proteins and binds to a wide range of ligands including members of the EF-hand containing S100 family of proteins. The annexin Ca^{2+}-binding site differs from that of the helix-loop-helix EF-hand in that only 5 of the seven co-ordination sites are provided by protein oxygens. Instead, the calcium ion is coordinated by three carbonyl oxygens in a short inter-helical loop, two carbonyl oxygens of an acidic residue are provided by another helix and water molecules, which can be substituted by phosphoryl moieties if the protein interacts with phospholipid containing membranes (reviewed in Gerke et al., 2005).

Some annexins can self-assemble into highly-ordered, tessellating two-dimensional arrays on lipid surfaces. By subtly re-organising the underlying lipids the proteins can alter the curvature and 'fuse-ability' of the underlying membrane. Other annexins have been shown to 'shepherd' anionic phospholipids together, corralling them into sub-domains. Some of these interactions are facilitated by cholesterol. In this way annexins can effectively 'un-mix' membranes and introduce concentration gradients in otherwise homogeneous arrays. This could have important consequences for the maintenance of signalling complexes and structural sub-domains of membranes. The different annexins have disparate calcium requirements for association with both the plasma membrane and other internal membranes. As calcium levels rise intracellularly in response to a range of signals the annexins will 'annexe' the membrane in an ordered manner determined by this calcium requirement. Thus many of the annexins may be considered to be a 'calcium-sensitive effectors'; with different annexins fulfilling this role on various subcellular membranes (Monastyrskaya et al., 2006).

In addition, several annexins have been shown to interact directly or indirectly with actin microfilaments and microtubules, thus annexins have the potential to form a structural link between the membrane and the underlying cortical cytoskeleton (reviewed in Gerke et al.; 2005 Hayes et al., 2004). As well as lateral inter-annexin interactions (either homotypic or heterotypic with other types of annexin)

the proteins can also form heteromeric complexes with their binding partners (Figure 1). This has probably been most intensely investigated for annexin 2 which forms a tetrameric complex with S100A10 via its N-terminal interacting domain. The protein can adopt a flexible, side to side configuration with S100A10 which might strengthen lateral interactions with membranes, or adopt a head-to-tail conformation which has been well characterised by crystallographic studies, in which the two concave surfaces come together, coordinated with two S100A10 proteins. In this way the protein could potentially interact with two closely approaching membranes,consistent with a role in vesicle fusion or budding. We stress here the roles of annexins as intracellular cytoplasmic proteins, but several annexins have apparently unrelated functions in the extracellular milieu. In fact, it is often in this context that they have come to the attention of medical researchers. Annexin 1 has been shown to be secreted by folliculo-stellate cells via a mechanism involving the adenosine 5'-triphosphate-binding cassette transporter A1 (Chapman et al., 2003, Omer et al., 2006). But the mechanism of annexin 'secretion' from other cells remains more controversial, and would appear to occur via non-conventional routes.

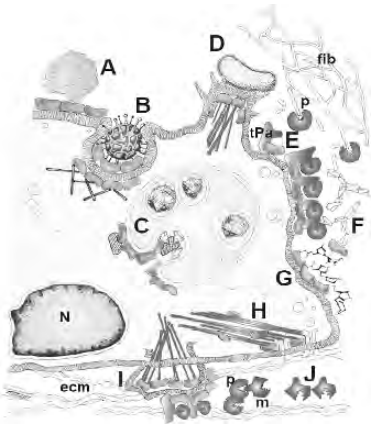

Figure 1. Schematic view of the putative roles of annexin 2 in disease pathology
A: Extracellular annexin 2 acts as a receptor for calcium oxalate crystals in collecting duct epithelial cells in Dent's disease. B: It acts as a receptor for respiratory syncytial virus and cytomegalovirus. C: It is necessary for maturation of the gag protein of HIV and virion maturation on the internal membranes of multivesicular bodies in macrophages. D: Annexin 2 is enriched in the PIP2 and actin-rich pedestals of non-invasive enteropathogenic E.coli. E: Annexin 2 acts as a 'fibrinolytic receptor' binding both tissue plasminogen activator (tPa) and Plasminogen (P) on the cell surface. Activated plamin (P) is then involved in degradation of fibrin (fib). F: Antibodies with specificity for annexin 2 (F) or the formation of Advanced Glycation Products (G) can interfere with the role of annexin 2 in fibrinolysis. Annexin 2 expression is often altered as normoplastic cells become cancer cells. This may reflect the role of annexin 2 in regulation of actin polymerisation. Dramatic changes in actin structure are associated with loss of focal adhesions (H) and the formation of invadopodia (I). Annexin 2 modulation of plamin activation (P) has knock-on effects on the activation of metalloproteinases (M) which degrade extracellular matrix (ECM) and encourage cellular migration. N is the nucleus. Annexin 2 is coloured green throughout (See Colour Plate 1)

At mildly acidic pH some annexins are proposed to be capable of undergoing conformational changes that would facilitate their insertion into membranes as pore complexes. It is conceivable that subsequent contortions could release the protein to the extra-cellular membrane. An alternative suggestion is that the proteins are 'budded-off' from the surface of the cell in association with small lipid vesicles which could then lyse and release the protein. Their high affinity for phospholipids in the presence of calcium is consistent with the retention of annexins on the cell surface though there is growing evidence for the presence of non-lipid annexin receptors on certain cell types.

The term 'annexinopathies' was first coined by Jacob H. Rand to describe the pathological sequelae in two disease states. The first of these involves over-expression of annexin 2 in a subset of patients having a hemorrhagic form of acute promyelocytic leukaemia (Tallman and Kwaan, 1992, Tallman et al., 1993; Menell et al., 1999). The second example concerns the under-expression of annexin 5 on placental trophoblasts in the antiphospholipid syndrome and in pre-eclampsia. In this chapter we discuss these first two observations before extending the list into a few new areas. Finally we will summarise some of the more conjectural suggestions linking annexins to a whole host of disease conditions.

2. ANNEXIN 2 IN A HAEMORRHAGIC FORM OF ACUTE PROMYELOCYTIC LEUKAEMIA (APL)

Acute myelocytic leukaemia is characterised by the accumulation of immature myelocytes in the bone marrow. This is the most common form of leukaemia found in adults, though it can affect people of all ages. Annexin 2 has been shown to be up-regulated in leukaemic cells in patients with acute promyelocytic leukaemia which is a subset of this disease in which the cells accumulate chromosomal abnormalities rendering them insensitive to vitamin A (Menell et al., 1999). This occurs due to disruption of the retinoic acid receptor alpha (RARA) gene by fusion with the PML tumour-suppressor gene-product following a t(15:17)(q22; q11) translocation (de The et al., 1990). This results in uncontrolled expansion of the pro-myelocytic lineage. The leukaemic cells enter the bloodstream and invade other organs where they continue to divide. A major complication of APL is a haemorrhagic disorder; bleeding and thrombosis can lead to premature death in approximately ten percent of patients despite otherwise successful treatment (Tallman and Kwaan 1992; Tallman et al., 1993).

Interestingly, cells containing the most common translocation produce more tissue plasminogen activator (tPA)-dependent plasmin than other cells and express higher levels of annexin 2 on their surface (Menell et al., 1999). Annexin 2 and its binding partner S100A10 have been shown to appear on the surface of monocytes, activated macrophages and endothelial cells where they accelerate the activation of plasmin from plasminogen by tPA. It is suggested that the proteins behave as a 'fibrinolytic receptor' bringing plasminogen and tPA into intimate association on the cell surface and releasing activated plasmin. There is currently some controversy as to which of the two proteins is the dominant factor in binding plasminogen (Hajjar et al., 1994;

Cesarman et al., 1994; MacLeod et al., 2003) indeed it is possible that both proteins do; but it is clear that the resultant plasmin may then participate in fibrinolysis, the degradation of fibrin clots. The process appears to be negatively regulated by a feedback mechanism involving angiostatin (a fragment of plasminogen and a powerful inhibitor of angiogenesis), which binds competitively to endothelial cell surface annexin 2 (Tuszynski et al., 2002), and thrombin (Peterson et al., 2003). The dominant function of thrombin is the conversion of fibrinogen into an active form that can assemble into fibrin filaments. It also activates the clotting factors XI, V, VIII and XIII. Thrombin is active in association with phospholipids on the surface of cells and its local accumulation is necessary for clotting. Interestingly treatment of human umbilical vein endothelial cells with both thrombin and the thrombin receptor-activating peptide (TRAP) resulted in increased surface expression of annexin 2 and S100A10 (Peterson et al., 2003), thus the very factors which promote and sustain fibrin formation and deposition facilitate its subsequent degradation by plasmin. Slightly counter-intuitively, recombinant annexin 2/S100A10 tetramers inhibit fibrinolysis by plasmin in vitro, but this may underline the importance of the cellular surface in regulating the process, or simply reflect a non-productive competitive steric hindrance at a shared binding site. Once activated, plasmin can subsequently cleave and activate other down-stream targets such as numerous matrix-metalloproteinases (MMPs), which are necessary for the remodelling of the extracellular matrix associated with angiogenesis. This would place annexin 2 and S100A10 at a nexus between the related axes of clotting, fibrinolysis and angiogenesis.

The fibrinolytic phenotype observed in cells containing the PML/RARA translocation could be induced in non-t(15:17) cells by over-expression of annexin 2 and inhibited in t(15:17) cells by annexin 2 specific antibodies. Treatment of cells with all trans-retinoic acid, which promotes the differentiation of pro-myelocytic cells and is commonly used in conjunction with chemotherapy to treat the disease; down-regulated annexin 2 and negated the pro-fibrinolytic phenotype of the cells (Menell et al., 1999). This condition thus represents a genuine annexinopathy in that the un-coordinated over-expression of annexin 2 on immature pre-myelocytes may be the causative factor in the haemorrhagic complications of APL. This is not completely clear cut, because it may be the case that annexin 2 expression is always high on pro-myelocytes which would imply that there is in fact no dis-regulation of the gene per se. The APL-RARA translocation site (15q22) is <14Mb from the annexin 2 gene itself (15q21-22) so it is possible that the translocation could affect expression of annexin 2 directly. Annexin 2 surface and mRNA expression do not appear to correlate consistently with pre-myelocyte status in cell lines (Olwill et al., 2005) but this may reflect subsequent changes accumulated in cell culture.

Up-regulation of annexin 2 has also been implicated in pro-B cell acute lymphoblastic leukaemia ALL (Matsunaga et al., 2004). This occurs as a result of a different translocation t(17;19)(q22;p13) and the expression of the fusion protein E2A-HLF is also associated with poor prognosis, hypercalcemia and haemorrhagic complications. Expression of E2A-HLF was sufficient to induce annexin 2 over-expression in leukaemic cell lines.

Recently two other annexins, namely annexin 1 and annexin 10 have been identified in proteomic screens for genes up-regulated in acute myeloid leukaemia (Lopez-Pedrera et al., 2006). Whether these changes have consequences for disease progression or pathogenesis remains to be seen.

3. ANNEXINS AND THE ANTI-PHOSPHOLIPID SYNDROME

The anti-phospholipid syndrome refers to a range of autoimmune conditions which are characterised by venous or arterial thrombosis, recurrent strokes, pulmonary embolism, recurrent pregnancy loss or obstetric complications and the presence of circulating antibodies with specificity to a range of phospholipids; including phosphatidylserine and cardiolipin. The syndrome is the leading cause of vascular thrombosis in children. It sometimes accompanies other autoimmune conditions such as systemic lupus erythematosus (SLE).

Recently it has become apparent that at least some of the auto-antibodies are in fact directed against phospholipid-protein complexes or against the lipid-binding proteins themselves: β2-glycoprotein I (β2 -GPI) and prothrombin being the major targets. β2 -GPI is a circulating plasma protein which binds to artherogenic-lipoprotein (Lp(a)) which is structurally homologous to plasminogen. It has been suggested that it could form a ternary complex with Glu-plasminogen and tissue-associated plasminogen activator, facilitating plasmin activation. As described already, endothelial surface-bound annexin 2/S100A10 complex functions as a 'fibrinolytic-receptor' by binding plasminogen and tPA. It has also been shown that annexin 2 forms a complex with β2-GPI on the surface of endothelial cells (Ma et al., 2000), allowing the latter protein to bind and generate an anti-coagulant environment at this surface.

Antibodies directed against β2 -GPI further enrich the protein on the cell surface but these promote a p38 Map-kinase signalling cascade which results in increased expression of tissue factor (TF) and reduced expression of thrombomodulin on the surface of cells. TF is a major initiator of coagulation and increased levels of TF expression have been measured on endothelial cells treated with anti-phospholipid antibodies and on monocytes both ex vivo and in vivo (Yasuda et al., 2005; Lopez-lira et al., 2005, Lopez-Pedrera et al., 2006). Thrombomodulin is a potent anti-coagulant protein which limits activation of thrombin, so the net result of circulating anti-phospholipid antibodies is to usurp the anti-coagulative, protective mechanism and initiate a pro-coagulation cascade.

Patients with APL have increased circulating titres of anti-annexin 2 antibodies (Cesarman-Maus et al., 2006). Anti-annexin 2 IgG enhances the expression of tissue factor on endothelial cells more than 6 fold, significantly blocks plasmin generation in a tPA-dependent generation assay (independently of beta-2-glycoprotein-I), and significantly inhibited cell surface plasmin generation on human umbilical vein endothelial cells. The exact interplay between β2-GPI and annexin 2 requires careful dissection but the importance of annexin 2 in the regulation of the coagulability of the environment is clear.

Despite the strength of the evidence for annexin 2 involvement in the development of cellular pathologies, Rand's initial description of the annexinopathies and APL syndrome in particular was not concerned with annexin 2, but rather with annexin 5. The surface availability of anionic charged phospholipids is critical for the initiation and maintenance of thrombosis. Annexin 5 prevents formation of prothrombinase and tenase complexes (intrinsic pro-coagulation complexes formed of factor VIIIa and factor IXa) by competing for these sites (Andree et al., 1992). It has also been shown to significantly displace preadsorbed anticardiolipin-β-GPI complexes from such membranes and to modify platelet aggregation and accretion into larger complexes under the conditions of blood flow (van Heerde et al., 1994). Significantly annexin 5 also attenuates the nucleating effects of core debris which can cause additional clots when they are released from ruptured plaques. A polymorphism in the Kozak sequence of the annexin 5 gene has been discovered that does not change the sequence of the protein but has been shown to correlate with an increase in the level of annexin 5 expression and abundance of the protein in the circulation. It has been suggested that this gives some protection against thromboembolic disease (González-Conejero et al., 2002), though this has not been confirmed by other groups (van Heerde et al, 2003).

Annexin 5 is expressed at high levels on the surface of syncytiotrophoblasts where its anti-coagulant activity is thought to protect the developing foetus. Annexin 5 has been proposed to form a 'shield' over the surface of the placenta, adopting a tessellating array on the exposed phospholipids and preventing the circulating pro-clotting factors from finding purchase. In a mouse model in which labelled annexin 5 was injected into Balb-c mice it accumulated on numerous foci on the apical surface of the trophoblasts. Antibodies with specificity to annexin 5 (which binds phosphatidylserine) are commonly associated with APL and it has been suggested that these antibodies or indeed anti-phospholipid antibodies could interfere with annexin 5 shielding of the trophoblast (Rand et al; 2003; 2004; Rand and Wu 2004; Rand et al., 2005, reviewed in Esposito et al., 2005). When the annexin 5 'shield' was disrupted in pregnant mice by the injection of annexin 5 antibodies, thrombosis and necrosis were seen on the foetal component of the placenta, and various degrees of foetal readsorption were observed (Wang et al., 1999).

The significance of anti-annexin 5 antibodies to the pathogenesis of this disease is not entirely uncontentious. A thrombotic association may only occur when anti-phospholipid antibodies are also present, so they may not be a useful independent marker of risk (Arnold et al., 2001). In this moderately sized study, there was not a single patient with antibodies against both annexin 5 and β2-GPI. This could suggest that there are two, independent routes to the disease: a β2–GPI/annexin 2 path and an aPL/annexin 5 path. A clear distinction between anti-β2-GPI and anti-annexin 5 was found in regard to late recurrent miscarriage (Zammiti et al., 2006) and no correlation was found between the presence of anti-annexin 5 antibodies and thrombosis in APL (de Laat et al., 2006). These authors do however find a positive correlation with the mutation in the Kozak sequence described above.

It is clear that there are still issues that need to be resolved in this field. Methodological differences have been highlighted by some of the authors to explain their differing conclusions, but there may be some specific property of annexins that renders them susceptible to this kind of analytical quandary. In various other autoimmune conditions it has been suggested that autoantibodies develop as a result of antigen creep from the so-called 'natural antibodies' with broad specificity for a range of common pathogens. Anti-annexin antibodies have been discovered in a wide range of autoimmune conditions; perhaps the charged, helix-rich structure of annexins, in complex with charged lipids just happens to represent a surface which can easily mimic antigens derived from pathogens. Perhaps the association of annexins with membrane surfaces, in inflammatory environments and on phagosomal and endosomal membranes (as will be discussed later) means that they feature regularly as the 'usual suspects' in immunological identity parades. That annexin-specific antibodies have the potential to be pathogenic is clear; whether they will be useful markers for autoimmune conditions is possible, and whether or not they are of clinical relevance still remains to be proven beyond reasonable doubt.

4. ANNEXINS AND DIABETES

Diabetes is a chronic condition that can present in two different ways; type-1 insulin-dependent diabetes or the more common type-2 insulin non-dependent diabetes. In both instances it is the resulting hyperglycemia that is thought to be primarily responsible for the disease facies. Although diabetes is a systemic condition that affects all parts of the body, for the purposes of this discussion the endothelium will be the main point of focus, since the most complete data regarding the role of annexins in diabetes have come from studies examining their actions in endothelial cells.

There are thought to be four major effects of hyperglycemia on endothelial cells: an increase in flux through the polyol pathway which leads to a depletion of NADPH and ultimately diminishes the cell's ability to produce protective anti-oxidants, an increase in conversion of glucosamine-6-phosphate to N-acetylglucosamine which leads to the formation of transcription factor-sugar adducts (which can affect transcriptional regulation), increased activation of protein kinase C isoforms and formation of Advanced Glycation End-products (AGE) via the Maillard pathway. An increase in reactive oxygen species (ROS) produced by the mitochondria in response to high glucose or increased fatty acid metabolism can be linked to all four pathways (reviewed in Brownlee 2005). Whether any of these cellular consequences of diabetes has an effect on any of the annexins is a novel area of research that is gaining momentum.

Although there have been a number of descriptions of annexin involvement in cell signalling pathways important in diabetes, the role of annexin 2 as a receptor for plasminogen and tissue plasminogen activator (tPA) is the most compelling. Diabetes has long been known to induce a hypercoagulable state and it is currently thought that a change in the ability of annexin 2 to perform its role in the formation

of plasmin plays some part in this. Annexins 1 and 2 are both early non-enzymatic glycation products (Ghitescu et al., 2001), and in cultured primary endothelial cells grown in high glucose and high insulin, plasmin formation is reduced, an effect that could be reversed by the addition of recombinant annexin 2 (Ishii, 2001). These observations led to the hypothesis that glycation of annexin 2 is sufficient to disrupt its ability to function in the generation of plasmin (Gugliucci and Ghitescu, 2002), although this has not been proven conclusively.

In another study, treatment of endothelial cells with high glucose was found to be sufficient to enhance binding of annexin 2 to a heat shock protein, HSP90α. This resulted in increased translocation of annexin 2 to the cell surface and a concomitant increase in plasmin production was observed (Lei et al., 2001). The major distinction between the two studies is the addition of insulin, since in both studies high glucose was used, but only in the study conducted by Ishii et al., were endothelial cells also exposed to high insulin. Insulin stimulation of cells leads to an increase in phosphorylated annexin 2 (Biener et al., 1996) though it is currently unknown if it is directly phosphorylated by the insulin receptor itself. The question of whether this phosphorylation event is sufficient to perturb tPA or plasminogen binding to annexin 2 has yet to be answered. There is however evidence to suggest that other modifications of annexin 2 can alter its binding capacity for such proteins. One such example is the effect of hyperhomocysteinemia, a condition known to adversely influence the microvascular complications of diabetes such as retinopathy (Goldstein et al., 2004). The presence of elevated levels of homocysteine has been shown to reduce the cell surface binding of tPA by 60%, and this has been attributed to the replacement of the cysteine 8 residue in the N-terminus of annexin 2 with a Homocysteine which does not favor tPA binding (Hajjar, 1993). It is also possible that phosphorylation of annexin 2 modifies its ability to act as a plasminogen/tPA receptor, especially since the tyrosine phosphorylation site of annexin 2 is only 15 residues away on tyrosine 23. This leads to the interesting possibility that the phosphorylation state of annexin 2 could have differing consequences in the context of type-1 and type-2 diabetes where there is an absence and increased presence of insulin respectively. Whether annexin 2 is directly involved in the pathogenesis of type-1 or type-2 diabetes is yet open to question, but it is most likely to have at least a significant ancillary role.

Further evidence for a role of annexins in diabetes comes from one study of a heterozygous annexin 7 knock-out mouse. In this study the annexin 7 null mouse was found to be embryonic lethal at day 10 but the heterozygote was viable and fertile. The heterozygous mice have enlarged islets of Langerhans with an 8–10 fold increase in vesicle insulin content at 6 weeks after birth. Abnormal calcium-mediated insulin secretion was observed in heterozygotes with a reduction in insulin secretion of 67% at 1mM Ca^{2+}, and a higher level of secretion at high calcium concentration 5mM Ca^{2+}. This was attributed to a decrease in the amount of IP_3 receptors in the heterozygotes that was confirmed by electron microscopy (Srivastava, 1999). These results however have not been reproduced in another annexin 7 knockout mouse which appears to have no defects in insulin metabolism

(Herr et al., 2001), and it remains a long-standing puzzle as to why an annexin 7 +/− mouse should have a deficiency of IP_3 receptors.

5. ANNEXINS AND HEART DISEASE

Annexins 1, 2, 4, 5, 6 and 7 have all been identified in the heart, though annexins 5 and 6 are by far the most abundant. Due to the fluctuating levels of submembranous calcium all annexins would be expected to exhibit dynamic on-off interactions with sarcolemal and sarcoplasmic reticulum membranes in cardiomyocytes upon exitation and calcium influx (reviewed in Camors et al., 2005).

Annexin 2 is localised in intra-myocardial capillaries, extracellular matrix and in endothelial cells of the coronary arteries but is undetectable in ventricular and atrial myocytes. In hypertensive guinea pigs at the onset of heart failure and in failing human hearts it was found to be significantly up-regulated and was localized to the interstitium between the cardiomyocytes and the coronary arteries (Trouve et al., 1999). In this region it might be involved in regulation of fibrinolysis and the coagulability of the endothelial surface of the heart blood vessels as previously described. Heart tissue derived from patients with end-stage congestive heart failure due to coronary artery disease showed increased levels of annexins 2 and 5 but significantly reduced expression of annexin 6 (Song et al., 1998).

Annexin 6 has been shown in vitro to be a modulator of the sarcoplasmic reticulum Ca^{2+}-release channel, the cardiac L-type channel and the Na^{+}/Ca^{2+} exchanger (Diaz-Munoz et al., 1990). Cardiomyocytes derived from an annexin 6 null mutant mouse show increased contraction and relaxation amplitudes and reduced 'time to peak relaxation', (Song et al., 2002). Heart-specific annexin 6 over-expression in a mouse resulted in dilation of the heart, acute diffusive myocarditis, moderate to severe fibrosis throughout the heart and frequency-dependent reduced shortening and rates of contraction of isolated cardiomyocytes (Gunteski-Hamblin et al., 1996) consistent with a role in negative inotopic calcium handling.

Sorcin (a member of the EF-hand superfamily of calcium binding proteins) has been shown to bind the N-terminal region of annexin 7 (Brownawell, 1997). Overexpression of sorcin affects cardiac contractility though such investigations have provided contradictory results, with some groups finding an increase in cell shortening (Seidler, 2003) whilst others have found a decrease (Meyers, 2003). These effects are thought to be mediated by sorcin's interactions with the sarcoplasmic reticulum Ca^{2+} channel, the ryanodine receptor (RyR) or the L-type calcium channel rather than via a direct effect on annexin 7 (Frank, 2005). These results are somewhat at odds with studies using another annexin 7 knockout mouse. Here the null mouse did not demonstrate embryonic lethality, indeed the mice are reported to be reasonably healthy and are fertile (Herr et al., 2001). However, although cardiomyocytes (taken at embryonic day 11.5–12.5) from the viable annexin 7 null mouse were found to have normal calcium homeostasis and complete expression of the components involved in excitation-contraction coupling, there was a decrease in the frequency-induced cell shortening. It was hypothesised that this might link to

a role for sorcin and consequently annexin 7 in the mature sarcomere, where there is a shift from calcium influx-mediated contraction to calcium-induced calcium release (CICR)-mediated contraction during development. The authors suggest this is the reason why there is no embryonic lethality of their null mouse, as the role for annexin 7 is restricted to the contraction components of the mature cardiomyocyte. It is possible therefore that annexin 7 and sorcin play a role in communication between the L-type Ca^{2+} channels and the RyR calcium release channels in the sarcoplasmic reticulum, a notion which is supported by the localisation of annexin 7 within the T-tubule system.

Annexin 5 has been shown to be associated in a complex with the cardiac Na^{+}/Ca^{2+} exchanger and caveolin-3 in both failing and non-failing hearts (Camors et al., 2006). The precise role of annexins in calcium homeostasis in the heart remains unclear. They may have direct regulatory roles on the calcium channels themselves, either via protein-protein interaction of through stabilisation or reorganisation of the membranes in which the channels operate. Alternatively they may regulate trafficking of calcium channels to and from the membrane, either as soluble complexes or in association with membranes. It is also possible that they contribute to calcium conductance directly, by adopting membrane spanning conformations to form atypical channels (reviewed in Moss and Morgan, 2004). In spite of a large and growing body of work involving the roles of annexins in normal heart function and in the diseased state, it remains unclear whether the changes in annexin expression and localisation are a cause or a consequence of cardiomyopathy.

6. ANNEXIN 1 AND INFLAMMATION

Annexin 1 is predominantly an intracellular protein initially identified as a major target for phosphorylation by the epidermal growth factor receptor (EGFR). It is expressed at high levels in many cell types and has a crucial role in EGF-mediated inward vesiculation of the EGFR (White et al., 2006). Signalling through the EGFR leads to stimulation of the MAP kinase pathway via an intermediate complex involving p21 ras and Grb2. Annexin 1 appears to competitively inhibit recruitment of this intermediate when cells are exposed to glucocorticoids, becoming phosphorylated and associating with EGFR-containing vesicles (Croxtall et al., 2000). Normal signalling through the MAP kinase pathway leads to relocalisation of the cytoplasmic phospholipase A_2 ($cPLA_2$) to membranes where it is involved in the production of the inflammatory second messenger arachidonic acid (AA). By inhibiting this pathway through increased annexin 1 recruitment, dexamethasone can moderate the pro-inflammatory stimulus. A small peptide derived from the N-terminus of annexin 1 can also uncouple EGF stimulation from AA production which lends promise to the development of annexin 1-based small anti-inflammatory drugs.

Consistent with much earlier work postulating a role for annexin 1 in mediating the effects of certain steroids, mice lacking annexin 1 are almost refractory to the

anti-inflammatory action of glucocorticoids (Hannon et al., 2003). Normally, glucocorticoid treatment results in up-regulation of annexin 1 expression, underscoring the importance of this protein in this pathway (Philipps et al., 1989, Morand et al., 2006). Other groups have shown that annexin 1 can regulate PLA_2 isoforms via a direct and specific interaction without a requirement for other proteins (Kim et al., 2001). The 19 mammalian PLA_2 isoforms are involved in a host of inflammatory processes including those associated with the central nervous system where they are highly expressed in astrocytes and neuronal cells, thus it is possible that annexin 1 might have a role in moderating such conditions as cerebral ischemia, Alzheimer's disease and neuronal injury due to cytotoxic agents.

Neutrophils express annexin 1 at high levels, with the protein being localised predominantly to the cytoplasm, the plasma membrane and to gelatinase containing granules. Fusion of these granules with the plasma membrane upon cell adhesion results in release of their contents and the appearance of annexin 1 on the cell surface. Annexin 1 is readily cleaved by extracellular proteases and the liberated amino terminus may then act as an 'autocoid', signalling via an interaction with the receptor for n-formylated peptides (nFPR receptors) (Gavins et al., 2003; Hayhoe et al., 2006). Annexin 1 inhibits diapedesis and extravasation in several models of inflammation (Perretti et al., 1996, 2001; Lim et al., 1998) and promotes leukocyte detachment through shedding of L-selectin and PSGL-1 (P-selectin glycoprotein-1 which is found on all leukocytes, primarily on the tips of their microvilli (Hayhoe et al., 2006). It appears that the N-terminus of annexin 1 can activate all the members of the FPR family, acting as a chemoattractant to both granulocytes and monocytes and also desensitizing these receptors to activation by other ligands. The balance between pro-and anti-inflammatory effects of the whole protein and the cleaved peptide will critically depend on concentration, presentation of the active moiety and the concentration of other ligands (Ernst et al., 2004).

It has been suggested that annexins 1 and 2 on the surface of neutrophils and macrophages could act as bridges between the phosphatidylserine exposed on the surface of apoptotic cells and the surface of the leukocytes. This could be particularly important in the non-phlogistic phagocytosis of effete lymphocytes (Fan et al., 2004). Annexin 1 null macrophages (from the knockout mouse) show diminished phagocytosis (Yona et al., 2006), but the authors note that these cells also show a reduction in the surface expression of the CD11b component of Mac-1, an important receptor in phagocytosis. This could reflect an annexin 1-dependent failure of trafficking of CD11b or may represent a downstream consequence of altered cell signalling. The authors also note the increased production of pro-inflammatory mediators by these cells upon phagocytosis of various targets. In in vivo experiments (the zymosan peritonitis model) macrophage extravasation, activation and migration (but not adhesion) was seen to be enhanced (Chatterjee et al, 2005) in the annexin 1 null mouse compared to wildtype controls. Annexin 1 expression was thought to be under the regulation of the pituitary adrenal axis (Buckingham and Flower, 1997), but in one model using adrenalectomized (glucocorticoids) or hypophysectomized

(pituitary factors) rats, over-expression and secretion of annexin 1 appeared to be independent of these pathways (Vergnolle et al., 1997).

Annexin 1 is up-regulated in multiple sclerosis and in an experimental model of the disease (experimental autoimmune encephalomyelitis-EAE) intracerebroventricular administration of annexin 1 proved to be neuroprotective. Annexin 1 is present in both macrophages and astrocytes localised in the lesions (Bolton et al., 1990; Huntinga et al, 1998). In experimental autoimmune neuritis (EAN), a model for human Guillain-Barre syndrome, increased annexin 1 expression was also observed in macrophages and T-cells in the inflamed sciatic nerve (Gold et al.,1999).

7. ANNEXIN 1 AND CYSTIC FIBROSIS

Cystic fibrosis is a chronic, fatal disease characterised by mutations in the CFTR gene, which codes for a cAMP-activated chloride channel, the most common mutation being a deletion of the phenylalanine at position 508. The disease is characterised by an increase in the concentration of secreted fluids on the surface of epithelia in the lung, pancreas and elsewhere. Patients become very susceptible to bacterial infections in the lung and damage to the pancreas can result in diabetes, a common complication. Liver damage is also common. Annexin 1 has been found to be down-regulated or differentially expressed in nasal cells of CF patients with a number of CFTR mutations and in cells from intestinal crypts, the lungs and pancreas (all cells known to express the CFTR) in the CFTR knock out mouse (Bensalem et al., 2005). Full-length annexin 1 can be detected in bronchoalveolar lavage (BAL) fluids from healthy individuals but in CF patients, in which neutrophils are often present in the BAL fluids, the protein appears to be cleaved by neutrophil elastase (Tsao et al., 1998). This would result in the release of the potentially immunomodulatory amino terminus of the protein.

It has been suggested that an increased production of arachidonic acid following down-regulation of annexin 1 in CF patients may explain some of the associated complications of the disease (Carlstedt-Duke et al., 1986; Strandvik et al., 1988). This is based on the observation that lymphocytes from CF patients show defective inhibition of AA production by dexamethasone. Increased AA production has been reported in CF and would influence chloride transport, mucus production and calcium homeostasis (and production of the eicosanoids). The reason why annexin 1 expression is reduced in these patients is at present unclear.

8. ANNEXINS AND BACTERIAL INFECTION

In addition to the problems discussed above, CF patients are particularly susceptible to infection with Pseudomonas aeruginosa and a recent study has suggested that extracellular annexin 2 on the surface of epithelial cells may act as a specific receptor for this bacterium (Kirschnek et al., 2005). Annexin 2 has also been

shown to be recruited to the actin-rich pedestals of non-invasive enteropathogenic E. coli (EPECs) (Rescher et al., 2004; Zobiack et al., 2002). These bacteria do not invade the cell, but rather form microcolonies on the cell surface, riding on an elevated 'cushion' of intimately associated plasma-membrane and underlying actin cytoskeleton. It is possible that the bacteria possess a membrane penetrating surface receptor which recruits annexin 2 directly, but the site of attachment is also coincident with a localised increase in phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$) production and this may be sufficient to bring annexin 2 to the membrane. In this context annexin 2 may stabilise or nucleate F-actin filaments at the plasma membrane (Hayes et al., 2004; Hayes et al., 2006). It is also possible that annexin 2 serves to stabilize the membrane in contact with the bacterial cells which is rich in 'raft-like' components (perhaps in the manner of a 'stalled' phagocytosis event) or is involved in some element of downstream signalling.

9. ANNEXIN 2 AND VIRAL INFECTION

In addition to possible roles in bacterial infection, annexin 2 is also implicated as a co-receptor for a number of viruses. It was identified as a potential receptor for respiratory syncytial virus (RSV) on Hep2 cells, binding to the viral G-protein, and indeed its expression was increased following viral infection (Raynor et al., 1999; Malhotra et al., 2003). Annexin 2 has also been identified as a co-receptor for cytomegalovirus (CMV) (Adlish et al., 1990; Taylor and Cooper, 1990; Raynor et al., 1999) and was localised to the surface of the mature virion by immunofluorescence (Wright et al., 1994) where it interacts directly with the viral glycoprotein B (Pitropaolo and Compton, 1997). Annexin 2 on the surface of the virion and on the host endothelium have both been implicated in the process of infection, though the direct role of annexin 2 in this regard remains speculative. It was not found to be necessary for CMV infection of fibroblasts (Pitropaolo and Compton, 1999), which suggests that the mode of action of annexin 2 is likely to be more complicated than the simple formation of a bridge between phospholipids on the viral and cellular membranes.

Recently a novel and more persuasive role has been ascribed to annexin 2 with regard to HIV maturation and infectivity. In $CD4^+$ macrophages HIV maturation occurs on the limiting membranes of multivesicular bodies, multi-lamellar structures which may be classified as sorting endosomes. Tissue macrophages (which include microglia of the central nervous system) are an important reservoir of HIV as they are less susceptible to the cytotoxic effects of the virus which normally matures on and buds from the plasma membrane of other $CD4^+$ cells. Proteomics studies have identified annexins, annexin-binding proteins and numerous components of the endosomal complex in the mature enveloped HIV-1 virus purified from monocyte-derived macrophages (Chertova et al., 2006). Annexin 2 was reported to form a complex with the Gag protein of HIV in an interaction that is necessary for complete Gag processing, maturation of the virion, and incorporation of CD63 (an intracellular tetraspanin protein associated with late endosomes and multivesicular

bodies) into the mature particle (Ryzhova et al., 2006). Interestingly Gag targeting to 'lipid raft-like' domains is dependent on the localised production of $PI4,5P_2$ and on the availability of cholesterol. So it is possible that annexin 2 actually shuttles Gag to these membrane subdomains.

Annexin 2 has also been implicated in other stages of infection of macrophages by HIV-1 (Ma et al, 2004). When annexin 2 expression was reduced with small interfering RNA in macrophages, HIV-1 infection was severely compromised. 'Blocking' antibodies with specificity for the N-terminus of annexin 2 also reduced HIV-1 infectivity. It appears that annexin 2 does not act at the initial stage of viral binding to the membrane (although the authors point out that it could act as a bridge between exposed phosphatidylserine on the viral envelope and the macrophage cell surface), rather it is involved in the production of fully infective particles, in tacit agreement with the previously described study. The authors showed that annexin 2 also bound SLPI (secretory leukocyte protease inhibitor) a known antagonist of HIV-1 infection, though the significance of this interaction is currently unknown.

Interestingly, over-expression of HIV tat-protein in Jurkat cells (a cell line derived from T-cells) resulted in down-regulation of numerous cytoskeletal elements including actin, tubulin and annexin 2 (Coiras et al., 2006). Again, the significance of these changes is as yet unclear, though one could hypothesise that this could allow the virus to remain dormant in a pool of T-cells.

10. DENT'S DISEASE

Mis-localisation of annexin 2 has recently been implicated in the pathogenesis of Dent's disease. This term is now used collectively to describe what was previously four conditions that affect kidney function: X-linked recessive nephrolithiasis with renal failure, X-linked recessive hypophosphatemic rickets, idiopathic low molecular weight proteinuria with hypercalciuria and nephrocalcinosis and Dent's disease. Patients with this condition present with low molecular weight proteinuria and hypercalciuria. Renal stones, nephrocalcinosis and renal failure are common late-stage developments. The condition has been attributed to abnormal acidification within endosomes of the proximal tubular cells. It is very rare and is usually caused by mutations in the voltage-dependent Cl^-/H^+ chloride antiporter CLCN5, but occasionally in the $PI4,5P_2$ 5-phosphatase, OCRL1 (oculocerebrorenal syndrome of Lowe protein 1).

Carr et al., (2006) showed that down-regulation of CLCN5 in a collecting-duct model system results in mis-localisation of annexin 2 from the cell cytoplasm to the cell membrane and onto the cell surface. Annexin 2 has been identified as the 'receptor' for calcium oxalate monohydrate crystals on the surface of MDCKI epithelial cells (dog kidney cells) (Kumar et al., 2003). The authors have shown that increased surface availability of annexin 2 results in increased binding of crystals to the cell, and that this could be inhibited by treating the cells with anti-annexin 2 antibodies.

11. ANNEXINS AND CANCER

As previously described, annexins are ubiquitous proteins, expressed to different degrees in different cell types. The annexin 'fingerprint' of cells may vary between quiescent and activated cells or indeed as cells undergo steps in developmental maturation. There have been many recent studies which have identified changes in annexin expression during neoplastic progression. Many of these studies depend on comparative proteomic or genomic analysis of tumour and 'normoplastic' tissues. In some cases clinical progression correlates well with increase or loss of annexin expression, in others it is the distribution of the protein which is altered. Such studies are useful in as much as they suggest that the level of annexin expression could be used as a prognostic indicator. Neoplastic changes are diverse and complex, in some cases they represent expansion of normally rare, multipotent clones of cells, sometimes they may represent 'de-differentiation' within a tissue, in others they represent invasion of one tissue (such as endocrine tissue) into another one. The annexin complement of the normoplastic and neoplastic cells may well represent the 'normal' annexin expression levels in these mislocated (spatially or temporally) cells. In most cases we do not know what the normal expression levels of any particular annexin are in such tissues, hence it is difficult to assess whether or not the observed expression level is inherently 'unusual'. Although the same could be said to be true of many proteins, the shear abundance of annexins means they are perhaps more likely to be picked up in such studies. We have only the most superficial idea of the cell/tissue-specific functions of many of the annexins so it may be premature in most cases to extrapolate from an observed correlation to a pathological role. The association of various annexins with particular neoplasis is summarized in Table 1.

Although earlier studies were largely dependent upon the use of only a single antibody in histological sections, and as such should be viewed with caution, many of these initial observations are now being verified by unbiased proteomic 'fishing' studies. It is clear from Table 1 that annexins often show either reduced or elevated expression levels (though not in the same cancer), suggesting that they are unlikely to fall into simple categories as tumour-supressor genes or oncogenes.

In this review we focus only on observations made using primary tissues and cells. There is an extensive literature examining the expression of annexins in cancer cell lines derived from a wide variety of sources, and the effects of modulating annexin expression on cell division, apoptosis, motility and 'invasiveness' in them. Such studies are vital if we are to further dissect the role of annexins in tumourigenesis, but real insights will only emerge with reference to primary cells as well. Many cells rapidly change their levels of annexins when cultured as their expression is dependent upon stress, growth rate and the presence or absence of growth factors. These gratuitous changes in the context of many other arbitrary changes seen in cell lines maintained for long periods in culture, means studies performed on cell lines alone should be viewed with caution.

Table 1. Tumour-associated changes in annexin expression

Disease	Annexin	Comments
Acute promyelocytic leukaemia	↑2;↑8	Haemorrhagic complications consistent with annexin 2 having a role in fibrinolysis (Menell et al., 1999); annexin 8 was found to be expressed at high levels in this leukaemia (Chang et al., 1992).
pro-B cell acute lymphoblastic leukaemia ALL	↑2	(Matsunaga et al., 2004).
B-cell Non-Hodgkin's lymphoma	↓1	(Vishwanatha et al., 2004)
Acute myeloid leukaemia	↑1, 10	(Lopez-Pedrera et al., 2006).
esophageal squamous cell carcinoma	↓1; ↓2	Loss off annexin 1 associated with poorly differentiated tumour foci. (Hu et al., 2004, Luo et al., 2004); (Zhang et al., 2004)
Head and neck Squamous cell Carcinoma	↑5; ↓1	Melle et al., 2003; Reduced annexin 1 expression correlates with histological grade. (Pedrero et al., 2004
Glioblastoma multiforme (Brain tumour)	↓7; ↑2	Loss of annexin 7 correlates with poor prognosis (Hung and Howng, 2003); Up-regulation of annexin 2 in high grade samples (Reeves et al., 1992)
Astrocytic brain tumour	↑2	Expression of Annexin 2 correlated with histological grade (Roseman et al., 1994)
Prostate Cancer	↓1,↓2, ↓5,↓4, ↓6,↓11	Progressive loss of annexins with increased histological stage. (Smitherman et al., 2004); Lehnigk et al., 2005); Annexin 1 expression reduced in prostatic adenocarcinoma and high-grade prostatic intraepithelial neoplasia (Kang et al., 2002; Patton et al., 2005); (Xin et al., 2003)
Renal Cancer	↑2;↑4	Upregulation of Annexin 2 in stress-induced renal carcinoma in a rat model. (Tanaka et al., 2004); Zimmerman et al., 2004; Shi et al., 2004)
Pancreatic Cancer	↑1; ↑4; ↑2	Bai et al., 2004; Shen et al., 2004; Annexin 2 up-regulated (Vishwanatha et al., 1993; Esposito et al., 2006)
Osteosarcoma	↓2	Annexin 2 expression inversely correlated to metastatic invasive potential (Gilette et al., 2004; Mintz et al., 2005)
Gastric Carcinoma	↑5	Wang et al., 2004); (Kang et al., 2006)
Breast cancer	↑11; ↓1; ↑8	(Hudelist et al., 2004); (Shen et al., 2005); Annexin A8 expression was associated with basal-cell-like breast cancers, associated with poor prognosis (Stein et al., 2005)
Nasopharyngeal carcinoma	↓1	(Rodrigo et al., 2005)
Thyroid	↓1	Down-regulation of annexin 1 associated with loss of differentiation (Petrella et al., 2006)

The ubiquitous expression of annexins means that they are superficially unattractive targets for drug development. Recently however it has been shown that Withaferin A, a steroidal lactone derived from Indian Ginseng which has been widely used in traditional Indian medicine, specifically binds to annexin 2 and has dramatic effects on cancer cell lines by modifying their actin cytoskeleton (Falsey et al., 2006). It is cytotoxic to cancer cell lines and is thought to alter the actin binding properties of annexin 2. Derivatives of this compound are finding uses as markers for angiogenesis and the compound itself has potent anti-angiogenic properties (Yokota et al., 2006; Mohan et al., 2004). This is congruent with the observation that the annexin 2 knockout mouse is incapable of pathological neo-angiogenesis, and with the proposed role of annexin 2 in the migration of new blood vessels (via tissue plasminogen activator, plasminogen and matrix metallo-proteinases MMPs) (Ling et al., 2004).

12. ANNEXINS AS REGULATORS OF TUMORIGENESIS?

Loss of heterozygosity (LOH) in the 10q21 region (human) has been found in 35% of prostrate tumours, the region which encodes for annexin 7 (Srivastava et al., 2001), and the protein has been shown to be down-regulated in samples of recurrent prostate carcinoma but not in androgen-stimulated prostate cancer (Smitherman et al., 2004). There are also significant alterations in hormone receptor status that correlate with this regional LOH, indicating that this could be a useful prognostic tool, and aid in the development of more specific treatments (Leighton et al., 2004).

In one study heterozygous $(+/-)$ annexin 7 mice exhibited a higher number of spontaneous tumours in thymus, spleen, liver and prostrate than littermate controls (Srivastava et al., 2003), leading to the suggestion that annexin 7 could act as a tumour suppressor gene. The same group have presented evidence of genomic instability in a hepatocellular carcinoma cell line derived from the annexin 7 $(+/-)$ mouse, supporting this notion. However there was an absence of phenotype in a second null $(-/-)$ annexin 7 mouse which was viable and fertile (Herr et al., 2001). Histological analysis of these animals revealed no significant abnormalities and no change in tumour susceptibility. No up-regulation at either an mRNA or protein level of annexins 1, 2, 4, 5, 6 or 11 was observed in brain, heart, liver and skeletal muscle, indicating there was no compensation by other members of the annexin protein family. The observed differences in the phenotypes of the two mice have been attributed to the different strategies used to knock-out the annexin 7 gene.

Another case involves annexin 6. Expression of this protein in A431 squamous epithelial cells reduced their proliferation rate in culture under conditions of limited serum (Theobald et al., 1994). When these cells were introduced subcutaneously into nude mice the developing tumours were significantly smaller, suggesting a tumour-suppressor activity of the protein (Theobald et al., 1995).

13. CONCLUSIONS

The review of literature in this chapter is far from complete, and for the sake of brevity we have had to largely exclude an extensive body of work based entirely on cultured cell lines. However, such studies are vital if we are to develop mechanistic models of how the annexins contribute to the pathology of the diseases with which they are increasingly becoming associated. Annexins are evolutionarily conserved proteins and appear for the most part to be cellular Ca^{2+} 'effectors'; structural and regulatory components of the cellular machinery that physically build connections between membranes and cytoskeleton and regulate and nucleate signalling complexes at these interfaces.

In this guise they may have many roles, for example regulating the dramatic changes in the structure of membrane subdomains in smooth muscle cells in response to changes in calcium (Draeger et al., 2005), or regulating flux through the endosomal compartments in response to growth factors (Gerke and Moss, 1997, Gruenberg and Stenmark, 2004). In some cases it is these roles that become deregulated in human disease. Neoplastic changes for example require increases in cellular transport, cell motility or loss of cell-cell contact, and these conditions are often associated with altered annexin expression or localisation. In other cases, the normal intracellular functions of annexins may be usurped by pathogens; as is the case with annexin 2 in viral and bacterial infectivity.

As well as their 'core' intracellular activities, many annexins have evolved other, specialised extracellular functions, such as the immunomodulatory action of annexin 1 or the pro-fibrinolytic activity of annexin 2. In this guise they contribute to homeostasis and repair; regulating and resolving infection and trauma, and will doubtless prove to be involved in many pathological processes. In the case of autoimmunity, when these mechanisms become disturbed, annexins are again implicated (for example in APL, SLE and diabetes) which underscores their importance as key regulatory factors.

The question remains as to whether we can definitively assign a disease state to a defect in annexin function. As explained at the beginning of this chapter, this requires a higher level of proof than has perhaps been achieved to date. Due to lack of space we have not discussed the potential roles of annexins in normal development, but it would appear that annexins have important roles at critical stages in this process. Mouse models however have shown that loss of some of the annexins is not incompatible with life, and annexin 1, 2, 4, 5 and 6 deficient mice are superficially healthy. In some cases however, the null mutations are accompanied by changes in the expression of other members of the annexin family, suggesting that compensatory changes have obfuscated the significance of this loss. It is possible that similar somatic changes occur in human in disease states.

Improvements in global genomic technologies for examination of the proteome or 'expressome' in normal or disease tissues are beginning to identify changes in annexin expression in the pathophysiological state. The identification of single nucleotide polymorphisms (SNPs) in annexin genes is also allowing various groups to assess the contribution of subtle changes in these proteins to quantitative trait loci.

Recently such a SNP in the annexin 2 gene has been associated with osteonecrosis and stroke associated with sickle cell disease (Baldwin et al., 2005, Sebastiani et al., 2005). This fits in with a role for annexin 2 in mineralization of bone and cartilage due to its involvement in the formation of matrix vesicles, a specialized form of exocytosis (Gillette and Nielsen-Preiss, 2004; Anderson 2003, Wang and Kirsh, 2002; Wu et al., 1996). It is in the coming together of the independent approaches of histology, cell biology, biochemistry and genetics that such results are so compelling. Rand identified two annexinopathies; before long we can safely predict that we will be able to add many more to this list.

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CHAPTER 2

CALPAINS AND HUMAN DISEASE

I. BERTIPAGLIA AND E. CARAFOLI

Department of Biochemistry, University of Padova, and Venetian Institute of Molecular Medicine, Padova, Italy

Abstract: Calpains, particularly conventional dimeric calpains, have claimed to be involved in the cell degeneration processes that characterize numerous disease conditions linked to dysfunctions of cellular Ca^{2+} homeostasis. The evidence supporting their involvement has traditionally been indirect and circumstantial, but recent work has added more solid evidence supporting the role of ubiquitous dimeric calpains in the process of neurodegeneration. The only disease condition in which a calpain defect has been conclusively involved concerns an atypical monomeric calpain: the muscle specific calpain-3, also known as p94. Inactivating defects in its gene cause a muscular dystrophy termed LGMD-2A. The molecular mechanism by which the absence of the proteolytic activity of calpain-3 causes the dystrophic process is unknown. Another atypical calpain, which has been characterized recently as a Ca^{2+}- dependent protease, calpain 10, appears to be involved in the etiology of type 2 diabetes. The involvement has been inferred essentially from genetic evidence. Also in the case of type 2 diabetes the molecular mechanisms that could link the disease to calpain 10 are unknown

Keywords: Calpain-3, muscular dystrophy, type 2 diabetes, calpain 10, neurodegeneration, protease

1. INTRODUCTION

An intracellular calcium dependent neutral proteolytic activity was described more than 40 years ago (Guroff, 1964). Calpains, as these proteases were later named, are widely distributed in the animal kingdom, from mammals to invertebrates, and have also been detected in fungi. They catalyse the limited proteolysis of proteins involved in numerous cell functions, among them cytoskeletal remodeling, signal transduction, cell cycle regulation, cell differentiation, apoptosis and necrosis, embryonic development and, in the central nervous system, long-term potentiation (Carafoli and Molinari, 1998), (Ono et al., 1998), (Wang, 2000). A number of pathological conditions have been associated with disturbances of the calpain system, among them: cataractogenesis, inflammation, arthritis, Alzheimer's

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(Saito et al., 1993) and Parkinson's diseases (Vanderklish and Bahr, 2000). The list could continue to include organ ischemia, stroke, brain trauma, various platelet syndromes, hypertension, liver dysfunction, and some types of cancer: Table 1 offers a panorama of the diseases that have most frequently been linked to calpains. A role for these proteases in the genesis of these conditions has been generally inferred from

Table 1. Pathologic conditions that have been associated with the calpains*

Diseases	Observations	References
LGMD-2A	This disease is associated with mutations in the gene encoding calpain 3 (*CAPN3*) and the probable loss of CAPN3 proteolytic activity.	Ono et al., 1998
Gastric cancer	This type of cancer is associated with down-regulation of *CAPN9*.	Yoshikawa et al., 2000
Type 2 diabetes mellitus	Mutations in intron 3 of *CAPN10* are associated with an increased incidence of type 2 diabetes in some populations.	Horikawa et al., 2000
Duchenne's and Becker's muscular dystrophies	These dystrophies are associated with the absence or deficiency of dystrophin, a membrane-associated protein, resulting in an increased Ca^{2+} level in muscle, loss of Ca^{2+} homeostasis, and inappropriate calpain activity.	Tidball and Spencer, 2000
Alzheimer's disease	There is an increased amount of m-calpain in the cytosolic but not the membranous fraction of the brain and in the neurofibrillary tangles of the brain.	Nixon and Mohan, 1999 Tsuji et al., 1998
Cataract formation	Ca^{2+} influx activates m-calpain, the predominant calpain in the lens, cleaving *a*- and *b*-crystallins but not *g*-crystallins; the crystallin fragments aggregate to form cataracts.	Shearer et al., 2000
Myocardial infarction	Ca^{2+} homeostasis is lost in ischemic areas, triggering inappropriate calpain activity; desmin and *a*-spectrin are degraded in ischemic heart tissue by synthetic calpain inhibitors; protein and mRNA levels of m-calpain and μ-calpain increase after myocardial infarction.	Papp et al., 2000 Sandmann, et al., 2001 Tsuji, et al., 2001 Yoshida, et al., 2001
Multiple sclerosis.	Levels of the 150-kD calpain-specific degradation product of *a*-spectrin increase 50% in human multiple sclerosis plaques; degradation of the 68-kD neurofilament protein is inhibited by a synthetic calpain inhibitor	Shields et al., 1999 Banik et al., 1997
Obsessive–compulsive disorder	Erythrocytes from patients with obsessive–compulsive disorder have significantly higher calpain activities than normal controls, a finding that could not be attributed to differences in memory function.	Mundo et al., 1997
Neuronal ischemia (stroke)	Calpastatin is degraded by calpain to a membrane-bound 50-kD polypeptide in ischemic brain tissue; calpains participate in both apoptosis and necrosis in tissue damage in ischemic areas.	Blomgren et al., 2001

* from Zatz and Starling, 2005

indirect evidence, and is thus not conclusively established. So far the only condition in which the linkage with a calpain mutation has been unambiguously proved is Limb-girdle muscular dystrophy type 2A (Richard et al., 1999). Type 2 diabetes mellitus (Horikawa et al., 2000) is the other disease for which relatively convincing evidence for a genetic linkage to a calpain, has been provided. We will therefore focus our attention on these two conditions. We will close this contribution with a brief discussion of the involvement of calpains in neuronal cell death, a process in which a role for calpains is suggested by increasingly convincing evidence.

2. PROPERTIES AND STRUCTURE OF CALPAINS

The first described calpains are the m- and μ-isoforms. They differ in sensitivity to calcium *in vitro,* as they are activated by millimolar and micromolar Ca^{2+}-concentrations respectively. They are heterodimers of two distinct subunits: an 80 kDa catalytic subunit also indicated as L-chain (large), and a regulatory 30kDa subunit or S-chain (small), each encoded by a different gene. The large subunit can be divided in four distinct domains:

Domain I is the N-terminal portion of the protein. The autocatalytic hydrolysis of this domain leads to the activation of the enzyme, and consequent dissociation of the 30K subunit (Yoshizawa et al., 1995).

Domain II is composed of two subdomains (IIa and IIb) and represents the catalytic core of the protease. A cys at position 115 (μ-calpain) or 105 (m-calpain), a His residue at position 272 (μ-calpain) or 262 (m-calpain) and an Asn residue at position 296 (μ-calpain) or 286 (m-calpain) form the catalytic triad characteristic of cysteine proteases such as papain or cathepsins B, L, or S. Domain II, however, shares only limited sequence homology with other cysteine proteases, and is likely to have evolved from a different ancestral gene.

Domain III shares no sequence homology with other known proteins, its structure resembling the C2 domains found in phospholipase C, protein kinase C and synaptotagmin (Rizo and Sudhof, 1998). In addition to linking the Ca^{2+}-binding domain of the molecule to the catalytic domain (domain II), domain III appears to be involved in phospholipids and Ca^{2+} binding (Tompa et al., 2001).

Domain IV is the C-terminal portion of the protein, and is also known as the calmodulin-like domain due to its similarity with calmodulin. It contains five EF-hand Ca^{2+}-binding motifs, the fifth of which does not bind calcium but is involved in the dimerization with the small subunit, through the interaction with the analogous EF-hand motif in domain VI. The small subunit is composed of two domains (V and VI) separated by a region containing a proline-rich stretch.

Domain V has high glycine content and is therefore a hydrophobic region that may be responsible for the interaction of calpain with the plasma membrane (Lee et al., 1990) (Kuboki et al., 1987) (Kuboki et al., 1990).

Domain VI is homologous to domain IV of the large subunit, and therefore has five EF-hands motifs, four of which bind calcium while the fifth is involved in the interaction with the large subunit. The crystal structures of domain IV (Blanchard

et al., 1996), and then of the entire heterodimeric m-calpain (Hosfield et al., 1999), has offered new insights into the functional properties of the protease (Figure 1). The x-ray structure of domain IV has revealed that the fifth EF-hand motif of the catalytic subunit indeed interacts with the EF-hand motif of domain VI of the small subunit. Crystallization at low and high calcium concentrations has demonstrated that the first four EF-hand motifs in domain IV and VI bind calcium with higher affinity than the fifth motif, which binds calcium only when crystallization is carried out at higher calcium concentration.

As mentioned above domain II is divided into two subdomains (IIa and IIb) containing the Cys and the His/Asn residues, respectively, which in the absence of calcium are held apart forming a wide gap that disrupts the catalytic triad, keeping the enzyme in an inactive state. To allow the formation of a functional catalytic complex, the distance between the residues in the triad must be reduced through

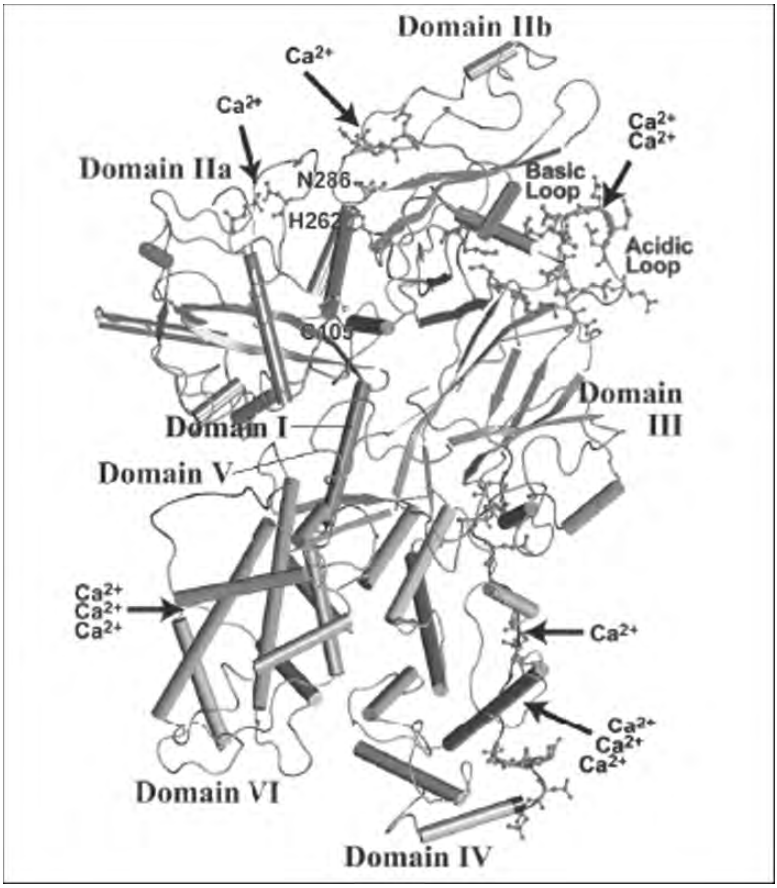

Figure 1. 3D structure of heterodimeric m-calpain, from Suzuki et al., 2004 (See Colour Plate 2)

a conformational change, which is produced by the binding of calcium. Since the calcium-linked conformational changes derived from the binding of the metal to domain IV are very limited, other calcium binding regions must be involved in the process. An "electrostatic switch" mechanism has been proposed (Strobl et al., 1999) as a possible mechanism of activation, where a putative calcium binding site is represented by the acidic loop in domain III which forms interdomain salt bridges with basic side chains in domain IIa. Binding of two or three calcium ions to this region would disrupt the electrostatic interaction, allowing domain IIa to move closer to domain IIb, and aligning the catalytic triad residues. Indeed, two non EF-hand calcium binding sites have been recently identified in domain IIa and IIb of μ-calpain. The binding of calcium to these regions induces a conformational change that brings the catalytic residues in close proximity to each other (Moldoveanu et al., 2002).

3. CALPAIN FAMILY

At least 14 calpain like DNA sequences have been identified in mammals, some of which not yet isolated as proteins. Some are tissue specific, others are expressed in a variety of tissues (Sorimachi et al., 1989) (Sorimachi et al., 1993). With few exceptions, very little is known on the properties of these (putative) calpains other than the sequence homology to conventional calpains (Dear and Boehm, 1999) (Dear and Boehm, 2001). The inclusion in the family is based on the sequence similarity with domain II of m- and μ-calpains. At least 38 calpain-like genes have been identified in a wide variety of species, including *Drosophila* (4 isoforms) (Pintér and Friedrich, 1988) (Pintér et al., 1992), *C. elegans* (12 isoforms), fungi/yeast (2 isoforms), *Trypanosoma brucei* (5 isoforms). A membrane intrinsic calpain has been identified in a variety of plants. These calpain-like genes produce proteins with widely divergent properties, which can be grouped in 11 "typical calpains", having a domain structure similar to the 80 kDa subunit of m- or μ-calpain, and 27 "atypical calpains" that lack the calmodulin-like EF-sequences in domain IV. The function of these "calpain-like" molecules is unknown: some of them do not have domain IV and might thus be calcium insensitive, other "atypical calpains", lack one or more residues of the catalytic triad and are probably proteolitically inactive (Sorimachi and Suzuki, 2001). Figure 2 shows the most important members of the calpain family.

3.1. Biochemical Properties of Calpains

3.1.1. Tissue and cell distribution

m- and μ-calpain are expressed in a variety of tissues and cell types, however, their mutual ratio varies with the tissue and, in the same tissues, with the species. For instance, human platelets and erythrocytes have no detectable m-calpain, while bovine platelets and smooth muscle have no detectable μ-calpain (Thompson and Goll, 2000). Several studies have led to the suggestion that calpains are capable of relocating in the cell in response to signals, for instance m-calpain has

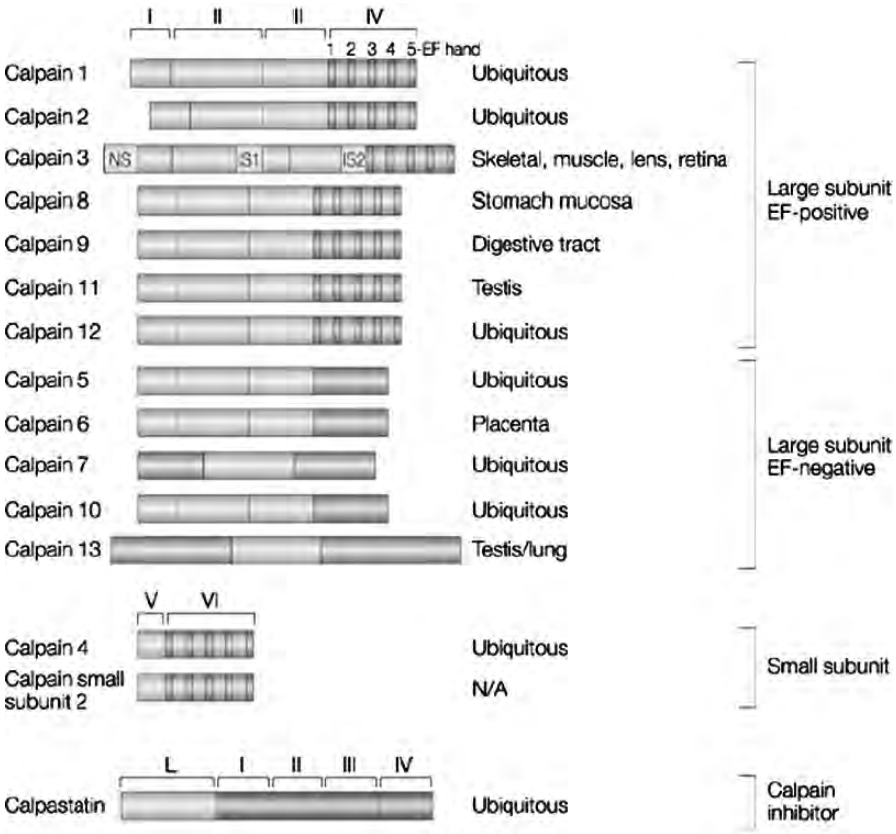

Figure 2. Domain organization of calpains from Branca, et al., 1999 (See Colour Plate 3)

been found at the plasma membrane in dividing (Schollmeyer, 1986) or fusing (Schollmeyer, 1986) myoblasts, and redistributes from the cytoplasm to the plasma membrane in COS-7 cells and in human lung tumor cells after stimulation with a Ca^{2+} ionophore (Gil-Parrado et al., 2003).

4. ACTIVATION UPON AUTOLYSIS?

Both m- and μ-calpain undergo rapid autoproteolysis when incubated with calcium. Autolysis of the large and the small subunits generates a 76kDa (μ-calpain) or 78 kDa (m-calpain) and an 18kDa fragment, respectively (Zimmerman and Schlapfer, 1991) (McClelland et al., 1989). Autolysis reduces the Ca^{2+}-requirement for half-maximal activity of μ-calpain from 3–50 to 0.5–2.0 μM Ca^{2+} and that of m-calpain from 400–800 to 50–150 μM Ca^{2+} (Suzuki et al., 1981). These observations have initially led to the suggestion that calpains could be proenzymes. However,

while a number of papers have claimed that autolysis was necessary for activation (Baki et al., 1995), other studies have found that both the cleaved and intact forms of the protease were capable of cleaving a casein substrate (Cottin et al., 2001). Eventually, the x-ray structure has shown that autolysis of m-calpain removed an a-helical NH_2 terminal fragment of the 80kDa subunit that did not block the active site, compatible with the idea that autolysis is not required for activation (Hosfield et al., 1999). The function of the autoproteolytic step is thus still unclear.

5. REGULATION OF CALPAIN ACTIVITY. THE CALCIUM REQUIREMENT PROBLEM

The calcium concentrations required for the activity of μ- and m-calpains are much higher than the 50–300 nM found in the cytosol of living cells. Efforts have thus been made to identify possible mechanisms to reduce the calcium requirement of the calpains, focusing on factors that might act as "activators". Extensive work on phospholipids has shown (Coolican and Hathaway, 1984) that phosphoinositides lower the Ca^{2+}-requirement of either m- or μ-calpain by three- to six-fold (Saido et al., 1992). Very high molar ratios of the phospholipids were required, making it unlikely that this mechanism would function under physiological conditions. It has been suggested that the calpains would interact with phospholipids at the plasma membrane, and that the interaction might lower the Ca^{2+}requirement of the protease. This is an interesting suggestion; however, calpains bind to proteins and not to phospholipids in the plasma membrane (Inomata et al., 1990). Isovelerylcarnitine has been claimed to reduce the Ca^{2+}-requirement for the activity of m-calpain to less than 10μM and to increase the specific activity of the protease (Pontremoli et al., 1990). A heat stable polypeptide (40–50kDa) isolated from human neutrophils (Pontremoli et al., 1988) and rat skeletal muscle (Pontremoli et al., 1990), has also been shown to reduce the Ca^{2+} concentration required for proteolytic activity of m-calpain. A dimeric protein of 30kDa, later identified as an acyl-CoA binding protein, has also been claimed to reduce the Ca^{2+}-requirement for m-calpain from a variety of sources (Melloni et al., 1998). Other activators have also been described, including nuclear DNA (Mellgren et al., 1993) and calpastatin subdomain A and C (see below) (Tompa et al., 2002). In summary, the mechanisms to reduce the calcium requirement of calpains are still obscure.

6. CALPASTATIN

Calpastatin was identified in experiments in which no calpain activity could be observed in crude skeletal muscle homogenates. After precipitation of calpain at pH 6.5 (Dayton et al., 1976), the supernatant was found to contain a heat-resistant factor with calpain inhibitory activity (Okitani et al., 1976), which was later named calpastatin. Early attempts to characterize and purify the factor were unsuccessful, due to the susceptibility of the protein to proteolytic degradation.

Another complicating factor was the existence of at least 9 different calpastatin polypeptides generated by different promoters and alternative splicing mechanisms from a single gene. Cloning and sequencing has eventually shown that calpastatin has four repeated, poorly homologous, inhibitory domains of approximately 140 aminoacids (domain I, II, III and IV) and an N-terminal domain L that has no inhibitory activity. Three subdomains were identified within each inhibitory domain. A conserved sequence in subdomain B appears to be essential for activity, but has little inhibitory activity by itself. Calpastatin is a very specific inhibitor, as it does not inhibit other proteases, including cystein proteases. Calpains bind to calpastatin in a calcium- dependent, reversible manner. Calpastatin domain A binds to domain IV of calpain and calpastatin domain C to domain VI of calpain.

7. CALPAIN-3

The first tissue-specific calpain, calpain-3 (or p94) was described in 1989 (Sorimachi et al., 1989). Calpain-3 is predominantly expressed in skeletal muscle, where its mRNA is 10 times more abundant than that of the ubiquitous calpains. The domain organization of calpain-3 is similar to that of the large subunit of the conventional calpains, but is characterized by three unique regions: NS at the N-terminal domain, IS1 in domain II and IS2 between domains III and IV. These inserts presumably contribute to calpain-3‘s peculiar properties and physiological function. NS comprises about 60 residues with numerous prolines, and becomes cleaved during the autolysis of calpain-3. Studies on NS-deletion mutants have shown that the absence of NS did not affect the activation of the proteolytic core of the protein (Diaz et al., 2004). IS1 is a 48 amino acid stretch situated just upstream of the active site His: together with IS2, it has been associated with the chronic instability of p94. IS1 has been recently shown to function as an internal propeptide that must be autoproteolytically removed from the vicinity of the active site before the enzyme can accept exogenous substrates and inhibitors (Diaz et al., 2004). The IS2 insert is rich in lysines and contains a nuclear localization signal (Sorimachi et al., 1990). Cells transfected with deletion mutants lacking the IS2 insert do not show clear nuclear localization signals, whereas those transfected with wild type variants do, indicating that the nuclear localization signal is functional. The finding that mutants lacking IS2 show increased stability to autoproteolysis suggests a role of IS2 in the regulation of the activity of the protease (Sorimachi et al., 1993).

8. AUTOLYSIS OF CALPAIN-3

A distinctive feature of calpain-3 is the propensity to autolysis: the half life of the protein *in vitro* is less than 30 minutes. Autoproteolysis is prevented by replacing the cys residue of the catalytic site with a ser, but is not affected by cysteine protease inhibitors such as calpastatin, E64 and Leupeptin (Sorimachi et al., 1993). The autocatalytic activity and the absence of effective inhibitors have made the

purification and characterization of calpain-3 difficult. Initially, the protein could not even be detected in rat skeletal muscle fractions, but eventually an affinity chromatography method allowed the isolation of the protein (Kinbara et al., 1998). Three auto-cleavage sites were identified within the IS1 region, producing fragments of 60, 58 and 55kDa, respectively (Kinbara et al., 1998). The autolytic cleavage sites of calpain-3 have been more precisely characterized in a deletion mutant, p94I-II, comprising only domains I and II. Incubation of p94I-II with 10mM $CaCl_2$ immediately initiated autoproteolysis, and two major fragments of 30 and 15 kDa appeared. They corresponded to cleavage sites located in the NS and IS1 regions, whereas two additional fragments corresponded to cleavage sites near the C-terminal ends of NS and IS1. All four sites had a pro or gly residue near the cleavage point (Rey and Davies, 2002).

9. EFFECT OF CALCIUM ON CALPAIN-3 AUTOLYSIS

The presence of a domain IV containing EF-hand motifs, of a C2-like domain and of the two non EF-hand calcium binding sites in domain IIa and IIb (Moldoveanu et al., 2002), suggests that calpain-3 does bind calcium. Nonetheless, early reports had indicated that it was rapidly degraded in the presence of EDTA (Sorimachi et al., 1993). The paradox was resolved by another study, that directly demonstrated calcium binding to calpain-3 using a gel overlay method, and showed that calpain-3 was stable in the presence of EGTA, autolytic cleavage already occurring at 500nM Ca^{2+}(Branca et al., 1999). Therefore, calpain-3 has a Ca^{2+}-affinity which is far higher than that of conventional calpains. Very recently, the calcium requirement for calpain-3 activity has been determined for two truncated isoforms: p94 I-II, comprising domain I and II and the two inserts NS and IS1, and p94I-IIΔNS/IS1 including domain I and II but lacking NS and IS1. The data have suggested cooperative binding of calcium at two sites, with half maximal activity values at 150 and 90 μM $CaCl_2$ (Diaz et al., 2006).

10. INTERACTION BETWEEN CALPAIN-3 AND CONNECTIN/TITIN

A yeast-two hybrid screening on human skeletal muscle cDNA library has shown that calpain-3 binds specifically to the N_2-line portion and the C-terminal of connectin. Connectin, also known as titin, is a giant filamentous protein expressed in all vertebrate striated muscles (Labeit et al., 1995). Single molecules of titin span half the sarcomere from the Z to the M lines and are thought to provide a template for sarcomere assembly during muscle cell development, to facilitate cell contraction by anchoring components of the thick and thin filament system, and to impart to the myofobrils the passive tension response to stretch (Gregorio et al., 1999). Further analysis has shown that calpain-3 interacts with the N2-line portion of titin through the IS2 region, and that only the full-length calpain-3 interacts with the C-terminus of connectin (Kinbara et al., 1997).

11. LGMD-2A

Autosomal recessive limb girdle muscular dystrophies are a clinically and genetically heterogeneous group of disorders involving at least ten recessive and three dominant forms indicated as LGMD2 and LGMD1, respectively (Walton and Nattrass 1954). LGMDs are characterized by progressive symmetrical atrophy of the shoulder and pelvic girdle muscles, and with the exception of LGMD-2A, they are all due to mutations in genes encoding structural proteins (Bushby, 1999). When it was discovered that mutations on the calpain3 gene (*CNP3*) caused LGMD-2A, it became clear that, in spite of similar clinical features, LGMDs have different pathogeneses (Zatz et al., 2000): LGMD-2A was the first identified dystrophic syndrome associated with an altered enzyme activity.

12. GENETICS AND ETIOLOGY

The gene whose mutations are responsible for this form of LGMD was located on chromosome 15 (Beckmann et al., 1991). The identification of LGMD-2A etiologic mutations came from mutation analyses conducted initially on a small inbred population of La Rèunion Island, and subsequently on other isolated ethnic populations such as the inter-related northern and southern Indiana Old Order Amish population (Richard et al., 1995). All the identified mutations fell inside gene *CPN3*, encoding calpain-3 (Sorimachi et al 1989). The gene is composed of 24 exons corresponding to a 3,5kb transcript (Sorimachi et al., 1996). The number of *CPN3* mutations found to affect the La Rèunion Island families led to the suggestion that LGMD-2A might be a digenic disease and that additional loci might be involved (Richard et al., 1995). However, further studies on other populations indicated that LGMD-2A was a monogenic recessive disease, and the high allelic heterogeneity found on La Rèunion Island became known as the Rèunion paradox. More than 280 distinct pathogenic mutations have now been documented (Fokkema et al., 2005): 65 missense, 55 frameshift, 36 splice sites, 38 insertions/deletions and a small number of apparently neutral polymorphic or unclassified variants. Missense mutations are the majority and show a non uniform distribution with clusters of hot spots around domains IIa, IIb and III and preserved regions around the IS1 and IS2 domains (Jia et al., 2001). In most cases the mutations lead to loss of proteolytic activity, while others seem to affect the ability of the protease to interact with titin (Ono et al., 1998). The interaction is believed to be important for the regulation of the protease activity (Sorimachi et al., 2000).

13. CLINICAL FEATURES

LGMD-2A is considered to be the most frequent form of recessive LGMD (Beckmann et al 1991), its frequency varying regionally between 10 and 50% and reaching 80% in some ethnic populations (Fanin et al., 2003). The age of onset ranges from 2 to 40 years, but the disease appears frequently during the second

decade of life. The course of the disease is slow but progressive, leading to loss of ambulation approximately one or two decades after diagnosis. Weakness of the limb girdle muscles manifests at first in the pelvic girdle, while involvement of the shoulder girdle usually occurs about 2–10 years later. Calf enlargement is frequently seen, involving the soleus and the medial head of the gastrocnemius and sparing the lateral head. There are no reports of cardiac or facial involvement and no intellectual impairment. The clinical phenotype is highly variable and attempts to derive genotype-phenotype correlations are difficult due to the high heterogeneity of *CANP3* mutations. It has only been possible to observe increased severity of the disease in patients carrying two inactivating alleles (Zatz et al., 2000). Biopsies show a typical dystrophic pattern consisting of necrosis, regeneration, fiber diameter variability, myofibrillar disorganization and fibrosis (Kawai et al., 1998) (Chae et al., 2001). Abundant and disorganized mitochondria have also been observed.

14. MOLECULAR MECHANISMS

The molecular mechanism of the disease has not been established, but a number of suggestions arising from experimental findings have been put forward.

NfkB/apoptosis NF-κBs are dimeric transcription factors that play a central role in the regulation of diverse biological processes, including cell growth and survival. In resting cells, NF-κB dimers are sequestered in the cytoplasm as latent complexes through binding inhibitors containing ankyrin repeat domains (ARD), called IκB (inhibitor of κB) proteins. The major pathway leading to NF-κB activation involves the induction of IκB degradation, that allows NF-κB dimers to accumulate in the nucleus to activate transcription (Xiao et al., 2006). A high incidence of apoptosis, associated with accumulation of Ikβα (a member of the IκB family) was observed in muscle biopsies from LGMD2A patients. Ikβαa was then reported to be cleaved by calpain-3 when the two where co-expressed in model cells, suggesting a role for calpain-3 in the NFKB signalling pathway, and therefore in the expression of survival genes (Baghdiguian et al., 1999). According to the suggestion, the absence of calpain-3 activity would lead to the accumulation of Ikβa, in turn leading to the stabilization of the Ikβα/NF-κB complex in the cytoplasm and to the inhibition of NF-κB signalling. Unfortunately, no conclusive evidence supporting this interesting suggestion has so far been produced and recent studies have instead demonstrated that NF-κB inhibition had no effect on muscle mass and pathology (Cai et al., 2004).

Filamin hypothesis Filamin C is a muscle-specific isoform of filamin that interacts with actin, myotilin, γ- and δ- sarcoglycans. It has been proposed to provide a signalling link between the sarcolemma and the Z-disks of myofibrils (Thompson, and Goll, 2000) (Stossel et al., 2001) (van der Ven et al., 2000). A recent report has associated C-terminal mutations of filamin C with a novel type of autosomal dominant myopathy with clinical features of LGMD, characterized by cytoplasmic aggregates that contain filamin C (Vorgerd et al., 2005). Calpain-3 has been found to cleave filamin C C-terminally both in *in vitro* and *in vivo* experiments

(Guyon et al., 2003) (Taveau et al., 2003). The calpain-3 mediated proteolysis of filamin C might play a role in the regulation of the protein ability to bind to sarcoglycans: indeed, the cleavage of filamin C inhibits its interaction with γ- and δ-sarcoglycans (Guyon et al., 2003). However, so far no evidence has been provided that the mechanism described above is functional in physiological conditions.

Dysferlin. Dysferlin is a member of the ferlin family, a group of vesicle-associated membrane proteins involved in the docking and fusion of vesicles in skeletal muscle cells (Bansal and Campbell, 2004). At least two autosomal recessive muscle diseases are related to mutations of the dysferlin gene: LGMD2B and Miyoshi pathology (Bashir et al., 1998) (Liu et al., 1998) (Illa et al., 2001). Recent data have demonstrated a role for dysferlin in the repairing of the muscle-fiber plasma membrane, indicating that dysferlin defects might lead to membrane damage and eventually to the dystrophic syndrome. Interestingly, a reduction of calpain-3 levels was reported in some patients affected by LGMD2B and Miyoshi myopathy (Anderson et al., 2000), and, in turn, a decreased content of dysferlin was occasionally found in patients with LGMD2A (Chrobakova et al., 2004). It has also been shown that calpain-3 co-immunoprecipitated with dysferlin. These data have led to the suggestion that calpain-3 may play a role in dysferlin-mediated plasma membrane repair. Unfortunately, recent work has cast doubt on the involvement of calpain-3 in membrane repairing as no membrane damage has been observed in calpain-3 knock-out mice (Fougerousse et al., 2003) (Kramerova et al., 2005).

Titin. The fact that full length calpain-3, although very unstable, can be detected in freshly prepared skeletal muscle homogenates might be explained by the presence in skeletal muscle of a stabilizing factor that prevents its autolysis. Titin is a plausible candidate for this function. As mentioned above, titin is a giant sarcomeric protein that interacts with calpain-3, through its N_2-line portion and C-terminal domain (Kinbara et al., 1997). The suggestion that titin could be involved in the regulation of calpain-3 activity is thus plausible. A mutation in the N2A region of titin causes a severe form of muscular dystrophy in mice (muscular dystrophy with myositis, mdm) (Garvey et al., 2002). The deletion affects the ability of calpain-3 to bind to the N2A region, and as a result the amount of calpain-3 decreases in the muscle homozygous mdm mice (Haravuori et al., 2001). The ability of titin to prevent calpain-3 autolysis has been investigated by coexpressing the latter with the 2NA titin fragment in insect cells: in this system the connectin fragment failed to prevent calpain-3 autolysis (Kinbara, et al., 1998). Recent studies have shown that N2A titin has a suppressive rather than an inhibitory effect on calpain-3, i.e. N2A titin fragment suppresses calpain-3 autolytic disassembly. While these data support the suggestion that titin might serve as an important regulator of calpain-3 activity, the fact that the severity and progression of the pathology does not change in double mutants with an mdm phenotype lacking calpain-3 indicates that altered calpain-3 activity is not a primary mechanism of mdm pathology (Ono et al., 2006).

PDLIM1. Work now in progress in our laboratory has identified PDLIM1 as a direct *in vivo* substrate of calpain-3. PDLIM1 is one of the proteins that were found to be differentially expressed during comparative proteomic analyses of calpain-3

knock out cultured muscle cells. In vitro experiments have then revealed that PDLIM1 is cleaved by the protease, and that a fragment of about 8 kDa is released from the C-terminal portion of the protein. PDLIM1 contains an N-terminal PDZ domain and a C-terminal LIM domain. The PDZ and LIM domains mediate protein association with the cytoskeleton and with proteins involved in signal transduction. PDLIM1 is most abundantly expressed in heart and skeletal muscles (Kotaka et al., 2001). It is associated through its LIM domain to the C-terminal calcium-insensitive EF hand region of a-actinin 2, thus localizing at the Z-disks of the sarcomere (Kotaka et al., 2000). The cleavage of the C-terminal portion of the protein would release the PDZ domain, allowing its interaction with other proteins. If these proteins were components of signal transduction pathways, PDLIM1 might play a role as a component of a "signaling complex" in the Z-line, acting together with calpain-3 in the modulation of muscle cell homeostasis. It could, for instance, transduce signals from external stimuli into internal responses.

15. CALPAIN-10

The human calpain-10 gene (chromosome 2q) contains 15 exons and is expressed in eight different alternatively spliced isoforms (calpain-10a-h), with calpain-10a being the most abundant (Goll, et al., 2003). CNP10 transcripts are ubiquitously expressed in mouse, rat and human tissues, with high expression levels in human heart and rat brain. The protein is present in high concentration in the water insoluble fractions of tissues, suggesting that a significant portion of it might be associated with membrane and/or cytoskeletal elements. For instance, in mouse skeletal muscle it is predominantly associated with the sarcolemma (Ma et al., 2001). Calpain-10 (CNP10) is an atypical calpain, in which the calmodulin-like domain IV is replaced by a domain structurally related to domain III. This domain, that does not contain calcium-binding EF-hand motifs, was initially termed domain T (Sorimachi and Suzuki, 2001), but is now called domain III'. The same domain organization, with the catalytic domain followed by a repeat of two domains III, is also found in two other atypical calpains, calpain-5 and 6. In addition to the absence of EF-hand domains, calpain-10 does not contain residues important for Ca^{2+}-binding in domain II, nor does it contain the acidic loops that are believed to be responsible for the 'calcium switch mechanism' that allow typical calpains to reach a conformationally active state. Thus, the Ca^{2+}-dependent activation mechanism must differ not only from that of typical calpains, it also differs from that of closely related calpain-5, which contain Ca^{2+}-binding sites in domains II and III. Ca^{2+}-mediated calpain-10 proteolytic activity could not be detected in animal tissues using zimography or fluorescence-labeled casein assays (Sokol, and Kuwabara, 2000). Enhanced expression levels and nuclear localization of calpain-10 were observed when mouse lens epithelial cells, where calpain-10 is expressed, were treated with 10μM ionomycin in the presence of calcium. This suggested a role for Ca^{2+}in the translocation of the protein to the nucleus and provided some evidence

for Ca^{2+}interaction with the protein)Ma et al., 2001) More recently, immunoblot analysis and activity assay revealed calpain 10 in fractions of kidney mitochondria.

The protease was found in all mitochondrial compartments, i.e., the outer membrane, intermembrane space, inner membrane, and matrix. The protease hydrolyzed a calpain substrate (SLLVY-AMC) in a reaction that was partially sensitive to calcium. The cleavage of the substrate was inhibited by active site-directed calpain inhibitors (calpeptin and E64), but not by inhibitors that bind to domain IV of typical calpains (PD150606). The Ca^{2+}-dependent proteolytic activity was blocked by ruthenium red, the inhibitor of the mitochondrial Ca^{2+}-uptake uniporter. Because ruthenium red blocks Ca^{2+} uptake across the mitochondrial inner membrane, it was suggested that the calpain activity was located in the mitochondrial matrix. Interestingly, the stimulation of the activity of Calpain-10 by Ca^{2+} appears to be a mediator of mitochondrial dysfunction through the cleavage of Complex I subunits and activation of mitochondrial permeability transition (MPT) (Arrington, et al., 2006).

16. TYPE 2 DIABETES MELLITUS

Type 2 or non insulin-dependent diabetes mellitus (T2DM) is the most common form of the disease, accounting for over 90% of the cases. It affects about 4% of the general population with a typical onset in adulthood, generally at age 40–60. The disease is characterized by defects in hepatic glucose production, insulin action and insulin secretion. Insulin resistance is an early feature, and is initially compensated by hyperinsulinemia, which ensures that glucose homeostasis is maintained. Deterioration of glucose homeostasis and overt T2DM develop when pancreatic β-cells become unable to provide sufficient compensatory hyperinsulinemia. At this point the hyperglycemia continues to worsen, compromising β-cells function, and eventually resulting in their failure)Harris et al., 2004).

17. THE GENETIC LINKAGE OF CALPAIN-10 TO T2DM

A major susceptibility locus for Type 2 diabetes, designated NIDDM1, was initially detected on chromosome 2 in a Mexican American population in Texas (Hanis et al., 1996). Positional cloning studies on the NIDDM1 region mapped the putative T2DM susceptibility gene to *CNP10,* which encodes calpain-10 (Horikawa et al., 2000). These studies discovered that a G→A polymorphism in intron 3 (UCSNP43) was significantly associated with T2DM. They also found that T2DM was associated with a high-risk haplotype defined not only by UCSNP43 but also by polymorphisms at UCSNP19 and UCSNP63. Association between *CNP10* polymorphisms and susceptibility to T2DM has then been found in a number of other ethnic groups including Finns and Germans (Horikawa et al., 2000). It has also been linked with a number of conditions associated with T2DM including obesity (Hoffstedt et al., 2002), polycystic ovary syndrome (Gonzalez et al., 2003), hypertension and dyslipidemia (Sugimoto et al., 2003). The mechanisms by which DNA polymorphisms in

CNP10 may lead to T2DM are still unclear, but it has been suggested that it might affect the transcriptional regulation of the gene.

18. CALPAIN-10 AND T2DM

Even if the linkage between calpain-10 and Type 2 diabetes remains obscure, the possibility that the protease participates in pathways that regulate glucose homeostasis is drawing increasing attention. Thus it has been reported that mutations of the calpain-10 gene influence both insulin secretion (Lynn et al., 2002) (Marshall et al., 2004) and insulin action (Baier et al., 2000) (Orho et al., 2002). Clinical studies on full heritage Pima Indians of Arizona, a population with the world's highest reported incidence of type 2 diabetes, have failed to indicate an association between a G/G genotype at UCSNP-43 and the increased occurrence of type 2 diabetes. A decreased rate of insulin stimulated glucose turnover was nevertheless observed, and reduced calpain-10 mRNA expression in skeletal muscle was found in G/G homozygotes (Baier et al., 2000). Elevated free fatty acids levels were also associated with mutations of the calpain-10 gene in a population of the Botnia region in Western Finland (Orho et al., 2002). They have been suggested to be involved in the reduced sensitivity to the antilipolytic effects of insulin, and in the worsening of insulin resistance. Studies reporting an involvement of protein kinase C (PKC) in skeletal muscle insulin resistance have offered some support to the suggestion. Free fatty acids activate PKC in vitro, leading to the hyperphosphorylation of the insulin receptor and to the downregulation of its tyrosine kinase activity. As a result, insulin resistance would increase (Griffin et al., 1999). PKC could also be involved in a different way: the kinase is a calpain substrate *in vitro,* therefore, decreased calpain-10 expression might upregulate its activity, increasing the phosphorylation of insulin receptors and decreasing insulin signaling (Orho et al., 2002). Other studies have linked calpain-10 to ryanodine-induced apoptosis of pancreatic β-cells, which plays an important role in the pathogenesis of diabetes mellitus: no ryanodine-mediated apoptosis was observed in transgenic mice in which the calpain-10 gene had been ablated. The data were supported by the finding that calpain inhibitors prevented ryanodine-induced apoptosis of β-cells (Johnson et al 2003) (Butler et al., 2003). Calpain-10 has also been found to coimmunoprecipitate with the plasma membrane SNARE-complex (N-ethylmaleimide-sensitive fusion protein attachment receptors), suggesting that it may trigger exocytosis in pancreatic β-cells. The protease could thus play a role in the coupling of stimuli with the secretory event in pancreatic β-cells (Marshall et al., 2005). Overexpression of calpastatin in skeletal muscle cells affected the function of the glucose transporter GLUT4: a severe resistance of the transporter to insulin stimulation was observed in transgenic mice overexpressing calpastatin (Otani et al., 2004). The glucose transporter was also found to be less responsive in contraction- stimulated muscles overexpressing calpastatin (Otani, et al., 2006). Calpain inhibition could thus result in the impairment of insulin- and contraction stimulated glucose transport. However, it remains to be established whether these effects are mediated by calpain-10 or by other components of the

calpain family. Quite simply, not enough is known on calpain-10 to draw convicing conclusions on this point. The same applies to the recent finding of significantly decreased insulin-stimulated glucose uptake in cultured human myotubes pre-treated with calpain inhibitors. Irrespective of whether calpain-10 has a role in the process, the finding is nevertheless compatible with the suggestion that calpain may have functions that could be relevant to the pathogenesis of insulin resistance in type 2 diabetes (Logie et al., 2005).

19. CALPAINS IN NEURODEGENERATION AND NEURONAL DEATH

The role of calpains in neuronal cell death has been traditionally considered very likely, and has been examined in various injuries and pathological conditions of the central nervous system. As expected, increased calpain activity has frequently been related to the development of neurological disorders involving impaired calcium homeostasis (Besse et al., 1994) (Blalock et al., 1999) (Romero et al., 2002). The importance of calpain activation as a final pathway in states of acute cell injury triggered by calcium influx is by and large accepted, particularly in the nervous system. For instance, in Alzheimer's disease a persistent abnormal level of calpain activation develops at an early stage of the disease (Saito et al., 1993). Alzheimer's disease is associated to the abnormal processing of amyloid precursor protein (APP), that leads to extracellular deposits of aggregates known as amyloid plaques. A secondary event is the generation of neurofibrillary tangles, composed of intracellular aggregates of the tau protein. The hyperphosphorylation of tau causes its dissociation from microtubules and its accumulation in the cytosol of neuronal cells (Buee et al., 2000) (Alvarez, et al., 1999). Calpains have been proposed to link these two events. Since calpains are involved in the activation of cdk5 and ERK/MAP kinases, their hyperactivation, triggered by the elevated intracellular calcium, might be responsible for the phosphorylation of the tau protein, and therefore for cytoskeletal disorganization and neuronal cell damage (Jordán et al., 1997), (Nath et al., 2000). A recently proposed model suggests that calpain might be directly responsible for APP proteolysis, and that its enhanced activity may be the main mechanism of amyloid plaque formation (Mathews et al., 2002). Interestingly, siRNA experiments have indicated that μ-calpain's gene silencing prevents APPs release, but additional work is clearly needed to confirm calpain's direct involvement in the formation of protein tau aggregates (Chen and Fernandez, 2005). Calpain activation has also been linked to Parkinson's disease. Inhibition of calpain activity prevents the neurological and behavioral defects in a Parkinson's mouse model (Mouatt-Prigent et al., 1996). Recent data have indicated the association between calpain activation and the loss of dopamine neurons in post mortem tissues in Parkinsons's disesase cases (Crocker et al., 2003). N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) evoked an increase in calpain-mediated proteolysis in nigral dopamine neurons in vivo. Inhibition of calpain proteolysis using either a calpain inhibitor (MDL-28170) or adenovirus-mediated overexpression of the endogenous calpain inhibitor protein,

calpastatin, significantly attenuated MPTP-induced loss of nigral dopamine neurons (Crocker et al., 2003). Calpain activation has also been reported in a number of in vivo and cell culture models of apoptosis which may be relevant to neurodegeneration. Members of the Bcl-2 protein family of cell death regulators can be process by calpains (Gil-Parrado et al., 2002), p53 activation has been linked to calpain inhibition (Sedarous et al., 2003), and calpain-mediated proteolytic activation of Bax has also been reported (Wood et al., 1998). Acute activation of calpains may lead to caspase independent apoptotic events (Lankiewicz et al., 2000), or to necrotic cell death triggered by calcium influx (Syntichaki et al., 2002). One of the mechanisms by which calpain may contribute to acute cell damage is the cleavage of essential cytoskeletal proteins such as neurofilaments, cain/cabin1 and fodrin. For example the cleavage of the calcineurin binding domain of cain/cabin1 would result in calcineurin activation, followed by calcium mediated cell-death (Kim et al., 2002). Cain/cabin1 is an endogenous inhibitor of calcineurin, a calcium-dependent serine-threonine phosphatase involved in various cellular functions including apoptosis. During apoptosis cain/cabin1 is cleaved by calpain at the carboxyl terminus, which correspond to the calcineurin-binding domain of cain/cabin1, preventing the latter from binding calcineurin. As a consequence, increased activation of calcineurin induces calcium-triggered cell death (Kim, et al., 2002). A recent report provides additional insights into the involvement of calpain in the molecular mechanisms of excitotoxic cell death in neurons. During excitotoxicity, Ca^{2+}influx, triggered by glutamate receptor stimulation, is followed by a second uncontrolled calcium increase that eventually leads to neuronal death. The secondary calcium overload is prevented by inhibition of calpain-mediated inhibitory cleavage of one of the isoforms of the plasma membrane Na/Ca exchanger (NCX3), which is particularly important for the regulation of calcium homeostasis in neurons. The function of this exchanger is to export calcium from neurons preventing the development of the calcium overload. Its cleavage by calpain would inhibit Ca^{2+} export, inducing neuronal death (Bano et al., 2005).

20. CONCLUSIONS

The list of pathological conditions that have been linked to the altered activity of the calpain system is very long. So far only LGMD-2A has been unambiguously linked to calpain interestingly, in the case of LGMD-2A it is the absence of calpain activity, not, as one would expect, its anomalous increase that causes the disease. Interest is now increasing in the involvement of the atypical calpain 10 in the etiology of diabetes type 2. Here, however, a word of caution is in order for more than one reason. One is the confused picture of the molecular/physiological aspects linking the down regulation of calpain-10 to the disease. Another is the paucity of information on calpain-10, the proteolytic activity of which has not even been conclusively demonstrated. Even if erroneous calpain activation has been found in a number of pathological conditions, the role played by calpains remains largely elusive. Serious difficulties have hampered the progress toward the elucidation of

the proteolytic pathway involved in these pathological processes. Among them is the existence of multiple calpain substrates, including cytoskeletal and regulator proteins, and the extensive cross-talk between different proteolytic systems. Since proteases modulate signal transduction pathways the precise definition of the *in vivo* role of calpains becomes further complicated. The identification of the physiological substrates of calpains, and of substrates specific for calpain isoforms, would be of great help in the understanding of the role played by calpains in diseases. Also of help would be the availability of specific inhibitors. They would not only help establishing unequivocally the physiological and pathological actions of calpains, but in a longer time perspective could possibly become useful tools in the therapy of disorders involving calpain dysfunction.

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CHAPTER 3

GELSOLIN AND DISEASES

L. SPINARDI[1,*] AND W. WITKE[2]

[1]*Direzione Scientifica, IRCCS Fondazione Ospedale Policlinico, Mangiagalli e Regina Elena, Via Francesco Sforza 28, 20122 Milano, Italy*

[2]*EMBL Monterotondo, Via Ramarini 32, 00015 Monterotondo, Rome, Italy*

Abstract: Gelsolin is a calcium-activated actin filament severing and capping protein found in many cell types and as a secreted form in the plasma of vertebrates. Mutant mice for gelsolin as well as clinical studies have shown that gelsolin is linked to a number of pathological conditions such as inflammation, cancer and amyloidosis. The tight regulation of gelsolin by calcium is crucial for its physiological role and constitutive activation leads to apoptosis. In the following we will give an overview on how gelsolin is regulated by calcium, and which clinical conditions have been linked to lack or misregulation of gelsolin

Keywords: Gelsolin, calcium, diseases, amyloidosis, inflammation, cancer

1. INTRODUCTION

Actin monomers (G-actin) reversibly associate and polymerize in a head to tail fashion to form long helical filaments (F-actin), whose ends are distinct in terms of dynamics (Wegner, 1976). The rapid assembly and disassembly of actin filaments plays an active role in a large number of cellular functions such as cell locomotion, cell differentiation and cytokinesis (Welch et al., 1997; Fishkind and Wang, 1995; Yamada and Geiger, 1997). The organization of the actin cytoskeleton is controlled by numerous actin-binding proteins, which affect the dynamics of filament turnover and allow remodeling of the actin cytoskeleton in response to appropriate signals (Way et al., 1991; Pollard and Cooper, 1986). Actin binding proteins have been identified from a wide range of organisms and have been classified according to their

*Laura Spinardi, E-mail: laura.spinardi@policlinico.mi.it

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in vitro activities: their ability for crosslinking, severing, capping and nucleation of actin filaments (see Figure 1: *The life cycle of actin filaments*).

Actin filament length in particular is regulated by proteins of the gelsolin and cofilin superfamilies (Carlier, 1998). The proteins of the gelsolin family are characterized by their ability to sever and cap actin filaments in a Ca^{2+}-dependent manner (Janmey et al., 1986) (Cunningham et al., 1991; Burtnick et al., 1997; Kwiatkowski, 1999; Yin and Stull, 1999; McGough et al., 2003). Family members include proteins such as villin (Bretscher and Weber, 1979), adseverin/scinderin (Maekawa and Sakai, 1990; Rodriguez Del Castillo et al., 1990), flightless I (Campbell et al., 1993) and the macrophage capping protein CapG (Yu et al., 1990; Prendergast and Ziff, 1991; Dabiri et al., 1992). Gelsolin and the family members are built by either three or six repeats of a conserved ancestral structural domain. Remarkably, the structure of this gelsolin subdomain is identical to the structure of the smaller cofilin family members, despite the fact that cofilin shows little sequence homology with gelsolin (Hatanaka et al., 1996), suggesting a common evolutionary origin

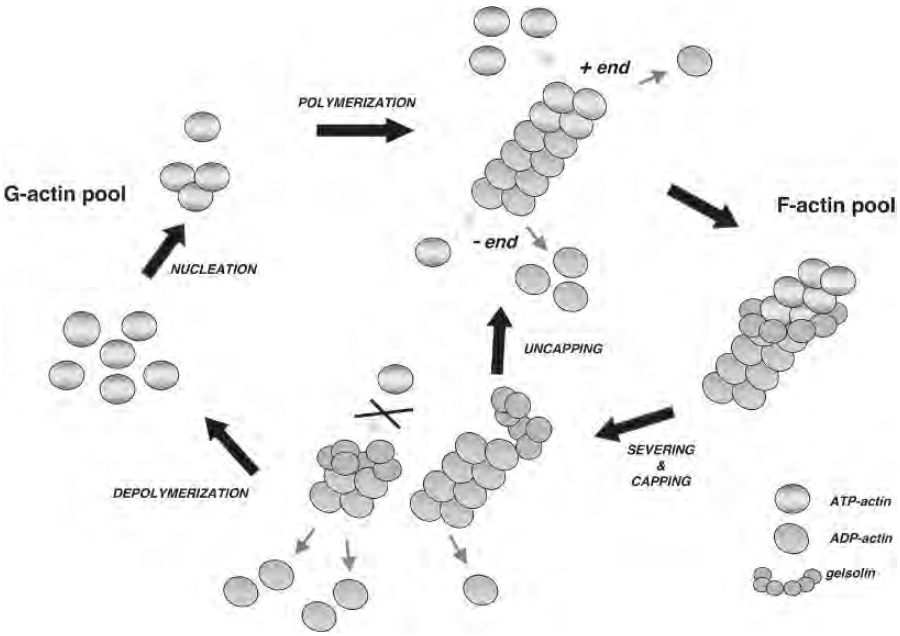

Figure 1. The life cycle of actin filaments.
Actin dynamics follows a complex kinetic that is regulated by actin binding proteins. ATP-bound globular actin (G-actin) first forms a trimer in a nucleation step after which rapid polymerization occurs into filamentous actin (F-actin). Due to the intrinsic ATP ase activity of actin the growing filament comprises an ATP-actin cap and an aged ADP-actin tail. Gelsolin preferentially binds to the ADP-actin containing filaments and severs them. Gelsolin then remains attached to the + end (barbed end) of filaments as a cap blocking further addition of actin monomers. Uncapping of gelsolin can liberate polymerization competent filament ends and thereby induce actin polymerization. (See Colour Plate 4)

or converging evolution for a protein domain required to sever actin filaments. Compared to gelsolin, villin, advillin, and supervillin all have C-, or N-terminal extensions, important for their roles in the formation of specialized cellular structures such as microvilli. The gelsolin family members have somewhat different biochemical activities, CapG for example can cap actin filaments but is lacking actin filament severing activity (Southwick and DiNubile, 1986), villin is also bundling actin filaments (Bretscher and Weber, 1980) and others show different sensitivity towards calcium (Kwiatkowski, 1999).

Gelsolin itself was discovered in 1979 by Yin and Stossel based on its ability to activate the gel-sol transformation of actin filaments in a calcium-dependent manner (Yin and Stossel, 1979; Yin et al., 1980). Gelsolin is composed of six domains, named S1-S6, that appears to have arisen from triplication, followed by duplication of an ancestral gene encoding a single domain protein of about 15 kDa (Kwiatkowski et al., 1986) (see Figure 2: *Gelsolin structure*).

A unique feature of gelsolin is that apart from the cytoplasmic protein found in most cell types, a secreted plasma form is generated mainly in muscle cells by alternative splicing, adding a 25 amino acid long amino-terminal leader peptide (Yin et al., 1984; Kwiatkowski et al., 1986). A minor cytoplasmic gelsolin isoform, named gelsolin-3, has been also identified, differing by an additional 11 amino acids at its amino-terminus (Vouyiouklis and Brophy, 1997). Cytoplasmic as well as secreted gelsolin are the most potent actin filament severing proteins identified to date. Severing is defined as the weakening of non-covalent bonds between actin

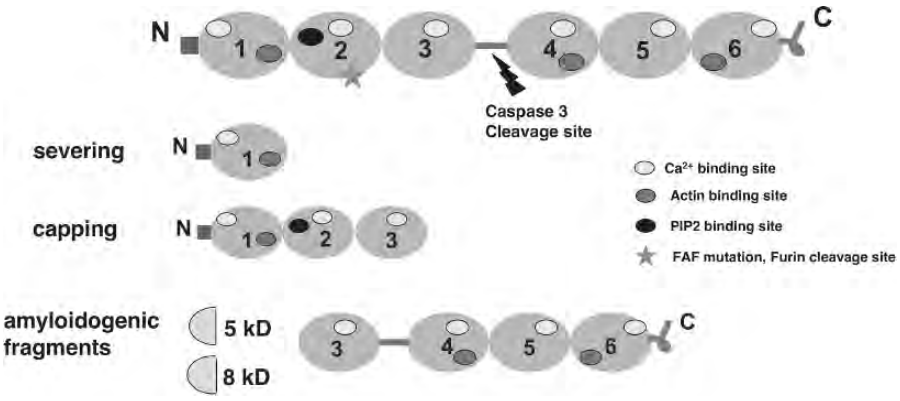

Figure 2. Gelsolin structure.
Gelsolin is composed of six domains, named S1-S6, that have arisen from of an ancestral gene encoding a protein of about 15 kDa. Gelsolin activity is regulated by calcium binding (yellow ovals) and by phosphatidylinositol 4,5-bisphosphate (PIP2) binding (black ovals). The isolated domain S1 contains severing activity, while domain S1-3 has capping activity. Biochemicals studies have identified three actin-binding sites (red ovals), a caspase-3 cleavage site (black arrow) and a furin cleavage site. In the Finnish type familial amyloidosis (FAF) a point mutations in gelsolin (orange star) activates the furin cleavage site eventually leading to aberrant intracellular cleavage and the production of amyloidogenic fragments. (See Colour Plate 5)

molecules within a filament leading to a break in the filament (see Figure 1: *The life cycle of actin filaments*). Severing is calcium dependent (see next chapter) and occurs rapidly upon binding of gelsolin to the side of an actin filament (Selden et al., 1998; McGough et al., 1998). After severing, gelsolin remains attached to the fast growing end (barbed end) of the filament forming a cap. As a result, a large number of very short actin filaments are generated which cannot reanneal or elongate at their barbed ends. In this way, the actin network is disassembled and can be rapidly re-assembled upon cell stimulation by selective uncapping of gelsolin and liberation of polymerization competent filament ends (Yin and Stull, 1999) (see Figure 1).

2. GELSOLIN IS A CALCIUM AND PHOSPHATIDYLINOSITIDE REGULATED MOLECULE

Two regulatory mechanisms are thought to modulate the activity of gelsolin *in vivo*. Calcium activates gelsolin to allow capping and severing of actin filaments, while phosphatidylinositol 4,5-bisphosphate (PIP_2) at the cell membrane keeps gelsolin sequestered in an inactive state. Upon hydrolysis of PIP_2 gelsolin is released into the cytoplasm and Ca^{2+} dependent activation can occur (Janmey et al., 1987; Allen, 2003). The importance of Ca^{2+}-regulated actin severing has been well documented during platelet activation (Witke et al., 1995; Goshima et al., 1999). To date gelsolin is the only known Ca^{2+}-dependent severing protein that by this mechanism can effectively increase the number of filaments (Sun et al., 1999).

Protein crystallography has identified a Ca^{2+}-binding site in each of gelsolin domains suggesting that free gelsolin can recruit up to six calcium ions (McLaughlin et al., 1993; Burtnick et al., 1997; Choe et al., 2002; Pope et al., 1995). Also each domain has different actin binding properties (Kwiatkowski et al., 1985; Chaponnier et al., 1986; Pope et al., 1991; Bryan and Hwo, 1986; Way and Matsudaira, 1993). In Figure 2 the Ca^{2+} and actin binding sites in gelsolin are schematically summarized. At submicromolar Ca^{2+} concentrations, domains S5 and S6 are occupied by calcium and gelsolin becomes activated to bind actin, however for full activation of severing activity higher calcium concentrations are required, most likely filling the sites on domains S1, S2 and S4 (Burtnick et al., 2004; Choe et al., 2002). Biochemical studies suggest that Ca^{2+} opens up gelsolin by inducing a conformational change in the C-terminus to expose the actin binding sites located at the N-terminus (Weeds et al., 1995; Pope et al., 1995). In calcium-free conditions, gelsolin exists as a compact folded structure that cannot bind actin as domain S4 and S6 remain tightly packed into an extended β-sheet blocking binding of actin to domain S2 (Weeds et al., 1995; Burtnick et al., 1997). Upon adding Ca^{2+} this structure opens, domain S6 swings away from S4 and forms new contacts with S5. Thereby gelsolin becomes activated so that the F-actin binding region in domain S2 is exposed allowing the molecule to make the initial contact with the actin filament. The gelsolin/EGTA crystal structure furthermore showed that the C-terminus of gelsolin has a tail extension made of a short helix. The tail helix is in close contact with the actin binding helix of S2 and

may act as a latch to inhibit actin binding by the N-half in the absence of Ca^{2+}. This model has been put forward as the "tail latch hypothesis"(Choe et al., 2002; Burtnick et al., 2004). The latch hypothesis suggests that Ca^{2+} binding to domain S6 induces a first conformational change in the gelsolin structure, releasing the tail latch and allowing binding of actin to domain S2. S2 binding to actin then directs the S1 domain to bind actin and induce severing. (Figure 3*: Gelsolin activation by Ca^{2+}*).

In agreement with this model, deletion of the last 20 residues in the C-terminus activates severing activity independent of Ca^{2+} (Kwiatkowski et al., 1989; Way and Matsudaira, 1993). The crystallographic analysis was further complemented by biochemical studies of proteolytic fragments which identified three actin-binding regions: a calcium-independent monomer binding fragment (S1), a calcium-independent filament-binding fragment (S2-3), and a calcium-dependent monomer-binding fragment (S4-6) (Bryan, 1988) (Way et al., 1990; Way et al., 1992; Pope et al., 1995; Pope et al., 1991).

These structural constrains have significant relevance *in vivo*. For example, an amino-terminal cleavage product of gelsolin (residues 1–352) comprising domain

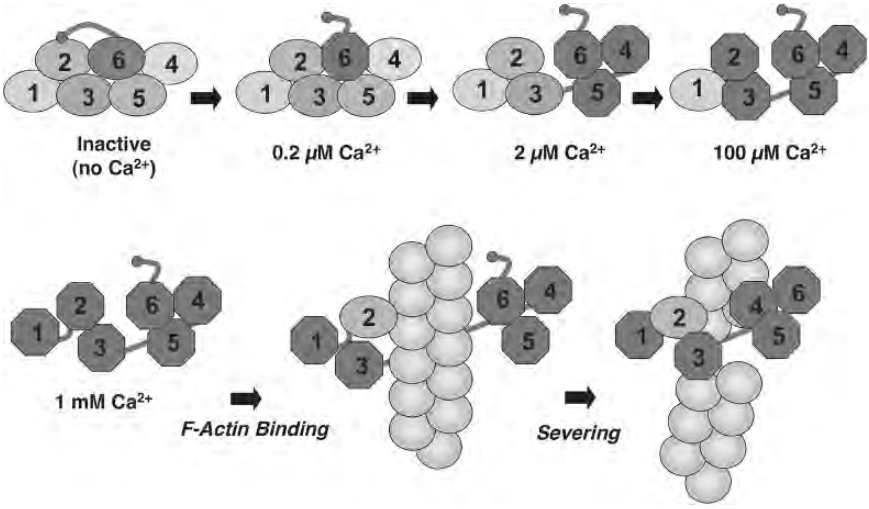

Figure 3. Gelsolin activation by Ca^{2+}.
Gelsolin is a Ca^{2+}-activated actin filament severing protein. Depending on the Ca^{2+}-concentration, gelsolin becomes gradually activated by controlled unfolding of the individual domains. Ca^{2+} opens up gelsolin by inducing a conformational change in the C-terminus (S4-6) to expose the actin binding sites on the N-terminus. This is called the "tail latch hypothesis".
In the presence of low calcium concentration, Ca^{2+} binds to the S6 domain, induces release of the C- tail domain and frees the actin binding sites present in domains S1-2. In order for severing to occur, higher concentrations of the ion are required. Additional calcium binding, most likely to domains S1, S2 and S4, fully activates the molecule, thus allowing severing to take place.
Activated gelsolin domains are shown as red hexagons and calcium ion concentrations required for each conformation change are indicated. F-actin is shown in yellow. (See Colour Plate 6)

1–3 no longer requires Ca^{2+} to sever actin filaments and to dismantle the membrane cytoskeleton (see Figure 2). Caspase-3 is such a specific cellular protease that is activated via the death receptor and mitochondrial apoptotic pathways (Kothakota et al., 1997; Kamada et al., 1998). Caspase-3 mediated cleavage of gelsolin induces constitutive filament severing and disruption of the actin cytoskeleton – a hallmark of apoptosis.

3. PATHOLOGY OF GELSOLIN DELETION IN THE MOUSE

Gelsolin is broadly expressed by cells of mesenchymal-epithelial origins both in the adult and in the embryonic tissues (Tanaka and Sobue, 1994; Teubner et al., 1994; Lueck et al., 1998; Arai and Kwiatkowski, 1999) (Spinardi and Witke, unpublished observation). Cytoplasmic gelsolin is highly expressed in platelets, macrophages and neutrophils, (Witke et al., 1995; Barkalow et al., 1996) but also in smooth muscle cells and osteoblasts (Spinardi and Witke, unpublished observation). However, muscle cells are the major source of the secreted plasma gelsolin (Kwiatkowski et al., 1988).

The generation of a mouse model lacking gelsolin was an important step to reveal and dissect the relevant biological functions of this protein. Surprisingly, gelsolin null mice lacking any cytoplasmic or plasma gelsolin were viable in a mixed background (Witke et al., 1995). However, cells from gelsolin null mice exhibited a number of defects in actin-mediated processes. Gelsolin null fibroblasts have exaggerated actin stress fibers, which is consistent with the inability to sever and remodel actin filaments (Witke et al., 1995). Furthermore, gelsolin null fibroblasts do not ruffle in response to growth factor, show impaired chemotaxis and delayed wound healing (Witke et al., 1995) (Azuma et al., 1998). In gelsolin null mice skin defects and impaired wound healing are amplified due to impaired keratinocyte function (Spinardi and Witke, unpublished observation). Gelsolin is required for rapid platelet activation and shape changes as shown by prolonged bleeding of mutant mice after injury. In conjunction with a delayed neutrophil extravasation these results highlighted a role for gelsolin particular in responses that require fast motility under stress conditions (Witke et al., 1995).

In neurons gelsolin was shown to regulate growth cone collapse and filopodia retraction (Lu et al., 1997). Interestingly, the alterations of F-actin levels in neurons lacking gelsolin prolonged the opening time of calcium channels, and as a result mutant mice showed increased susceptibility to glutamate induced excitotoxicity (Furukawa et al., 1997).

In immune cells it was demonstrated that gelsolin plays a role in Fc receptor- and integrin-but not in complement- mediated phagocytosis (Arora et al., 1999; Arora et al., 2004) (Serrander et al., 2000; Witke et al., 2001). Analysis of phagocytosis in CapG/gelsolin double-null mice, further suggested distinct and non-overlapping functions of CapG and gelsolin in the phagocytic processes of macrophages (Witke et al., 2001).

Using primary cells from gelsolin null mice it was shown that gelsolin functions as a downstream effector of the GTP-binding protein Rac (Azuma et al., 1998). Once activated, Rac promotes dissociation of gelsolin from capped actin filaments, allowing actin remodeling to proceed (Arcaro, 1998). Rac activation is also a first step in Fc receptor- and integrin-mediated phagocytosis (Caron and Hall, 1998) which was shown to be affected in gelsolin null cells.

4. GELSOLIN IN HUMAN DISEASES

Since its discovery as an actin depolymerizing factor, gelsolin has been implicated in a number of disease pathways such as amyloidosis, inflammation, and oncogenic transformation (Kwiatkowski, 1999). In the following section we will summarize the knowledge on the role of gelsolin in pathological processes and discuss potential therapeutic strategies.

4.1. Amyloidosis is Caused by Point Mutations in Gelsolin

Protein folding disorders are now recognized to contribute to a large number of human diseases including those involving amyloid fibril deposition in various organs (Aridor and Balch, 1999; Kelly, 1996). The name amyloid comes from the early mistaken identification of the fibril substance as starch (*amylum* in Latin), based on crude histological iodine-staining techniques. For some time it was debated whether or not amyloid deposits were fatty deposits or carbohydrate deposits until it was finally resolved that it was rather an extracellular deposition of proteins. Amyloidoses represent a group of late-onset degenerative disorders caused by the misfolding and self-assembly of a single protein. It is believed that the general mechanism of amyloidosis is similar for all amyloid diseases, including Alzheimer's and diabetes II (Carrell and Gooptu, 1998; Zerovnik, 2002).

One type of familial amyloidosis first identified in the Finnish population is caused by deposition of gelsolin (Maury et al., 2000; Maury et al., 2001). This Finnish type familial amyloidosis (FAF) is a hereditable autosomal dominant amyloid polyneuropathy, characterized by corneal lattice dystrophy, progressive cranial and peripheral neuropathy as well as skin changes (Chen et al., 2001; Maury et al., 2001).

The major protein constituents of the amyloid are 5 and 8 kDa internal fragments of the actin-binding protein gelsolin (see Figure 2). Sequencing of these fragments revealed amino acid substitutions at a single residue (D187N, or D187Y) when compared to wild type gelsolin (Maury, 1991). These mutations lead to an aberrant intracellular cleavage of secreted gelsolin between residues 172 and 173 resulting in the production of the amyloidogenic 173–243 fragment. The 172/173 cleavage appears to be due to disruption of the Cys188–Cys201 disulfide bond in gelsolin and occurs at highest frequency in neuronal cells, consistent with the pattern of symptoms in this disease (Paunio et al., 1998).

The production of the D187N and D187Y variants of gelsolin are due to aberrant processing by furin, a Ca^{2+}-dependent protease known as proprotein convertases. Normally, wild-type gelsolin is stabilized by Ca^{2+} binding in the trans-Golgi and the furin consensus site is hidden. However, the mutant gelsolins are not able to bind Ca^{2+} in the S2 domain, resulting in the destabilization of the structure and the exposure of the furin cleavage site (Isaacson et al., 1999; Huff et al., 2003). Furin cleavage then liberates an amyloidogenic fragment that assembles into amyloid fibrils (Page et al., 2004) (see Figure 2. *Gelsolin structure*). Although the role of plasma gelsolin is still not well understood, it is clear that a mutation in the circulating gelsolin form is the basis for the disease. Interestingly, plasma gelsolin isolated from homozygous FAF patients lacks both, actin severing and nucleating activities (Weeds et al., 1993). In contrast, cytoplasmic gelsolin is not cleaved in these patients and the cellular actin modulating function of intracellular gelsolin is not affected (Kangas et al., 1999).

4.2. Gelsolin and Inflammation

Inflammation is part of the first line response of the immune system to infection. Inflammation is characterized by two main components: an exudative response and a cellular response. The exudative response involves the local recruitment of fluid (edema, swelling), containing proteins such as fibrin and immunoglobulins. The cellular response involves the release of cytokines from resident cells such as fibroblasts and the invasion of white blood cells into the inflamed tissue. Leukocytes take on an important role in inflammation by clearing the site from bacteria and cellular debris. In chronic inflammation an increased extravasation of neutrophils, monocytes, activated T cells, and macrophages to the inflamed site will persist.

Gelsolin was shown to play an important role in the cellular response of inflammation. For example, gelsolin null mice respond more slowly to an induced inflammatory stimulus such as intraperitoneal thioglycollate instillation (Witke et al., 1995).The slower kinetics of leukocyte accumulation in gelsolin null mice were similar to those observed in P-selectin knockout mice (Mayadas et al., 1993) and most likely due to an impaired emigration of leukocytes from blood vessels (Witke et al., 1995). The blunted inflammatory response in gelsolin null mice is restricted to the early acute phase which suggests that gelsolin could represent a potential target for anti-inflammatory therapy (Witke et al., 1995).

The ethiology of chronic inflammatory diseases differs from acute inflammation and is still poorly understood. One example is the chronic rheumatoid arthritis (RA), which is characterized by a chronic destructive arthropathy. In RA and other inflammatory bone diseases there is intense crosstalk between the skeleton and the immune system by the use of common cytokines and transcriptional pathways. A newly established research area – 'osteoimmunology' – attempts to unravel this skeletal/immunological axis and several signaling pathways involved in inflammatory bone loss have been identified (Wagner and Eferl, 2005). One pathway

leading to rheumatoid arthritis (RA) involves gelsolin and cytoskeletal organization. Recent studies in the mouse as well as in patients have confirmed that gelsolin expression is lost in rheumatoid synovial fibroblasts leading to severe alterations in cytoskeletal organization (Aidinis et al., 2005). Furthermore, induction of RA in gelsolin null mice resulted in the exacerbation of the disease symptoms, suggesting that loss of gelsolin plays an important role in the pathophysiology of the disease.

Crohn's diseases, is another example of chronic inflammation of the intestine, in which actin reorganization and gelsolin might play an important role. Smooth muscle from Crohn's patients change their phenotype from a contractile form to a migratory form that correlates with an increased expression of gelsolin (Ehrlich et al., 2000).

One important conclusion from all these studies is that there might be conditions where modulating gelsolin expression or activity might be beneficial. Since the etiology and progression is different in the different diseases, one might want to inhibit gelsolin activity during acute type of inflammation, while boosting gelsolin expression might ameliorate the symptoms in chronic inflammation like RA.

4.3. Gelsolin and Cancer

A number of studies have suggested a role for gelsolin as a tumor suppressor gene mainly in breast cancer and carcinomas (Mullauer et al., 1993; Asch et al., 1996; Mielnicki et al., 1999; Onda et al., 1999; Fujita et al., 2001). Gelsolin expression is also downregulated in 60–90% of tumors during carcinogenesis of breast, colon, stomach, bladder, prostate, and lung (Asch et al., 1999; Kuzumaki et al., 1997; Tanaka et al., 1995; Dosaka-Akita et al., 1998; Prasad et al., 1997; Sagawa et al., 2003).

In these cancer types downregulation of gelsolin during tumor progression was observed and overexpression of gelsolin reverted the transformed phenotype in cell culture and mouse models (Sagawa et al., 2003). However, in a subset of breast tumors overexpressing the tyrosine kinase receptors erbB-2 and EGFR, gelsolin overexpression was described to correlate with negative prognoses (Thor et al., 2001). Similarly it has been observed that high level expression of gelsolin in early stage non-small-cell lung cancer occurs in 14% of patients, and provided a highly significant negative prognostic factor (Shieh et al., 1999). These somewhat contradictory results could for example be explained by an increased invasive potential of transformed cells when gelsolin expression is high (De Corte et al., 2002). A direct correlation of motility with expressed levels of gelsolin was shown in cell culture (Cunningham et al., 1991).

However it should be noted that under normal housing conditions and without challenging the animals, the gelsolin null mice do not show any increased incidence of tumor formation (Witke W, Kwiatkowski DJ, unpublished observation), suggesting that the loss of gelsolin in cancer is perhaps a downstream event, or that loss of gelsolin has to go together with the loss of other tumor promoting genes in order to cause transformation.

5. GELSOLIN AS A THERAPEUTIC MOLECULE?

When necrosis and cell death occurs, cytoplasmic actin is released into the blood stream where it can interact with components of the haemostatic and fibrinolytic systems, or polymerize and form filaments because of the favorable ionic conditions. An actin scavenger system must therefore exist that helps to remove free actin from the circulation and plasma gelsolin was discussed as one important component of this system. A depletion of the actin scavenger system upon continued tissue damage and actin release may result in severe organ damage. Several studies reported gelsolin depletion in a variety of tissue injury syndromes such as acute liver failure, septic shock, acute lung injury, cardiac infraction and myonecrosis (Janmey et al., 1986; Suhler et al., 1997) (Dahl et al., 1999). Recent data showed that intravenous infusion of recombinant gelsolin completely prevented the increase in pulmonary microvascular permeability in burn-injured rats, suggesting that the exogenous administration of recombinant gelsolin could be beneficial to prevent inflammation-induced pulmonary microvascular dysfunction (Rothenbach et al., 2004).

Another condition where cell debris becomes life threatening is the obstruction of airways by viscous sputum in patients suffering from cystic fibrosis (CF). It has been shown that human plasma gelsolin can rapidly decreased the viscosity of CF sputum samples *in vitro* (Vasconcellos et al., 1994; Sheils et al., 1996; Davoodian et al., 1997; Ismailov et al., 1997), suggesting that in CF patients gelsolin may have therapeutic potential as a mucolytic agent.

These data provide evidence for an important protective function of circulating gelsolin during systemic inflammatory events, and suggest that therapeutic use of recombinant gelsolin could be a promising strategy for treatment of specific pathological condition.

6. CONCLUDING REMARKS

While the early work on gelsolin has mainly focused on the calcium regulated remodeling of actin filaments, recent data have begun to elucidate the importance of gelsolin-mediated function in pathological conditions. Several strategies are currently explored to test whether gelsolin itself can be used as a therapeutic molecule or whether it can serve to target and restrict cell migration for example in cancer and metastasis.

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CHAPTER 4

GUANYLATE CYCLASE-ACTIVATING PROTEINS AND RETINA DISEASE

W. BAEHR[1] AND K. PALCZEWSKI[2]

[1]*Department of Ophthalmology and Visual Sciences, University of Utah, Salt Lake City, Utah 84112-5339, USA*

[2]*Department of Pharmacology, Case School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106-4965, USA*

Abstract: Detailed biochemical, structural and physiological studies of the role of Ca^{2+}-binding proteins in mammalian retinal neurons have yielded new insights into the function of these proteins in normal and pathological states. In phototransduction, a biochemical process that is responsible for the conversion of light into an electrical impulse, guanylate cyclases (GCs) are regulated by GC-activating proteins (GCAPs). These regulatory proteins respond to changes in cytoplasmic Ca^{2+} concentrations. Disruption of Ca^{2+} homeostasis in photoreceptor cells by genetic and environmental factors can result ultimately in degeneration of these cells. Pathogenic mutations in GC1 and GCAP1 cause autosomal recessive Leber congenital amaurosis and autosomal dominant cone dystrophy, respectively. This report provides a recent account of the advances, challenges, and possible future prospects of studying this important step in visual transduction that transcends to other neuronal Ca^{2+} homeostasis processes

Keywords: Calcium, rod and cone photoreceptors, rod outer segments (ROS), retina, guanylate cyclase (GC), guanylate cyclase-activating proteins (GCAPs), EF-hand motif, Ca^{2+}-binding protein, dominant cone dystrophy, Leber Congenital Amaurosis (LCA), cone dystrophy

1. THE PHOTOTRANSDUCTION CASCADE AND Ca^{2+} FEEDBACK

The rod phototransduction cascade is well researched and has been extensively reviewed (Polans et al., 1996; McBee et al., 2001; Baylor and Burns, 1998; Pugh, Jr. et al., 1999; Burns and Arshavsky, 2005). In darkness, the cytoplasm of the rod cell contains high levels of cGMP (1–10 μM) maintaining a number of CNG channels in an open state. Within milliseconds after a light flash, cGMP-gated (CNG) cation channels close and photoreceptor cells hyperpolarize. Open CNG

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channels allow an inward flow of cations (primarily Na^+ and Ca^{2+}), called the "dark current", across the plasma membrane. Single cell recordings with suction pipettes can measure this current, providing an extremely useful method to analyze phenotypes in genetically engineered rods (reviewed in (Burns and Baylor, 2001)). The main task of rhodopsin is to capture a photon and to activate the visual cascade. The activated state of rhodopsin, R*, acts as a guanine nucleotide-exchange factor (GEF), generating T*-GTP, the activator of a cGMP-specific phosphodiesterase (PDE). PDE* in turn rapidly hydrolyzes cGMP, thereby reducing the cytoplasmic level of cGMP and causing the CNG channels to close. CNG channel closure terminates the influx of cations, whereas the Na^+, Ca^{2+}/K^+ exchanger (NCKX) continues to extrude Ca^{2+}, producing hyperpolarization of the photoreceptor cell. The consequence of channel closure and NCKX activity is a rapid drop in internal free Ca^{2+}, leading to activation of guanylate cyclase-activating proteins (GCAPs) to form Ca^{2+}-free GCAPs, which can stimulate a membrane bound guanylate cyclase (GC) to accelerate production of cGMP from GTP (Figure 1). This is an important step in the recovery of photoreceptors to the dark state, which requires the re-opening of CNG channels. Additional key steps for full recovery involve quenching excitatory intermediates of the rod cascade (R*, T*, and PDE*). Deactivation of

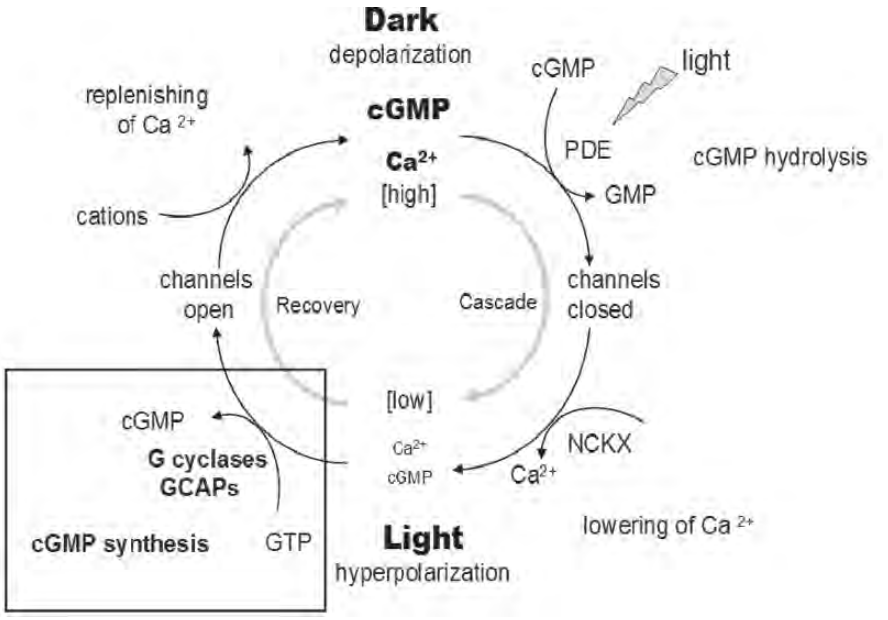

Figure 1. Negative cGMP/Ca^{2+} feedback loop. Light activates PDE which rapidly hydrolizes cGMP. Closure of cGMP-gated channels and continued activity of the $Na^+/Ca^{2+},K^+$ exchanger NCKX lowers intracellular Ca^{2+} leading to activation of GCs by GCAPs and cGMP re-synthesis (boxed area). Consequently, channels open and Ca^{2+} rises again to dark levels in a time course of less than one second (depending on light intensity)

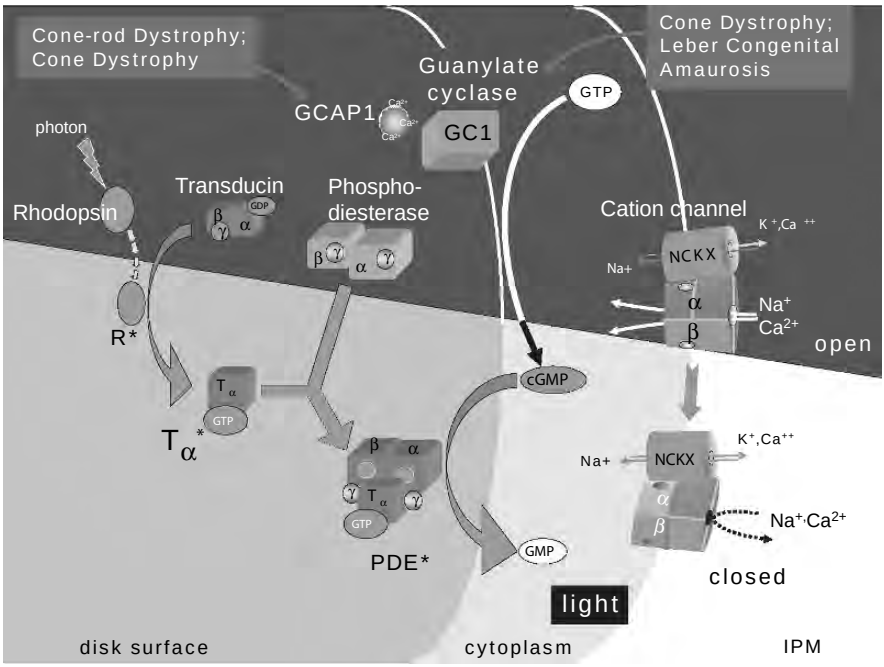

Figure 2. The rod phototransduction cascade and human diseases associated with mutations in GC1 and GCAP1. The main proteins of the cascade are shown in gray. GC1 and GCAP1 represent the main components of the Ca^{2+}-regulated GC in rods and cones responsible for cGMP synthesis. IPM = Interphotoreceptor Matrix (See Colour Plate 7)

PDE*/T*-GTP is initiated by hydrolysis of GTP bound to T, which in turn is accelerated by a GTPase-accelerating protein (GAP) consisting of RGS9/Gß5/R9AP subunits, in conjunction with PDEγ (Krispel et al., 2006; Lyubarsky et al., 2001). Conversion of T*-GTP to T-GDP leads to inactivation of PDE by re-association of catalytic subunits with their γ-subunits. In this review, we explore the link to retinal diseases of GC1 and GC2 (Imanishi et al., 2002; Tucker et al., 1999; Tucker et al., 2004; Yang et al., 1999) and their Ca^{2+} dependent activators GCAP1 and GCAP2 (Palczewski et al., 2004; Newbold et al., 2002; Dizhoor, 2000) (Figure 2).

2. DIVERSITY OF GCs AND GCAPs

In mice and humans, two guanylate cyclases have been identified in the retina (termed GC-E and GC-F in mice, retGC1 and retGC2 in humans) (Lowe et al., 1995). For simplicity, in this review we will refer to these as GC1 and GC2, respectively. *In situ* hybridization of the human GC1 and GC2 shows localization in the photoreceptor cell bodies and inner segments (Imanishi et al., 2002) By immunocytochemistry, GCs are present in murine rods (Yang and Garbers, 1997; Hallett et al., 1996), and human/primate rods and cones (Liu et al., 1994; Imanishi et al., 2002).

There are also reports associating GC1 with the photoreceptor axoneme and microtubules (Fleischman et al., 1980; Hallett et al., 1996). Photoreceptor GC1 was also detected in the pineal gland and olfactory bulb (Duda and Koch, 2002), and in significant amounts in the cochlear nerve and in the organ of Corti (Seebacher et al., 1999).

In mice, only GCAP1 and GCAP2 are expressed, whereas in humans a third GCAP (GCAP3) is also present. *In situ* hybridization studies of GCAP mRNAs in bovine and monkey retinas revealed nearly identical expression patterns in the myoid regions of rod and cone photoreceptors (Otto-Bruc et al., 1997b; Imanishi et al., 2002). Immunocytochemical studies in mice demonstrated that GCAP1 is present in rods and cones, whereas GCAP2 is predominantly in rods and may also be present in cells of the inner retina where its function is unknown (Otto-Bruc

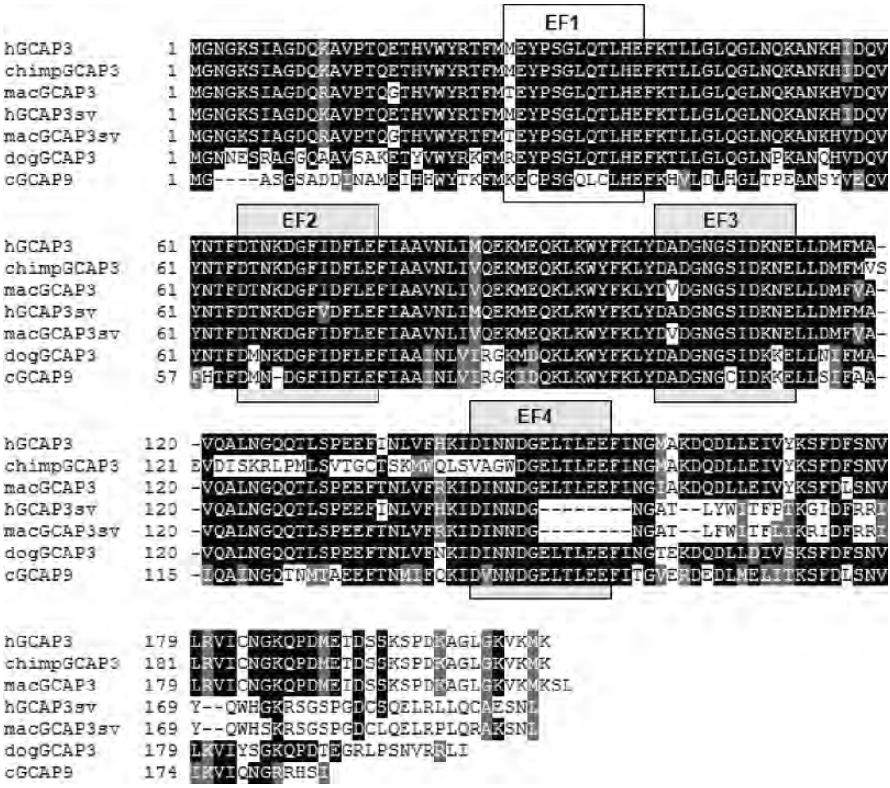

Figure 3. Alignment of cloned and predicted mammalian GCAP3 sequences, in comparison with a predicted chicken GCAP9 sequence identified in chicken genomic library. Prefix h, human; chimp, chimpanzee (Pan troglodytes); mac, macaque (Macaca mulatta); dog (Canis familiaris); c, chicken (Gallus gallus). hGCAP3 (Acc.: O95843); chimpGCAP3 (Acc.: XP_516639); macGCAP3 (Acc.: XP_001102050); dogGCAP3 (Acc.: XP_545090); cGCAP3 (Acc.: XP_425532); hGCAP3sv (splice variant) (Acc.: AAD19945); macGCAP3 (Acc.: XP_001101961)

et al., 1997b; Howes et al., 1998; Cuenca et al., 1998). ESTs encoding human GCAP1 have been cloned from other sources suggesting the presence of GCAP1 at low levels in other tissues (Palczewski et al., 2004). Most likely due to genome duplications, the diversity of GCAPs is more complex in lower vertebrates (teleost and pufferfish) where as many as 8 GCAP genes have been identified (Imanishi et al., 2004). Based on genome searches and EST analyses, the mouse and human genomes do not harbor GCAP4-8 genes. GCAP3 was originally only identified in human and teleosts (Imanishi et al., 2002), and a GCAP3 splice variant of unknown function was identified only in human (Haeseleer et al., 1999). Due to the progress in genome sequencing of many mammalian species, we also identified GCAP3 sequences in dog, macaque and chimpanzee and a GCAP3 splice variant in macaque (Figure 3). A sequence related to mammalian GCAP3, but distinct from GCAP4-8 and GCAP1-2 was identified in the chicken genome. We tentatively termed this sequence chicken GCAP9 (Figure 3).

3. GC STIMULATION BY GCAPs

Photoreceptor GCs have all the structural features characteristic of a membrane GC including a signal sequence, a large N-terminal extracellular domain, a single membrane-spanning region, a kinase-like homology domain, a hinge or dimerization domain, and a C-terminal catalytic domain. As a hallmark of biological function, photoreceptor GCs respond to changes in $[Ca^{2+}]_{free}$ mediated by Ca^{2+}-binding proteins called GCAPs. GCAPs stimulate GC in low $[Ca^{2+}]$ (light), whereas GCAPs are inactive at high $[Ca^{2+}]$ (dark). GCAPs interact with an intracellular domain of GC, because deletion of the extracellular domain has no effect on GCAP stimulation of GC. Deletion of the kinase-like domain diminished the stimulation by GCAP1 (Duda et al., 1996; Laura et al., 1996). Dimerization of GC1 may be an essential step for activation (Yu et al., 1999), possibly by forming homomers (Yang and Garbers, 1997).

In vitro experiments showed that GCAP1 effectively stimulates mostly GC1, whereas GCAP2 and GCAP3 stimulate both GC1 and GC2 (Haeseleer et al., 1999) (Figure 4). A mutation associated with a cone-rod dystrophy in the

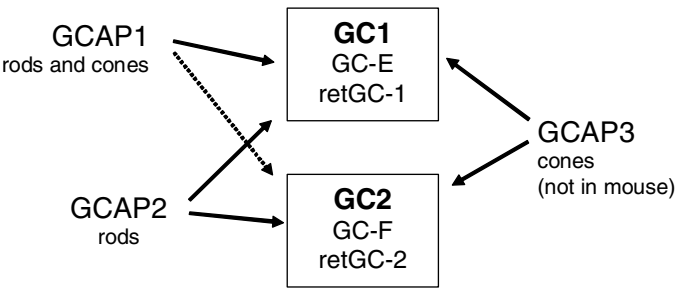

Figure 4. Cellular localization and cross-stimulation of GC1 and GC2 by the three GCAPs

Table 1. Selected pathogenic mutations in GC1 (Additional mutations can be found in (Hanein et al., 2004)). LCA1, Leber congenital amaurosis type 1; ar, autosomal recessive; ad, autosomal dominant; RP, retinitis pigmentosa; CORD, cone-rod dystrophy; IRP, juvenile isolated RP; fs, frameshift; mis, missense; splice, splice site mutation; non, nonsense mutation

Exon	DNA mutation	AA change	type	Disease	Domain	References
2	nt460delC		fs	LCA1	Ext	(Perrault et al., 1996; Rozet et al., 2001)
2	nt693delC		fs	LCA1	Ext	(Perrault et al., 1996; Rozet et al., 2001)
2	52-99dup48bp		ins	LCA1	Ext	(Rozet et al., 2001)
2	226-239del14bp		fs	LCA1	Ext	(Rozet et al., 2001)
2	387delC		fs	LCA1	Ext	(Rozet et al., 2001)
2		M1I	mis	LCA1	Ext	(Rozet et al., 2001; Perrault et al., 2000)
2		W21 R	mis	LCA1	Ext	(Rozet et al., 2001)
2		L41 F	mis	LCA1	Ext	(Rozet et al., 2001; Perrault et al., 2000)
2		C105Y	mis	LCA1	Ext	(Tucker et al., 2004)
2		H109P	mis	LCA1	Ext	(Rozet et al., 2001)
2		N129K	mis	LCA1	Ext	(Perrault et al., 2000)
3		R313C	mis	LCA1	Ext	(Perrault et al., 2000)
		L325P	mis	LCA1	Ext	(Tucker et al., 2004)
7		R540C	mis	LCA1	Kin	(Rozet et al., 2001)
8	T1767C	F589S	mis	LCA1	Kin	(Perrault et al., 1996)
ivs8	IVS9-1G/T		splice	LCA1	Kin	(Perrault et al., 2000)
ivs8	IVS9-1 G/A		splice	LCA1	Kin	(Perrault et al., 2000)
ivs8	IVS0+2T/A		splice	LCA1	Kin	(Perrault et al., 2000)
9	1805-1829del25bp		fs	LCA1	Kin	(Rozet et al., 2001)
10		P701S		arRP		(Booij et al., 2005)
12		P768W		LCA		
13		E837D	mis	adCORD	dim	(Kelsell et al., 1998; Payne et al., 2001; Perrault et al., 1998)
13		R838C(H,A,S)	mis	adCORD	dim	(Kelsell et al., 1998; Payne et al., 2001; Perrault et al., 1998; Van Ghelue et al., 2000)
13		T839M	mis	adCORD	dim	(Payne et al., 2001; Downes et al., 2001b; Perrault et al., 1998)
		R838H, 838C		adCORD	dim	(Ito et al., 2004b)
	C2636T	Q855ter	non	LCA1	dim	(El-Shanti et al., 1999)
		P858S		LCA1	dim	(Tucker et al., 2004)
	T2817C,G2822C	I915T, G917R	mis	adCORD	dim	(Ito et al., 2004a)
15		L954P		LCA1		(Tucker et al., 2004)
15		R976L	mis	LCA1	cat	(Rozet et al., 2001; Perrault et al., 2000)
15	2943deIGc. 3043+4A>T		fs	arRP	cat	(Perrault et al., 2005)
16		R995W	mis	LCA1	cat	(Rozet et al., 2001)

17	3078-3079delGA		fs	LCA	cat	(Rozet et al., 2001)
17		H1019P	mis	LCA	cat	(Rozet et al., 2001)
18		P1069R	mis	IRP	cat	(Booij et al., 2005)
18	3236insACCA			arRP	cat	(Perrault et al., 2005)

human GC1 gene (R838C, Table 1) was shown to dramatically reduce stimulation by GCAP2 whereas increasing the affinity for GCAP1 and altering the Ca^{2+}-sensitivity (Tucker et al., 1999). These and other results suggest that GCAP1 and GCAP2 may have distinct but overlapping contact sites on GC1. Several extensive reviews summarize the regulation of GCs by GCAPs *in vivo* and *in vitro* (Koch et al., 2002; Pugh, Jr. et al., 1997; Hurley and Dizhoor, 2000; Palczewski et al., 2000; Palczewski et al., 2004). Based on these studies, two mechanisms emerged describing the stimulation of GC in the recovery phase of phototransduction. One model assumes changes within the flexible central helix of GCAP1 upon Ca^{2+} dissociation, causing relative reorientation of two structural domains containing a pair of EF-hand motifs and thus switching its targets, GCs, from an inactive (or basal activity) to an active conformation. The second model proposes that dimerization of GCAPs is part of the mechanism by which this protein regulates GCs (Olshevskaya et al., 1999b).

An important factor in GC regulation is ATP (Duda et al., 1993), a nucleotide that causes an increase in the V_{max} of GC (Otto-Bruc et al., 1997a; Gorczyca et al., 1994; Yamazaki et al., 2006; Yamazaki et al., 2005; Sitaramayya et al., 1995). We found that ATP doubled the affinity of retGC1 for GCAP1 in reconstituted systems composed of purified GCAP1 and washed ROS or recombinant photoreceptor GC1 (Otto-Bruc et al., 1997a). Dizhoor et al. found that ATP potentiates stimulation of GC by GCAP2 and that neither the extracellular nor the transmembrane domains of GC1 participate in its regulation by ATP (Laura et al., 1996). These data suggest strongly that ATP is a key component of GC1 regulation by GCAPs. ATP influences the rate of cGMP production by increasing the rate of catalysis and affinity for GCAP1.

4. PHOTORECEPTOR GC GENES, KNOCKOUTS AND RETINA DISEASE

Null mutations in the GC1 gene (*GUCY2D, chromosome 17q*) have been linked to Leber congenital amaurosis type 1 (LCA1) (Perrault et al., 1996), and missense mutations have been linked to dominant cone-rod dystrophy (Kelsell et al., 1998) (Figure 5; Table 1). The GC2 (*GUCY2F*) gene on Xq22 (Yang et al., 1996) has not been linked to a disease phenotype. In a naturally occurring animal model that is null for GC1 (*rd* chicken), both rods and cones degenerate rapidly starting one week post-hatch (Semple-Rowland et al., 1998). Thus, expression of a functional GC1

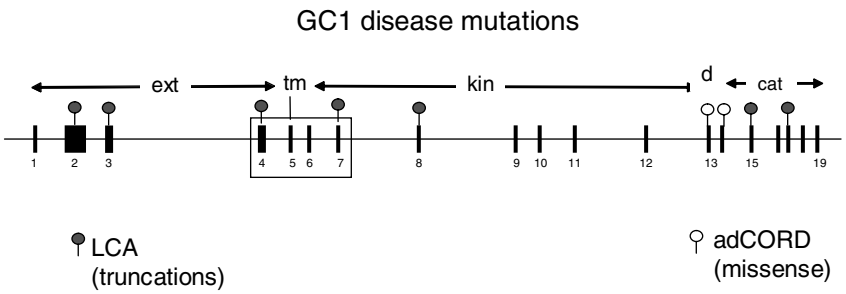

Figure 5. Cartoon of the human GC1 gene and GC1 disease mutations. In general, null mutations are associated with recessive LCA, whereas missense mutations in the dimerization domain are associated with dominant cone dystrophy

gene in chicken retina is essential for survival of rods and cones. In GC1 knock-out mouse models, rod photoreceptor function is preserved but cones are non-functional and degenerate (Yang et al., 1999; Coleman et al., 2004). In mice, therefore, GC1 is essential only for cones, whereas GC2 (or yet another unidentified GC) can substitute, in part, for the loss of GC1 in rods (Yang et al., 1999). Mutations in GC1 that are associated with CORD6 are restricted to the dimerization domain. Important missense mutations of the dimerization domain in exon 13 include E837D, R838C, and T839M (Table 1). It is speculated that these mutations lead to the production of a mutant cyclase that interferes with normal protein dimerization or with Ca^{2+} dependent regulation of GC by GCAPs.

5. GCAP KNOCKOUT AND TRANSGENIC MICE: ROLES OF GCAP1 AND GCAP2

GCAP1 and GCAP2 genes are organized in a tail-to-tail array on mouse chromosome 17 (Figure 6) facilitating a double knockout (dko) with one construct. The phenotype of GCAPdko in rods and cones consisted of a delayed return to the dark-adapted state, consistent with a defect in GC stimulation (Mendez et al., 2001; Pennesi et al., 2003). Absence of GCAPs in photoreceptors, however, did not cause retinal degeneration indicating that GCAP genes are not essential for the development or the survival of photoreceptors.

We provided evidence that transgenic GCAP1 can rescue the $GCAP^{-/-}$ phenotype and that GCAP1 supports the generation of normal flash responses in rods in the absence of GCAP2 (Howes et al., 2002). In contrast, when GCAP2 was expressed on the $GCAP^{-/-}$ background, normal rod flash responses were not restored (Mendez et al., 2001). We also analyzed these mice for restoration of the cone response (Pennesi et al., 2003). These studies revealed that, similar to rods, deletion of GCAP1 and GCAP2 delays the recovery of light responses in cones and that GCAP1 restores the recovery of cone responses in the absence of GCAP2.

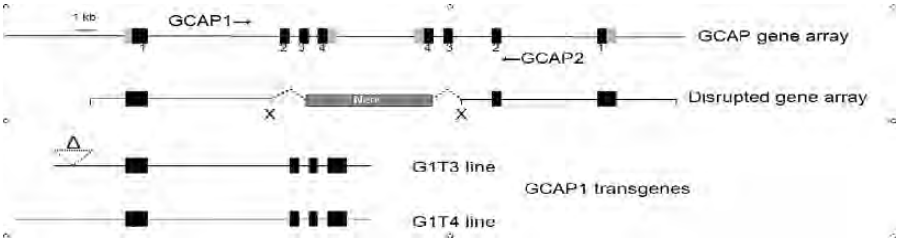

Figure 6. GCAP1 and GCAP2 genes are arranged in a tail-to-tail array on mouse chromosome 17 (top). This arrangement facilitates to knockout both genes simultaneously (second from top). Two transgenic lines were generated (G1T3 and G1T4) to express transgenic GCAPs on a GCAPs knockout background

6. GCAP MUTATIONS AND RETINA DISEASE

The GCAP1 gene has been linked to autosomal dominant cone or cone-rod dystrophies (adCD, adCORD) in several unrelated families (Figure 7), and the GCAP2 gene was recently linked to autosomal dominant RP in a single small family (Sato et al., 2005). The Y99C mutation is located adjacent to the EF3-hand motif (Payne et al., 1998) and three mutations (I143NT, E155G, L151F) have been described in or adjacent to the EF4-hand motif (Wilkie et al., 2001; Nishiguchi et al., 2004; Sokal et al., 2004; Jiang et al., 2005). A fifth mutation (P50L) is located between

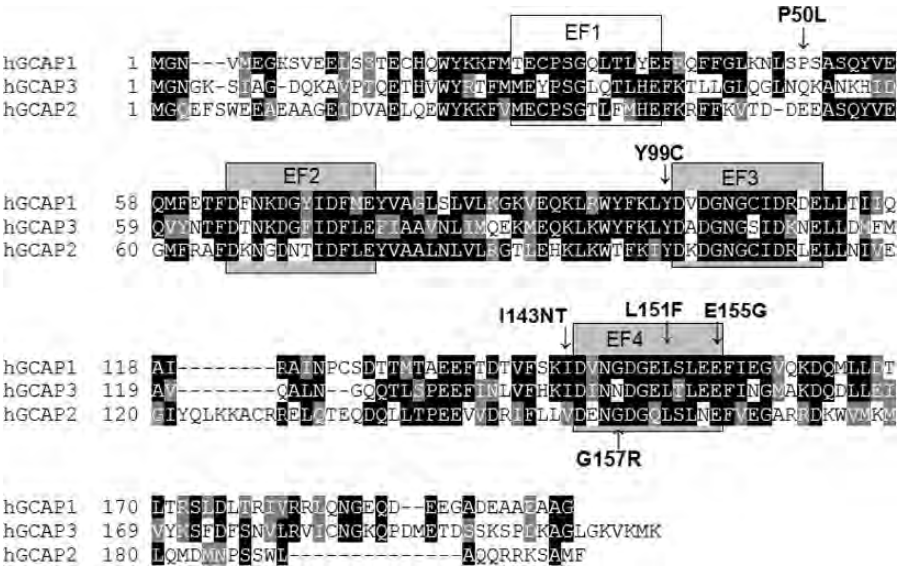

Figure 7. Sequence alignment and positions of mutations associated with disease. Functional EF-hand motifs are shaded. Positions of mutations are indicated by vertical arrows. Conserved residues are printed white on black or gray background. In GCAP1, Y99C is located adjacent to EF3 and I143NT, E155G, L151F are located in EF4. For GCAP2, G157R is located in EF4

EF1 and EF2, and does not affect Ca^{2+} binding (Newbold et al., 2001). A single identified mutation in GCAP2 is located within the EF4-hand motif (Sato et al., 2005) (Figure 7).

6.1. The GCAP1(Y99C) Mutation

Patients with autosomal dominant cone dystrophy have reduced visual acuity and experience a complete loss of color vision. In a four-generation British family (27 members, 7 affected), dominant cone dystrophy was mapped to chromosome 6p21.1, the locus of the GCAP1/GCAP2 gene array (Payne et al., 1998). A missense mutation (A→G) at codon 99 in exon 2 of the gene encoding GCAP1 (*GUCA1A*) was identified. The mutation replaced Tyr at position 99 with Cys (Y99C), a change that was absent in over 200 unrelated controls. The same mutation was later identified independently in two ancestrally related families (Downes et al., 2001a). The Y99C mutation in GCAP1 has been reported to cause both cone-rod dystrophy and isolated macular dysfunction (Michaelides et al., 2005). The authors suggested that the phenotypic variation indicated an intrafamilial heterogeneity of retinal dysfunction that can be observed in persons harboring the same mutation. The residue Tyr99 immediately precedes the EF3-hand motif, one of three functional EF-hand high affinity Ca^{2+}-binding sites in GCAPs. Residues flanking the hydrophilic Ca^{2+}-binding loop are invariantly hydrophobic (Falke et al., 1994). Substitution by a smaller polar Cys leads to changes in the orientation of the N-terminal a-helix and a decrease in affinity for Ca^{2+}. When Tyr99 was replaced by Trp, a hydrophobic residue, biological activity of mutant GCAP1 was unchanged (Sokal et al., 1999). Analysis of the EF3-hand motif Ca^{2+} binding kinetics with the Y99W mutant (W3Cys-), exploiting the intrinsic Trp fluorescence of Trp99, showed a significant increase in the Trp fluorescence intensity of W3-GCAP1(w^-) in the presence of high Ca^{2+}, reflecting a conformational change (Sokal et al., 1999). The change in fluorescence provided a convenient tool to measure the kinetics of Ca^{2+} binding using a stopped flow setup. The results showed that GCAP1 rapidly binds Ca^{2+} ($k_1 > 2\times10^8 M^{-1}s^{-1}$) and rapidly loses its bound Ca^{2+}($k_{-1} = 72s^{-1}$ at 37°C) at the EF3-hand motif, thereby displaying thermodynamic and kinetic properties that are compatible with its involvement early in the phototransduction response. Thus, the EF3-hand motif is a key region for conversion of GCAP1 from activator to inhibitor consistent with mutations in this region being causative of cone dystrophy.

6.2. The GCAP1(P50L) Mutation

The GCAP1(P50L) mutation was also suggested to be associated with autosomal cone dystrophy (Downes et al., 2001a; Newbold et al., 2001). Amino acid residue Pro50 is located in a variable region between the EF1-hand and EF2-hand motifs (Figure 7), and not conserved in other GCAPs. We found that biochemical properties of recombinant GCAP1(P50L) were largely identical to WT GCAP1, activating

photoreceptor GC at [Ca^{2+}] < 100 nM and inhibiting it at micromolar concentrations (Sokal et al., 2000). We also found a reduced capacity to bind free Ca^{2+} which can lead to changes in cone photoreceptor Ca^{2+}homeostasis (Sokal et al., 2000). Newbold *et al*. demonstrated that the P50L mutant was less stable and more susceptible to proteolysis (Newbold et al., 2001). The biochemical mechanism leading to dominant cone dystrophy with this mutation in GCAP1 is unclear.

6.3. The GCAP1(E155G) Mutation

An A464G transition in the *GUCA1A* gene, not seen in 200 normal controls, was identified in a large dominant cone dystrophy pedigree of 67 individuals, of whom 33 were found to be affected (Wilkie et al., 2001). The age at onset of decreased visual acuity and color vision defects was 8–24 years. This transition changes amino acid Glu155 in the EF4-hand motif to Gly, and is predicted to affect Ca^{2+} coordination at the EF4-hand motif. Ca^{2+} binding at the EF4-hand motif does not affect structural changes of GCAP1 to the same extent as it does at the EF3-hand motif, as shown measuring intrinsic fluorescence as a function of Ca^{2+} using a Trp at position 142 (Sokal et al., 1999). However, it is expected to exert similar dominant effects on GC1 stimulation as does GCAP1(Y99C). The residue Glu155 of GCAP1 is invariant in all GCAPs (Palczewski et al., 2004); an invariant Glu at position 12 of the EF-hand loop, contributing both of its side-chain oxygen atoms to the metal-ion coordination, has been shown to be essential for Ca^{2+} coordination (Nakayama et al., 1992; Falke et al., 1994).

6.4. The I143NT Mutation

A novel GCAP1 mutation, I143NT (substitution of Ile at codon 143 by Asn and Thr) affecting the EF4-hand Ca^{2+}-binding loop was identified in a heterozygote father and son with autosomal dominant cone degeneration. Both patients had much greater loss of cone function versus rod function; previous histopathologic evaluation of the father's eyes at autopsy (age 75 years) showed no foveal cones and only a few, scattered cones remaining in the peripheral retina. Biochemical analysis showed that the GCAP1-I143NT mutant adopted a conformation susceptible to proteolysis, and the recombinant mutant protein inhibited GC only partially at high Ca^{2+} concentrations. Properties of the GCAP1-I143NT mutant protein suggest that it is incompletely inactivated by high Ca^{2+} concentrations as should occur with dark adaptation.

6.5. The GCAP1(L151F) Mutation

GCAP1(L151F) mutations affecting the EF4-hand motif linked to dominant cone dystrophy and cone-rod dystrophy were identified in two unrelated families. In one family (Sokal et al., 2004), affected family members experienced dyschromatopsia, hemeralopia, and reduced visual acuity by the second to third decade

of life. Electrophysiology revealed a non-recordable photopic response with later attenuation of the scotopic response. GCAP1-L151F stimulation of photoreceptor GC was not completely inhibited at high physiological [Ca^{2+}], consistent with a lowered affinity for Ca^{2+} binding to the EF4-hand motif. These results showed that a conservative L151F mutation in the EF4-hand motif of GCAP1 is associated with adCORD. Although there is a conservative substitution, molecular dynamics suggests a significant change in Ca^{2+} binding to the EF4-hand and EF2-hand motifs and changes of the shape of L151F-GCAP1.

In a second family (Jiang et al., 2005) of 24 individuals at risk for disease in a five generation family, 11 members were affected. Clinical presentations included photophobia, color vision defects, central acuity loss, and legal blindness with advanced age. The disease phenotype was observed in the second and third decades of life and segregated in an autosomal dominant fashion. An electroretinographic analysis was consistent with cone dystrophy. Mutational analysis and direct sequencing revealed a C451T transition in GUCA1A, corresponding to a L151F mutation in GCAP1.

6.6. The GCAP2(G157R) Mutation

All GCAP1 mutations identified to date and linked to dystrophy are in high-affinity Ca^{2+}-binding sites (exception P50L), affecting association/dissociation of Ca^{2+}. Recently, a G157R mutation in the EF4-hand motif of GCAP2 (Figure 7) was reported to be associated with autosomal dominant RP in 3 families of Japanese origin (Sato et al., 2005). Gly157 is a key conserved residue in the EF4-hand motif, and its replacement by R is expected to influence Ca^{2+} binding, similar to that observed for the EF3/4-hand motif mutations in GCAP1. The mutation is a strong candidate for causing dominant RP for the following reasons. First, in GCAP2, the EF4-hand motif is more important for Ca^{2+}-dependent stimulation of GC than the EF3-hand motif that contributes to Ca^{2+} sensitivity of GC only weakly (Olshevskaya et al., 1999a). For example, a GCAP2(Y104C) mutation, corresponding to GCAP1(Y99C) linked to disease, has no effect on Ca^{2+} sensitivity (Dizhoor et al., 1998). Second, the mutation is predicted to affect severely the affinity of Ca^{2+} to the EF4-hand motif. Third, GCAP2 is expressed predominantly in rods, thus mutations will affect rods consistent with an RP-like phenotype. Fourth, the G157R mutation segregated with RP in Japanese patients and was not present in over 100 control subjects.

7. THE BIOCHEMICAL PHENOTYPE OF CONE DYSTROPHIES ASSOCIATED WITH EF-HAND MOTIF MUTATIONS

Cone-specific degeneration is consistent with high expression levels of GCAP1 in cone outer segments. All mutations alter the Ca^{2+} sensitivity of GCAP1, leading to constitutive stimulation of GC1 at high $[Ca^{2+}]_i$, limiting its ability to fully inactivate GC1 under physiological dark conditions (Figure 8). Persistent stimulation of GC

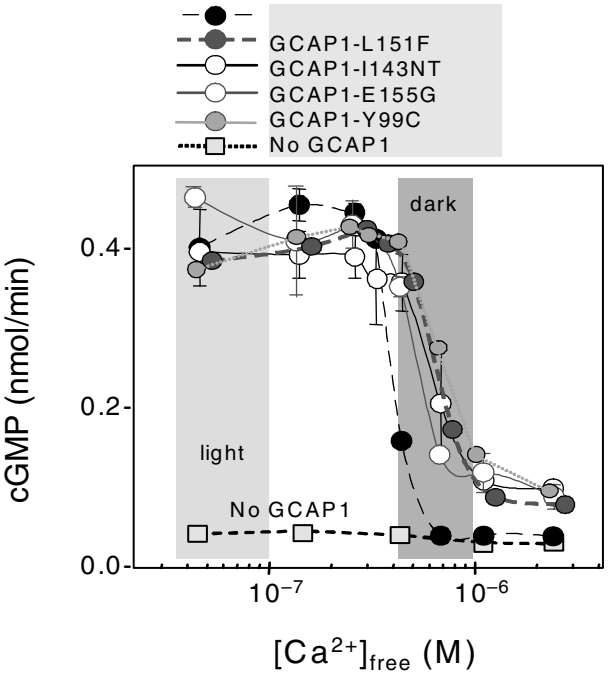

Figure 8. Stimulation of GC activity in ROS membranes by normal and mutant GCAP1s as a function of Ca^{2+}. The dark shaded area indicates low $[Ca^{2+}]_{free}$ expected in the dark-adapted photoreceptors, the lighter shaded area reflects high $[Ca^{2+}]_{free}$ expected in the light. Mutant GCAP1s are active at Ca^{2+} concentrations which inactivate normal GCAP1 (See Colour Plate 8)

by the mutant proteins is predicted to lead to elevated levels of cGMP in the dark-adapted retina, which in turn causes a higher percentage of cGMP-gated channels in the plasma membrane to be opened. The altered physiological cGMP levels may be subtle and thus cause relatively slow retinal degeneration. The reason for the mostly cone-specific degeneration in response to this physiological defect is unknown. GCAP1 may be more active in cones than rods, or, alternatively, it may reflect other differences in cGMP metabolism between rods and cones. Animal models with homologous dominant GCAP1 mutations will be helpful to address this uncertainty. A mouse line expressing GCAP1(Y99C) was generated and shown to shift the Ca^{2+} sensitivity of GCs in photoreceptors, keeping it partially active at 250 nM free Ca^{2+}, the normal resting Ca^{2+} concentration in darkness (Olshevskaya et al., 2004).

8. GCAP MUTATIONS AND EFFECTS ON STRUCTURE

GCAPs belong to the EF-hand motif-containing superfamily of Ca^{2+}–binding proteins from the calmodulin superfamily (Lewit-Bentley and Rety, 2000; Bhattacharya et al., 2004). The structures of several members of the "recoverin branch" of the EF-hand

motif-containing superfamily have been solved. These include the NMR structure of GCAP2 (Ames et al., 1999), crystal structure of GCAP3 (Stephen et al., 2006), and the crystal structures of recoverin (Flaherty et al., 1993), neurocalcin (Vijay-Kumar and Kumar, 1999), frequenin (Bourne et al., 2001), calcineurin (Griffith et al., 1995) and the Ca^{2+}–and–Integrin–Binding protein (CIB) (Padmanabhan et al., 2004). These proteins display remarkable structural similarities despite a relatively modest 40–60% sequence similarity. The crystal structure of GCAP3 was determined recently (Stephen et al., 2006) and shows three EF-hand Ca^{2+} binding motifs whereas, as expected for a GCAP family member, Ca^{2+} binding to the EF1-hand motif is disabled as observed in the NMR structure of GCAP2 (Ames et al., 1999). The loop connecting the two helices in the EF1-hand motif is one amino acid shorter than the canonical EF loop and this domain is missing three of the Ca^{2+} coordinating side chains (Figure 9).

GCAPs arrange their four EF-hand motifs into two domains encompassing the EF1-hand and EF2-hand motifs from the N-terminal domain connected by a linker to a C-terminal domain composed of the EF3-hand and EF4-hand motifs (Figure 9). This arrangement resembles the two lobe structure of calmodulin (Lewit-Bentley and Rety, 2000; Bhattacharya et al., 2004). The structures of the C-terminal domains of calmodulin and GCAP3 are superimposable (Stephen et al., 2006). However, the relative arrangement of the two domains is quite different because in GCAPs the central helix is kinked whereas it is more extended in calmodulin. Thus, calmodulin adopts a dumbbell shape; GCAPs have a compact structure with all

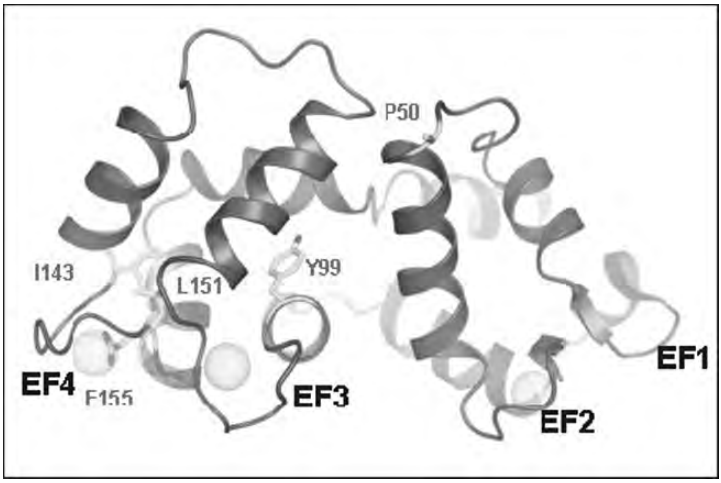

Figure 9. The structure of GCAP3 (Stephen et al., 2006). Residues that correspond to pathogenic mutations in GCAP1, the structure of which is unknown, are shown in yellow with the side chains. Ca^{2+} is shown as yellow spheres. Molecular graphics representations were created with PYMOL (Warren L. DeLano "The PyMOL Molecular Graphics System." DeLano Scientific LLC, San Carlos, CA, USA. http://www.pymol.org) (See Colour Plate 9)

four EF-hand motifs in a tandem array. This compact arrangement is typical of recoverin and other Ca^{2+}–binding proteins in the "recoverin branch" of the EF-hand motif-containing superfamily.

The interface between GCs and GCAPs has been investigated extensively by biophysical methods (Hurley and Dizhoor, 2000; Krylov et al., 1999; Laura and Hurley, 1998; Sokal et al., 2002; Krylov and Hurley, 2001). However, no direct structural analysis of the complex has been obtained yet. Most of the pathogenic mutations occur at the EF3-hand and EF4-hand motifs, dis-regulating Ca^{2+} inactivation (Figure 9). This is in excellent agreement with biochemical data which show that these two EF-hand motifs are critical for conformational changes that inactivate GCAPs (Rudnicka-Nawrot et al., 1998; Sokal et al., 1999). Whether these residues form the interface in the complex with GC is unclear, although evolutionary trace analysis suggests this as a possibility (Imanishi et al., 2004).

9. OUTLOOK

Further research will be necessary to provide more details about the regulation of GCs by GCAPs, specifically on the structural level. Of particular interest will be finding a cure for retinal dystrophies associated with mutations in the GC1 and GCAP genes. GC mutations causing the most devastating blindness, LCA, may be treated by gene transfer if implemented before onset of retina degeneration. Viral gene transfer therapies of retinal diseases by an adeno-associated virus (AAV) vector have been very successful in several recessive animal models (Acland et al., 2001; Bennett et al., 1999; Bennett et al., 1998; Batten et al., 2005; LaVail et al., 2000; Haire et al., 2006; Williams et al., 2006). Cone and cone-rod dystrophies linked to GCAP1 mutations may be treatable by small molecules that inactivate GCAP1. The search for such molecules is underway.

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CHAPTER 5

PATHOLOGIES INVOLVING THE S100 PROTEINS AND RAGE

C.W. HEIZMANN*, G.E. ACKERMANN AND A. GALICHET

Division of Clinical Chemistry and Biochemistry, Department of Pediatrics, University of Zurich, Switzerland

Abstract: The S100 proteins are exclusively expressed in vertebrates and are the largest subgroup within the superfamily of EF-hand Ca^{2+}-binding proteins

Generally, S100 proteins are organized as tight homodimers (some as heterodimers).

Each subunit is composed of a C-terminal, 'canonical' EF-hand, common to all EF-hand proteins, and a N-terminal, 'pseudo' EF-hand, characteristic of S100 proteins. Upon Ca^{2+}-binding, the C-terminal EF-hand undergoes a large conformational change resulting in the exposure of a hydrophobic surface responsible for target binding

A unique feature of this protein family is that some members are secreted from cells upon stimulation, exerting cytokine- and chemokine-like extracellular activities via the Receptor for Advanced Glycation Endproducts, RAGE. Recently, larger assemblies of some S100 proteins (hexamers, tetramers, octamers) have been also observed and are suggested to be the active extracellular species required for receptor binding and activation through receptor multimerization

Most S100 genes are located in a gene cluster on human chromosome 1q21, a region frequently rearranged in human cancer

The functional diversification of S100 proteins is achieved by their specific cell- and tissue-expression patterns, structural variations, different metal ion binding properties (Ca^{2+}, Zn^{2+} and Cu^{2+}) as well as their ability to form homo-, hetero- and oligomeric assemblies

Here, we review the most recent developments focussing on the biological functions of the S100 proteins and we discuss the presently available S100-specific mouse models and their possible use as human disease models

In addition, the S100-RAGE interaction and the activation of various cellular pathways will be discussed. Finally, the close association of S100 proteins with cardiomyopathy, cancer, inflammation and brain diseases is summarized as well as their use in diagnosis and their potential as drug targets to improve therapies in the future

Keywords: Calcium, EF-hand, S100 proteins, human diseases, RAGE

*Claus W. Heizmann, Division of Clinical Chemistry and Biochemistry, Department of Pediatrics, University of Zurich, Steinwiesstrasse 75, 8032 Zurich, Switzerland, E-mail: Claus.Heizmann@kispi.unizh.ch

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1. INTRODUCTION

S100 proteins (named because of their solubility in 100% ammonium sulfate solution) constitute the largest family within the EF-hand Ca^{2+}-binding proteins superfamily and display unique properties (Marenholz et al., 2004a). S100 proteins are small, acidic proteins containing a classical Ca^{2+}-binding EF-hand at the C-terminus and a S100-specific EF-hand at the N-terminus (Fritz and Heizmann, 2004; Bhattacharya et al., 2004; Vallely et al., 2002; Ikura and Ames, 2006; Zimmer et al., 2005). Most S100 proteins show a cell- and tissue-specific expression pattern indicating specialised biological functions. S100 proteins form homo- and heterodimers and even oligomers, which also contribute to their functional diversity. S100 proteins act intracellularly as Ca^{2+}-signalling molecules, but some members are secreted from the cells and act in a cytokine-like manner through their binding to the receptor for advanced glycation end products (RAGE) or other receptors (Ramasamy et al., 2005).

In addition to their affinity for Ca^{2+}, some S100 proteins display high affinity towards Zn^{2+} or Cu^{2+} ions, which could influence their biochemical activities. S100 proteins are involved in a variety of cellular processes such as cell cycle regulation, cell growth, cell differentiation or motility. Interestingly of more than 20 human genes, 16 are tightly clustered in a region of the human chromosome 1q21. The S100 gene cluster also exists in mouse and is structurally conserved during evolution (Ridinger et al., 1998). Generally, interspecies homology between the known mouse and human S100 cDNAs ranges from 79.6 to 95%. Based on these findings, an official nomenclature was introduced (Table 1) (Marenholz et al., 2004a; Marenholz et al., 2006). The S100 genes structure is highly conserved and in most cases contains three exons and two introns, the first exon being non-coding.

A related gene family is present in the same gene cluster and encodes for proteins containing an S100-like domain fused to a larger peptide. These proteins include trichohyalin, filaggrin, and repetin, which are multidomain proteins involved in epidermal differentiation (Marenholz et al., 2001; Huber et al., 2005) and are classified as a separate family.

Recently, S100 proteins have received increasing attention due to their close association with several human diseases including cancer, neurodegenerative disorders, and cardiomyopathies. Therefore they have become increasingly valuable for diagnostic purposes (Table 2) and are also useful as markers to improve clinical management and are considered as having a potential as drug targets to improve therapies (Heizmann, 2005).

2. NOMENCLATURE

The plethora of names given to the first members of the S100 family resulted in considerable confusion in this field of research and prompted Schaefer et al. (Schafer et al., 1995) to introduce an official nomenclature of the S100 family,

Table 1. Nomenclature and chromosomal location of the S100 genes

Approved gene symbol	Approved gene name	Previous symbols and aliases	Chromosomal location	Sequence Accession ID
S100A1	S100 calcium binding protein A1	S100A, S100-alpha	1q21	NM_006271
S100A2	S100 calcium binding protein A2	S100L, CaN19	1q21	NM_005978
S100A3	S100 calcium binding protein A3	S100E	1q21	NM_002960
S100A4	S100 calcium binding protein A4	Calvasculin, metastasin, murine placental homolog, calcium placental protein (CAPL), MTSI, p9Ka, 18A2, pEL98, 42A	1q21	NM_002961
S100A5	S100 calcium binding protein A5	S100D	1q21	NM_002962
S100A6	S100 calcium binding protein A6	Calcyclin (CACY), 2A9, PRA, CABP	1q21	NM_014624
S100A7	S100 calcium binding protein A7	Psoriasin 1 (PSOR1), S100A7c	1q21	NM_002963
S100A7A	S100 calcium binding protein A7A	S100A15, S100A7L1	1q21	NM_176823
S100A7L2	S100 calcium binding protein A7-like 2	S100A7b	1q21	–
S100A7P1	S100 calcium binding protein A7 pseudogene 1	S100A7L3, S100A7d	1q21	–
S100A7P2	S100 calcium binding protein A7 pseudogene 2	S100A7L4, S100A7e	1q21	–
S100A8	S100 calcium binding protein A8	Calgranulin A (CAGA), CGLA, P8, MRP8, CFAG, LIAg, 60B8AG	1q21	NM_002964

(*Continued*)

Table 1. (Continued)

Approved gene symbol	Approved gene name	Previous symbols and aliases	Chromosomal location	Sequence Accession ID
S100A9	S100 calcium binding protein A9	Calgranulin B (CAGB), CGLB, P14, MRP14, CFAG, LIAg, 60B8AG	1q21	NM_002965
S100A10	S100 calcium binding protein A10	Annexin II ligand (ANX2LG), calpactin I, light polypeptide (CAL1L), p11, CLP11, 42C	1q21	NM_002966
S100A11	S100 calcium binding protein A11	Calgizzarin, S100C	1q21	NM_005620
S100A11P	S100 calcium binding protein A11 pseudogene	S100A14	7q22-q31	–
S100A12	S100 calcium binding protein A12	Calgranulin C (CAGC), CAAF1, CGRP, p6, ENRAGE	1q21	NM_005621
S100A13	S100 calcium binding protein A13		1q21	NM_005979
S100A14	S100 calcium binding protein A14	BCMP84, S100A15	1q21	NM_020672
S100A16	S100 calcium binding protein A16	S100F, DTIP1A7, MGC17528	1q21	NM_080388
S100B	S100 calcium binding protein B	S100-beta	21q22	NM_006272
S100G	S100 calcium binding protein G	Calbindin 3 (CALB3), CaBP9K, CABPI	Xp22	NM_004057
S100P	S100 calcium binding protein P		4p16	NM_005980
S100Z	S100 calcium binding protein Z	S100-zeta	5q13	NM_130772

Approved by the HUGO Gene Nomenclature Committee and the European Calcium Society.
Reference: 1. Marenholz et al., Biochim. Biophys. Acta – Molecular Cell Research (2006), in press.

Table 2. Involvement of S100 Proteins in Human Diseases

Proteins	Association with Diseases
S100B	Developmental brain dysfunction, learning and memory deficits
	Alzheimer's disease, blood brain barrier dysfunction
	Down syndromebrain trauma and ischemia, schizophrenia, depression
S100A1	Cardiomyopathies
S100A2	Cancer, tumour suppression
S100A4	Cancer, metastasis
S100A6	Cancer, amyotrophic lateral sclerosis (ALS)
S100A7	Psoriasis
S100A8/A9	Inflammation, cystic fibrosis
	Wound healing, juvenile rheumatoid arthritis
S100A10/A11	Cancer
S100A12	Inflammation, Kawasaki disease, Mooren's ulcer
S100P	Cancer

References: Marenholz et al., Biochem. Biophys. Res. Commun. 2004;322:1111–1122; Heizmann et al., Frontiers Bioscience 2002;7:d1356–1368; Foell et al., Arthritis & Rheumatism 2004;50:1286–1295; Donato et al., Microsc. Res. Tech. 2003;60:540–551; Kwon et al., Frontiers Bioscience 2005;10:300–325; Foell et al., Lancet 2003;361:1270–1272; Hetzel et al., Psychopharmacol. 2005;178:161–166.

which was recently updated (Marenholz et al., 2004a; Marenholz et al., 2006). One of these genes (S100A15) had already been published with the approved gene symbol S100A16 (Marenholz and Heizmann, 2004) (see Table 1). Although carrying the S100-specific EF-hand motif at the N-terminus, proteins of the 'fused' gene family (approved gene nomenclature: HRNR, hornerin and TCHHL1, and trichohyalin-like 1) should not be classified as S100 proteins but as a separate family.

Four S100 gene symbols have now been changed according to the functional relevance and phylogenetic relationship of the genes. S100A7L1/S100A15 was renamed S100A7A to indicate its high homology with S100A7. S100A15 has been withdrawn as an official gene symbol and will not be used for any future S100A genes. Because S100A7L3 and S100A7L4 are non-coding genes, their new gene symbols are S100A7P1 and S100A7P2, respectively. Finally, CALB3 (calbindin 3) has been renamed S100G, to make its affiliation to the S100 family more obvious. http://www.gene.ucl.ac.uk/nomenclature/ index.html.

3. PROTEIN STRUCTURES, METAL BINDING, AND INTERACTIONS WITH TARGET PROTEINS

Structural analyses have revealed a typical architecture of the S100 proteins. Each S100 protein is composed of two EF-hand Ca^{2+}-binding domains connected by a central hinge region (Fritz and Heizmann, 2004; Bhattacharya et al., 2004;

Vallely et al., 2002; Ikura and Ames, 2006). The C-terminal EF-hand contains the classical Ca^{2+}-binding motif, common to all EF-hand proteins (see above). This Ca^{2+}-binding motif has a typical sequence signature of 12 amino acids and is flanked by helices H_{III} and H_{IV}. The N-terminal EF-hand is different from the classical EF-hand motif and is characteristic of the S100 proteins. Therefore, this EF-hand, with a 14 amino acid consensus sequence motif, is flanked by helices H_I and H_{II} and is called the 'S100-specific' or 'pseudo' EF-hand.

Upon Ca^{2+}-binding, S100 proteins undergo a conformational change which is mainly due to a large reorientation of helix H_{III} of the classical EF-hand, whereas helix H_{IV} , engaged in the dimer interface, does not move. The N-terminal EF-hand, flanked by helices H_I and $H_{II,}$ exhibits only minor structural changes upon Ca^{2+}-binding. The Ca^{2+}-induced conformational change opens the structure and exposes a wide hydrophobic cleft formed by residues of the hinge region, helix H_{III}, and the C-terminal loop region. This hydrophobic surfacerepresents the interaction site of S100 proteins with their target proteins. However, S100-target protein interaction mechanism also presents some diversity, as it was revealed by the structure of six S100-peptide complexes. In four of these structures, the rat or human S100B was complexed with peptides derived from Ndr-kinase (Heizmann, 2005), p53 (Ramasamy et al., 2005), and CapZ (Huber et al., 2005). Comparison of the structures revealed differences in the orientation of the peptides as well as the type of interaction with S100B. Similarly, the structures of the S100A10/annexin II (Rety et al., 1999) and S100A11/annexin I (Rety et al., 2000) complexes are similar to each other but different from other S100-target protein complexes (Bhattacharya et al., 2004).

Some S100 proteins e.g. S100A9 have long and flexible C-terminal extensions, which might be required for target interaction independently of calcium binding.

In general, the C-terminal end exhibits the highest sequence variation and therefore may contribute to the specificity of the different S100 proteins (Marenholz et al., 2004a; Bhattacharya et al., 2004; Wilder et al., 2006).

Generally, dimeric S100 proteins bind four Ca^{2+} (K_d = 20–500 μM) with strong positive cooperativity. In addition to binding to Ca^{2+}, a number of S100 proteins also bind Zn^{2+} with a wide range of affinities (K_d = 4 nM to 2 mM) (Heizmann and Cox, 1998). Among the S100 proteins, S100A3 displays the highest affinity for Zn^{2+} (K_d = 4 nM) and, interestingly, the lowest affinity for Ca^{2+} (K_d = ~20 mM) (Fritz et al., 1998), implying that S100A3 functions as a Zn^{2+} rather than a Ca^{2+}-signalling protein. Spectroscopic studies and crystal structure of metal-free S100A3 (Fritz et al., 2002) allowed the identification of one preformed Zn^{2+} binding site (distinct from the EF-hand) in the C-terminus of each subunit in which the Zn^{2+}-ion is coordinated by one histidine and three cysteine residues.

Recently, the structure of the Zn^{2+}-bound S100A2 was characterised in more detail (Koch et al, submitted). The binding of Zn^{2+} leads to the formation of a novel tetrameric structure different from other S100 proteins. This finding implies that, under physiological conditions, Zn^{2+} may control the cellular activity of S100A2.

Cu^{2+} can also binds to S100 proteins as it was reported for S100B (K_d = 0.46 μM) (Nishikawa et al., 1997) and S100A5 (K_d = 4 μM) (Schafer et al., 2000).

S100A16 protein is a unique member of the S100 protein family with only one functional Ca^{2+}-binding site located in the C-terminal canonical EF-hand (Sturchler et al., 2006). Surprisingly, the Ca^{2+}-binding affinity of the mouse C-terminal EF-hand is very low, whereas the affinity in the human protein is 2-fold higher. The weak binding of Ca^{2+} to the C-terminus of the mouse S100A16 may be due to the unusual C-tail consisting of additional Q QEC/S repeats. S100A16 also binds Zn^{2+}, and binding studies indicate that Ca^{2+} and Zn^{2+} do not bind to the same sites and that the exposed hydrophobic patches are different.

Multimeric forms of S100 proteins appear to be associated with their extracellular activity. Larger assemblies than dimers were reported for S100A12 (Moroz et al., 2002), S100A4 (Novitskaya et al., 2000; Novitskaya et al., 2000)and S100B (Barger et al., 1992; Ostendorp et al., 2005). It has been proposed that such polymeric forms of S100 proteins trigger aggregation of RAGE, thereby activating intracellular signal cascades.

4. BIOLOGICAL FUNCTIONS AND PATHOLOGIES

S100 proteins are involved in a large number of cellular activities such as signal transduction, cell differentiation, regulation of cell motility, transcription and cell cycle progression (Marenholz et al., 2004a; Heizmann et al., 2002) through modulation of their subcellular localization and their interaction with various target proteins in a Ca^{2+}-(and possibly also in a Zn^{2+}- and Cu^{2+}-) dependent as well as Ca^{2+}-independent manner.

Understanding the biological functions of S100 proteins will crucially depend on the identification of their target proteins. During the last decade, a large number of possible interactors have been described involving enzymes, cytoskeletal elements as well as transcription factors.

Many of the target protein interactions have been characterised on the biochemical level using in vitro assay systems and are summarised in recent reviews (Heizmann et al., 2002; Marenholz et al., 2004a; Deloulme et al., 2003; Zimmer et al., 2003; Zimmer et al., 2005; Donato, 2003; Santamaria-Kisiel et al., 2006). Despite this large amount of biochemical data, very little is known about the physiological functions of S100 proteins. This can be ascribed to the fact that experiments using whole organisms are still scarce.

4.1. S100B

S100B protein represents 0.2% of the total brain proteins and is mainly synthesised by astrocytes, oligodendrocytes, and Schwann cells and to a lesser extent by neurons (Chan et al., 2003; Donato, 2003; Vives et al., 2003; Zimmer et al., 2003; Van Eldik and Wainwright, 2003). The 3-dimensional structure of the S100B dimer has been determined by NMR (Drohat et al., 1998; Drohat et al., 1999; Kilby

et al., 1996). In addition to Ca^{2+}, S100B can also bind Zn^{2+} at a site distinct from the EF-hand Ca^{2+}-binding domains. In several cases, binding of Zn^{2+} can affect Ca^{2+}-binding and target binding affinities (Baudier et al., 1986; Baudier and Gerard, 1986; Wilder et al., 2003). S100B also binds copper, which might have a neuroprotective function (Shiraishi and Nishikimi, 1998; Nishikawa et al., 1997). S100B has been shown to interact in vitro with more than 15 proteins including p53 (Fernandez-Fernandez et al., 2005; Delphin et al., 1999; Wilder et al., 2006), NDR kinase (Bhattacharya et al., 2003), and TRTK-12 (Inman et al., 2002). S100B is involved in various cellular functions such as cell metabolism, cell motility, cell-death, or synaptic plasticity. S100B is also secreted by astrocytes and exerts either a trophic or a toxic effect on neurons depending of its concentration: at nanomolar concentration, S100B stimulates neurite outgrowth and enhances survival of neurons during development and after glucose deprivation damage, whereas a micromolar concentration of S100B induces apoptosis (Huttunen et al., 2000). As with S100A1, RAGE has been suggested to be the multiligand receptor mediating the extracellular effects observed with S100B (Hofmann et al., 1999).

S100B protein is not essential for survival since $S100B^{-/-}$mice present only mild phenotypes (Table 3). They show an enhanced spatial and fear memory associated with strengthened neuronal plasticity (Nishiyama et al., 2002). In addition, enhanced epileptogenesis has been reported (Dyck et al., 2002), probably because of abnormalities in calcium-handling in astrocytes, as evidenced by increased Ca^{2+} transients upon KCl and caffeine treatment in S100B-deficient neonatal cerebellar glia cells (Xiong et al., 2000). Transgenic mice overexpressing S100B exhibit enhanced explorative activity, reduced anxiety and impaired learning and memory capabilities (Bell et al., 2003; Gerlai et al., 1995; Gerlai and Roder, 1996; Winocur et al., 2001). Interestingly, in humans, higher S100B concentration has been detected after brain trauma and ischemia, making S100B a potential diagnostic protein in traumatic brain damage (Rothermundt et al., 2003). An increased concentration of S100B has also been found in cases of neurodegenerative diseases, such as Alzheimer's disease (Griffin et al., 1989), or in patients with Down syndrome and in some psychiatric disorders such as schizophrenia (Table 2).

4.2. S100A1

S100A1 binds Ca^{2+} with moderate affinity (Kd = 20–50 μM), which induces a conformational change for interaction with other proteins (Heizmann and Cox, 1998; Heizmann et al., 2003; Marenholz et al., 2004b; Santamaria-Kisiel et al., 2006; Zimmer et al., 2003). Ca^{2+}-binding strongly activates S-nitrosylation of S100A1 at its single cysteine residue (Cys85) and results in alterations of the 3-dimensional protein structure (Zhukova et al., 2004). In addition, S-glutathionylation of S100A1 at the same site increases Ca^{2+} affinity of the C- and N-terminal EF-hands 10- and 10,000-fold, respectively (Goch et al., 2005). These findings suggest that besides its involvement in Ca^{2+} signalling, S100A1 may play a role in the intracellular redox response.

Table 3. Genetically engineered S100 mouse models

Transgene	Mouse model	Pheontype
S100B	Overexpression	Female specific hyperactivity, lack of habituation to novelty, reduced T-maze spontaneous alternation rate, abnormal exploratory behavior Enhanced astrocytosis and neurite proliferation Impaired learning and memory, increased dendrite density, enhanced age-related loss of dendrites Inhibitory effect on cardiac hypertrophy Increased susceptibility to hypoxia-ischemia Increased apoptosis after myocardial infarction Enhanced neuroinflammation and neuronal dysfunction induced by amyloid-β
	Knock-out	Increased synaptic plasticity, spatial memory and fear memory Chronic gliosis Decreased susceptibility to hypoxia-ischemia Enhanced epileptogenesis Enhanced Ca^{2+} transients Enhanced hypertrophy, decreased apoptosis and improved hemodynamics after myocardial infarction
S100A1	Overexpression	Increased myocardial contractile performance
	Knock-out	Impaired cardiac contractility in response to β-adrenergic stimulation and hemodynamic stress Reduced anxiety-related responses and increased exploratory drive in male KO mice Prolonged Q-T intervals, reduced Ca^{2+}-induced Ca^{2+} release upon β-adrenergic stimulation.
S100A4	Overexpression	Enhanced tumour development and metastasis Increased right ventricular systolic pressure, right ventricular hypertrophy, reduced ventricular elastance and decreased cardiac output.
	Knock-out	Tumour development and reduced apoptosis Delayed tumour uptake, decreased tumour incidences, no metastasis after transplantation of highly metastatic mammary carcinomas. Enhanced astrocyte migration after demyelination
S100A8	Knock-out	Embryonic lethal
S100A9	Knock-out	No functional abnormalities. Absence of S100A8 protein. Myeloid cells with reduced density. No functional abnormalities *in vivo*. Absence of S100A8 protein. Reduced response to chemoattractant stimuli *in vitro*. Stimulation of myeloid cells with ATP results in a stronger increase of intracellular free Ca^{2+}. No emigration of CD4-CD8 double-negative thymocytes upon tetrachlorodibenzo-p-dioxin exposure.

(*Continued*)

Table 3. (*Continued*)

Transgene	Mouse model	Pheontype
		Reduced inflammatory-induced IP_3-mediated Ca^{2+} release *in vitro*.
S100A10	Overexpression	Hyperactive, reduced anxiety-related distress, reduced depression-like behavior, increased serotonin 1B receptors.
	Knock-out	Increased anxiety-related distress, increased depression-like behavior, decreased response to sweet reward, reduced serotonin 1B receptors.
S100A11	Knock-out	No obvious abnormalities

References: Bell et al., Neuropsychopharmacology 2003;28:1810–1816; Gerlai et al., Behav. Brain Res. 1993;55:51–59; Gerlai et al., Behav. Neurosci. 1994;108:100–106; Gerlai et al., Behav. Brain Res. 1993;59:119–124; Gerlai et al., J. Psychiatry Neurosci 1995;20:105–112; Gerlai et al., Neurobiol. Learn Mem. 1996;66:143–154; Gerlai et al., Learn Mem. 1995;2:26–39; Roder et al., 1996a;60:31–36; Roder et al., Physiol. Behav. 1996b;60:611–615; Reeves et al., Proc. Natl. Acad. Sci USA 1994;91:5359–5363; Whitaker-Azmitia et al., Brain Res. 1997;776:51–60; Winocur et al., Neurobiol. Learn Mem. 2001;75:230–243; Parker et al., Can. J. Appl. Physiol. 1998;23:377–389; Wainwright et al., Ann. Neurol. 2004;56:61–67; Tsoporis et al., Circulation 2005;111:598–606; Craft et al., Glia 2005;51:209–216; Nishiyama et al., Proc. Natl. Acad. Sci USA 2002a;99:4037–4042; Nishiyama et al., Neurosci Lett. 2002b;321:49–52; Dyck et al., Brain Res. Mol. Brain Res. 2002;106:22–29; Xiong et al., Exp. Cell Res. 2000;25:281–289; Most et al., J. Biol. Chem. 2003;278:33809–33817; Du et al., Mol. Cell Biol. 2002;22:2821–2829; Ackermann et al., Biochim. Biophys. Acta 2006; in press; Ackermann et al., unpublished results; Davies et al., Oncogene 1996;13:1631–1637; Ambartsumian et al., J. Dairy Res. 2005;72: Spec. No., 27–33; Merklinger et al., Circ. Res. 2005;97:596–604; El Naaman et al., Oncogene 2004;23:3670–3680; Grum-Schwensen et al., Cancer Res. 2005;65:3772–3780; Fang et al., Acta Neuropathol. (Berl.) 2006;111:213–219; Passey et al., J. Immunol. 1999;163:2209–2216; Hobbs et al., Mol. Cell Biol. 2003;23:2564–2576; Manitz et al., Mol. Cell Biol. 2003;23:1034–1043; Nacken et al., Int. J. Biochem. Cell Biol. 2005;37:1241–1253; Temchura et al., Eur. J. Immunol. 2005;35:2738–2747; McNeill et al. Cell Calcium 2006; Svenningsson et al., Science 2006;311:77–80; Mannan et al., Mol. Reprod. Dev. 2003;66: 431–438.

Protein extraction from tissues revealed that S100A1 forms homodimers and heterodimers with S100B (Baudier and Gerard, 1986), mostly by hydrophobic interaction (Deloulme et al., 2003; Isobe et al., 1983). The 3-dimensional solution structures of apo- and Ca^{2+}-bound S100A1, the S100A1 homodimer and S100A1/S100B heterodimer were later determined by NMR spectroscopy (Rustandi et al., 2002; Wright et al., 2005). Using yeast two-hybrid techniques, S100A1 was further found to form heterodimers with S100A4 and S100P (Tarabykina et al., 2000; Wang et al., 2000a; Wang et al., 2004). The physical interaction of S100A1 with S100A4 or S100P was additionally demonstrated in living mammalian cells using fluorescence resonance energy transfer (FRET) (Wang et al., 2005; Wang et al., 2004). Moreover, S100A1 and S100A4 were shown to behave mutually antagonistic towards each other's activities in vivo, i.e. S100A1 reduced the metastasis-inducing capabilities of S100A4, whereas S100A4 attenuated S100A1-triggered host muscle cell proliferation in a rat model of mammary tumour transplantation (Wang et al., 2005).

Using quantitative northern blotting, S100A1 was demonstrated to be most prominently expressed in the heart, and with decreasing levels in the kidney, liver, skin, brain, lung, stomach, testis, muscle, small intestine, thymus and spleen (Zimmer et al., 2005). S100A1 was additionally detected in the submaxillary and thyroid glands (Kiewitz et al., 2000b). S100A1 was reported to be present in the cytoplasm of astrocytes, neurons, skeletal and cardiac myocytes, kidney and mammary cells (Ackermann et al., 2006; Benfenati et al., 2004; Kato and Kimura, 1985; Wang et al., 2000a). In addition, undifferentiated and differentiated skeletal muscle (L6S4) and neuroendocrine (PC12) cells were found to express S100A1 protein, which was predominantly located in the cytoplasm (Zimmer and Landar, 1995). In the rat heart muscle cell line H9c2, S100A1 and the transcription factor MyoD localize both primarily in the nucleus, but are also found in the cytoplasm. Nuclear staining with affinity-purified S100A1 antibodies was also observed in adult skeletal muscle, although the bulk of the protein was cytoplasmic (Haimoto and Kato, 1987).

S100A1 was shown to interact with at least 24 different proteins in a Ca^{2+}-dependent and -independent manner (Santamaria-Kisiel et al., 2006). These include the sarcomeric, myosin-associated giant kinase twitchin (Heierhorst et al., 1996), its vertebrate ortholog titin (Yamasaki et al., 2001), the transcription factor MyoD (Baudier et al., 1995), connexin 43 (Donato, 2001; Zimmer et al., 1995), adenylate cyclase (Fano et al., 1989a), sarcoplasmatic reticulum Ca^{2+}-ATPase 2a (SERCA2a) (Kiewitz et al., 2003), phospholamban (PLB) (Kiewitz et al., 2003), ryanodine receptor 1 (RyR1) (Treves et al., 1997) and ryanodine receptor 2 (RyR2) (Most et al., 2003b), in order to mention a few muscle-specific examples.

S100A1 was also reported to interact with protein domains capable of binding calmodulin (Baudier et al., 1987), which implies the participation of S100A1 in calmodulin-mediated signalling.

S100A1 is the most abundant S100 protein found in striated muscle and predominates in myocardial tissue (Kato and Kimura, 1985). Besides its cytoplasmic occurrence, S100A1 was reported in these cells to associate with the sarcolemma, sarcoplasmatic reticulum (SR), contractile filaments, intercalated discs, outer mitochondrial membrane and other intracellular membrane structures (Arcuri et al., 2002; Donato et al., 1989; Haimoto and Kato, 1988; Sorci et al., 1999). However, the exact location of S100A1 on the contractile elements of the sarcomere is still controversial (Maco et al., 2001; Zimmer, 1991).

S100A1 was found to stimulate Ca^{2+}-induced Ca^{2+} release (CICR) in skeletal muscle terminal cisternae (Fano et al., 1989b; Marsili et al., 1992). In the presence of nanomolar Ca^{2+}-concentrations, S100A1 was observed to increase the channel open probability of purified rabbit skeletal muscle RyR1 (Treves et al., 1997). Biosensor measurements indicated that S100A1 binds RyR1 in the presence of milli- and nanomolar Ca^{2+}-concentrations, and affinity chromatography revealed binding of the RyR1 to S100A1-conjugated Sepharose in the presence of nanomolar Ca^{2+}-concentrations (Treves et al., 1997). In line with these results, S100A1 was shown to enhance SR Ca^{2+} release and contractile performance in murine skinned

skeletal muscle fibers (Most et al., 2003c). Additionally, S100A1 was reported to reduce Ca^{2+} sensitivity and to enhance Ca^{2+} cooperativity in skinned single rabbit muscle fibres (Adhikari and Wang, 2001).

Expression of S100A1 in primary cultures of rabbit cardiomyocytes improves contractility, augments intracellular Ca^{2+} transients, increases SR Ca^{2+} uptake and decreases myofilament Ca^{2+} sensitivity (Most et al., 2001). These results were confirmed in neonatal and adult rat cardiomyocytes overexpressing S100A1, with the additional findings that the SR Ca^{2+} load was increased and contractility as well as Ca^{2+} cycling improved, also after β-adrenergic stimulation (Most et al., 2005; Remppis et al., 2002). In a following study, the introduction of S100A1 at a concentration of 100nM into rabbit cardiomyocytes via a patch clamp pipette similarly resulted in increased Ca^{2+} transient amplitudes and enhanced Ca^{2+} re-uptake via SERCA2a (Kettlewell et al., 2005). However, higher and lower concentrations of S100A1 were ineffective. In addition, L-type Ca^{2+} current amplitudes, Ca^{2+} efflux rates via the Na^{+}/Ca^{2+} exchanger (NCX) and SR Ca^{2+} load were unaffected by S100A1 infusion. The authors conclude that S100A1 enhances fractional SR Ca^{2+} release and uptake, consistent with the additional findings that S100A1 stimulates RyR2 activity and co-immunprecipitates with SERCA2a and RyR2. However, Völkers et al. reported that S100A1 decreases Ca^{2+} spark frequencies (Volkers et al., 2006).

S100A1 is released into the blood during ischemic periods and can serve as a marker for myocardial ischemia together with creatine kinase isoenzymes, myoglobin, troponin I and T (Kiewitz et al., 2000a). Extracellular S100A1 was reported to be endocytosed into the endosomal compartment of neonatal rat cardiomyocytes and to inhibit apoptosis via activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2) (Most et al., 2003a). Endocytosed S100A1 was further demonstrated to result in decreased diastolic Ca^{2+}-concentrations and a diminished SR Ca^{2+} load (Most et al., 2005). In rodent cardiomyocytes, the extracellular application and subsequent endocytotic internalization of S100A1 resulted in increased L-type Ca^{2+} currents (I_{Ca}^{2+}), accelerated fast inactivation kinetics of I_{Ca}^{2+} and a shift of the *I*/V relationship of I_{Ca}^{2+} to negative potentials (Reppel et al., 2005).

In engineered heart tissue, overexpression of S100A1 increases isometric force development and Ca^{2+} sensitivity at rest and upon β-adrenergic stimulation (Most et al., 2001; Remppis et al., 2004). Intracoronary delivery of adenovirus containing cytomegalovirus promoter-driven S100A1 after myocardial infarction in rats resulted in improved left ventricular function at rest as well as upon β-adrenergic stimulation and attenuated the hypertrophic response (Most et al., 2004; Pleger et al., 2005). The improved cardiac performance was attributed to increased cardiomyocyte shortening, elevated Ca^{2+} transients and SR Ca^{2+} loads, due to enhanced SR Ca^{2+} uptake and reduced SR Ca^{2+} leak. In addition, S100A1 gene transfer into failing cardiomyocytes was associated with a reduction of elevated Na^{+}-concentrations, restoration of energy supply and reversal of the activated hypertrophic fetal gene expression program (Most et al., 2004). On the transcription level, co-expression of S100A1 in neonatal rat cardiomyocytes resulted in the inhibition of the β-adrenergic

activation of hypertrophic response genes, such as α-skeletal actin, β-myosin heavy chain and S100B (Tsoporis et al., 2003).

Transgenic overexpression of S100A1 in the mouse myocardium resulted in improved cardiac performance (i.e. increased left ventricular systolic ejection pressure, contractility and relaxation rates) at rest and after β-adrenergic stimulation (Most et al., 2003b). Consistent with these in vivo findings, cardiomyocytes isolated from transgenic S100A1 mice showed increased shortening, Ca^{2+} transient amplitudes, SR Ca^{2+} load and CICR (Most et al., 2003b). The fact that chronic cardiac overexpression of S100A1 in the mouse did not result in hypertrophy suggests that the up-regulation of S100A1 in a pig model of cardiac hypertrophy was compensatory and not causative (Ehlermann et al., 2000). In rat models of cardiac hypertrophy induced by aortic banding or coronary artery ligation, levels of S100A1 were however reduced (Tsoporis et al., 2003). Similarly, levels of S100A1 protein were found to be diminished in patients with end stage heart failure (Remppis et al., 1996). In a mouse model of gene targeting by homologous recombination, S100A1-deficient mice failed to augment left ventricular systolic pressure, contraction and relaxation rates, and showed reduced fractional shortening in response to β-adrenergic stimulation (Du et al., 2002). In the state of chronic pressure overload, these mice again were not able to increase contraction or relaxation rates, although the hypertrophic response to thoracic aorta constriction was normal. These findings were paralleled by a reduced Ca^{2+} sensitivity during contraction, but broadly intact β-adrenergic signalling. In another mouse model of S100A1 deficiency, generated by gene trap mutagenesis, left ventricular systolic pressure, contraction and relaxation rates were not reduced in response to β-adrenergic stimulation (Ackermann et al., 2007, unpublished results). The role of S100A1 in the cardiomyocytes is summarised in Figure 1.

In the neuroendocrine PC12 cell line, S100A1 protein was found to be up-regulated in response to nerve growth factor (NGF)-induced differentiation (Zimmer and Landar, 1995). Increased S100A1 protein levels were also documented during cell differentiation of the glial C6 cell line (Zimmer and Van Eldik, 1989). Ablation of S100A1 in PC12 cells by an antisense strategy resulted in enhanced neurite outgrowth in response to NGF, elevated levels of α-tubulin and reduced cell proliferation at high cell densities (Zimmer et al., 1998). S100A1-deficiency in PC12 cells was also associated with reduced basal $[Ca^{2+}]_i$ levels and enhanced ionomycin-induced Ca^{2+} release (Zimmer et al., 2005). In addition, S100A1 was demonstrated to inhibit brain microtubule assembly (Donato, 1988; Donato, 1991).

The microtubule-associated-protein tau is a component of the neurofibrillary tangles in Alzheimer's disease and a target of S100A1. PC12 cells devoid of S100A1 were shown to be more resistant to Aβ(25–35) peptide-mediated cell death and have lower levels of intracellular amyloid precursor protein (APP) (Zimmer et al., 2005).

S100A1-deficient mice present a normal brain morphology and cytoarchitecture and exhibit increased explorative activity and reduced anxiety-related responses (Ackermann et al., 2006). Levels of α-tubulin and APP in whole brain extracts of

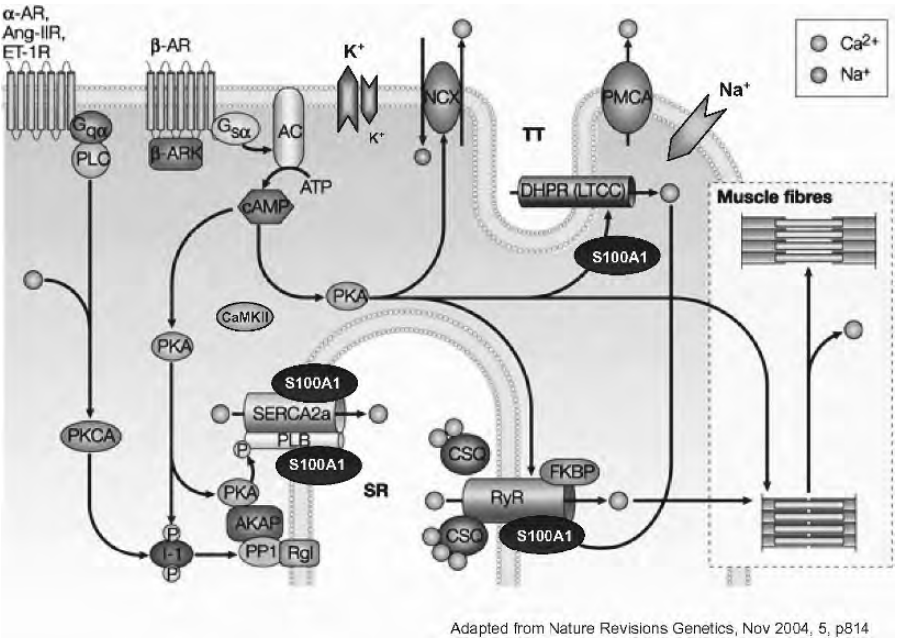

Figure 1. Calcium cycling in the heart: The role of S100A1
Legend: S100A1 occurs in the cytoplasm and was shown to physically interact and functionally modulate the sarcoplasmatic reticulum calcium ATPase (SERCA2a) and the ryanodine receptor (RyR). In addition, S100A1 can bind phospholamban (PLB). Our preliminary in vivo results indicate that loss of S100A1 results in reduced Ca^{2+}-induced Ca^{2+} release and prolonged Q-T intervals upon (-adrenergic stimulation (Ackermann et al. 2006, unpublished data). Abbreviations: adenylate cyclase (AC), A-kinase anchoring protein (AKAP), angiotensin II receptor (Ang-IIR), alpha and beta adrenergic receptor (α/β-AR), beta adrenergic receptor kinase (β-ARK), calcium/calmodulin-dependent protein kinase II (CaMKII), calsequestrin (CSQ), dihydropyridine receptor (DHPR) or L-type calcium channel (LTCC), endothelin-1 receptor (ET-1R), FK506-binding protein (FKBP), G-proteins (Gqα and Gsα), phosphatase inhibitor 1(I-1), sodium/calcium exchanger (NCX), phospholipase C (PLC), plasma membrane calcium ATPase (PMCA), protein kinase A (PKA), protein kinase C alpha (PKCA), protein phosphatase 1 (PP1), PP1 regulatory subunit 3A (Rgl), sarcoplasmatic reticulum (SR), transverse tubule (TT)(See Colour Plate 10)

S100A1-deficient mice are unchanged when compared to wild-types as determined by immunoblotting (Ackermann et al., 2006, unpublished results).

4.3. S100A2

S100A2 is a homodimeric Ca^{2+}- and Zn^{2+}- binding protein (Rustandi et al., 2000) (Bhattacharya et al., 2004). S100A2 protein was first detected in lung and kidney and is mainly expressed in a subset of tissues such as breast epithelia and liver (Heizmann et al., 2002; Fritz and Heizmann, 2004; Franz et al., 1998; Glenney et al., 1989; Zhang et al., 2002b).

Interestingly, S100A2 encoding cDNA was first identified as a novel tumour suppressor gene by subtractive hybridisation between normal and tumour-derived human mammary epithelial cells (Lee et al., 1991). Expression studies showed that S100A2 gene is markedly down-regulated in several tumour tissues of various origins such as melanomas (Maelandsmo et al., 1997) and breast carcinoma (Pedrocchi et al., 1994). Moreover, growth factors were reported to alter S100A2 gene expression at late G_1/S phase, indicating that S100A2 expression is cell cycle-regulated (Lee et al., 1992). Site-specific DNA methylation of the S100A2 gene promoter region in normal versus tumourigenic breast cancer cell lines indicated repression of gene expression in tumour cells, thus suggesting a role for S100A2 in suppression of tumour cell growth and possibly inhibition of tumour progression (Wicki et al., 1997).

The S100A2 protein was found to translocate from the cytoplasm to the nucleus during the S phase of the cell cycle in two different oral carcinoma cells (i.e. FADU and SCC-25) (Mueller et al., 2005). Co-immunoprecipitation experiments and electrophoretic mobility shift assays showed a Ca^{2+}-dependent interaction of S100A2 with the tumour suppressor p53 and the characterisation of this interaction indicated that S100A2 binds to the p53 C-terminal end. Furthermore, S100A2-p53 interaction increased p53 transcriptional activity. S100A2 was shown to interact at the same p53 binding site as S100B (Rustandi et al., 2000; Delphin et al., 1999; Wilder et al., 1998).

S100A2 was found to be oxidised in keratinocytes subjected to oxidative stress (Zhang et al., 2002b). Normal human keratinocytes displayed strong nuclear and moderate cytoplasmic S100A2 immunoreactivity. H_2O_2 treatment of normal human keratinocytes caused a transregulation of S100A2 from the nucleus to the cytoplasm.

These results highly suggested that S100A2 oxidation and translocation is associated with early cellular responses to oxidative stress.

It has been found that ΔNp63 (a mutant lacking the NH_2-terminal TA^2 transactivation domain of p53) may play an oncogenic role in the tumourigenic pathway of squamous cell cancers. To gain more insight into this pathway, the global gene expression pattern in cancer cells expressing ΔNp63 gene was examined by oligonucleotide microarray (Hibi et al., 2003). It was found that S100A2 is a target of the ΔNp63 pathway and that S100A2 induction was strictly dependent on ΔNp63 expression. These results suggest that S100A2 is a novel downstream mediator of ΔNp63.

4.4. S100A3

S100A3 is unique for its poor Ca^{2+}-binding (K_d = 4–35 mM) but exceptionally high Zn^{2+}-binding affinity ((K_d = 4 nM), this high affinity for Zn^{2+} being attributed to the unusual high Cys content of S100A3. These results imply that S100A3 might be a Zn^{2+}- rather than a Ca^{2+}-signalling molecule. The protein is highly expressed in fast-proliferating hair root cells and astrocytoma pointing toward a function in cell cycle control (Kizawa et al., 2002). S100A3 has a very selective expression

in human hair (Boni et al., 1997; Kizawa et al., 1998; Takizawa et al., 1999). The protein was found to be mainly expressed in the cuticular and cortical cells of the hair follicle . S100A3 might therefore play an important role in hair shaft formation.

The high resolution structure (Fritz et al., 2002) revealed a large distortion of the C-terminal canonical EF-hand, which most likely abolishes Ca^{2+}-binding. The crystal structure of S100A3 allowed the prediction of one putative Zn^{2+}-binding site in the C-terminus of each subunit of S100A3 involving Cys and His residues in the coordination of the metal ion. Zn^{2+}-binding induced a large conformational change in S100A3, pertubing the hydrophobic interface between two S100A3 subunits.

4.5. S100A4

S100A4 is implicated in tumour progression and metastasis (Helfman et al., 2005; Kiryushko et al., 2006; Jenkinson et al., 2004; Pedersen et al., 2004).

The 3-dimensional structure was determined in the apo state by NMR spectroscopy (Vallely et al., 2002). As other S100 proteins, S100A4 is a symmetrical homodimer held together by non-covalent interactions. A preliminary X-ray structure of human S100A4 confirmed this solution structure (Zhang et al., 2002a). Larger assemblies than dimers were reported for S100A4 (Novitskaya et al., 2000) probably interacting/activating RAGE.

S100A4, like some other S100 proteins, is able to relocate upon cellular stimulation, allowing its interaction with different partner proteins in a spatial and temporal manner. These include liprin β1, a member of the family of LAR transmembrane phosphatase –interacting proteins (Kriajevska et al., 2002), annexin II (Semov et al., 2005), p53 (Grigorian et al., 2001), methionine aminopeptidase 2 (Endo et al., 2002), or myosin-IIA heavy chain (Li and Bresnick, 2006).

Genetically modified mouse models have been used to study the mechanisms of S100A4-mediated metastasis (Ambartsumian et al., 2005). The combined results support the view that S100A4 plays an important role in tumour development and metastasis. Interestingly, in mice deficient for S100A4, the expression of the S100A5 flanking gene was activated in some cells that normally express S100A4 (El-Naaman et al., 2004). Although it could be ruled out that the oncogenic action of S100A4 is due to S100A5, it nevertheless indicates a crosstalk compensation mechanism between neighbouring S100 genes. This was also supported by recent finding of a mutually antagonistic action of S100A4 and S100A1 in carcinoma cells (Wang et al., 2005).

The expression of S100A4 has been detected in various human tumours and high S100A4 levels have been repeatedly correlated with a reduced patient survival and poor prognosis (Helfman et al., 2005).

We investigated the relevance of S100A4 to clinical cancer in several archival colon cancer specimens, without metastasis at the time of surgery (Stein et al.,

2006). Remarkably, S100A4 mRNA levels were found to be higher in the primary tumours, which later developed distant metastases. Patients whose tumours were heterozygous for activating β-catenin mutation were identified, and the tumours showed both nuclear β-catenin staining and high S100A4 expression. All these patients developed metastases in the liver. This was the first demonstration that mRNA expression of S100A4, determined in primary tumours in a quantitative manner, is of value for the prediction of metastatic cancer.

The results showed an interconnection of two previously unconnected molecular pathways which play important roles in tumour progression and metastasis, the β-catenin/TCF signalling pathway and S100A4, that controls motility and invasiveness. The finding demonstrated that β-cateninTCF directly regulates the expression of the S100A4, and that β-catenin-induced effects on cell migration and invasion are mediated by S100A4 in colon cancer cells. New therapeutic strategies aimed at disrupting this regulation and the function of S100A4 protein may be of particular value for prevention of colon cancer metastasis.

4.6. S100A5

Homodimeric S100A5 binds four Ca^{2+}-ions with strong positive cooperativity and an affinity 20–100 fold higher than the other S100 proteins studied under identical conditions (Schafer et al., 2000). S100A5 also binds two Zn^{2+}-ions and four Cu^{2+} ions per dimer and Cu^{2+}-binding strongly impairs the binding of Ca^{2+}; however, none of these ions change the α-helical-rich secondary structure. After covalent labelling of an exposed thiol with 2-(4'-(iodoacetamide)anilino)-naphtalene-6-sulfonic acid, Cu^{2+}binding, but not Ca^{2+} or Zn^{2+}, strongly decreased its fluorescence. In light of the 3-dimensional structure of S100 proteins, our data suggest that in each subunit the single Zn^{2+} site is located at the opposite side of the EF-hands. The two Cu^{2+}-binding sites probably share ligands of the EF-hands. However, the potential role of S100A5 in copper homeostasis is unknown.

The expression of S100A5 is restricted to a few specific areas of the brain: namely the olfactory bulb, the brainstem, and the spinal trigeminal tract (Schafer et al., 2000; Chan et al., 2003). S100A5 was found to be overexpressed in astrocytic tumours (Camby et al., 2000) suggesting a role of S100A5 as marker of recurrence in certain meningiomas (Hancq et al., 2004a). S100A5 was also found in the human kidney where it is proposed to play a functional role in the ascending limb of the loop of Henle in the nephron (Teratani et al., 2002). No target protein for S100A5 has been yet described.

Interestingly, when the expression of the S100A5 and S100A3 genes was analysed in S100A4 null mice, an alteration of the expression of S100A5 was observed. This raised the possibility of a compensatory mechanism of the S100A4 activity in mice and should be considered when other S100 null mice are investigated.

4.7. S100A6

S100A6 was initially identified by Baserga and coworkers as a cDNA clone encoding an mRNA specie whose level is induced when quiescent/growth-arrested human fibroblasts are stimulated to progress from the G_0 to the G_1 phase of the cell cycle (Ferrari et al., 1987; Calabretta et al., 1986). The S100A6 has been purified to homogeneity and found to be expressed in high levels in muscle, lung, kidney, spleen, and brain (Kuznicki et al., 1989a; Filipek et al., 1990; Kuznicki et al., 1989b).

The 3-dimensional structure of apo-S100A6 was the first S100 protein structure determined (Potts et al., 1995) and revealed a new dimeric fold, the unique architecture common to all S100 proteins but fundamentally different from calmodulin. Refined structural analysis (Otterbein et al., 2002) of the Ca^{2+}-free and Ca^{2+}-bound states have contributed to our understanding of target binding and cooperativity of metal-binding in the S100 family.

A Ca^{2+}-dependent translocation of S100A6 was found in endothelial cells (Hsieh et al., 2002) and neuroblastoma cells (Filipek et al., 2002b) enabling the protein to interact with different target proteins localised in distinct cell compartments.

These include annexin (Minami et al., 1992; Tomas and Moss, 2003; Farnaes and Ditzel, 2003) at the nuclear envelope suggesting a role of this complex in cell division.

S100A6 was also found to specifically interact with the Siah-1-interacting protein (SIP), a component of a novel ubiquitinylation (Filipek et al., 2002a) pathway regulating β-catenin degradation (Matsuzawa and Reed, 2001). Several other S100 proteins also bind to the C-terminus of SIP in a Ca^{2+}-dependent manner suggesting that protein ubiquitinylation can be regulated by S100 proteins (Lee et al., 2004).

In the brain, S100A6 is restricted to some subpopulations of neurons and astrocytes (Yamashita et al., 1999). Interestingly, S100A6 is overexpressed in astrocytes associated with the neurodegenerative lesions of amyotrophic lateral sclerosis (ALS) (Hoyaux et al., 2002) as well as in patients with Alzheimer's disease (AD), and in two different AD mouse models (Boom et al., 2004). A deregulation of S100A6 expression was also found in certain tumour tissues (Stulik et al., 2000; Luu et al., 2005; Hancq et al., 2004b; Cross et al., 2005) and in patients with acute myeloid leukemia (Murphy et al., 1988).

4.8. S100A7

S100A7 proteins (for nomenclature see Table 1) have various functions in the epidermis (Kulski et al., 2003; Eckert et al., 2004; Eckert and Lee, 2006). The human S100A7 was first identified as a protein upregulated in psoriatic skin (Madsen et al., 1991; Madsen et al., 1992). Recently, the homologous S100A7A (previous symbol S100A15) was identified and its expression was found to be upregulated in lesional skin (Wolf et al., 2006). S100A7 and S100A7A are co-expressed

in psoriasis probably participating in keratinocyte maturation, proliferation, and inflammation.

The crystal structure of S100A7 revealed that this protein, in contrast to other S100 proteins, probably binds only one calcium ion per monomer and this binding does not induce large conformational changes of the protein (Boeshans et al., 2006; Brodersen et al., 1998).

The structures also revealed a Zn^{2+}-binding site in the protein. The coordination of the zinc ion is similar to the pattern seen in certain metalloproteases (Brodersen et al., 1999). Sequence comparison suggests that this zinc-binding site is present in a number of the remaining members of the S100 family (Fritz and Heizmann, 2004). The structure of S100A7, crystallised in the absence of zinc, further showed that loss of zinc resulted in a reorganisation of the adjacent empty and distorted EF-hand loop, similarly to a calcium-loaded EF-hand.

In the intracellular compartment, S100A7 interacted with epidermal fatty acid-binding protein (E-FABP) (Hagens et al., 1999; Ruse et al., 2003). Although E-FABP is involved in lipid metabolism and transport, the role of the intracellular S100A7-E-FABP interaction is not known. Interestingly, S100A7 is translocated to the cell periphery when keratinocytes are stimulated. In addition, S100A7 is found extracellulary in psoriatic plaque exudate, as its binding partner E-FABP.

S100A7 proteins are of major interest as they are overexpressed in wound healing, inflammatory skin diseases, and skin- and other types of cancer diseases (Webb et al., 2005; Emberley et al., 2004).

4.9. S100A8/S100A9

S100A8 and S100A9 are characterised by a unique expression pattern with strong prevalence in cells of myeloid origin (Roth et al., 2003; McCormick et al., 2005). These proteins were first discovered in body fluids of children with cystic fibrosis.

The X-ray structure of monodimeric human S1008 (Ishikawa et al., 2000) and S100A9 proteins (Itou et al., 2002) has been reported.

S100A9 is different from other S100 proteins because of its long C-terminus, which is extremely flexible. S100A8 and S100A9 tend to form homo- and heterodimers in the absence of Ca^{2+} and associate to higher-order oligomers in a Ca^{2+}-dependent manner (Leukert et al., 2006; T. Vogel, 2006). The formation of tetramers may also be triggered by Zinc, suggesting that Zn^{2+} or Ca^{2+} might activate different cellular pathways (Yui et al., 2002).

Intracellularly, S100A8/S100A9 complexes play an important role in myeloid maturation, cell trafficking and arachidonic acid (AA) metabolism. S100A8 and S100A9, present in high concentrations in the cytoplasm of neutrophils and monocytes, are able to translocate and are secreted after activation of a novel pathway requiring an intact microtubule network (Rammes et al., 1997).

Extracellular S100A8/A9 heterodimer exhibits cytokine-like functions, e.g. enhancing leukocyte recruitment to inflammatory sites and AA transportation to target cells (Vandal et al., 2003; Kerkhoff et al., 1999; Eue and Sorg, 2001).

S100A8/S100A9 seem to bind and activate various surface receptors such as heparin sulfate proteoglycans (Srikrishna et al., 2001), CD36 (Kerkhoff et al., 2001), and RAGE (Hermani et al., 2006).

The functions of S100A8 and S100A9 pro-inflammatory proteins were investigated in two different mouse models. Inactivation of the S100A9 gene showed no obvious phenotype (Hobbs et al., 2003; Manitz et al., 2003) (Table 3). However, reduced migration of S100A9-deficient neutrophils and decreased surface expression of CD11b, which belongs to the integrin family, were observed in vitro upon stimulation (Manitz et al., 2003). In addition, chemokine-induced down-regulation of the cytosolic Ca^{2+}-level was detected. Obviously, these in vitro effects are compensated by alternative pathways in vivo.

In contrast, mice lacking S100A8 displayed a severe phenotype and provided the first evidence of a nonreduntant function of a S100 protein (Table 3). Lack of S100A8 expression caused early resorption of the mouse embryo by the mother, suggesting that S100A8 protects the implanting embryo from maternal rejection (Passey et al., 1999).

S100A8/A9 proteins are involved in inflammatory diseases. Their expression is low in healthy people whereas, during inflammation, specific cell populations release homo- or hetero-S100 protein complexes depending on the phase and the type of inflammation. Tests have been developed to detect S1008 and S100A9 in body fluids of patients with rheumatoid arthritis for the discrimination of active and nonactive osteoarthritis from rheumatoid arthritis. S100A8 and S100A9 are also associated with chronic inflammatory diseases including bowel disease and chronic periodontitis, and both proteins are involved in wound repair by reorganisation of the keratin cytoskeleton in the injured epidermis. In addition, some of the therapeutic effects of retinoids in inflammatory and hyperproliferative skin diseases might be associated with repression of S100A8 function.

As a consequence of the various proinflammatory properties of S100 proteins, strategies targeting these molecules by in vivo administration of S100 antibodies are a novel option for anti-inflammatory therapies. Another approach is the inhibition of the release of these cytokine-like molecules at sites of inflammation.

In clinical practice, laboratory measures used to monitor those inflammatory diseases are C-reactive protein and erythrocyte sedimentation rate. Recent studies have shown that the use of S100A8/S100A9 proteins as markers of synovial inflammation is even superior to C-reactive protein and erythrocyte sedimentation rate (Kane et al., 2003).

Furthermore, overexpression of S100A8 and S100A9 was also reported in various types of cancers contributing to their development and progression. They were suggested to be novel diagnostic markers when measured in the serum of patients with prostate cancer and benign prostate hyperplasia (BPH) (Hermani et al., 2005).

4.10. S100A10

New data have been reported about the structure (Rety et al., 1999; Menke et al., 2004) and functions of S100A10 (van de Graaf et al., 2003; Yamada et al., 2005; Kwon et al., 2005; Donier et al., 2005; Svenningsson et al., 2006).

The annexin II-S100A10 complex was found to reorganise the actin cytoskeleton, causing the association of the E-cadherin and nectin systems to form adherens junctions in kidney cells (Yamada et al., 2005). Furthermore, it has been shown (Kwon et al., 2005) that the annexin A2-S100A10 complex stimulates the conversion of plasminogen to plasmin and that loss of S100A10 from the cell surface results in a dramatic loss of plasmin generation, suggesting that this protein complex is a major regulator of plasmin production.

Recently the yeast two-hybrid system was applied to identify sensory neuron proteins that interact with the Acid-Sending Ion Channel (ASIC1a) (Donier et al., 2005). ASICs are voltage-independent H^+-gated ion channels implicated in mechanosensation, learning and memory, and anxiety-like behaviour.

S100A10 was found to interact physically with ASIC1a. It was suggested that its primary role to regulate ASIC1a activity is to enhance cell surface expression of ASIC1a (Donier et al., 2005). Besides this specialised role in enhancing ASIC1a functional expression, S100A10 is also known to traffic the Transient Receptor Potential Channels (TRPV5 and TRPV6) to the plasma membrane presenting the facilitation of Ca^{2+} inward currents (van de Graaf et al., 2003).

Most interestingly, the serotonin 1B receptor was found to colocalise/ interact with S100A10, thereby increasing the recruitment of this receptor to the cell surface (Svenningsson et al., 2006). Abnormality in serotonin signalling has been implicated in the pathophysiology of depression. S100A10, a new molecule to brighten the mood (Sharp, 2006), is decreased in animal models of depression and in brain tissues from depressed patients. $S100A10^{-/-}$ mice exhibit a depression-like phenotype and have reduced responsiveness to 5-HT_{1B} agonists. These and other results indicate an important modulation of 5-HT_{1B} receptor functions by S100A10 which can associate with depression-like states (Svenningsson et al., 2006).

4.11. S100A11

The structure of S100A11 has been determined by NMR spectroscopy and crystallography (Dempsey et al., 2003; Rety et al., 2000; Sakaguchi et al., 2004; Sakaguchi et al., 2003; Sakaguchi et al., 2005). A novel mode of interaction of the annexin I tetramer has been proposed (Rety et al., 2000) suggesting it is important for membrane organisation during fusion events.

S100A11 is homodimeric and interacts in a Ca^{2+}-dependent manner with annexin I to coordinate membrane association (Pan et al., 2006).

It has been recently demonstrated (Sakaguchi et al., 2004; Sakaguchi et al., 2003; Sakaguchi et al., 2005) that S100A11 is a key mediator for growth inhibition of normal human epidermal keratinocytes (NHK) triggered by high Ca^{2+} or TGFβ.

The pathways for high Ca^{2+}- and TGFβ-induced growth inhibition of NHK cells is linked to PKCα-dependent phosphorylation of S100A11. This enables a more differentiated regulation of growth inhibition (Sakaguchi et al., 2005). Exposure of cells to high Ca^{2+} concentration or to TGFβ results in S100A11 phosphorylation ($^{[10]}$Thr). Phosphorylated S100A11 binds then to nucleolin and is transferred to the nucleus. This leads to the liberation of Sp1 and the induction of p21 expression. TGFβ activates smad proteins and this leads to an increased affinity of Sp1 to the proximal p21 promoter (Sakaguchi et al., 2004; Sakaguchi et al., 2005).

Just recently, an S100A11-deficient mouse was generated to analyse the role of S100A11 during mouse spermatogenesis. Although S100A11 is highly expressed in Sertoli cells of the testis, knockout mice were fertile and showed no gross abnormalities in testis morphology. So far, no phenotypic abnormalities could be observed (Mannan et al., 2003).

4.12. S100A12

F. Guignard et al. detected a protein that was immunologically related to S100A8 and S100A9 (Guignard et al., 1995). Human S100A12 was then purified, its proteins sequence determined by tandem mass spectrometry (Ilg et al., 1996) and its nucleotide sequence localised on human chromosome 1q21 (Wicki et al., 1996a). S100A12 is mainly expressed in granulocytes but also in keratinocytes and psoriatic lesions (for rev. see (Moroz et al., 2003b; Roth et al., 2003).

The Ca^{2+}-loaded crystal structure of dimeric S100A12 has been determined (Moroz et al., 2002) and the two residues Glu 4 and Glu 8, that are highly conserved in other S100 proteins, possibly take part in target binding. The structure of the human S100A12-copper complex was reported (Moroz et al., 2003a) showing that each monomer binds a single copper ion that is probably essential in early immune responses.

A number of S100A12 binding partners have been identified including aldolase, annexin V, and S100A9 (Hatakeyama et al., 2004) and it was suggested that S100A12 may have a Ca^{2+}-dependent chaperone-like function.

Similar to other S100 proteins, S100A12 was found to translocate from the cytosol to the membrane of neutrophiles after Ca^{2+} activation. There is accumulating evidence that S100A12 may be involved in defence mechanisms against microorganisms and parasites. S100A12 was also found to induce neurite formation when added to cultured hippocampal cells. S100A12 extracellular activities may be transmitted through its interaction with RAGE, generating a cascade of intracellular events (Mikkelsen et al., 2001).

The S100A12 molecule, interacting with the extracellular domaine of RAGE, might be a S100A12-hexamer built up of 3 dimers bridged by intermolecular Ca^{2+}-binding sites (Moroz et al., 2002). It is suggested that this hexameric molecule has the capacity to interact with the extracellular domains of RAGE resulting in receptor oligomerisation and signal propagation.

S100A12 is associated with several pathological states including psoriasis (together with S100A7), inflammation, Mooren's ulcer, an autoimmune disease of the human cornea, and Kawasaki's disease, an acute multisystem vasculitits, occurring in children usually under 5 years of age (Table 2).

4.13. S100A13

The cDNA of human and murine S100A13 was first identified by screening expressed sequence tag databases of human chromosome 1q21 (Hsieh et al., 2002). The human S100A13 gene was shown to neighbour S100A1 on chromosome 1q21, at least 35 kilobase pairs apart from the subgroup of the closely linked S100A2-S100A3-S100A4-S100A5-S100A6 genes. Mouse S100A13 cDNA shows the lowest sequence identity among S100 cDNAs when compared to the human cDNA.

Expression of S100A13 mRNA has been so far detected in skeletal muscle, heart, kidney, pancreas, ovary, spleen, and small intestine (Wicki et al., 1996b). Comparison of human S100A13 protein sequence with other S100 proteins revealed interesting differences. The second EF-hand contains an unusual lysine in the –x position which might influence Ca^{2+} binding properties (Ridinger et al., 2000). Furthermore, the last eleven C-terminal amino acids contain six lysine and two arginine residues. This positively charged C-terminal region could potentially be involved in specific protein interactions as it was reported for the positive C-terminal tail of syntaxin, which interacts with synaptotagmin (Shao et al., 1997).

S100A13 differs from the other family members in its very broad expression pattern and the absence of a surface-exposed hydrophobic patch in the Ca^{2+}-saturated state. Its 3-dimensional structures (Sivaraja et al., 2005) deposited in the RCSB Protein Data Bank supports the unique properties of this S100A13 protein.

In addition, NMR experiments (Arnesano et al., 2005) showed that Ca^{2+}-binding to S100A13 leads to a conformational change, thus creating a novel binding site for Cu^{2+}-ions on a solvent exposed location. This would be in agreement with the findings that intracellular S100A13 may be involved in the formation of a Cu^{2+}-dependent interleukin-1α-S100A13 complex, facilitating the export of both proteins (Mandinova et al., 2003).

The fact that the two antiallergic drugs, amlexanox and cromolyn bind S100A13 suggests that S100A13 may be involved in degranulation of mast cells (Oyama et al., 1997; Shishibori et al., 1999). In endothelial cells, S100A13 is translocated in response to a rise in Ca^{2+} (Hsieh et al., 2002), using a different pathway from that used by S100A6. This suggests that different S100 proteins use distinct translocation pathways and end up in specific subcellular components in order to fulfil their distinct tasks.

4.14. S100A14

S100A14 was identified and characterised by analysing a human lung cancer cell line subtraction cDNA library (Pietas et al., 2002). It encodes an mRNA present

in several normal human tissues of epithelial origin with the highest expression level in colon. The full-length cDNA predicts a protein of 104 amino acids with 68% homology to S100A13. The deduced amino acid sequence of human and mouse S100A14 contains two EF-hand Ca^{2+}-binding domains, a myristoylation and a glycosylation sites and several potential protein kinase phosphorylation sites. Examination of the intracellular distribution of S100A14 using two human lung carcinoma and an immortalised monkey cell lines revealed a pronounced cytoplasmatic staining. S100A14 was shown to be overexpressed in ovary, breast and uterus tumours and down-regulated in kidney, rectum, and colon tumours. This pattern suggests a distinct regulation with potentially important functions in malignant transformation.

4.15. S100A15 (see S100A7)

According to the official nomenclature (Table 1) the S100A15 gene symbol has been withdrawn and replaced by the new approved gene symbol, S100A7L1.

4.16. S100A16

S100A16 is the most recently identified member of the S100 EF-hand Ca^{2+}-binding protein family. The biochemical characterisation of human and mouse recombinant S100A16 proteins has been reported recently (Sturchler et al., 2006). Both S100A16 proteins form homodimers and bind one Ca^{2+}-ion at the C-terminal EF-hand. The human protein exhibits a two fold higher Ca^{2+}-affinity (Kd $\sim$ 430μM). Trp fluorescence shifts indicate conformational changes in the ortholog proteins upon Ca^{2+}-binding, while formation of a hydrophobic patch, implicated in target protein recognition, only occurs in the human S100A16 protein.

S100A16 mRNA was found in many tissues (Marenholz and Heizmann, 2004) including the brain, where it was found to be astrocyte specific (Sturchler et al., 2006). In glioblastoma cells, S100A16 was found to accumulate within nucleoli and to translocate to the cytoplasm in response to Ca^{2+}-stimulation, suggesting a possible role of S100A16 in ribonucleoprotein complex processing, gene silencing, or cell cycle progression.

Like other members of the family, S100A16 lacks the canonical nuclear localisation signal. Nuclear import may require interaction with transporter proteins or phosphorylation, as it was described for S100A11 (Hollstein et al., 1991), or may occur via facilitated diffusion pathways, as observed for calmodulin (Hibi et al., 2003; Moll et al., 1995). Interestingly, a recent study identifies phosphorylated S100A16 protein in nucleoli of HeLa cells (Raybaud-Diogene et al., 1996), indicating a possible function of phosphorylation in S100A16 nuclear import.

4.17. S100RVP

S100RVP was identified as an androgen-responsive gene in the rat ventral prostate (RVP). This protein is abundantly expressed in the cytoplasm and nucleus of

epithelial cells. The protein shows a high homology to S100A13 and S100A7. S100RVP is suggested to play a role in calcium homeostasis in the prostate (Oram et al., 2006).

4.18. S100P

The crystal structure of the human S100P in the Ca^{2+}-bound form has been resolved (Zhang et al., 2003). S100P, localises on human chromosome 4p16 and is expressed in several malignant neoplasms (Arumugam et al., 2005; Arumugam et al., 2004; Wang et al., 2006). It has been suggested that S100P plays a major role in the aggressiveness of pancreatic cancer possibly mediated through RAGE activation.

Dimeric S100P was found to bind and activate ezrin, a membrane/F-actin cross-linking protein (Koltzscher et al., 2003) possibly influencing cell morphology.

4.19. S100Z

Gribenko et al. isolated a human cDNA from human prostate encoding a 99-amino acid S100 protein, designated S100Z. It was reported to be capable of interacting with S100P. S100Z is a dimeric, predominantly α-helical protein, with two Ca^{2+}-binding sites. S100AZ is expressed in various tissues with highest levels in spleen and leukocytes. S100Z gene expression appears to be deregulated in some tumour tissues (Gribenko et al., 2001).

4.20. S100G (CALBINDIN 3, CaBP9K)

Whereas S100 proteins form homo-, heterodimers, and polymers in solution, calbindin occurs as a monomer. This structural difference is also reflected in S100G function as a Ca^{2+} buffer or transcellular Ca^{2+} transporter (van der Eerden et al., 2005) versus the signalling activities of the other S100 proteins (Julenius et al., 2002; Bertini et al., 2002; Nelson et al., 2002) of the strictly conserved . The dimerisation plane of S100 proteins is composed of strictly conserved hydrophobic residues, which are missing in S100G. S100G is expressed in a number of tissues, such as intestine, uterus, placenta, kidney, and bone tissues (Choi et al., 2005) under the regulation of 1,25-dihydroxy vitamin D3.

5. TWO S100-LIKE PROTEINS

5.1. p26olf from Frog

Frog p26olf is a S100-like Ca^{2+}-binding protein found in olfactory cilia. It consists of two S100-like domains aligned in series and has a total of four Ca^{2+}-binding sites (known as EF-hands) (Miwa and Kawamura, 2003). This protein localises in the cilia of olfactory epithelium and interacts with a frog β-adrenergic receptor kinase (βARK)-like protein in a Ca^{2+}-dependent manner. Through the βARK-dependent

phosphorylation, p26olf has been suggested to have some role(s) in olfactory signal transduction.

5.2. Dicalcin from Xenopus

A homologous protein (named dicalcin) was detected in Xenopus eggs. When compared with other S100 members, the C-terminal half of Xenopus dicalcin shows the highest homology (~45%) with rabbit S100A11 and lesser homology (~38%) to rat S100A1. Immunohistochemical studies revealed that Xenopus dicalcin is localised prominently in the vitelline envelope and the cytoplasm of the cortex of both the animal and the vegetal hemispheres of Xenopus eggs. Xenopus dicalcin binds to several soluble proteins and two vitelline envelop proteins in a Ca^{2+}-dependent manner. These results suggest that Xenopus dicalcin is a novel S100-like protein in eggs and may to be involved in the fertilisation process.

6. S100 PROTEINS AND RAGE SIGNALLING

Several S100 proteins present extracellular functions (Fritz et al., 2002; Marenholz et al., 2004a). Although active secretion has only been demonstrated for S100B, S100A4, S100A8/S100A9 and S100A12 (Rammes et al., 1997; Stary et al., 2006; Van Eldik and Zimmer, 1987; Ye et al., 2004), it cannot be excluded that some members of the S100 protein family, similarly to amphoterin, could be released by injured or necrotic cells and then act as signalling molecules (Scaffidi et al., 2002). S100 protein-driven biological functions are mediated through their interaction with specific receptors located at the cell surface and S100B, S100A1, S100A2, S100A4, S100A6 and S100A12 have been shown to interact with the receptor for advanced glycation end-products (RAGE) (Hofmann et al., 1999; Huttunen et al., 2000; Reddy et al., 2006b; Leclerc et al., unpublished).

RAGE is a member of the immunoglobulin-like cell surface receptor superfamily and its gene is located on the human chromosome 6p21.3 in the MHC class III locus near the junction to class II (Neeper et al., 1992; Vissing et al., 1994). RAGE is composed of three extracellular immunoglobulin domains, (a "V" followed by two "C" type domains), a single spanning transmembrane region and a short cytosolic domain (Figure 2) (Neeper et al., 1992). Recently, additional RAGE isoforms lacking the transmembrane and cytosolic regions (endogenous secreted RAGE or esRAGE) or the "V" immunoglobulin domain (N-truncated RAGE or NtRAGE) were identified in the human brain (Ding and Keller, 2005b). Whereas the human RAGE isoforms are produced by differential splicing, mouse RAGE isoforms are likely to be produced by carboxy-terminal truncation (Ding and Keller, 2005b; Hanford et al., 2004). The presence of various RAGE proteins suggests that they display specialized functions. For example, NtRAGE is expected to exhibit different ligand affinities compared to the full-length RAGE as it lacks the "V" domain involved in ligand interaction (Ding and Keller, 2005a; Ding and Keller, 2005b; Kislinger et al., 1999; Yonekura et al., 2003). In contrast, esRAGE, released into

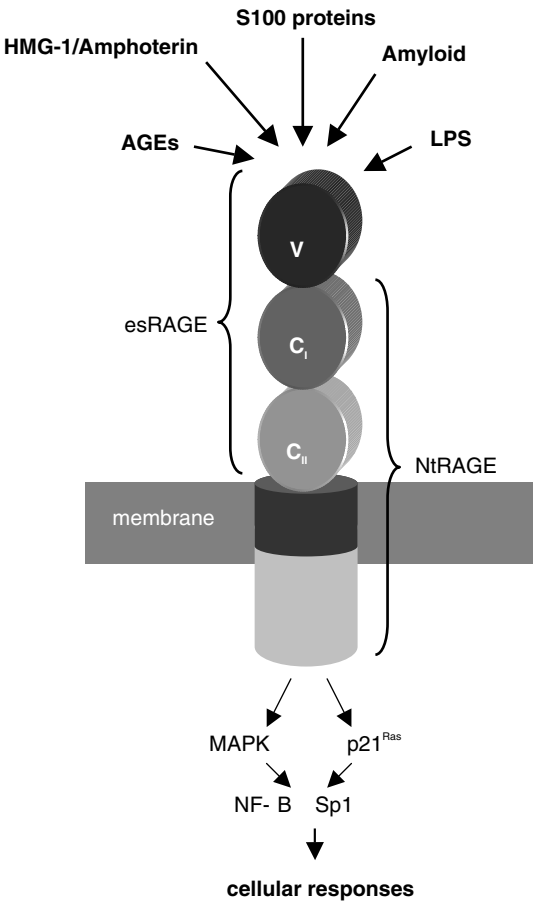

Figure 2. The RAGE signalling network. The RAGE ligands (S100 proteins, Amyloid-β or AGEs, LPS or Amphoterin) bind to full-length RAGE or N-truncated RAGE (NtRAGE) and recruit different signalling pathways, $p21^{ras}$and mitogen-activated protein kinases (MAPK). Depending on the pathways recruited, RAGE ligand-signal leads to the activation of transcription factors like NF-κB or Sp1 and to cellular responses. In contrast, binding of the RAGE ligands to the endogenous secreted RAGE (esRAGE) abolishes membrane-localized RAGE activation and protect cells against cellular activation

the extracellular space, can interact with all ligands and sequester RAGE ligands in a concentration-dependent manner prior to their interaction with full-length RAGE (Deane et al., 2003; Taguchi et al., 2000; Yan et al., 2000). RAGE can be activated by amyloid-β (Aβ), amphoterin, LPS or advanced glycation endproducts (AGEs) as well as by S100 proteins (Hori et al., 1995; Neeper et al., 1992; Yan et al., 1996). RAGE is expressed at high levels during development, especially in the central nervous system. In contrast, RAGE expression decreases to low level in most adult cell types like neurons, smooth muscle cells, mononuclear phagocytes,

cardiac myocytes, hepatocytes, renal glomerular epithelial cells, Muller and bipolar ganglion cells of the retina. The notable exception is lung epithelial cells, where its expression remains high (Li and Schmidt, 1997; Schmidt et al., 2001; Shirasawa et al., 2004). Although RAGE expression is very low in most adult tissues, the specific accumulation of RAGE ligands, observed in a large number of pathophysiological processes, results in the up-regulation of the receptor. This leads to sustained activation and has been suggested to be the starting point of chronic cellular activation and tissue damage (Bierhaus et al., 2004; De Vriese et al., 2006; Pachydaki et al., 2006).

RAGE functions during brain development by promoting cell migration and neurite outgrowth (Chou et al., 2004; Hori et al., 1995; Huttunen et al., 2000). Furthermore, involvement of RAGE in neurite outgrowth was also demonstrated in vivo in a sciatic nerve crush model. Blockade of RAGE-ligand interaction with esRAGE or specific antibodies directed against RAGE, S100 proteins or amphoterin reduced the functional regeneration of the peripheral nerve (Rong et al., 2004a). However, $RAGE^{-/-}$ mice present neither any obvious neuronal deficit nor behaviour abnormalities, suggesting that the presence of other cellular mechanisms might ensure normal development when RAGE function is impaired (Bierhaus et al., 2004). In vitro studies as well as functional characterization of the $RAGE^{-/-}$ mice have revealed the involvement of the RAGE-ligand network in several human pathologies such as diabetes, cancer, neurodegenerative diseases, proliferative retinal disease, inflammation, atherosclerosis and myocardial injury (Arumugam et al., 2004; Bucciarelli et al., 2006; Bucciarelli et al., 2002; Cecil et al., 2005; Ehlermann et al., 2006; Liliensiek et al., 2004; Lu et al., 2004; Pachydaki et al., 2006; Rong et al., 2004b; Yan et al., 1996). In these pathological contexts, RAGE-ligand interaction at the cell surface results in diverse cellular responses varying from cytokine secretion, increased cellular oxidative stress and apoptosis (Schmidt et al., 2001). Furthermore, RAGE engagement by a ligand triggers the activation of key signalling pathways involving $p21^{ras}$, Rac, p44/42, Akt, JNK or p38 MAP kinases as well as NF-κB and resulting in the modulation of the expression of target genes (Figure 2) (Du Yan et al., 1997; Huttunen et al., 1999; Huttunen et al., 2000; Reddy et al., 2006a; Riuzzi et al., 2006; Taguchi et al., 2000; Yan et al., 2000). More particularly, we have shown that RAGE-S100 protein interaction differentially affects cellular functions depending on specific S100 protein and its concentration. These effects are mediated through the recruitment of a large set of signalling pathways, some being generally associated with S100-mediated cellular responses and other specifically activated in the presence of a particular S100 protein (Figure 3) (E. Leclerc, C.W. Heizmann and A. Galichet, personal communication). In addition, it is also important to mention that RAGE-ligand interaction can have very different consequences depending on the cell type. RAGE-Aβ interaction increases microglia activation and macrophage colony-stimulating factor secretion whereas it promotes apoptosis in neurons (Lue et al., 2001; Sousa et al., 2001). S100B protects LAN-5 neuroblastoma cells against Aβ-induced apoptosis in a RAGE-dependent manner,

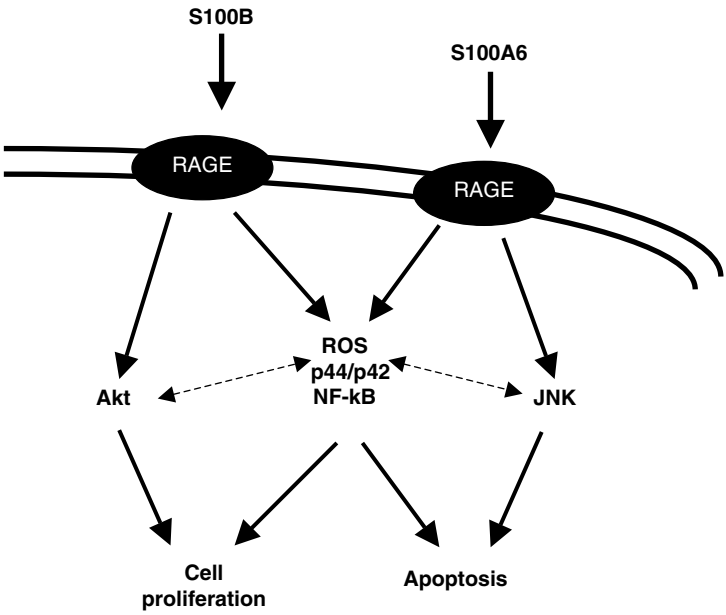

Figure 3. RAGE-mediated S100 proteins biological functions and transduction pathways. Various members of the S100 proteins family bind to RAGE. S100B activates cell proliferation in a concentration-dependent manner through the formation of reactive oxygen species (ROS) which then activate the Akt (Protein kinase B) and the extracellular signal-regulated kinases (erk) pathways, leading to changes in the activity of NF-κB transcription factor. In contrast, S100A6 induces cellular apoptosis by production of ROS, activation of Erk pathway and changes in NF-κB transcriptional activity but also involves the c-JUN NH_2-terminal kinase (JNK). JNK and Erk activation are dependent on ROS formation

although this study did not provide information on the involvement of RAGE in Aβ toxicity or on the potential physical competition between the two ligands for the RAGE receptor which could also explain S100B protection (Businaro et al., 2006).

Endogenous secreted soluble RAGE (esRAGE) binds to the diverse RAGE ligands. It could therefore participate to the removal and detoxification of the ligands involved in human diseases by acting as a decoy. The potential use of esRAGE as a therapeutic target was investigated in animal models and results of these studies consistently indicated that administration of esRAGE reduced or limited the development of most RAGE-dependent human disorders. Specifically, esRAGE suppressed Alzheimer's disease-associated pathology and reduced the transport of Aβ peptide across the blood-brain barrier in an Alzheimer's disease mouse model (Deane et al., 2003; Lue et al., 2001). In addition, injection of esRAGE in mice blocked the development of tumours and metastasis as well as inflammation and prevented diabetes-associated impaired wound healing (Goova et al., 2001; Hofmann et al., 1999; Taguchi et al., 2000). esRAGE also emerged as a disease marker and clinical studies revealed a specific decrease of circulating

esRAGE level in patients with hypertension, Alzheimer's disease or reduced inflammatory response. In contrast, elevated esRAGE level was found in patients with acute lung injury or renal diseases (Geroldi et al., 2006). A second blockade approach of the RAGE-ligand interaction has been investigated in diabetic and Alzheimer's disease mouse models using specific antibodies directed against RAGE and has proven to be successful (Flyvbjerg et al., 2004; Jensen et al., 2006; Lue et al., 2001). However, the use of such approaches needs a perfect understanding of the RAGE-ligand interaction structure as well as the development of antibodies specifically directed against the binding site of a particular ligand on RAGE.

The $RAGE^{-/-}$ and wild type mice present similarly increased immune responses following the induction of delayed-type hypersensitivity (DTH), which generates the production of RAGE ligands like S100 proteins. In contrast, treatment of both wild type and $RAGE^{-/-}$ mice with esRAGE reduces this adaptive immune response (Liliensiek et al., 2004). Similarly, administration of esRAGE to diabetic wild type mice completely restored pain perception, whereas pain perception was only partly restored in diabetic $RAGE^{-/-}$ animals (Bierhaus et al., 2004). These results strongly indicate that RAGE ligands, sequestered by esRAGE, can coactivate receptors other than RAGE which are also involved in these pathological mechanisms. Indeed, it was reported that S100A8/S100A9 bind to heparin sulphate glycosaminoglycans on human HMEC-1 cells devoid of RAGE. Similarly, AGEs can bind to receptors like AGE-R1 and AGE-R2 (He et al., 2000; Robinson et al., 2002; Stitt et al., 1999). Furthermore, Aβ peptide can interact with cell surface binding sites such as type A scavenger and a-7 nicotinic acetylcholine receptors (Paresce et al., 1996; Wang et al., 2000b). In addition, RAGE-mediated leukocyte recruitment is dependent on the interaction between RAGE and the β2-integrin Mac-1, and the binding of RAGE to Mac-1 is enhanced by S100 proteins but not by AGEs (Chavakis et al., 2003). The difference in the outcome between different cell types in contact with the same RAGE ligand could also arise from cell-type specific expression of coreceptors. Altogether, this reveals the high complexity of the RAGE network involved in human disease and highly suggests that ligand recognition, RAGE-ligand interaction as well as the biological significance of RAGE activation must be tightly regulated in cells.

7. CONCLUSION

Many vital physiological functions and metabolic processes are regulated by Ca^{2+} and a large number of human diseases are linked to an altered Ca^{2+} homeostasis. The Ca^{2+}-signal within cells is transmitted by many Ca^{2+}-binding proteins, including the S100 proteins family. S100 proteins have received increased attention recently because of their close association with human diseases and their use in diagnosis. S100 proteins are also considered (together with their extracellular receptor RAGE) to have a potential as drug targets to improve therapies in the future.

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CHAPTER 6

THE CALCIUM-SENSING RECEPTOR: PHYSIOLOGY, PATHOPHYSIOLOGY AND CaR-BASED THERAPEUTICS

Physiology and pathophysiology of CaR

E.M. BROWN, M.D.*

Division of Endocrinology, Diabetes and Hypertension, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, 221 Longwood Avenue, 02115 Boston, MA, USA

Abstract: The extracellular calcium (Ca_o^{2+})-sensing receptor (CaR) enables the parathyroid glands and other CaR-expressing cells to sense alterations in the level of Ca_o^{2+} and to respond with changes in function that are directed at normalizing the blood calcium concentration. In addition to the parathyroid gland, the kidney is a key site for Ca_o^{2+}-sensing that enables it to make physiologically relevant alterations in divalent cation and water metabolism. Several disorders of Ca_o^{2+}-sensing arise from inherited or acquired abnormalities that "reset" the serum calcium concentration upward or downward. Inactivating mutations produce a benign form of hypercalcemia when present in the heterozygous state, termed Familial Hypocalciuric Hypercalcemia (FHH), while homozygous mutations produce a much more severe hypercalcemic disorder resulting from marked hyperparathyroidism, called Neonatal Severe Hyperparathyroidism (NSHPT). Activating mutations cause a hypocalcemic syndrome of varying severity, termed autosomal dominant hypocalcemia or hypoparathyroidism. Inactivating or activating antibodies directed at the CaR produce the expected hyper- or hypocalcemic syndromes, respectively. "Calcimimetic" CaR activators and "calcilytic" CaR antagonists have been developed. The calcimimetics are currently in use for controlling severe hyperparathyroidism in patients receiving dialysis treatment for end stage renal disease or with parathyroid cancer. Calcilytics are being evaluated as a means of inducing a "pulse" in the circulating parathyroid hormone (PTH) concentration, which would mimic that resulting from injection of PTH, an established anabolic form of treatment for osteoporosis

Keywords: Seven transmembrane receptor, calcium-sensing receptor, calcium homeostasis, calcimimetic, calcilytic, familial hypocalciuric hypercalcemia, autosomal dominant hypoparathyroidism, acquired hypoparathyroidism, osteoporosis, hyperparathyroidism

*Edward M. Brown, M.D., Division of Endocrinology, Diabetes and Hypertension, Brigham and Women's Hospital, EBRC223A, 221 Longwood Ave., Boston, MA 02115, E-mail: embrown@partner.org

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1. INTRODUCTION

The calcium-sensing receptor (CaR) is a seven transmembrane receptor (7TM receptor–also termed G protein-coupled receptor) that is expressed widely throughout the body (Brown et al, 1993). It plays key roles in the maintenance of a nearly constant extracellular ionized calcium concentration (Ca_o^{2+}), particularly in the chief cells of the parathyroid gland. Here it regulates the synthesis and secretion of PTH as well as parathyroid cellular proliferation (Tfelt-Hansen and Brown, 2005), inhibiting all three when Ca_o^{2+} is high and stimulating them when Ca_o^{2+} is low (Tfelt-Hansen and Brown, 2005). PTH plays a central role in the acute regulation of Ca_o^{2+} and through its regulation by the CaR, maintains Ca_o^{2+} within a narrow range (1.1–1.3mM). Both very high and very low levels of Ca_o^{2+} can lead to serious clinical sequellae and in some instance can be life-threatening. Even minute alterations in Ca_o^{2+} from its normal level (e.g., of a few percent) promote immediate physiologic responses, especially reciprocal changes in PTH secretion that will normalize the level of Ca_o^{2+}. The CaR, cloned over a decade ago, is a central element in the maintenance of this delicate state of calcium homeostasis. It serves as the body's thermostat for Ca_o^{2+}, functioning as a "calciostat" and informing the parathyroid glands of the precise level of Ca_o^{2+}.

The CaR was cloned using the expression-cloning technique in Xenopus laevis oocytes (Brown et al, 1993). Analysis of its nucleotide and amino acid sequences place the CaR within family C of the superfamily of seven transmembrane, G protein-couple receptors (GPCRs). Other members of this family are the G protein -coupled, so-called metabotropic receptors for glutamate (mGluRs) and for gamma-aminobutyric acid (GABA), as well as GPCRs for sensing pheromones, taste, and odorants (in fish). Recently, another member of family C, GPRC6A, has been found to share several pharmacological properties with the CaR (Wellendorph and Brauner-Osborne, 2004; Wellendorph et al, 2005). Like the CaR, GPRC6A is sensitive towards certain L-amino acids, although unlike the CaR, which senses predominantly aromatic amino acids, GPRC6A is most responsive to basic amino acids (Wellendorph et al, 2005). Subsequent studies showed that this receptor is also activated by high concentrations of extracellular calcium (e.g., 10–20 mM) and calcimimetics (Pi et al, 2005), allosteric activators of the CaR that will be discussed later. These data have implicated GPRC6A as a second calcium-sensing receptor (CaSR2).

The physiological relevance of the CaR in humans was proven by the identification of inherited disorders caused by mutations in the receptor leading to either loss- or gain-of-function (Hendy et al, 2000). Heterozygous (e.g., the mutation is present in only one allele) gain-of-function mutations cause a form of autosomal dominant hypoparathyroidism (ADH). Heterozygous loss-of-function mutations are the cause of a disorder called familial hypocalciuric hypercalcemia—FHH, also termed familial benign hypocalciuric hypercalcemia (FBHH), which typically manifests as asymptomatic hypercalcemia with relative or absolute hypocalciuria. When present in the homozygous or compound heterozygous state, in contrast, inactivating CaR mutations produce neonatal severe primary hyperparathyroidism

(NSHPT), a severe, sometimes lethal disease if it is left untreated. Mouse models with disruption of one or both CaR genes produce biochemical and phenotypic features closely resembling those observed in FHH and NSHPT, respectively. Thus our increasing understanding of inherited disorders of calcium-sensing as well as animal models with knockout of the receptor have illuminated not only the pathophysiology but also the physiology of the CaR and is reviewed in a later section.

CaR expression is greatest in the parathyroid glands, calcitonin-secreting C-cells of the thyroid gland, and kidney, but the CaR is also found in the two other key organs that participate in calcium homeostasis: gut and bone (Brown and MacLeod, 2001). This review will focus on the structure and function of the CaR, its role in normal physiology and in various disorders of Ca_o^{2+}–sensing, and the development of CaR-based therapeutics.

2. BIOCHEMICAL AND PHYSIOLOGICAL FEATURES OF THE CaR

This section briefly introduces key aspects of the structure of the CaR and its downstream signaling pathways to provide sufficient background information to understand the molecular basis for both normal mineral ion homeostasis and for inherited diseases of the CaR. We also present an update regarding the growing number of naturally occurring and pharmacologic ligands of the receptor. The use of the latter as CaR-based therapeutics for various disorders of Ca_o^{2+}–sensing is covered in greater depth in section F, following the description of these ligands and disorders.

2.1. Structure and Signaling Pathways of the CaR

The 5.3-kb clone of the CaR isolated by expression cloning, when expressed in the oocytes, exhibited the same pharmacological properties as the Ca_o^{2+}-sensing mechanism previously characterized in dispersed bovine parathyroid cells, the prototypical calcium-sensing cell (Brown et al, 1993). The use of nucleic acid hybridization-based cloning then enabled the CaR to be cloned from humans (Garrett et al, 1995), rats (Riccardi et al, 1995), mice, rabbits (Butters et al, 1997) and, more recently, the dogfish shark (Nearing et al, 2002) and bony fish (Loretz et al, 2004). The nucleic acid sequences of the mammalian receptors are at least 85% identical to that of the original bovine parathyroid CaR. The amino acid sequences show even greater similarity [>90% identity using http://www.ncbi.nlm.nih.gov/BLAST/]. Therefore, only a limited amount of divergence from a putative primordial calcium-sensing receptor has taken place throughout evolution, and the functionally important structural features have presumably been retained.

The CaR is a member of family C II of the superfamily of seven transmembrane (7TM) receptors, also termed G protein-coupled receptors (GPCRs) (Brown and MacLeod, 2001). 7TM receptors are by far the largest group of cell surface receptors. They are very important in clinical medicine, since the 7TM receptors represent

the targets of about 50% of currently available drugs. The human CaR comprises 1078 amino acid residues and has three structural domains, as do all 7TM receptors (Figure 1). It has an unusually large extracellular domain (ECD) (612 residues), which is characteristic of the family C GPCRs; a transmembrane domain (TMD) of 250 amino acids containing the 7 membrane spanning helices, and an intracellular, C-terminal tail (ICD) of 216 amino acids (Figure 1). The receptor exhibits substantial N-linked glycosylation, which is important for the normal level of cell membrane expression of the receptor but does not appear to modify the function of the

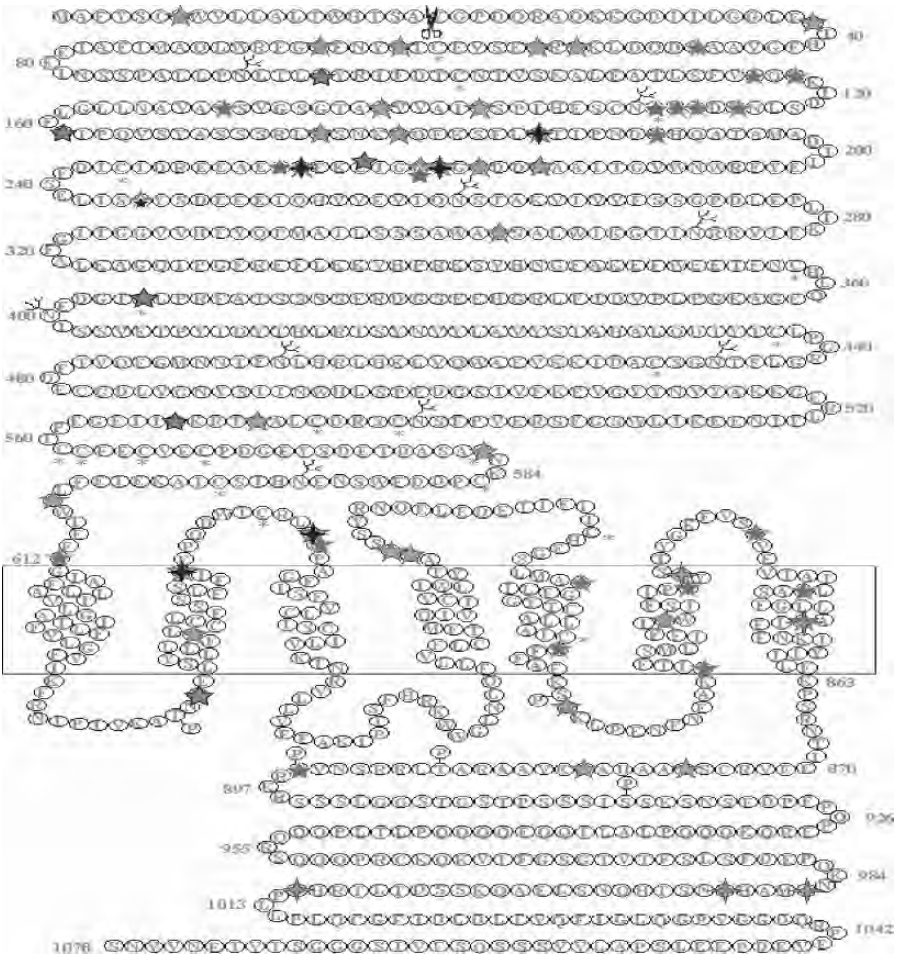

Figure 1. Topology of the CaR showing naturally occuring mutations
Scissors indicate end of signal peptide, circled P's at amino acids T646, S794, S915, S895, T888 are protein kinase C sites, blue symbols are polymorphisms, green are inactivating mutations, red are activating mutations, and black on top of green are two inactivating mutations (See Colour Plate 11) reproduced with permission from G.N. Hendy, Ph.D.

receptor per se (Rav et al, 1998). The cell surface form of the CaR is primarily a dimer, and the two monomers within the dimeric CaR are linked by disulfide bonds involving cysteine residues 129 and 131 within each monomer (Bai et al, 1998a). The location(s) of the Ca_o^{2+}-binding sites in the CaR have not been fully elucidated, but recent evidence suggests that one potential binding pocket is present within the cleft between the two lobes (Silve et al, 2005). The ECD of each CaR monomer probably contains more than one binding sites for Ca_o^{2+}, because the Hill coefficient for the activation of the receptor by Ca_o^{2+} is 3, 4; consistent with the presence of positive cooperativity amongst at least this number of binding sites within the dimeric CaR. The TMD is also apparently involved in Ca_o^{2+}-sensing, since a mutant CaR lacking the ECD also responds to Ca_o^{2+} and other polyvalent cations (Hu et al, 2005). The receptor contains five protein kinase C (PKC) phosphorylation sites (Figure 1) (Bai et al, 1998b). The PKC phosphorylation sites are part of a negative feedback system, whereby phosphorylation of one or more of the PKC sites, particularly T888, inhibits CaR-mediated activation of phospholipase C (PLC), which is a major downstream mediator of the receptor's biological responses. PLC is an important participant in CaR-mediated activation of PKC, particularly the calcium-dependent forms of the enzyme. Studies carried out prior to the cloning of the CaR demonstrated that incubation of parathyroid chief cells with high levels of Ca_o^{2+} inhibited hormone-dependent cAMP accumulation and activated PLC and, consequently, IP_3 production, documenting that the CaR activates both $G\alpha_q$ and $G\alpha_{i/o}$ (Brown, 1991). The ICD binds to the scaffolding proteins, filamin-A and caveolin-1 (anti-caveolin antibodies co-immunoprecipitate the CaR, but direct binding of caveolin-1 to the CaR has not been demonstrated) (Hjalm et al, 2001; Kifor et al, 1998); both of these proteins also bind signaling partners activated by the CaR, such as components of the MAPK pathways (see below). The interaction of the CaR with filamin-A was recently shown to protect the CaR from intracellular degradation (Zhang and Breitwieser, 2005); this finding may explain why some studies have found that the CaR exhibits limited internalization following binding to its ligands.

Studies utilizing a heterologous system of human embryonic kidney (HEK) cells with or without stably transfected CaR (HEK-CaR) have uncovered a plethora of intracellular signaling pathways regulated by the receptor (Awata et al, 2001; Brown and MacLeod, 2001). Most are also active in CaR-mediated signaling in other cells expressing the CaR endogenously. In HEK-CaR cells as well as in parathyroid cells, the CaR activates phospholipases (PL) A_2, C, and D. PLC hydrolyzes phosphatidylinositol bisphosphate to produce IP_3, which, in turn, activates the IP_3 receptor in the endoplasmic reticulum (ER), thereby releasing calcium from its internal stores within the ER; the attendant influx of calcium into the cytosol causes spikes in the cytosolic free calcium concentration (Ca_i^{2+}). An early step in the biosynthesis of polyphosphoinositides is conversion of phosphatidylinositol (PI) to phosphatidylinositol 4-phosphate (PI-4P) by phosphatidylinositol 4-kinase. The CaR stimulates phosphatidylinositol 4-kinase via $G\alpha_q$ in parallel with its concomitant activation of PLC in HEK-CaR cells (Huang et al, 2002). Another key group of intracellular

signaling pathways linked to the CaR is the mitogen-activated protein kinases (MAPKs). Activation of MAPKs takes place via phosphorylation by their respective upstream kinases. MAPKs are key intracellular signaling pathways that often produce changes in gene expression, i.e., in cell cycle regulation. But MAPKs can also regulate processes close to the cell membrane, such as the activity of potassium channels and the secretion of peptides. We have previously demonstrated that the CaR in parathyroid cells and HEK-CaR cells stimulates the activity of MAPKs (Kifor et al, 1997). Handlogten et al. also showed that the CaR activates MAPK in HEK-CaR cells; they used HEK cells stably transfected with a dominant-negative CaR (Arg796Trp) as a control (Handlogten et al, 2001). They likewise demonstrated that the CaR stimulates PLA_2 through $G\alpha_q$, PLC, calmodulin, and calmodulin-dependent kinase, but not through Ga_s or MAPK in HEK-CaR cells. The explanation(s) underlying the differing results of these two studies concerning the role of MAPK in PLA_2 activation has not yet been clarified. In cells that express the CaR at lower levels than parathyroid cells and HEK-CaR cells, including testicular cancer cells, MAPK and phosphatidylinositol 3-kinase (PI3-kinase), components of an important prosurvival pathway, have been shown to be activated by the CaR (Tfelt-Hansen et al, 2004).

2.2. Agonists of the Calcium-sensing Receptor

The CaR behaves in a promiscuous manner with regards to the considerable number of ligands that regulate it. CaR agonists are described as type I or type II (Nemeth et al, 1998b). Type I agonists are direct agonists, while type II serve as allosteric modulators, requiring the presence of calcium to stimulate the CaR; the type II modulators left-shift the calcium dose-response curve by sensitizing the receptor to this or other type I agonists. The type I ligands comprise a variety of polycations, both inorganic and organic, and their potencies generally parallel the number of positive charges that they possess. The rank order of potency for the stimulation of the CaR by various inorganic di- and trivalent cations is as follows: $Gd^{3+} \geq La^{3+} \gg Ca^{2+} = Ba^{2+} > Sr^{2+} > Mg^{2+}$ (Brown et al, 1990; Nemeth and Scarpa, 1987; Shoback et al, 1988). The best characterized type I organic polycationic CaR agonists are neomycin, spermine, and amyloid β-peptides (Brown et al, 1993; Quinn et al, 1997; Ye et al, 1997). Neomycin and gadolinium are often utilized to demonstrate that an action of Ca_o^{2+} is likely to be mediated through the CaR; however, they are far from specific for the CaR and newer, more specific approaches, such as the use of pharmacological activators or inhibitors of the receptor, dominant negative constructs or RNA silencing, offer great advantages in this regard. Slight alterations in Ca_o^{2+} (50–100 micromolar) within the physiological range *in vivo* regulate the CaR's activity, but the affinity of the receptor for its principal physiological regulator, calcium, is far lower than that of other GPCRs for their ligands. The CaR's low affinity, when considered within the context of the levels of calcium within the bodily fluids (e.g., millimolar) as well as the steepness of the curve relating Ca_o^{2+} to CaR activity, make the CaR a excellent "calciostat" for informing CaR-expressing cells of the precise level of Ca_o^{2+} within their immediate vicinity. The Hill coefficient, which provides a measure of how well the CaR responds to small alterations in the

concentrations of its agonists, is 3 to 4 in HEK-CaR cells (Bai et al, 1996), as noted above. In dispersed parathyroid cells *in vitro*, the CaR is even more sensitive to changes in Ca_o^{2+}: PTH release is maximal at 0.75 mM and fully suppressed at just below 2 mM ionized Ca_o^{2+} (Brown, 1991). HEK-CaR cells as well as other types of cells, which, in general, express the CaR at lower levels than do the chief cells of the parathyroid gland, e.g. leydig cancer cells derived from the testis, exhibit higher EC_{50} values ($\backsim$3–4 mM) (Bai et al, 1996; Sanders et al, 2000).

As noted earlier, type II agonists are allosteric modulators of the CaR, e.g., they potentiate the action of Ca_o^{2+} on the receptor. They comprise two classes: small molecule drugs and amino acids. Drugs that allosterically stimulate the CaR are called "calcimimetics" (Nemeth et al, 1998b). NPS R-467 and NPS R-568 and AMG 073 are calcimimetics that have been used in various experimental studies and clinical trials and, more recently, as treatments for the secondary hyperparathyroidism, which is not uncommonly encountered in patients with end stage renal disease receiving dialysis therapy (Block et al, 2004; Goodman et al, 2002). AMG 073 (also called cinacalcet or sensipar) is currently the drug of choice, because NPS R-467 and NPS R-568 are degraded by a cytochrome P-450 enzyme, CYP2D6 (Amgen, unpublished data). Five to seven percent of the general population expresses CYP2D6, which has reduced enzymatic activity, thereby resulting in higher blood levels and delayed metabolic clearance in this segment of the population. Calcimimetics interact with the CaR's TMD and in some manner increase the apparent affinity of the receptor for calcium. More details on CaR-based therapeutics and their application to human disease are given in Section F.

Some L-amino acids also act as type II agonists, in contrast to the respective D-amino acids, which are several-fold less potent in activating the receptor (Conigrave et al, 2000). This CaR's ability to be activated by both extracellular calcium and L-amino acids may permit it to sense nutrients in the gut, for example. Available data indicate that amino acids bind to the ECD of the receptor, interacting with a binding pocket homologous to those binding GABA and glutamate in the $GABA_B$ receptors and mGluRs, respectively (Silve et al, 2005; Zhang et al, 2002). This may be pharmacologically important, since L-phenylalanine and the calcimimetic NPS R-467 have synergistic actions in stimulating the CaR. Finally, calcilytics represent another type of pharmacological agent acting on the CaR, as described in more detail in Section F. They antagonize the action of Ca_o^{2+} on the receptor, and are being studied for use in the treatment of osteoporosis, as they stimulate a pulse of endogenous PTH secretion, which has the potential to exert an anabolic action on bone, similar to that of once daily injected PTH (Gowen et al, 2000).

2.3. Physiology

It is crucial for the body that Ca_o^{2+} is maintained within a narrow range (1.1–1.3 mM). Both very low and very high levels of Ca_o^{2+} can be dangerous and even life-threatening. Even very small alterations in the level of Ca_o^{2+}, on the order of

a few percent, produce immediate physiologic responses, which include alterations in PTH secretion that restore Ca_o^{2+} to its normal level. Rapid alterations in Ca_o^{2+} are more hazardous than slowly developing ones; thus the rapidity of the PTH response is essential as a homeostatic defense against both hypo- and hypercalcemia.

Parathyroid hormone (PTH), calcitonin, and $1,25(OH)_2D_3$ are the three most important Ca_o^{2+}-regulating hormones (Bringhurst et al, 1998). As noted earlier, there is a functionally critical inverse relationship between Ca_o^{2+} and PTH, a calcium-elevating hormone. This relationship is mediated by the CaR (Ho et al, 1995; Schwarz et al, 1993). In contrast, high Ca_o^{2+} stimulates the secretion of calcitonin (CT), a Ca_o^{2+}-lowering hormone; this action of calcium on CT secretion is likewise mediated by the CaR (Fudge and Kovacs, 2004). The rapidity with which the secretion of PTH and CT respond to changes in extracellular calcium usually normalizes Ca_o^{2+} within minutes to hours. Available data have demonstrated that the CaR is expressed not only in the organs that secrete calcium-regulating hormones (e.g., the parathyroid glands and C-cells of the thyroid glands), but also in target tissues for these hormones. These latter tissues regulate Ca_o^{2+} by translocating calcium ions into or out of the bodily fluids, and include the kidney, which expresses the CaR at robust levels in certain nephron segments, as well as bone and intestine, which express the receptor at lower levels (see below). By acting on both hormone-secreting and hormone-responsive tissues through its own cell surface receptor, Ca_o^{2+} acts in effect, as another Ca_o^{2+}-regulating "hormone" (in this case Ca_o^{2+}–lowering) or "first messenger". Elevations in the extracellular ionized calcium concentration stimulate the CaR and lower Ca_o^{2+} by enhancing CT secretion, promoting urinary calcium excretion and suppressing PTH release. In the remainder of this section, we will provide a brief discussion of the CaR's known and putative roles in parathyroid gland, kidney and bone, three out of the four main organs involved in calcium homeostasis. For a discussion of the CaR's localization and potential roles along the gastrointestinal tract, see (Hebert et al, 2004).

2.3.1. Parathyroid (PT) glands

The most important function of the CaR in minute-to-minute Ca_o^{2+} homeostasis lies in the CaR-mediated inhibition of PTH secretion. The steep inverse sigmoidal curve relating Ca_o^{2+} and PTH release was described long before the CaR was cloned. Studies utilizing dispersed parathyroid cells to investigate Ca_o^{2+}–regulated PTH release are mainly limited to those performed within a few hours of isolating the cells, because these cells, particularly bovine parathyroid cells, exhibit a marked, loss of their expression of the CaR on the cell membrane over 24–48 hours (Brown et al, 1995; Mithal et al, 1995). The receptor is normally expressed at high levels on the surface of the parathyroid chief cells. The principal functions of the CaR in the parathyroid are shown in Table 1. Relatively little is known about the control of its expression on the cell surface, but of note, the expression of the CaR in rat parathyroid and kidney was increased by $1,25(OH)_2D_3$, while Ca_o^{2+} had no effect (Brown et al, 1996). In more recent studies, however, raising Ca_o^{2+} increased CaR expression in avian parathyroid gland (Yarden et al, 2000), and a

Table 1. Key Roles of CaR in Parathyroid and Kidney

- Parathyroid
  (1) Inhibit PTH secretion
  (2) Inhibit PTH gene expression
  (3) Inhibit parathyroid cellular proliferation
- Kidney
  (1) Proximal tubule—blunt PTH-induced phosphaturia
  (2) MTAL—inhibit NaCl reabsorption
  (3) CTAL—inhibit reabsorption of Ca^{2+} and Mg^{2+}
  (4) IMCD—inhibit vasopressin-elicited water reabsorption

calcimimetic elevated the expression of the receptor in pathological parathyroid glands (Mizobuchi et al, 2004). The upregulation of the receptor following its activation could clearly serve as a positive feedback loop contributing to CaR-mediated actions in the parathyroid gland. Of note in this regard, Ca_o^{2+} also regulates the expression of the vitamin D receptor (VDR) (Bajwa et al, 2005), which could potentiate the action of vitamin D on the level of CaR expression and also enhance the biological actions of the CaR. Another case where there is upregulation of parathyroid CaR expression is in sheep subjected to burn injury, which was accompanied by a decrease in set-point for suppression of PTH secretion by calcium (Murphey et al, 2000). This increase in CaR expression and the associated change in Ca_o^{2+}–regulated PTH secretion may be a factor that contributes to the hypocalcemia and relative hypoparathyroidism in human burn patients. It might also participate in the hypocalcemia accompanied by inappropriately normal PTH levels, which can be observed in severely ill patients with inflammatory states.

In bovine parathyroid cells, the CaR is located on the cell membrane in caveolin-1-containing rich membrane domains (Kifor et al, 1998) that are called caveolae and can serve as "message centers" where several different types of signaling molecules are concentrated. The signaling systems downstream of the CaR that contribute to the regulation of PTH secretion are not known with certainty. However, a recent report showed that knockout of both G_q and G_{11} in mice resulted in biochemical features similar to those caused by homozygous knockout of the CaR in mice and humans, suggesting a critical role for these G proteins in CaR-induced suppression of PTH secretion (Wettschureck et al, 2006). Another report showed that activation of the CaR increases ERK1/2 activity via the PKC pathway and, to a lesser extent, the PI-3K pathway, in normal parathyroid cells. Thus, while more work is needed, a pathway comprising CaR $\rightarrow$ $G_{q/11}$ $\rightarrow$ PLC $\rightarrow$ PKC $\rightarrow$ ERK1/2 may participate in the modulation of PTH secretion by Ca_o^{2+}.

2.3.2. *Expression, function and regulation of the CaR in the kidney*

The kidney plays several critical roles in calcium homeostasis. The CaR is widely expressed along essentially the whole nephron. The cellular localization and putative function(s) of the CaR in the kidney seem to depend upon the region of the nephron in which the receptor resides (Ward and Riccardi, 2002). The CaR's

expression along the nephron has been studied using *in situ* hybridization as well as reverse transcriptase-polymerase chain reaction (RT-PCR) of micro-dissected nephron segments (Riccardi et al, 1996). Later, the cellular localization and regional distribution of receptor protein along the nephron was examined using immunofluorescence (Riccardi et al, 1998). One outcome of these studies was the recognition that the polarity of CaR protein varies along the nephron. In the proximal tubule the receptor is present on the apical surface of the proximal tubular epithelial cells. On the contrary, in the cells of the cortical thick ascending limb (CTAL), the receptor is localized in the basolateral membrane. Similarly, basolateral staining for the CaR was observed in the medullary thick ascending limb (MTAL), macula densa, and the distal convoluted tubule (DCT). In the cortical collecting duct, immunostaining for the CaR is located on some intercalated cells, while in the inner medullary collecting duct (IMCD) the receptor has primarily an apical distribution.

There has been relatively little work characterizing the factors that regulate the CaR's expression in the kidney. A recent report demonstrated that in rat kidney, C-cell and parathyroid *in vivo* as well as in a human proximal tubule cell line *in vitro*, transcription of the CaR gene was increased about two-fold following 8 and 12 h of treatment with $1,25(OH)_2D_3$ (Canaff and Hendy, 2002), acting via two promoters that lie upstream of the CaR gene. A low phosphate diet had no effect on CaR expression along the nephron in one study (Caride et al, 1998), while Riccardi et al. demonstrated *in vivo* in rats that a low phosphate diet as well as treatment with PTH downregulated CaR protein in the proximal tubule (Riccardi et al, 2000). Thus CaR expression in the proximal tubule of the rat kidney is modulated by $1,25(OH)_2D_3$ and PTH and, perhaps, by dietary phosphate. One other study on the regulation of the CaR's expression along the nephron demonstrated that the level of CaR protein in purified apical endosomes isolated from IMCD, which also contain aquaporin-2, the water channel that traffics to the apical membrane in response to vasopressin and enhances water reabsorption, was reduced in rats rendered hypercalcemic by vitamin D administration (Sands et al, 1998). To summarize, the CaR's roles along the nephron include: 1) diminishing the inhibitory effect of PTH on renal phosphate reabsorption in the proximal tubule (Ba et al, 2003); 2) inhibiting renal tubular reabsorption of calcium in the CTAL (Motoyama and Friedman, 2002); and 3) reducing urinary concentrating ability in the IMCD by antagonizing the action of vasopressin (Sands et al, 1997).

2.3.3. Bone

Abundant data indicate that Ca_o^{2+} inhibits the formation and activity of osteoclasts and stimulates the activity of osteoblasts. The first evidence for the existence of a G protein-coupled, cation-sensing mechanism in osteoblasts was presented shortly after the cloning of the CaR (Quarles et al, 1994). Since then some, but not all studies have found that the CaR is expressed in various osteoblastic cell lines and primary osteoblasts (Chang et al, 1999; Chattopadhyay et al, 2004;

Pi et al, 1999). A interesting study demonstrated that osteoblasts from CaR knockout mice still had a promitogenic response to Ca_o^{2+}, supporting the presence of a calcium-sensing mechanism other than the full length CaR. This mechanism could potentially be represented by the newly cloned GPRC6A, although the latter is responsive to calcimimetics, and the actions of extracellular calcium on the CaR knockout osteoblasts were not (Pi et al, 2005). The CaR is also present in articular and hypertrophic chondrocytes (Chang et al, 1999). Utilizing a type II CaR agonist in organ culture (fetal rat metatarsal bones) to study the possible role of the CaR in bone growth, Wu et al. (Wu et al, 2004) demonstrated that the receptor modulates chondrogenesis in the growth plate and enhances longitudinal bone growth.

The CaR is expressed by some osteoclasts (Kanatani et al, 1999) and by monocytes, which are of the same lineage as osteoclast precursors (Yamaguchi et al, 2000). In addition to inhibiting the formation and activity of osteoclasts, high extracellular calcium has been shown to promote osteoclast apoptosis (Lorget et al, 2000). However, the calcimimetic, AMG 073, produced none of the actions of elevated extracellular calcium on osteoblast proliferation or osteoclast formation and resorption in one study (Shalhoub et al, 2003). One possible mechanism that has been suggested to mediate calcium-sensing in osteoclasts is a plasma membrane, ryanodine-like receptor that couples to increases in the intracellular calcium concentration (Zaidi et al, 1999). Therefore, although the CaR and other calcium-sensing mechanisms may participate in the regulation of bone cell and cartilage function, further studies are clearly required to clarify the divergent results observed in the studies to date.

3. DISORDERS OF CALCIUM-SENSING THAT INVOLVE THE CaR

3.1. Clinical and Genetic Features of Familial Hypocalciuric Hypercalcemia (FHH) [OMIM 14598]

The principal disorders of extracellular calcium sensing are listed in Table 2. FHH is typically a benign form of hypercalcemia (Law Jr. and Heath III, 1985; Marx et al, 1981a). The diagnosis of FHH can be made in a patient with mild-to-moderate, PTH-dependent hypercalcemia averaging approximately 2.75 mM (total calcium), an autosomal dominant pattern of inheritance of a similar degree of hypercalcemia on family screening, and an inappropriately reduced rate of urinary calcium excretion in the face of hypercalcemia. Several families, however, have been identified with more marked hypercalcemia, averaging 3 and 3.4 mM. Several affected neonates from some of these kindreds have manifested a neonatal severe hyperparathyroid-like state (Bai et al, 1997). It is thought that the mutant receptors harboring these mutations may in some cases exert a dominant negative action on the wild type partner in mutant-wild type heterodimers.

Because the disorder is benign in most cases, patients with FHH are frequently not diagnosed until a routine measurement of the blood calcium concentration shows

Table 2. Disorders of Calcium-Sensing

- Conditions with reduced sensitivity to Ca_o^{2+}
  (1) FHH/NSHPT
  (2) Primary hyperparathyroidism
  (3) Inactivating antibodies to the CaR
- Conditions with increased sensitivity to Ca_o^{2+}
  (1) Autosomal dominant hypocalcemia
  (2) Activating antibodies to the CaR

an unexpectedly high value, or family screening is carried out owing to the birth of a child with NSHPT (Marx et al, 1982). Patients with FHH commonly have normal serum levels of PTH despite their hypercalcemia, although in about 15–20% of cases, PTH levels are frankly elevated (Heath, 1989b).

The hypercalcemia in FHH, when viewed in the context of a normal level of PTH that is inappropriately high for that serum calcium concentration, reflects the presence of a right-shifted set-point for Ca_o^{2+}-regulated PTH release (Auwerx et al, 1984). An additional important finding is the fact that there is typically a normal or even frankly reduced urinary calcium excretion in spite of the coexistent hypercalcemia (Marx et al, 1981a). This alteration in renal calcium handling reflects "resistance" of the kidney to the usual hypercalciuric action of hypercalcemia and is the equivalent of the resistance of PTH secretion to the normal inhibitory effect of high calcium in FHH. Of note, administration of a loop diuretic (e.g., ethacrynic acid) promotes renal excretion of calcium in hypoparathyroid subjects with FHH (Attie et al, 1983). This observation points towards a key role of the thick ascending limb—the site where this class of diuretics acts–in the anomalous renal calcium handling in FHH.

Short of carrying out mutational analysis, the most useful means of distinguishing FHH from other forms of hypercalcemia, particularly primary hyperparathyroidism, is to determine the ratio of the renal clearance of calcium to that of creatinine (Ca/Cr). A value less than 0.01 is found in about 80% of individuals with FHH, while a similar proportion of cases of primary hyperparathyroidism have levels higher than this (Fuleihan et al., 2002). Another biochemical finding in patients with FHH is their capacity to concentrate their urine normally, in contrast to patients with primary hyperparathyroidism, in whom maximal urinary concentration elicited by dehydration is reduced (Marx et al, 1981b). While this finding is not used diagnostically, it likely reflects renal resistance of FHH patients to the hypercalcemia-induced diminution in urinary concentrating ability that is observed in other forms of hypercalcemia. Individuals with FHH usually manifest serum magnesium concentrations that are in the upper normal range or mildly elevated. Finally, while differentiating FHH from primary hyperparathyroidism is usually straightforward, a recent study of the genetic basis for familial isolated hyperparathyroidism showed that four of 22 unrelated probands harbored inactivating mutations of the CaR (Warner et al, 2004). Therefore the clinician should bear in mind that FHH can be an underdiagnosed but important cause of familial isolated hyperparathyroidism (Simonds et al, 2002;

Warner et al, 2004). Moreover this study points out that there can be overlap in the clinical presentations of FHH and primary hyperparathyroidism, particularly in familial forms of the latter.

Additional FHH families have also highlighted the existence of atypical presentations of this condition that initially led to a diagnosis of primary hyperparathyroidism. One such kindred manifested hypercalcemia, high PTH levels, hypercalciuria and even renal stone formation, but was then shown to harbor an inactivating FHH mutation (Carling et al, 2000). Subtotal parathyroidectomy in most affected family members provided long-term remission of their biochemical abnormalities, demonstrating that parathyroid surgery, while typically ineffective in curing hypercalcemia in FHH, may be appropriate in an occasional kindred. Recent studies have described the presence of single and multiple parathyroid "adenomas" in several patients with FHH, although the parathyroid glands in FHH typically are of normal size and histology, or in some cases exhibit mild chief cell hyperplasia with one or more enlarged glands (Burski et al, 2002; Carling et al, 2000). A few cases of parathyroid lipohyperplasia have been described in FHH, and some authors have suggested that finding should prompt a search for inactivating mutations of CaR (Fukumoto et al, 2001).

While FHH most commonly presents as an asymptomatic form of hypercalcemia, a few kindreds exhibit more severe hypercalcemia. Nevertheless, even in these cases the natural history of the disorder is usually so benign that the great majority of these patients should be followed without intervention, with a few exceptions (see above). In the unusual individual with FHH and symptomatic hypercalcemia, the new calcimimetics–described in more detail below—could potentially provide a useful form of treatment; indeed the use of this treatment was recently described in this setting.

The mutations in the CaR gene in FHH or NSHPT cause a loss-of-function of the CaR that result in a rightward shift in the set-point for Ca_o^{2+}-regulated PTH secretion. In 1972, Foley et al. (Foley Jr et al, 1972) first described the characteristic clinical features of the hereditary condition now known as familial hypocalciuric hypercalcemia (FHH) (it was initially called familial benign hypercalcemia). Nearly two decades later, linkage analysis showed that the predominant locus of the FHH disease gene resided on the long arm of chromosome 3 (band q21–24) in four large FHH families (Chou et al, 1992). However, FHH is not always linked to chromosome 3q. Notably, two families with a clinical features similar to FHH showed linkage to the short and long arms of chromosome 19 (Heath et al, 1993; Lloyd et al, 1999), respectively; one of these was called the Oklahoma variant and exhibited a tendency for the biochemical abnormalities to be progressive with time (Lloyd et al, 1999). FHH that is linked to these latter two loci may be present in a minority of the $\backsim$30% of FHH cases without an identifiable mutation in the CaR gene. The remaining cases of FHH without an identifiable mutation presumably harbor mutations in regulatory regions of the CaR gene that control its expression, but this remains to be shown directly.

Since the initial discovery of three unique missense mutations in the CaR gene in 1993 in the families with FHH (Pollak et al, 1993), more than 150 additional mutations have been described. Most are unique to individual families, although a few apparently unrelated kindreds have identical mutations (e.g., gly552arg) (see web site http://www.casrdb.mcgill.ca). Most are missense mutations and reside in the first half of the ECD or within the TMD of the receptor (Hauache, 2001). However, truncation, insertion, deletion and splice site mutations have also been described (Carling et al, 2000; D'Souza-Li et al, 1998; Hendy et al, 2000; Janicic et al, 1995). An unusual type of mutation was described in an FHH family that exhibited a mutation in the acceptor splice site at position-1 of intron 2 of the CaR gene, which resulted in a frame shift and a truncated protein of 153 amino acids (D'Souza-Li et al, 1998). Although the mRNA for the receptor was stable, the truncated protein, lacking any of the transmembrane domain, was never found on the cell membrane. Individual mutations are associated with distinct phenotypes: In some instances, a mutant receptor exerts a dominant-negative action on the remaining wild type receptor within mutant-wild type heterodimers, as noted above, thereby rendering the phenotype more severe (Bai et al, 1997). Such a dominant-negative action presumably reflects the specific properties of the mutant receptor protein residing on the cell surface. In the case of mutant receptors exerting a dominant negative action, normally functioning, wild type CaR homodimers would only represent about one-fourth of all the cell surface receptors (reflecting the theoretically expected ratio of 1:2:1 of wild type homodimer, wild-type-mutant heterodimer, and mutant homodimers, respectively). Truncations in the CaR gene, in contrast, may simply reduce the number of normally functioning CaRs expressed on the cell surface, producing so-called haploinsufficiency, which results from a decrease in receptor expression/activity due to loss of one CaR allele, analogous to mice heterozygous for knockout of the CaR gene (Ho et al, 1995), where levels of the CaR protein are about 50% of normal. It should be kept in mind while evaluating the literature describing in vitro characterization of mutant CaRs that the cells used for these experiments differ substantially from the chief cells of the parathyroid glands or CaR-expressing kidney cells. Thus the experimental data likely represent only an approximation of how the receptors would function in their native environment.

3.2. Clinical and Genetic Features of Neonatal Severe Primary Hyperparathyroidism (NSHPT) [OMIM 239200]

NSHPT in most cases presents within the first six months of life. Affected infants have severe, symptomatic, PTH-dependent hypercalcemia, along with the bony changes of severe hyperparathyroidism. Infants with NSHPT can exhibit polyuria, dehydration, hypotonia, and failure to thrive (Brown et al, 1997; Eftekhari and Yousefzadeh, 1982; Grantmyre, 1973; Heath, 1989a; Marx et al, 1985). A prominent feature of the disease is the associated hyperparathyroid bone disease, which can be associated with multiple fractures. Rib fractures can in some cases produce a

"flail chest" syndrome that causes respiratory difficulties, owing to a decreased capacity of the affected infant to expand its chest wall and generate the negative intrathoracic pressure needed for normal respiration (Grantmyre, 1973).

The mass of the parathyroid glands in NSHPT is generally increased several-fold, and they exhibit prominent chief cell hyperplasia. Biochemical evaluation shows hypercalcemia, hyperparathyroidism, and relative hypocalciuria (Cole et al, 1997). Total serum calcium concentrations range from moderately elevated (e.g., 3–3.25 mM) to levels as high as 7.7 mM in the most severely affected cases (Brown, 2000; Heath, 1989a). PTH levels are often 10-fold higher than the upper limit of normal. Early diagnosis is critical, as untreated NSHPT can be a devastating neurodevelopmental disorder, which in some cases is lethal without parathyroidectomy to alleviate the hyperparathyroidism and hypercalcemia (Cole et al, 1997). As noted earlier, the most severe cases of NSHPT develop ribcage deformities, as well as rachitic changes, skeletal undermineralization, and fractures of the long bones and other skeletal sites (Eftekhari and Yousefzadeh, 1982; Grantmyre, 1973).

The older literature on NSHPT (e.g., prior to 20 years ago) describes substantial mortality in infants with NSHPT; therefore, treatment for the condition in its severe form has traditionally been total parathyroidectomy (Eftekhari and Yousefzadeh, 1982). However, more recently, a broader clinical spectrum for NSHPT has become apparent; particularly given the availability of genetic testing of the CaR gene. As a result a number of studies have now shown that some infants have milder hyperparathyroidism and a substantially milder clinical presentation and natural history (Heath, 1989a; Pearce et al, 1995b). This latter form of the disease might better be termed neonatal hyperparathyroidism (NHPT), to emphasize this milder phenotype in these infants, most of whom harbor heterozygous inactivating CaR mutations. In these latter cases, the condition can revert with time to a phenotype resembling FHH with medical management alone (Heath, 1989a). Therefore, at the moment parathyroidectomy should be reserved for the most severely affected infants, in whom intensive medical therapy (e.g., with aggressive hydration and, if appropriate, bisphosphonates) has failed to stabilize the patient, and there is concern for the infant's survival.

Recent reports have described patients with homozygous mutations in the CaR gene, who escaped detection until adulthood, at which time they did not have the usual symptoms and signs of hypercalcemia and were only identified serendipitously by routine biochemical screening. One such patient, a 35-year-old woman had two copies of the missense mutation pro39ala from related parents. She was asymptomatic, despite a serum calcium concentration of 3.75 to 4.25 mM (Aida et al, 1995). Another such patient, who was homozygous for a distinct inactivating CaR mutation was likewise not diagnosed until adulthood (Fukumoto et al, 2001). Both mutations produced relatively mild defects of their function when expressed heterologously, perhaps enabling a sufficient of control PTH release by calcium to be compatible with a relatively normal life, despite quite marked hypercalcemia. Indeed the seeming lack of hypercalcemic symptoms in the face of moderate to severe hypercalcemia supports the notion that at least some of these symptoms

are mediated by the CaR. That is, these patients seem to be resistant not only to the effects of calcium on parathyroid and kidney but also to the development of hypercalcemic symptoms. In these patients a calcimimetic might represent a means of lowering the serum calcium concentration–assuming the mutant CaRs were responsive to the drug–thereby providing not only a diagnostic test to determine whether the patient obtained any symptomatic benefit from parathyroidectomy but also, potentially, an effective long term, medical therapy.

NSHPT is most commonly an autosomal recessive condition; that is, the CaR genes from both of the parents is mutated (e.g., homozygous FHH). Pollak et al. studied 11 kindreds with FHH, in whom consanguineous unions engendered four infants with NSHPT (Pollak et al, 1994b). It should be recognized, however, that NSHPT is quite uncommon in FHH families considered as a whole. In one case of NSHPT, two distinct mutations—one a mutation in exon 7 from the mother and the other a mutation in exon 4 from the father—caused the disease, e.g., as a result of the compound heterozygosity in the proband, who thereby lacked any normal CaRs (Kobayashi et al, 1997). In theory, NSHPT can result from (1) homozygosity from a consanguineous FHH union, (2) two mutant alleles of the CaR gene arising from two distinct FHH kindreds or (3) from a de novo mutational event, with or without an inherited, mutant parental allele (Pearce et al, 1995b). In addition, an investigation of a girl with phenotypic NSHPT and her family revealed a single mutant allele (present in exon 6, Gly552Arg) in her CaR gene, while her sister, despite having the same genotype, had phenotypic FHH (Schwarz et al, 2000). Thus factors leading to this degree of phenotypic variation are still only partly understood.

3.3. Clinical and Genetic Features of Autosomal Dominant Hypoparathyroidism (OMIM) [#601298]

Patients with this inherited form of hypocalcemia/hypoparathyroidism are commonly asymptomatic, similar to the majority of patients with FHH. Some patients, however, can exhibit neuromuscular irritability, seizures and basal ganglia calcification. Patients generally exhibit mild to moderate hypocalcemia, with serum PTH levels that are inappropriately low given the hypocalcemia, e.g., within the lower half of the normal range or frankly subnormal (Pollak et al, 1994a). Affected individuals often exhibit relative or absolute hypercalciuria, with normal or frankly elevated urinary calcium excretion, respectively, in spite of their low serum calcium concentration. Some studies have shown that renal calcium excretion in ADH is higher than that in typical hypoparathyroidism. However, not all studies have shown this difference in renal calcium excretion (Okazaki et al, 1999; Yamamoto et al, 2000). During febrile episodes patients, particularly children, with ADH may present with symptoms of hypocalcemia and, in some cases, seizures. It is important to prevent renal complications, including nephrocalcinosis, nephrolithiasis, and renal impairment, during treatment of ADH patients with calcium and vitamin D (Pearce et al, 1995a). These renal complications are generally seen in a setting in which

the clinician has tried to correct the serum calcium concentration to or close to the normal range. Treatment with calcium supplements and vitamin D metabolites should be reserved for those patients with symptomatic ADH; the goal should be to increase the serum calcium concentration only to a level sufficient to render the patient asymptomatic (Lienhardt et al, 2001). Renal excretion of calcium requires monitoring in treated patients in order to minimize the risk of urinary complications. If a serum calcium concentration high enough to ameliorate symptoms cannot be achieved with calcium and vitamin D supplementation without inducing frank hypercalciuria (generally 4 mg/kg/24h), it may be necessary to co-administer a hypocalciuric agent, such as a thiazide diuretic or injectable PTH (Winer et al, 1998).

ADH is a rare syndrome, although in index cases it may comprise a sizeable fraction of cases of idiopathic hypoparathyroidism, perhaps representing as many as a third of such cases (Lienhardt et al, 2001). Patients with this condition harbor an activating or gain-of-function mutation of the CaR gene that resets the set-point of Ca_o^{2+}-regulated PTH secretion leftward and lowers renal calcium reabsorption. Within a year after the cloning of the CaR, Finegold et al. (1994) showed linkage of ADH to a locus on chromosome 3 q13—the same locus containing the gene for the CaR. Shortly afterward, a heterozygous missense mutation, Glu127Ala, was shown to be the cause of ADH in an unrelated family (Pollak et al, 1994a). Since these first reports, more than 30 mutations have been characterized causing ADH (see CaR Database at **http://www.casrdb.mcgill.ca/**). Most of these are missense mutations within the CaR's ECD and TMD. When expressed in heterologous systems, these mutations cause a left-shift in the activation of the CaR by Ca_o^{2+}, and they only rarely induce constitutive activation of the receptor (Baron et al, 1996; D'Souza-Li et al, 2002; Hauache, 2001; Pearce et al, 1996; Pollak et al, 1994a). A recent report identified a family with a large deletion of 181 amino acids within the C-terminus of the CaR, which increased the sensitivity of the receptor for Ca_o^{2+} (Lienhardt et al, 2000). This family contained the only individual to date known to be homozygous for an activating mutation, but this individual exhibited a phenotype very similar to that of the heterozygous family members. Thus one mutated allele may be enough to induce a maximal shift in the set-point of Ca_o^{2+}-regulated PTH secretion, and the presence of the second mutated allele does not alter the biochemical properties of the receptor dimers any further, perhaps due to a "dominant positive" effect of the mutant receptor on its wild type partner within the heterodimeric CaR. Another activating mutation of the CaR changed a cysteine at amino acid 129 to a serine (Cys129Ser) (Hauache, 2001; Hirai et al, 2001). Because this cysteine participates in dimerization of the CaR, this result suggests that this cysteine constrains the receptor in its inactive state.

4. AUTOIMMUNE DISEASES

Recent studies have identified autoimmune, acquired forms of hypo- and hypercalcemia analogous to ADH and FHH, respectively, but which result from activating and inactivating antibodies, respectively, rather than activating and inactivating mutations.

Although these conditions are rare, they are important to be aware of in considering the differential diagnosis of the inherited diseases of calcium homeostasis just described.

4.1. Anti-CaR Antibodies and PTH-Dependent Hypercalcemia

Kifor et al. (2003) found autoantibodies to the CaR in four patients who had a clinical picture resembling that of FHH in the setting of other autoimmune conditions (e.g., Hashimoto's thyroiditis and sprue). The patients' sera stimulated PTH secretion and inhibited high calcium-stimulated inositol phosphate accumulation and MAPK activation, presumably owing to antibody-mediated inhibition of the CaR. Further studies of a larger number of patients are required to determine the incidence of autoimmune, PTH-dependent hypocalciuric hypercalcemia in the presence of various types of autoimmunity.

4.2. Anti-CaR Antibodies and Hypoparathyroidism

Idiopathic hypoparathyroidism is a condition with hypocalcemia caused by insufficient PTH secretion to maintain normocalcemia that is of unknown cause (e.g., hypomagnesemia or prior neck surgery). Blizzard et al. reported the presence of autoantibodies to the parathyroid glands in the sera of patients with idiopathic hypoparathyroidism in 1966 (Blizzard et al, 1966). They showed that 38% of 74 patients with idiopathic hypoparathyroidism had demonstrable anti-parathyroid antibodies, compared with only 6% of 245 healthy controls, thereby establishing the existence of parathyroid autoimmunity. A later study showed that autoantibodies to the parathyroid glands in patients with sporadic, adult-onset hypoparathyroidism bound to the cell surface of dispersed human parathyroid cells and inhibited PTH release (Posillico et al, 1986). This result supported the presence of a cell surface moiety that participated in regulating PTH secretion, which in retrospect may have been the CaR. Li et al. (Li et al, 1996) more recently reported that 14 of 25 patients with autoimmune hyperparathyroidism had antibodies directed at the CaR. In contrast, none of 50 control patients with other autoimmune diseases and 22 normal subjects had antibodies to the receptor. Recently, Kifor at al. reported two patients with hypoparathyroidism and anti-CaR antibodies that activated the receptor as assessed by simulation of MAPK activity and inositol phosphate accumulation and inhibition of PTH release (Kifor et al, 2004). Two further studies have shown conflicting results concerning the presence of anti-CaR antibodies in patients presenting with autoimmune hypoparathyroidism. One study examined 90 patients with autoimmune polyendocrine syndrome type 1 and found no anti-CaR autoantibodies (Soderbergh et al, 2004). Another study evaluated 51 patients with idiopathic hypoparathyroidism–most with only hypoparathyroidism–and 45 healthy controls; Forty nine percent of the patients had serologic evidence of anti-CaR antibodies (Goswami et al, 2004), and there was an association between antibodies to the CaR and HLA-DR, suggesting an autoimmune component to

the disease. Of note, the two patients in the study of Kifor et al. with activating autoantibodies to the CaR also had Graves' disease and Addison's disease, respectively, further supporting the hypothesis of an autoimmune disease. It remains to be determined whether the differences in the results of these two studies reflect the fact that the incidence of anti-CaR antibodies in patients with type 1 APS and those with isolated hypoparathyroidism differs or is due to other factors.

5. CaR-BASED THERAPEUTICS

The development of allosteric activators ("calcimimetics") (Nemeth et al, 1998b) and antagonists ("calcilytics") (Gowen et al, 2000) of the CaR has made possible CaR-based therapy of disorders of extracellular calcium homeostasis (Table 3). AMG073 (known as Cinacalcet hydrochloride or Sensipar) has recently been approved by the Food and Drug Administration (FDA) for use in treating secondary hyperparathyroidism in patients receiving dialysis therapy for end stage kidney disease (Block et al, 2004) as well as in parathyroid cancer (see website: www.amgen.com). The drug also shows efficacy in mild primary hyperparathyroidism, as described below, but has not yet received FDA approval for this indication (Peacock et al, 2005).

Beneficial effects of calcimimetics in treating 2^o HPT were first reported in animal models in which this condition was induced by subtotal nephrectomy. In these studies parathyroidectomy or NPS R-568 slowed the progression of renal failure and decreased heart-risk factors (Ogata et al, 2003). In both these animal models and *in vitro*, the calcimimetics decreased circulating PTH levels (Fox et al, 1999; Roussanne et al, 2001; Wada et al, 1998; Wada et al, 2000). It is noteworthy that NPS R-568 suppressed not only PTH release but also parathyroid cellular growth in rats with experimentally induced renal insufficiency that were receiving a normal-phosphate diet (Wada et al, 2000), indicating that the receptor regulates parathyroid growth as well as secretion. This study found no induction of parathyroid cell apoptosis.

Table 3. Use of CaR-Based Therapeutics

- Calcimimetics
  - (1) Approved by FDA
    - (a) Secondary hyperparathyroidism in dialysis patients
    - (b) parathyroid cancer
  - (2) Not yet approved
    - (a) Primary hyperparathyroidism
    - (b) Possibly FHH/NSHPT or inactivating CaR antibodies
- Calcilytics
  - (1) Not yet FDA-approved
    - (a) Osteoporosis
    - (b) Possibly activating CaR mutations or antibodies

Several studies have examined the efficacy of calcimimetics in treating 2^o HPT in human subjects on dialysis treatment (Antonsen et al, 1998; Block et al, 2004; Goodman et al, 2002; Ohashi et al, 2004). These studies demonstrated that both NPS R-568 and cinacalcet are effectively in lowering serum levels of PTH as well as serum total and ionized calcium concentrations, and, in studies of long enough duration, the calcium-phosphate product (e.g., the product of the calcium and phosphorous concentrations in the serum, which provide an index of the risk of pathological calcification). Because the calcimimetic-induced decrease in serum PTH at times induces hypocalcemia, it may be necessary to administer an active analogue of vitamin D along with the calcimimetic. The utility of an analogue of vitamin D in this setting makes physiological sense as a replacement for the decreased synthesis of $1,25(OH)_2D_3$ by the damaged kidneys.

In a recent 26 week multicenter study of 741 patients with end stage renal disease receiving hemodialysis, administration of cinacalcet reduced mean PTH concentration by 43% in patients, while PTH increased by 9% in the placebo group (Block et al, 2004). As has been observed in other clinical trials, the only consistent adverse effect in cinacalcet-treated subjects was generally self-limited nausea and vomiting. Notably, the serum calcium-phosphate product decreased by 15% in the patients receiving cinacalcet and was unchanged in the placebo group. The use of some traditional modes of treatment for 2^o HPT, e.g., vitamin D analogues and calcium-containing phosphate binders, can lead to hypercalcemia and/or hyperphosphatemia, which are associated with an increased risk of death as well as greater arterial stiffness, and calcification of the coronary arteries, aorta and cardiac valves. Thus the cinacalcet-induced decrease in the calcium-phosphate product might be expected to reduce these complications and perhaps prolong life in dialysis patients. A recent small meta analysis summarized the initial evidence for beneficial long term effects of cinacalcet, showing that adminstration of the drug reduces risk of parathyroidectomy, fracture and cardiovascular hospitalization and also improves quality of life (Ogata et al, 2006). There is a potential risk of low turnover bone disease if doses of cinacalcet are utilized that produce too great a decrease in serum PTH; further studies are needed to assess the frequency of this complication.

A recent, 52 week trial of cinacalcet in 78 patients with mild primary hyperparathyroidism found that 73% of the patients treated with the drug achieved normocalcemia, while only 5% of controls reached this endpoint (Peacock et al, 2005). There was no increase in urinary calcium excretion, suggesting that there was no excess activation of the CaR in the kidney that might have promoted hypercalciuria. Despite the biochemical improvements in these subjects, however, there was no increase in bone mineral density, unlike what is seen following surgical removal of parathyroid tumors in PHPT. Therefore, cinacalcet is safe and efficacious for treating the hypercalcemia in patients with primary hyperparathyroidism, although it may not be as effective as parathyroid surgery in treating patients with reduced bone mineral density. However, cinacalcet hasn't yet been approved by the FDA for the treatment of PHPT, except in the case of parathyroid cancer, for which

there is no other effective therapy of the biochemical abnormalities. Administration of the calcimimetic in the latter setting enables some amelioration of the severe hyperparathyroidism that not uncommonly is the cause of death in this condition. Based on experience in a small number of patients, there is about a 60% response rate, although progression of the disease eventually overwhelms the drug's efficacy in most cases (see www.amgen.com).

Calcium receptor antagonists, so-called calcilytics, have been developed, and their clinical utility is being explored. In the presence of the calcilytic, a higher than usual calcium concentration is needed to suppress PTH levels to a given extent (Gowen et al, 2000; Nemeth et al, 1998a). As a result, the calcium receptor reads normocalcemia as hypocalcemia and secretes a pulse of PTH. Parathyroid hormone, when administered in a once daily regimen, exerts an anabolic action on the skeleton, and is currently being used in the setting of osteoprosis (Neer et al, 2001). Once daily adminsitration of the calcilytic, therefore, could promote a similar effect owing to release of endogenous PTH, if the pharmacokinetics of the endogenous PTH secretion mimicked sufficiently well that resulting from exogenous PTH. Proof-of-principle has been obtained in a rat model of bone loss resulting from removal of the ovaries, but further studies, particularly those in humans, are needed to establish the role of calcilytics in the treatment of osteoporosis (Gowen et al, 2000).

6. SUMMARY AND FUTURE ISSUES

The CaR is a membrane bound 7TM receptor expressed in all of the tissues regulating extracellular calcium homeostasis. It "senses" even minute (on the order of a few percent) alterations in the level of calcium in the blood; thus it acts as the body's "calciostat". The CaR, in turn, regulates the functions of the cells that express it so as to normalize the level of blood calcium concentration. CaR-mediated control of the release of PTH plays an especially important role in calcium homeostasis, since it directly or indirectly regulates the functions of all of the tissues that are involved in regulating blood calcium. Patients who have loss-of-function mutations in the CaR gene exhibit a form of hypercalcemia that is accompanied by absolute or hypocalciuria. In the heterozygous form, it produces a benign hypercalcemic condition, FHH. In the homozygous form (NSHPT), the hypercalcemia may be lethal if it is not treated surgically. Gain-of-function mutations produce a generally benign state of hypocalcemia with relative or absolute hypercalciuria, ADH; It will be of interest in future studies to collect detailed clinical information in large kindreds to study the possible implications of the CaR's altered sensitivity to calcium in these patients and the resulting alterations in the levels of the serum calcium concentration. This could be even more relevant than previously considered, since the CaR is expressed in numerous organs, such as the breast brain, intestine and cardiovascular system, which are not thought to be involved in systemic calcium metabolism. Finally, symptomatic patients may derive benefit from the new calcimimetic CaR activators and, perhaps in the future, calcilytics.

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CHAPTER 7

PHYSIOLOGICAL ROLES OF THE Ca^{2+}/CaM-DEPENDENT PROTEIN KINASE CASCADE IN HEALTH AND DISEASE

J. COLOMER AND A.R. MEANS

Department of Pharmacology and Cancer Biology, Duke University Medical Center

Abstract: Numerous hormones, growth factors and physiological processes cause a rise in cytosolic Ca^{2+}, which is translated into meaningful cellular responses by interacting with a large number of Ca^{2+}-binding proteins. The Ca^{2+}-binding protein that is most pervasive in mediating these responses is calmodulin (CaM), which acts as a primary receptor for Ca^{2+} in all eukaryotic cells. In turn, Ca^{2+}/CaM functions as an allosteric activator of a host of enzymatic proteins including a considerable number of protein kinases. The topic of this review is to discuss the physiological roles of a sub-set of these protein kinases which can function in cells as a Ca^{2+}/CaM-dependent kinase signaling cascade. The cascade was originally believed to consist of a CaM kinase kinase that phosphorylates and activates one of two CaM kinases, CaMKI or CaMKIV. The unusual aspect of this cascade is that both the kinase kinase and the kinase require the binding of Ca^{2+}/CaM for activation. More recently, one of the CaM kinase kinases has been found to activate another important enzyme, the AMP-dependent protein kinase so the concept of the CaM kinase cascade must be expanded. A CaM kinase cascade is important for many normal physiological processes that when misregulated can lead to a variety of disease states. These processes include: cell proliferation and apoptosis that may conspire in the genesis of cancer; neuronal growth and function related to brain development, synaptic plasticity as well as memory formation and maintenance; proper function of the immune system including the inflammatory response, activation of T lymphocytes and hematopoietic stem cell maintenance; and the central control of energy balance that, when altered, can lead to obesity and diabetes. Although the study of the CaM-dependent kinase cascades is still in its infancy continued analysis of the pathways regulated by these Ca^{2+}-initiated signaling cascades holds considerable promise for the future of disease-related research

Keywords: calmodulin dependent protein kinase, kinase cascade, cell cycle regulation, apoptosis, long term memory, T cell activation, hematopoiesis, neurite growth, metabolism regulation

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1. INTRODUCTION TO THE CALMODULIN KINASE CASCADE

A considerable number of diseases and physiological disorders, ranging from cancer to neuronal dysfunction to immunological conditions, are related to defects in signaling cascades initiated by changes in intracellular calcium (Ca^{2+}). This is due to the fact that Ca^{2+} is a very prominent second messenger not only in all cells of the human body, but also in all eukaryotic organisms. Therefore, all cells regulate intracellular Ca^{2+} levels exquisitely (Klee and Means, 2002). Free intracellular Ca^{2+} ions are rapidly bound by several proteins known as "calcium sensors", which will change their conformation upon Ca^{2+} binding. One of the most pervasive calcium sensors is calmodulin (CaM), a small, acidic protein (17 kDa) with 4 Ca^{2+} binding motifs of the EF-hand type, that interacts with a great number of proteins, including those involved in regulating Ca^{2+} homeostasis, cyclic nucleotide metabolism and protein phosphorylation/dephosphorylation (Chin and Means, 2000). Indeed, CaM regulates protein phosphorylation through the activation of numerous protein kinases as well as the protein phosphatase 2B (also known as calcineurin). Some of the CaM-dependent protein kinases target a very specific substrate and are known as "dedicated kinases", while others target a wide range of proteins that contain consensus phosphorylation sequences and are known as "multifunctional kinases" (Chow and Means, 2006). The multifunctional kinase family is comprised of the CaM-dependent protein kinase I (CaMKI) subfamily (α,β,γ and δ genes), CaMKII subfamily (α,β,γ and δ genes) and CaMKIV (one gene), as well as the CaMKK subfamily (α and β, genes). The CaMKII subfamily members associate in 8–12 unit oligomers, are activated by Ca^{2+}/CaM binding and are characterized by intrasubunit phosphorylation at threonine 286 (T^{286}) or T^{287}, depending on the isoform (Saitoh et al., 1985; Saitoh et al., 1987), a modification that generates kinase activity that is autonomous (independent of Ca^{2+}/CaM). CaMKII is a very important regulatory kinase but is not a component of a CaMK cascade, and its regulation has been reviewed in detail elsewhere (Hudmon and Schulman, 2002), so is not considered herein. However, all members of the CaMKI subfamily as well as CaMKIV are activated by phosphorylation of a critical threonine in their activation loop by CaMKKα or CaMKKβ, constituting what has been recently dubbed a CaMK cascade (Soderling, 1999; Means, 2000). Interestingly, in addition to CaMKI and CaMKIV, CaMKKs can also phosphorylate and activate additional substrates such as AMPK (Hawley et al., 1995; Okuno et al., 2000; Hamilton et al., 2002). We will address the potential physiological functions of such CaMK cascades in this chapter.

2. THE CAMKK SUBFAMILY

Currently, two genes coding for distinct CaMKK isoforms have been identified, namely Camkk1 and Camkk2, which encode CaMKKα and CaMKKβ, respectively. Figure 1 presents a schematic representation of these two isoforms as well as a comparison with the other multifunctional kinases, the CaMKI isoforms and

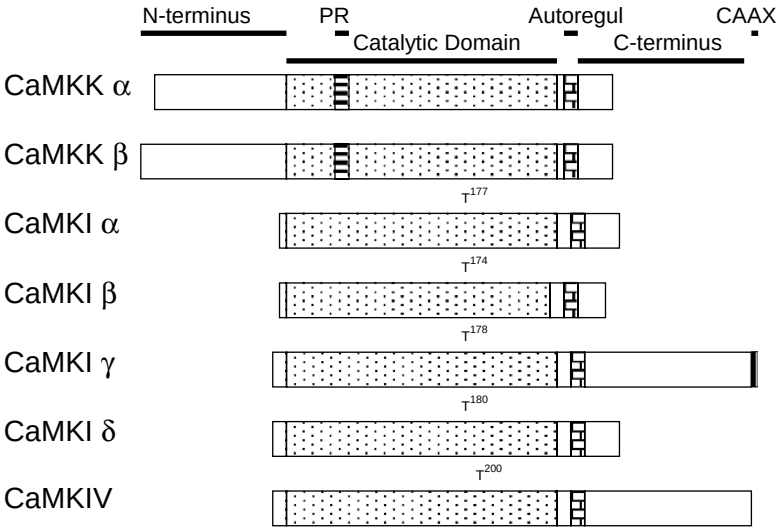

Figure 1. Schemaic representation of the proteins involved in the CaMK cascade, aligned by functional domain. Dotted box, catalytic domain; horizontal striped box, RP insert; vertical striped box, CAAX insert; brick box, autoregulatory domain; white boxes, unknown or undefined domains. T, CaMKK target threonine. See text for more details

CaMKIV. Both CaMKKα and CaMKKβ have a fairly long N-terminal domain of between 120–165 residues, followed by a kinase domain consisting of between 260–290 residues, the autoregulatory domain, which is comprised of a 30–40 residue autoinhibitory segment/calmodulin binding domain and ends with a C-terminal domain of unknown function that varies between 50–80 residue (Tokumitsu et al., 1995; Anderson et al., 1998; Tokumitsu et al., 1999). Interestingly, the catalytic domain of the CaMKKs contains a subdomain rich in arginine and proline residues, called the RP domain. This RP region is inserted between subdomains II and III of the kinase domain and, in the case of CaMKKα, has been suggested to be required to bind and activate CaMKI and CaMKIV, but not other potential substrates such as PKB (Akt) or synthetic peptides (Tokumitsu et al., 1999). All CaMKKs require Ca^{2+}/CaM for maximal kinase activity (Tokumitsu and Soderling, 1996), although CaMKKβ exhibits substantial constitutive activity in the absence of Ca^{2+}/CaM whereas CaMKKα is completely dependent on Ca^{2+}/CaM binding for activity (Edelman et al., 1996; Anderson et al., 1998). CaMKKs also undergo autophosporylation, although the process is very slow, substochiometric and does not seem to affect catalytic activity (Anderson et al., 1998; Tokumitsu et al., 1999). The Camkk2 gene produces several splicing isoforms depending on cell type, which increases the number of isoforms of CaMKKs (Hsu et al., 2001; Ishikawa et al., 2003). All members of the CaMKK subfamily have a molecular weight between 54–68 kDa and are located mainly in the cytoplasm, although on occasion CaMKKβ has been reported to be present in the nucleus

(Sakagami et al., 2000; Nakamura et al., 2001). At the organismal level, both CaMKKα and CaMKKβ are expressed primarily in brain (although they vary in relative abundance in different parts of the brain), in some peripheral tissues such as thymus, spleen and testis, and in many other cells and tissues at low levels (Tokumitsu et al., 1995; Kitani et al., 1997; Anderson et al., 1998; Sakagami et al., 2000; Hsu et al., 2001; Vinet et al., 2003). To a certain extent, the expression pattern of CaMKKβ seems to parallel that of CaMKIV to a greater extent than does CaMKKα (Anderson et al., 1998). Table 1 presents a summary of the expression patterns of all of the kinases that participate in a CaM kinase cascade including the CaMKKs.

Table 1. Tissue distribution of the different members of the CaMK cascade. Underdlined are the tissues with most abundant expression of each gene are in italics

Gene	Tissue distribution	Reference
Camkk1	Brain (parietal cortex, hippocampus, brainstem, mesencephalon and thalamus, caudate-putamen and cerebellum) Thymus, spleen, many other tissues	(Tokumitsu et al., 1995; Anderson et al., 1998; Sakagami et al., 2000; Vinet et al., 2003)
Camkk2	Brain (parietal cortex, cerebellum (granule cells), caudate-putamen, hippocampus, olfactory bulb, brainstem, mesencephalon and thalamus) Thymus, testis, spleen, lungs, many other tissues	(Kitani et al., 1997; Anderson et al., 1998; Sakagami et al., 2000; Hsu et al., 2001; Vinet et al., 2003)
Camk1α	Brain (hippocampus, cortex, brain stem and olfactory bulb) Adrenal gland, liver, lung, every tissue tested	(Picciotto et al., 1993; Picciotto et al., 1995; Sawamura et al., 1996)
Camk1β	Brain (hypothalamus, hippocampus, cortex, cerebellum, septum, thalamus, amygdala, olfactory bulb) Uterus, ovary, testes, heart, skeletal muscle, pancreas, placenta, liver, lung, spleen	(Naito et al., 1997; Ueda et al., 1999; Gardner et al., 2000; Loseth et al., 2000; Rina et al., 2001)
Camk1γ	Brain (amygdale, ventromedial hypothalamus, olfactory bulb, hippocampus, pineal gland)	(Nishimura et al., 2003; Takemoto-Kimura et al., 2003)
Camk1δ	Brain (Hippocampus, cortex, amygdala, spinal cord) Granulocytes (Polymorphonuclear leucocytes)	(Verploegen et al., 2000; Sakagami et al., 2005)
Camk4	Brain (parietal cortex, cerebellum (granular cells), caudate putamen, hippocampus, olfactory bulb, brainstem, mesencephalon and thalamus) Thymocytes, Testis, Ovary	(Ohmstede et al., 1989; Means et al., 1991; Sakagami et al., 1992; Sun et al., 1995; Anderson et al., 1998; Sakagami et al., 1999; Wu et al., 2000; Wu and Means, 2000; Wang et al., 2001)

3. THE CAMKI SUBFAMILY

Four distinct genes encoding CaMKI isoforms have been identified so far and are known as Camk1α, Camk1β (also Pnck), Camk1γ (also ClickIII) and Camk1δ (also CKLiK), and each gene produces one or more splice variants. As shown in Figure 1, all CaMKI family members have a similar domain structure, consisting of a short N-terminal sequence (up to 23 residues) followed by the kinase domain, the autoregulatory domain and a 30–50 C-terminal domain of unknown function (150 residues in the case of CaMKIγ) (Haribabu et al., 1995; Yokokura et al., 1995; Naito et al., 1997; Gardner et al., 2000; Ishikawa et al., 2003; Takemoto-Kimura et al., 2003). All isoforms require Ca^{2+}/CaM for kinase activity and also contain a conserved T in the activation loop (T^{174} to T^{180}, depending on the isoform). Phosphorylation of this T by a CaMKK is usually required for maximal CaMKI activity (Haribabu et al., 1995; Naito et al., 1997; Ishikawa et al., 2003; Nishimura et al., 2003; Takemoto-Kimura et al., 2003) although this depends on the substrate (Hook et al., 1999). Overall, all members of the CaMKI subfamily have molecular weights between 38–42 kDa, except for CaMKIγ, which is 53 kDa. This is due to a longer C-terminal domain which ends in a CAAX sequence that targets CaMKIγ to membranes of the sarcoplasmic reticulum and the Golgi (Takemoto-Kimura et al., 2003). The other three isoforms are localized primarily in the cytoplasm, although some splice variants, such as CaMKIβ2 and CaMKIδ, have been reported to translocate into the nucleus in response to cell stimulation (Ueda et al., 1999; Rina et al., 2001; Sakagami et al., 2005). Lastly, various CaMKI isoforms are expressed in brain (although heterogeneously in different portions of the brain) as well as many other tissues (Picciotto et al., 1993; Picciotto et al., 1995; Sawamura et al., 1996; Naito et al., 1997; Ueda et al., 1999; Gardner et al., 2000; Loseth et al., 2000; Verploegen et al., 2000; Rina et al., 2001; Nishimura et al., 2003; Takemoto-Kimura et al., 2003; Sakagami et al., 2005). Interestingly, CaMKIα appears to be ubiquitously expressed in mammalian tissues (Picciotto et al., 1993; Picciotto et al., 1995; Sawamura et al., 1996).

3.1. CaMKIV

Only one gene encoding CaMKIV has been identified, although it produces several mRNAs due to differential splicing as well as the use of an alternative transcriptional initiation site (Sakagami and Kondo, 1993). The two protein isoforms, CaMKIVα and CaMKIVβ, are identical, except that the latter contains a 28 residue N-terminal extension, which has no known function. A schematic representation of the CaMKIV protein is shown in Figure 1. Similar to the CaMKI subfamily, CaMKIV has a short N-terminal sequence, followed by the kinase domain, the autoregulatory domain, and a C-terminal domain of unknown function (Means et al., 1991; Cruzalegui and Means, 1993; Bland et al., 1994). CaMKIV requires Ca^{2+}/CaM to initiate kinase activity and also contains a conserved T in the activation loop (T^{200} in the human CaMKIV), which is phosphorylated by a CaMKK. Activation loop phosphorylation

is necessary for maximal CaMKIV activity (Selbert et al., 1995; Tokumitsu et al., 1995) and generates autonomous activity, which is required for its transcriptional activation functions. All CaMKIV isoforms have a molecular weight between 65–67 kDa and translocate between nucleus and cytoplasm in response to cell stimuli that result in an increase in intracellular Ca^{2+} (Bito et al., 1996; Sakagami et al., 1999; Lemrow et al., 2004). Interestingly, the Camk4 gene also encodes calspermin, which is a distinct Ca^{2+}/CaM-binding protein of unknown function that is expressed exclusively in testis (Ono et al., 1989). The transcript is initiated by use of an alternate promoter that is located in the 10th intron of the gene and is regulated by cAMP (Means et al., 1991; Sun et al., 1995). Regarding CaMKIV distribution in tissues, it is expressed primarily in brain (with the highest levels in the cerebellum) but also present in hematopoietic stem and progenitor cells, T cells, testis and ovary (Ohmstede et al., 1989; Means et al., 1991; Sakagami et al., 1992; Sun et al., 1995; Anderson et al., 1998; Sakagami et al., 1999; Wu et al., 2000; Wu and Means, 2000; Wang et al., 2001; Kitsos et al., 2005). As mentioned previously, the CaMKIV expression pattern resembles that of CaMKKβ suggesting that CaMKKβ may primarily serve to activate CaMKIV in cells that express both proteins.

In this section we have only presented a brief overview of the cast of characters that constitute the CaM kinase cascades. For a much more thorough discussion of the biochemical properties of CaMKKs as well as CaMKI and CaMKIV, we refer the reader to a recent review by Chow and Means (Chow and Means, 2006).

3.2. Inhibitors of the CaMK Cascade

The present chapter focuses on the physiological functions of the CaMK cascade that have been identified to date. The definition of these functions has relied heavily on the use of pharmacological inhibitors, overexpression of constitutively active or dominant/negative mutants of the various CaM kinases, and a variey of model organisms that include mice, worms and fungi null (or transgenic) for specific members of the CaMK cascade.

The only currently available small molecule inhibitor of the CaMKK subfamily is STO-609, which acts as a competitive antagonist for ATP (Tokumitsu et al., 2002). Whereas, STO-609 is a relatively selective inhibitor of the CaMKKs, it is not specific, as it also targets other kinases at higher concentrations than normally used to inhibit the CaMKKs (Tokumitsu et al., 2002; Hawley et al., 2005). However, STO-609 does not inhibit CaMKI or CaMKIV. The small molecules most frequently used as inhibitors of CaMKI and CaMKIV (and CaMKII) are KN-62 and KN-93, which are thought to act as competitive antagonists of Ca^{2+}/CaM although the mechanism involved is not known (Tokumitsu et al., 1990; Sumi et al., 1991). Unfortunately, these compounds can neither distinguish between CaMKI, CaMKII and CaMKIV nor inhibit autonomously active versions of CaMKII and CaMKIV. In addition, the KN molecules can inhibit molecules unrelated to the CaM kinases (such as ion channels) but have no effect on the CaMKKs. A detailed discussion on

the usefulness and limitations of these pharmacological compounds can be found in Chow and Means (Chow and Means, 2006).

Overexpression of dominant/negative (kinase-inactive), constitutively active or wild type forms of the different CaMKKs and CaMKs has been used to either inhibit or stimulate various signaling pathways in which these CaM kinases are suspected to be involved. Such approaches have certainly contributed to our appreciation of the panoply of physiological processes in which the CaM kinase cascade may participate. However, the overexpression approach also has potentially serious limitations in terms of specificity. The advantages and disadvantages of overexpression are also discussed in detail by Chow and Means (Chow and Means, 2006). In sum, several approaches, pharmacological and protein expression, as well as gene deletion, inactivation or modification, should probably be used in combination to evaluate the role of these kinases in cells and organisms. Even so it is prudent to interpret the results obtained with caution.

4. THE CAMKS, THE CELL CYCLE AND CANCER

There is compelling evidence that Ca^{2+}/CaM and various multifunctional CaMKs are important in cell cycle regulation, (Kahl and Means, 2003). Proper control of the cell cycle is necessary to avoid hyperproliferative diseases such as cancer. The cell cycle progresses through a series of checkpoints to ensure fidelity of DNA replication and cell division. Overexpression of CaM can abrogate some of these checkpoints and shorten the cell division cycle. In addition, many human cancers have elevated levels of CaM that could contribute to the proliferative defects characteristic of these diseases. More recent work has revealed that CaM controls the activites of several CaM kinases that contribute to cell cycle regulation. CaMKII is important for the G2/M transition whereas the CaM kinase cascade is involved in regulating the G1/S transition (Kahl and Means, 2003).

4.1. CaMKII Regulation of G2/M in Vertebrate Cells

Considerable evidence exists supporting a role for CaMKII in regulating the G2/M transition. The first such data were derived from studies in which a consitutively active form of CaMKII was transiently overexpressed in a mouse cell line and found to block cells in G2 of the cell cycle. Similar results were obtained in human HeLa cells several years later (Planas-Silva and Means, 1992; Beauman et al., 2003). On the other hand, a CaMKII specific inhibitory peptide also blocks cells in G2, presumably by preventing the phorphorylation of the Cdc25 phosphatase by CaMKII (Patel et al., 1999). In addition, CaMKII is absolutely required for the resumption of the cell cycle in *Xenopus* eggs arrested in metaphase of the second meiotic division due to the inactivation of CyclinB/cdk1 (Lorca et al., 1993). In this case a critical substrate of CaMKII is XErp1/Emi2 (Liu and Maller, 2005). Similarly, CaMKII is required for resumption of meiosis following fertilization of mouse eggs (Knott et al., 2006). These collective results suggest that the timing of CaMKII activation

is critical in G2/M progession for two reasons. First, it must remain inactive in order for cells to progress from G2 into meiosis/mitosis and second, it must become activated in order for cells to enter and complete meiosis/mitosis.

4.2. The CaMK Cascade and Cell Cycle in *Schizosaccharomyces Pombe*

A link between CaMKI and cell cycle regulation has been reported to occur in the fission yeast *Schizosaccharomyces pombe*. *S. pombe* contains at least two CaM-dependent kinases with considerable similarity to the mammalian CaMKs: CMK1 (40% identity) (Rasmussen, 2000), and CMK2 (also 40% identity to CaMKI and 35% identity to CaMKII) (Alemany et al., 2002). Whereas CMK2 does not have a phosphorylatable T in the activation loop and cannot be activated by phosphorylation (Sanchez-Piris et al., 2002), CMK1 does contain a T in the activation loop at position 192 (T^{192}), which, when mutated to an acidic residue ($T^{192}D$), increases its *in vitro* activity 15X compared to the wild type CMK1 (Rasmussen, 2000), indicating that CMK1 is the yeast CaMKI. Although the CMK1-kinase has yet to be described in yeast, these data suggest the presence of a CaMKK/CaMK1 cascade in *S. pombe*.

A role for CMK1 in cell cycle regulation in *S. pombe* is supported by two observations. First, Cmk1 mRNA levels are tightly regulated in synchronized *S. pombe* cells, increasing 6X between G1 and S phase, followed by a rapid decline at the end of the S phase (Rasmussen, 2000). Second, overexpression of the mutant CMK1 $T^{192}D$ causes cell cycle arrest and changes in cell morphology (Rasmussen, 2000). At first glance, such results could be interpreted to implicate a negative role for CMK1 in the G1/S transition, since it is a gain of function mutation that blocks such a transition. On the other hand, like described above for the role of CaMKII in G2/M, an equally plausible interpretation of the data is that the timing of the activation and inactivation of CMK1 could be important in the regulation of the G1/S transition.

Of course, the *S. pombe* study is far from complete and several issues remain to be addressed. First, the CMK1 activator equivalent to the mammalian CaMKK must be cloned in order to prove that *S. pombe* does, in fact, have a functional CaMKK cascade. Second, both the CMK1 and its activator must be deleted in order to convincingly demonstrate a requirement of the cascade in the cell cycle. Nevertheless, the data obtained in *S. pombe* to date are consitent with a role for CMK1 in regulation the G1/S transition of the cell cycle.

4.3. The CaMK Cascade and Cell Cycle in *Aspergillus Nidulans*

Aspergillus nidulans, a filamentous fungus, also contains three homologues of mammalian CaMKs, called CMKA, CMKB and CMKC. CMKA is most similar to CaMKII, CMKB to CaMKI (43% identity to *S. pombe* CMK1, 35% identity to rat CaMKI) and CMKC the CaMKKs (Joseph and Means, 2000). Indeed, CMKC activates CMKB kinase *in vitro* by 73X and this activation is entirely dependent on phosphorylation of T^{179} in the activation loop of CMKB, which is equivalent to T^{177}

in human CaMKI (Haribabu et al., 1995). Confirming the relationship, mutation of T^{179} to alanine ($T^{179}A$) completely abrogates the ability of CMKC to activate CMKB (Joseph and Means, 2000). Therefore in *Aspergillus nidulans* the CaMK cascade is comprised of CMKB and CMKC.

The functional role of the CaMK cascade in *Aspergillus* is based on loss of function studies. The deletion of the Cmkb gene is lethal, therefore the consequences of loss of function was assessed by disrupting the Cmkb promoter, which results in a 1–2 h delay in CMKB protein expression upon spore germination (Joseph and Means, 2000). This delay in expression results in a 2 h delay in nuclear division after germination (both for the first and the second nuclear divisions) which is consistent with a similar increase of the duration of the cell cycle (Joseph and Means, 2000). Together these results clearly demonstrate that CMKB regulates the cell cycle.

Fortunately, deletion of the Cmkc gene is not lethal, therefore it could be used to address the function of CMKC in cell division during spore germination. The absence of CMKC results in the failure of 30% of the germlings to undergo the first nuclear division. The ones that do complete this division are dramatically delayed compared to the control germlings (2–4 h). In addition, the time required to complete the second nuclear division is similarly delayed (Joseph and Means, 2000). Regarding the mechanism responsible for the increased nuclear division time, both the deletion of Cmkb promoter and of the Cmkc coding sequence result in a delay of the activation as well as in a decreased maximal activity of $NIMX^{cdc2}$, the cyclin dependent kinase in *Aspergillus* that triggers the entry into S phase (Joseph and Means, 2000). Therefore, the CaMKK cascade in *Aspergillus*, comprised of CMKB and CMKC, is required for proper activation of $NIMX^{cdc2}$ and thus, the timing of nuclear division after germination.

Interestingly, the overexpression of wild type CMKB in *Aspergillus* has no effect on the cell cycle, whereas overexpression of the constitutively active mutant of CMKB, CMKB $T^{179}D$ (CMKB T^{179} is the residue phosphorylated by CMKC in *Aspergillus*) lengthens the nuclear division cycle in both the wild type and Cmkc-null strains (Joseph and Means, 2000) in a similar way to how CMK1 affects the cell cycle of *S. pombe* (Rasmussen, 2000). The mechanism responsible for this phenomenon is not clear, but again the data are compatible with the idea that the timing of CMK activation and inactivation is critical in order to execute the G1/S transition in both *Aspergillus* and *S. pombe*.

4.4. The CaMKI Cascade and Cell Cycle in Cultured Mammalian Cells

Two reports presented evidence that CaMKI and its activators CAMKKα and CaMKKβ are important in G1 progression of human cells in culture. In one study, WI-38 normal diploid fibroblasts were used whereas the other study used MCF-7 human breast cancer cells together with the non-tumorigenic MCF-10A clone.

WI-38 cells can be arrested in G0 by serum starvation and are syncronously released back into the cell cycle upon re-addition of serum. Treatment of quiescent

WI-38 cells with the CaMK inhibitor KN-93 inhibits re-entry to DNA synthesis. Since WI-38 cells express CaMKIα, CaMKIIγ and CaMKIIδ (Tombes and Krystal, 1997; Kahl and Means, 2004), but not CaMKIV (Kahl and Means, 2004), 1over-expression of kinase-inactive mutants of CaMKI*α* (dn-CaMKI) and CaMKII (dn-CaMKI) were used to question which CaMK might be the target of KN-93. Whereas dn-CaMKI replicated the KN-93-induced block at the G1/S boundary, dn-CaMKII did not, suggesting that CaMKIα is required for G1 progression in WI-38 cells (Kahl and Means, 2004). The precise mechanism by which CaMKIα regulates the G1/S transition is not clear, but the kinase is required for activation of the fully assembled, nuclear cdk4/cyclin D complex (Figure 2). In the presence of KN-93 or dn-CaMKI, the complex is not activated so Rb is not phosphorylated and G1 progression is arrested due to the inability of E2F to activate the transcription of genes that are required for the entry into S phase (Kahl and Means, 2004). Remarkably, this role for the CaMKI cascade in human fibroblasts is very similar to its requirement in *Aspergillus*. In the latter case, CMKB and CMKC are required to activate $NIMX^{cdc2}$, which is the cdk required for the G1/S transition in this fungal species (Joseph and Means, 2000).

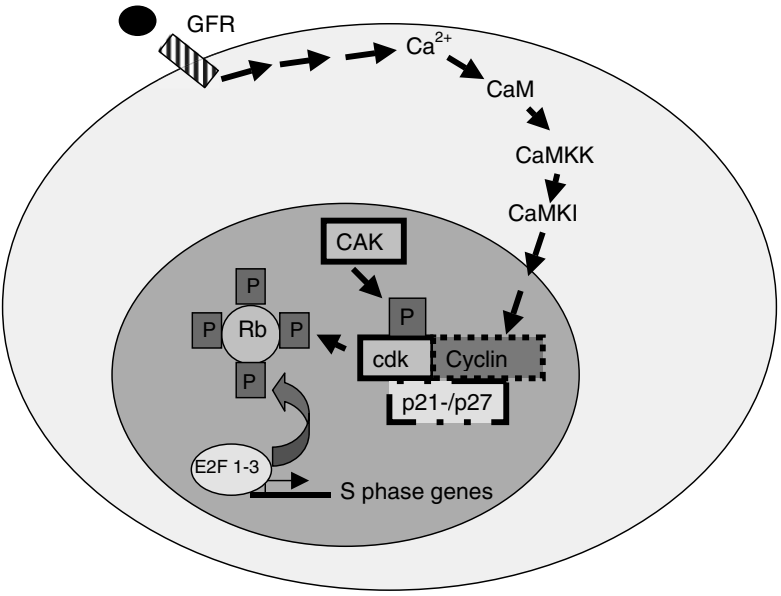

Figure 2. Schematic representation of the activation of the cdk4/cyclin D complex during G1. Evidence shows that CaMKIα, which can be activated by either CaMKKα or β, activates the cdk4/cyclin D complex, leading to the phosphorylation of Rb and therefore de-repression of E2F transcription factor members. The mechanism by which CaMKI regulates cdk4/cyclin D remains to be determined. GFR, receptor for a growth factor; CAK, cdk activating kinase; p21/p27, members of the inhibitory Cip and Kip families, respectively; E2F 1-3, transcription factors 1-3 of the E2F family; Rb, Retinoblastoma protein; P, phosphorylation event

The subsequent study confirming a role for the CaMKI cascade in cell cycle regulation in human cells was carried out in the human breast tumor cell line, MCF-7, and the immortalized but not transformed human breast epithelial cell line, MCF-10A. As found in WI-38 cells, the cell cycle of both MCF-7 and MCF-10A cell lines was inhibited in G1 by KN-93, although the tumorigenic MCF-7 cells were blocked better than the non-tumorigenic MCF-10A cells. Also similar to WI-38 cells, both MCF cell lines express CaMKIα, CaMKIIγ and CaMKIIδ (but not CaMKIV), in addition to at least one CaMKK isoform (one in MCF-10A cells, two in MCF-7 cells) (Rodriguez-Mora et al., 2005). This study used siRNA to evaluate which CaMK was the target of KN-93 whose inhibition led to cell cycle arrest in G1. CaMKIα-specific siRNA resulted in the accumulation of cells in G1, but CaMKIIγ- or β-specific siRNAs did not, again confirming a role for CaMKIα in G1 progression (Rodriguez-Mora et al., 2005). Moreover, both CaMKKα- and CaMKKβ- specific siRNAs also blocked the MCF-7 cells in G1, a result entirely consistent with a CaMKK/CaMKI cascade regulating passage through G1. Again in a manner similar to WI-38 cells, CaMKIα is required to induce Rb phosphorylation through the activity of cdk4/cyclin D (Rodriguez-Mora et al., 2005). Thus, both MCF-7 and WI-38 cells require a CaMKIα cascade in order to activate cdk4/cyclin D, phosphorylate Rb and proceed from G1 into S phase of the cell cycle, consistent with the model depicted in Figure 2.

4.5. The CaMK Cascade and Apoptosis in Mammalian Cells

Another characteristic of cancer cells is their decreased susceptibility to apoptotic cell death. Interestingly, a CaM kinase cascade is also required for cell survival, although the mechanism is still speculative. The first evidence that a CaMK might be pro-survival, came from an *in vivo* study in which mouse thymocytes specifically expessed a kinase-inactive form of CaMKIV (dn-CAMKIV) due to regulation of the transgene by the thymocyte-specific lck promoter. The transgenic mice showed a markedly reduced thymus, a correspondingly lower number of thymocytes and an increased percentage of thymocytes undergoing apoptosis compared to control mice (Anderson et al., 1997). Although this phenotype was not produced in mice globally deleted for the CaMKIV gene, such mice do show an inability to activate memory T cells. Both the transgenic thymocytes and the memory T cells from the CaMKIV null mice shared a common phenotype in that they could not be activated by engagement of the T cell receptor (TCR). Interestingly, depletion of CaMKIV from isolated human peripheral T cells also prevents TCR-mediated activation of these cells. In the Camk4$^{-/-}$ mouse, hematopoietic stem cells (HSC) and cerebellar granule cells (CGC) are reduced in number due to increased apoptosis (Kitsos et al., 2005). The HSCs from the Camk4 null mice have lower levels of the pro-survial protein Bcl-2 and cannot repopulate the bone marrow upon transplantation into irradiated recipient mice (Kitsos et al., 2005). The pathways by which CaMKIV regulates cells of the immune system are discussed in more detail in a subsequent section.

As mentioned briefly in the preceding paragraph, CaMKIV has an anti-apoptotic effect in CGCs, which robustly express this kinase. Culturing isolated CGCs in low KCl (5 mM) was previously shown to induce apoptotic cell death that correlates with calpain-mediated proteolysis of CaMKIV (Tremper-Wells and Vallano, 2005). Apoptosis could be prevented by over-expression of a constitutively active mutant of CaMKIV and stimulated by a kinase-inactive mutant of CaMKIV (dn-CaMKIV), confirming that the cytoprotective function requires the kinase activity of CaMKIV. Moreover, the decrease in CaMKIV protein correlates with decreased phosphorylation of CREB on S^{133}, which regulates (among many other genes) the expression of the neuroprotective brain-derived neurotrophic factor (BDNF) (See et al., 2001). Indeed, CREB and CaMKIV can regulate the expression of BDNF in neurons, and a CaMKIV-sensitive element in the BDNF promoter has been defined (Shieh et al., 1998; Shieh and Ghosh, 1999). Evaluation of the role of CaMKIV in neuronal apoptosis in $Camk4^{-/-}$ mice is currently underway in our laboratory.

The CaMKIV/CaMKI activator CaMKKα has also been implicated in apopotosis but through a PKB pathway instead of a CaMKIV pathway. Yano et al. showed that CaMKKα could phosphorylate and activate PKB *in vitro* and in neuronal cell cultures that pathway was suggested to protect the cells from apoptosis (Yano et al., 1998). In this study, CaMKKα was suggested to phosphorylate PKB in response to stimuli that increased intracellular Ca^{2+}, which in turn results in the phosphorylation of the pro-apoptotic factor BAD on S^{136} and its sequestration by 14-3-3, therefore promoting survival of the neurons (Yano et al., 1998). In addition, it has been reported that CaM and presumably a CaMK (based on the use of the CaM inhibitor W7 and the CaMK inhibitor KN-93) regulate the activation of PKB induced by hydrogen peroxide (Howe et al., 2002). These obervations suggest the possibility that a CaMK cascade may function upstream of the PKB-dependent apoptotic pathway.

4.6. Concluding Remarks on the CaMK Cascade, the Cell Cycle and Cancer

The role of the CaMKK/CaMKI cascade in the cell cycle, as well as the role of the CaMKK/CaMKIV in apoptosis prompted Rodriguez-Mori et al. to propose that the CaMK cascade is a promising target for cancer therapy (Rodriguez-Mora et al., 2005). Current evidence strongly supports a regulatory function for CaMKK/CaMKI (and CaMKII) at specific stages of the cell cycle (as discussed above) and circumstantial evidence suggests that such a role may be more prominent in cancer cells. For example, the human breast cancer-derived cell line MCF-7 is more susceptible to CaMK siRNA blockade of the cell cycle than is the related but non-transformed cell line MCF-10A (Rodriguez-Mora et al., 2005). Moreover, hepatocarcinoma cells of both rat and human origin express higher levels of CaMKK and CaMKIV than do normal hepatocytes from the corresponding species (Tamura et al., 2000). A dual role of the CaMKK cascade in cell cycle regulation and cell survival could indicate

appropriately specific CaMK inhibitors as a means to decrease cancer cell proliferation as well as to sensitize cancer cells to currently employed chemotheraputic drugs (Rodriguez-Mora et al., 2005). In spite of this encouraging evidence, the details of the mechanism by which the CaMK cascade regulates cell cycle remain ill-defined, and further study is necessary to clarify the specific target(s) of CaMKI that is(are) relevant to activation of the cdk4/cyclin D complex, as well as the precise mechanism by which CaMKIV regulates cell survival.

5. THE CAMK CASCADE REGULATES CREB ACTIVATION AND SYNAPTIC PLASTICITY IN NEURONAL CELLS

Neurons from mammals abundantly express most of the members of the CaMK cascade, as shown in Table 1. Neurons are specialized to receive stimuli from and send them to other cells, and such stimuli can be either chemical (such as neurotransmitters) or electrical. The ultimate function of neurons is to compute data that can be either processed to produce a desired task or stored as memory, which can be retrieved later. The mechanisms by which memories are stored and retrieved are not well understood, but memory formation involves synaptic plasticity. Here synaptic plasticity is defined as changes in the synaptic strengths between two neurons. Synaptic plasticity has been best studied in hippocampal neurons, and there are two well-studied examples of such plasticity. The first one is a long-lasting increase in synaptic strength, called long term potentiation (LTP), which is produced by a brief, high frequency activation of excitatory synapses. The second type of synaptic plasticity is a long-term decrease of synaptic strength, called long term depression (LTD), which is produced by two inputs that are negatively correlated on the same dendritic tree. (Sejnowski T, 1989). As we discuss below, LTP and LTD in certain types of neurons is dependent on the CaMK cascade.

The LTP process, which may last for several hours or even days, as well as the LTD process, require new gene transcription and protein synthesis in their late phases (reviewed by Kandel, 2001). Indeed, both in invertebrates (*Aplysia, Drosophila*) and vertebrates the transcription factor cAMP-dependent response element binding protein (CREB) becomes activated after LTP induction, which results in the transcription of several immediate early genes, which act on other genes to promote growth of new synaptic connections (Kandel, 2001). Although many genes have been identified whose expression is dependent on CREB (Zhang et al., 2005), the specific ones that are important for LTP or LTD activation have not yet been defined (Lonze and Ginty, 2002). Today there is a vast amount of data supporting the critical role of CREB in long-term memory formation in mammals, ranging from changes in CREB phosphorylation at S^{133} in neurons after LTP induction to loss-of-function mouse models such as mice hypomorphic for the Creb gene and mice with inducible expression of a CREB inhibitor (reviewed by Lonze and Ginty, (Lonze and Ginty, 2002)).

CREB transcriptional activity is regulated by phosphorylation, and the most critical phosphorylation site is S^{133} in the KID domain. S^{133} can be phosphorylated

by a variety of kinases, including PKA, RSK 1–3, MSK 1–2 and MAPKAPK2, which are activated by the ras/ERK pathway, the SAPK2/p38MAPK, an unidentified kinase downstream of PI3K/AKT and several CaMKs (reviewed by Lonze and Ginty (Lonze and Ginty, 2002)). Among the CaMKs that have been shown to phosphorylate CREB, at least CaMKI (Sheng et al., 1991; Sun et al., 1996), CaMKII (Dash et al., 1991; Sheng et al., 1991) and CaMKIV (Enslen et al., 1994; Matthews et al., 1994; Bito et al., 1996) can phosphorylate CREB at S^{133} *in vitro*, although CaMKII phosphorylates an additional site, S^{142}, that results in inhibition of CREB transcriptional activity instead of activation (Sun et al., 1994). Among the several CaMK isoforms expressed in neurons, only CaMKIV and a few splice variants of CaMKIβ and δ localize to the nucleus, where CREB is localized, suggesting that CaMKIV is the most likely CaMK to be physiologically relevant as a physiological CREB kinase that is activated in response to a Ca^{2+} signal (Bito et al., 1996). The Ca^{2+}-dependent protein kinase is more important to initially phosphorylate CREB on S^{133} following an activating stimulus whereas the ERK pathway seems critical to maintain CREB phosphorylation for up to 1 h after the initial signal (Wu et al., 2001). The CaMKIV cascade is not only important for CREB phosphorylation in neurons of mammals, but also in neurons of more simple organisms, suggesting that it is an evolutionary conserved mechanism to regulate neuronal activity.

5.1. The CaMK Cascade in the Nervous System of *Caenorhabditis Elegans*

Caenorhabditis elegans, a nematode utilized as a model for complex organisms due to the convenience of genetic manipulation, has a CaMK cascade consisting of CKK-1 and CMK-1. CKK-1 is highly homologous to the mammalian CaMKKs, including the presence of an arginine/proline rich insert (RP-insert) in the catalytic domain. Even the CKK-1 C-terminal domain (which includes the autoinhibitory and CaM binding domains) is 54% identical to the mammalian CaMKKα. Moreover, CKK-1 shares many properties with mammalian CaMKK, including Ca^{2+}/CaM-dependent activition and its ability to phosphorylate the activation loop T of both CMK-1 and CaMKIV, indicating CKK-1 is a *bona fide* CaMKK (Tokumitsu et al., 1999). CMK-1 is 60% identical to both rat and human CaMKI and is of a similar molecular weight (39,066). However, CMK-1 has some features characteristic of mammalian CaMKIV, such as its nuclear localization and V_{max} for the peptide syntide-2 (Eto et al., 1999). Another similarity between CMK-1 and CaMKIV is the ability of both of them to phosphorylate CREB in mammalian cells. Indeed, co-expression of the *C. elegans* CaMK cascade proteins (both CKK-1 and CMK-1) into mammalian COS-7 cells results in the Ca^{2+}-dependent phosphorylation and transcriptional activation of mammalian CREB (Eto et al., 1999).

A physiological role for the CaMK cascade in activating CREB transcriptional activity in neurons *in vivo* has been demonstrated in *C. elegans*. As in mammalian neurons, *C. elegans* neurons also express a gene homologous to creb1, called crh-1 (Kimura et al., 2002). CRH-1 must be phosphorylated at S^{129} (the equivalent site

to S^{133} in mammalian CREB) for maximal transcriptional activity, and CMK-1 can phosphorylate this site *in vitro*. Moreover, the presence of CKK-1 dramatically enhanced the CMK-1-dependent phosphorylation of CRH-1 *in vitro* nominating CRH-1 as a good candidate substrate for the CKK-1/CMK-1 cascade (Kimura et al., 2002). Therefore, the existence of a CKK-1/CMK-1/CRH-1 cascade was evaluated in *C. elegans in vivo*. All three components of the CKK-1/CMK-1/CRH-1 cascade were co-expressed in head neurons (Kimura et al., 2002). In addition, CMK-1, but not the other two components of the cascade, is expressed in AIY and AIZ interneurons, which together with AFD neurons comprise the thermosensory circuit (Satterlee et al., 2004). These differences in expression patterns between the members of the cascade may be relevant to CRH-1 independent functions of CMK-1. Next, the ability of the CKK-1/CMK-1 cascade to activate CRH-1 *in vivo* in *C. elegans* was tested using a reporter construct in which the CRH-1 responsive element (CRE) regulated expression of GFP. A series of experiments was then conducted which conclusively proved that the CKK-1/CMK-1/CRH-1 cascade serves a physiological signaling function in *C. elegans* (Kimura et al., 2002). This physiological function is likely to involve sensing or integrating environmental stimuli as nematodes null for the crh-1 gene show behavioral defects in response to food-rich media (Kimura et al., 2002).

As indicated above, there is also evidence supporting CRH-1-independent signaling pathways of CMK-1 *in vivo*. AFD neurons express the cmk-1 gene but not the crh-1 gene, and cmk-1 null worms show down-regulation of the gcy-8 gene in AFD neurons, whereas expression of this gene was not altered in worms null for crh-1 (Satterlee et al., 2004). Interestingly, the requirement of CMK-1 for gcy-8 is also independent of CKK-1, since the CMK-1 T^{179}A mutant rescued gcy-8 expression in cmk-1 mutant worms and worms lacking the ckk-1 gene did not show down-regulation of gcy-8 in AFD neurons (Satterlee et al., 2004). As AFD neurons are involved in mediating thermo-sensitivity, the response of worms to temperature changes was analyzed in cmk-1 null worms. These nematodes showed a weakened response to temperature change that could be rescued by re-expression of cmk-1 in the AFD neurons (Satterlee et al., 2004). It is unclear whether the weakened response was due to a defect in sensing the temperature change or in remembering/storing the initial cultivation temperature to which the AFD cells compare temperature change. If the latter proves to be the case then this would be an example of CMK-1 regulated memory storage in *C. elegans* that is independent of the CREB homologue (Satterlee et al., 2004).

5.2. The CaMK Cascade and CREB Regulation in Mammalian Cells

Strong evidence also exists that the CaMKK/CaMKIV cascade regulates CREB activity in mice. As mentioned at the beginning of this section, several CaMKs can phosphorylate CREB at S^{133} and activate its transcriptional function *in vitro* and in cultured mammalian cells. However, it required analysis of mice null for the Camk4

gene or its activator Camkk2 together with transgenic mice that express a kinase-inactive mutant of Camk4 gene in neurons to confirm that the CaMKIV/CaMKK cascade was physiologically relevant for CREB activation in the context of the whole organism. Ho et al. reported that cortical neurons from Camk4$^{-/-}$ mice have low basal levels of CREB phosphorylated at S^{133} and the increase in CREB phosphorylation after stimulation with KCl is impaired (Ho et al., 2000). Similarly, neurons present in the CA1 region of hippocampal slices from Camk4$^{-/-}$ mice fail to increase CREB phosphorylation at S^{133} after glutamate stimulation (Ho et al., 2000). More importantly, a stimulus such as restraint stress, which has been shown to stimulate CREB-phosphorylation in the cortex and hippocampus of mice (Melia et al., 1994), fails to increase CREB phosphorylation in the hippocampus (but not the amygdala) of Camk4$^{-/-}$ mice, revealing that CaMKIV is required for CREB activation *in vivo* in specific areas of the brain (Ho et al., 2000). Fear conditioning also stimulates CREB phosphorylation in particular areas of the mouse brain, such as the basolateral nucleus of the amygdala and the CA1 region of the hippocampus. Importantly, Camk4$^{-/-}$mice subjected to a fear conditioning regimen showed no increase or a markedly blunted increase in CREB phosphorylation in the basolateral region of the amygdala, somatosensory neurons, insular cortex neurons, the ACC and the CA1 region of the hippocampus, indicating that CaMKIV is involved in CREB activation that contributes to the memory of fear (Wei et al., 2002). In an independently created Camk4$^{-/-}$ mouse strain, placing mice in an unfamiliar environment, which stimulates CREB phosphorylation in the cerebellum of wild type mice, failed to increase CREB S^{133} phosphorylation in cerebellar neurons (Ribar et al., 2000). In the latter study, CREB phosphorylation was even decreased in the cerebellum of Camk4$^{-/-}$ mice prior to the environmental stimulus (Ribar et al., 2000). Taken together these experiments show clearly that CaMKIV is required to phosphorylate and activate CREB in a variety of neuronal types in the intact brain of mammals in response to physiological stimuli.

The relevance of CaMKIV-dependent phosporylation of CREB was also addressed using a different mouse model, in this case a transgenic mouse that specifically expresses a kinase-inactive CaMKIV mutant (dn-CaMKIV) in post-natal forebrain. Hippocampal slices prepared from this model show a significant reduction of CREB S^{133} phosphorylation in response to stimulation with membrance depolarizing amounts of KCl or glutamate (Kang et al., 2001). However, in the absence of stimulation, the basal level of CREB S^{133} phosphorylation was normal, a result that is different from that found in the Camk4$^{-/-}$ mice (in which basal CREB phosphorylation is decreased). Of course, this apparent difference could be the result of the mechanism used to impair CaMKIV activity. The total absence could result in no CREB phosphorylation even under non-stimulated conditions, whereas the dn-CaMKIV may only prevent the increase in endogenous CaMKIV activity that occurs in response to actute stimulation. In this instance, perhaps the dn-CaMKIV functions by sequestering the endogenous CaMKK, which is required to activate CaMKIV in response to a rise in Ca^{2+} (Chow et al., 2006). In support of this possibility, CREB phosphorylation in hippocampus from mice null for

Camkk2 is similar to that in hippocampus from wild type mice but show a failure to increase CREB S^{133} phosphorylation in response to environmental stimuli such as water-maze training (Peters et al., 2003). In addition, Camkk1$^{-/-}$ mice also show decreased CREB S^{133} phosphorylation in response to environmental stimuli such as the fear conditioning protocol. In particular, CREB phosphorylation fails to increase specifically in the lateral nucleus of the amygdala, compared to wild type mice (Blaeser et al., 2006). At any rate all of these studies support an important role for the CaMKKβ or α/CaMKIV/CREB cascade in neurons present in the brain that are activated in response to multiple types of neuro-excitatory stimulation and support the concept that only the phosphorylated, autonomously active form of CaMKIV is capable of entering the nucleus and phosporylating CREB (Chow et al., 2005).

5.3. The CaMK Cascade, LTP and Memory

Since CREB has been shown to be required for neuronal functions such as LTP and long-term memory formation and the CaMKK/CaMKIV cascade is required for CREB phosphorylation and activation, it follows logically that the CaMKK/CaMKIV would be required for LTP and long-term memory. This hypothesis has been addressed by analyzing LTP and some types of long-term memory in mice lacking either CaMKIV or CaMKK or mice expressing the dn-CaMKIV. Camk4$^{-/-}$ mice show normal gross morphology of the hippocampus and cortex (Ho et al., 2000). The hippocampus, which is important for memory, undergoes LTP and LTD, depending on the type of stimulation received. Treatment of CA1 hippocampal neurons with low frequency stimulation (LFS) induces LTD in hippocampal slices prepared from wild type or Camk4$^{-/-}$ mice. However, tetanic stimulation of the CA1 neurons, which results in LTP, did not result in LTP in hippocampus from Camk4$^{-/-}$mice, indicating that CaMKIV is involved in LTP but not LTD in the hippocampus. The requirement of CaMKIV for LTP was specific for the late phase, which requires protein synthesis (Ho et al., 2000). Because CA1 neurons have been implicated in spatial memory, Camk4$^{-/-}$ mice were subjected to spatial learning and memory tests such as the Morris water navigation task and the radial arm maze. Surprisingly, neither test showed differences between wild type and Camk4$^{-/-}$mice (Ho et al., 2000). The reasons why the LTP defect did not result in altered spatial learning and memory are not clear, although memory tasks that involve emotionality (such as neophobia and walking initiation, which showed some differences between wild type and Camk4$^{-/-}$ mice) may require CaMKIV (Ho et al., 2000).

However, a different mouse model of CaMKIV inhibition, the forebrain-specific transgenic dn-CaMKIV mouse, yielded somewhat different results. Spatial memory was tested using the hidden platform and the visible platform versions of the Morris water maze task. Both tests showed impaired spatial memory formation in dn-CaMKIV mice compared to controls, since dn-CaMKIV mice did not remember the location of the platform as well as wild type mice (Kang et al., 2001). Tests

that reflect locomotion and emotional behavior (anxiety) such as the open-field test and the elevated plus maze, did not show any differences between dn-CaMKIV and control mice, therefore it was concluded that only spatial memory requires CaMKIV (Kang et al., 2001). In order to distinguish between a problem with memory formation and memory retention in the dn-CaMKIV mice, the contextual fear conditioning test was used, and the results showed that the initial formation of the memory was similar between dn-CaMKIV and control mice (as measured 24 h after the cue), but the retention of the memory was impaired in dn-CaMKIV mice (Kang et al., 2001). On the other hand, pyramidal CA1 cells from dn-CaMKIV mice show normal pre-synaptic function, normal early LTP but decreased late LTP (Kang et al., 2001), as seen in the Camk4$^{-/-}$ mice (Ho et al., 2000). Although both models, Camk4$^{-/-}$ and dn-CaMKIV mice, show that LTP in the CA1 neurons of the hippocampus require CaMKIV, the actual long term memory phenotype that was predicted is only manifest in the dn-CaMKIV. The reason for this discrepancy is unknown, but one major difference between these two models is the time during development at which CaMKIV function is lost: from the very beginning of embryogenesis in the case of Camk4$^{-/-}$ mice versus from immediately after birth in the case of dn-CaMKIV mice. This difference in timing the absence of CaMKIV during development might promote the activation of compensatory mechanisms in the case of Camk4$^{-/-}$ mice that fail to occur in the dn-CaMKIV mice. This possibility could be explored by the use of conditional Camk4$^{-/-}$mice that would allow deletion of the Camk4 gene at various times during development.

The potential of CaMKIV has been analyzed not only for spatial memory in the hippocampus, but also in other regions of the brain that account for different types of memory. Because LTP has been associated with fear memory formation as well, the role of CaMKIV in LTP in the various neuron types involved in fear conditioning was examined, using tissue slices and electrical stimulation to induce LTP. The amygdala neurons of Camk4$^{-/-}$ mice showed significantly reduced LTP whereas no LTP could be measured in ACC neurons, somatosensory neurons or insular cortex neurons, indicating that CaMKIV is required to mediate LTP in multiple neuron types during fear memory formation (Wei et al., 2002). Using tests to assess fear memory formation based on the association of a neutral conditioned stimulus (such as tone) that has been paired with an aversive unconditioned stimulus (such as a foot shock) and with the context in which the animals were conditioned, the study shows that Camk4$^{-/-}$ mice had an impaired fear memory response, which is one type of long term memory (Wei et al., 2002).

The CaMKIV activator CaMKK would be predicted to have a role on neuronal function as well, since it is required to activate CaMKIV. This hypothesis was initially tested using mice lacking the Camkk2 gene (which encodes the CaMKKβ enzyme). Camkk2$^{-/-}$ mice show certain types of long term memory (LTM) deficiencies such as those required for social transmission of food preference (eating cued food 24 h after training) and spatial memory (hidden platform version of the Morris water maze, 6 days after training), but there was no difference between wild type and Camkk2$^{-/-}$ mice in the spatial memory after 10 days of training, suggesting

that CaMKKβ is involved in accelerating spatial memory formation (Peters et al., 2003). In addition, no other LTM based in hippocampus such as cued, conditional or trace fear conditioning, or any form of short term memory was altered in Camkk2$^{-/-}$ mice (Peters et al., 2003). Using hippocampus slices stimulated with electrodes, this study showed that late (but not early) long term potentiation (LTP) was decreased in hippocampal neurons of the Camkk2$^{-/-}$ mice compared to wild type control mice (Peters et al., 2003). Overall, the phenotypes displayed by Camkk2$^{-/-}$ mice are more similar to the transgenic mice overexpressing dn-CaMKIV than Camk4$^{-/-}$ mice. For example, basal CREB phosphorylation was normal in Camkk2$^{-/-}$ and dn-CaMKIV neurons but failed to increase upon stimulation, whereas Camk4$^{-/-}$ neurons show decreased CREB phosphorylation at S^{133}both in the basal state and upon stimulation. The reason for a LTM-only phenotype of Camkk2$^{-/-}$ mice may be due to the requirement of transcription and new protein synthesis in LTM (Silva and Giese, 1994) since CaMKK is required to activate the transcription activation capabilities of CaMKIV (Chow et al., 2005).

Interestingly, two groups have recently analyzed memory formation in mice null for the Camkk1 gene that encodes CaMKKα (Blaeser et al., 2006; Mizuno et al., 2006). Both groups report defects in memory envoked by fear conditioning. Both spatial and contexural fear memory required input from the hippocampus. The studies on loss of CaMKKβ show it to be important in spatial but not contextural fear memory. Contrariwise, Mizuno et al, reveal that mice null for CaMKKα are deficient in contexural but not spatial fear memory. Since CaMKKα and CaMKKβ are quite similar enzymes and are both efficient CaMKI and CaMKIV activating kinases, it is comforting to learn that at least some function of these two CaMKKs are not redundant.

5.4. The CaMK Cascade, ERK and Neuronal Plasticity

Besides the role of CaMKK/CaMKIV in regulating synaptic plasticity, the extracellular signal-regulated kinases (ERK) also are important in learning, synaptic plasticity and dendrite growth (for recent reviews see (Sweatt, 2004; Thomas and Huganir, 2004). ERK can be activated in response to stimulation of a variety of membrane receptors and can cross-talk with several other signaling pathways including a Ca^{2+}/CaM/CaMK pathway (Agell et al., 2002; Schmitt et al., 2004). However, the components of the pathway that link the initial Ca^{2+} rise in stimulated neurons to activation of the ERK pathway that is relevant for synaptic plasticity remains enigmatic. Recent evidence points to CaMKK/CaMKIα as likely components based on the observation that NMDA receptor stimulation of hippocampal neurons, which induces a cytosolic Ca^{2+} rise, results in ERK phosphorylation, and STO-609, or dn-CaMKIα (but not a dn-CaMKIV, or the naturally ocurring CaMKII inhibitory protein, CaMKIIN) prevents the activation (Schmitt et al., 2005). The addition of STO-609 to hippocampal slices stimulated to induce early-LTP or late LTP prevented or attenuated the process, leading to the suggestion that one of the CaMKKs is involved in both LTP phases (Schmitt et al., 2005). Since

Camkk2$^{-/-}$ mice only show a deficiency in late-LTP, but not early-LTP (Peters et al., 2003), and Camkk1$^{-/-}$ mice show normal LTP in the hippocampus (in spite of showing a deficiency in fear) (Blaeser et al., 2006; Mizuno et al., 2006), it is possible that CaMKKα in involved in LTP only in certain conditions or that STO-609 has an effect on LTP by inhibiting an unrelated protein. ERK activation is necessary for phosphorylation of the translation factors eIF4E and 4E-BP1, based on studies in transgenic mice expressing dn-MEK1, indicating that the ERK pathway contributes to proteins synthesis during late-LTP (Kelleher et al., 2004). Similarly, ERK activation mediated by the CaMK cascade (inhibitable by STO-609 or dn-CaMKIα) also results in phosphorylation of both eIF4E and 4E-BP1, suggesting that the CaMK cascade might be involved in the regulation of translation during LTP (Schmitt et al., 2005) (Figure 3). However, because STO-609 can inhibit several other proeins kinases as higher concentrations than required to inhibit the CaMKKs (Hawley et al., 2005), this concept needs to be addressed with alternative

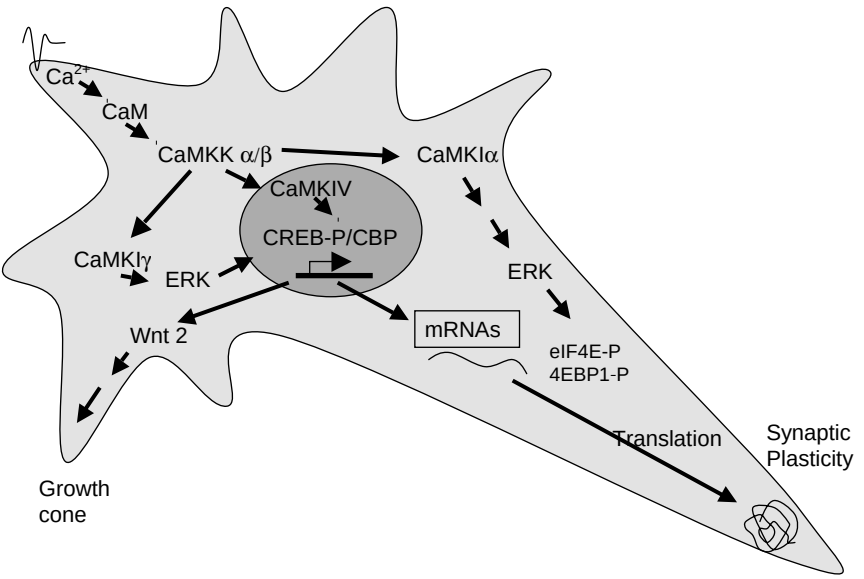

Figure 3. Neuron representing different CaMK cascade pathways that might be activated after electrical stimulation of the cell. Electrical stimulation (represented as a wave on the top left corner) induces a surge of Ca^{2+}, which binds to calmodulin and activates CaMKK α or β, which in turn activate CaMKIV (both activate CaMKIV, although in different neuron types), which proceeds to induce the expression of some genes, possibly by CREB phosphorylation. On the other hand, a CaMKK has been proposed to activate CaMK1α, which would crosstalk with the MAPK/ERK pathway and enhance translation of the mRNAs whose synthesis is regulated by the CaMKIV pathway. The production of such proteins is necessary for synaptic plasticity. Also, there is evidence that the CaMKK/CaMKI γ pathway is involved in dendrite growth, in particular stimulating the growth cone of the dendrites through activation of Wnt 2 expression. This figure is a summary of data collected from the different mouse models and neuron types studied, and details of some of the CaMKK/CaMKI pathways still require clarification

tools such as siRNA and/or genetically altered mice to deplete additional members of the CaMK cascade such as CaMKKα and CaMKIα.

5.5. The CaMK Cascade in the Cerebellum

Camk4$^{-/-}$ mice also showed anomalies associated with the cerebellum, such as ataxia, as reflected by deficiencies in balance, coordination and sensorimotor function in the strain generated in our laboratory (Ribar et al., 2000). However, in a Camk4$^{-/-}$ strain created in the Chatila laboratory, whereas differences in walking initiation were found, no differences in locomotor activity, ledge, inclined screen, rotating rod or balance were identified (Ho et al., 2000). One type of neuron that transiently expresses CaMKIV in the cerebellum is the Purkinje cell (Wang et al., 2001), and in Camk4$^{-/-}$ mice Purkinje cells were severely affected. Microscopic analysis revealed considerably fewer Purkinje cells in the Camk4$^{-/-}$ mice, and the remaining cells were smaller, immature (Ribar et al., 2000), or had reduced dendritic arbors (Ho et al., 2000).

Purkinje cell function was also analyzed in Camk4$^{-/-}$ mice. Analysis of cerebellar slices revealed that Purkinje cells from Camk4$^{-/-}$ mice remain innervated by multiple climbing fibers, which result in graded postsynaptic currents. These characteristics are usually restricted to immature Purkinje cells. The synaptic electrical properties of Purkinje cells are also altered by the absence of CaMKIV. On one hand, postsynaptic currents due to parallel fiber interaction are faster and have larger amplitude in cerebellar slices prepared from Camk4$^{-/-}$ relative to wild type mice. On the other hand, postsynaptic currents due to climbing fiber interaction have smaller amplitude in Camk4$^{-/-}$ slices compared to wild type ones (Ribar et al., 2000). Short term plasticity of the synapses is also altered in Purkinje cells from Camk4$^{-/-}$ mice, in a manner that favors synaptic facilitation rather than depression in response to stimulation of paired climbing fibers, and the frequency of postsynaptic currents is reduced (Ho et al., 2000; Ribar et al., 2000). Moreover, Purkinje cells stimulated with glutamate/depolarization (which induces LTD in response to subsequent glutamate stimulation in wild type Purkinje cells), fails to induce LTD in Purkinje cells from Camk4$^{-/-}$ mice. This failure occurs during the late phase of LTD, which requires protein synthesis, indicating that CaMKIV is required for the expression of proteins involved in the late phase of LTD in Purkinje cells (Ho et al., 2000). As only some of these physiological differences are common to the independently generated Camk4$^{-/-}$ strains, whereas each strain also exhibits unique behavioral phenotypes, it seems likely that genetic background differences may be responsible (C57BL6/J in the Chatila strain and a mixed C57BL6/J:129/Sv in the Means strain). It is important to note that Purkinje cells most highly express CaMKIV at the time of birth, and these levels decrease as the mouse matures to adulthood (Sakagami et al., 1992), suggesting that CaMKIV may be more relevant during the development and/or maturation of Purkinje cells than to the function of mature Purkinje cells in adult mice. Regardless of the differences between strains

of mice and the molecular mechanisms involved, it is clear that CaMKIV is required for LTP and long term memory formation in several types of neurons.

5.6. Concluding Remarks on the CaMK Cascade Relating to Memory

The similarities between the roles of CREB and CaMKKβ/CaMKIV in LTP and memory formation, together with the fact that the CaMK cascade results in CREB phosphorylation after stimulation, support the existence of a functional CaMKK/CaMKIV/CREB pathway in neurons (See Figure 3). Additional supporting evidence is provided by the observations that the major function of CREB is to regulate gene transcription, which results in the synthesis of new proteins required for the late phase of LTP (reviewed by Kandel, 2001). The stage in the late phase of LTP that is blocked by the protein synthesis inhibitor anisomycin, is exactly the same stage in the late phase of LTP blocked in neurons from dn-CaMKIV mice, suggesting that dn-CaMKIV ultimately prevents CREB-directed gene transcription (Kang et al., 2001). Moreover, this is also the same stage in the late phase of LT that is blocked in neurons from Camk4$^{-/-}$ mice (Ho et al., 2000). Finally, a study in which adenovirally-mediated over-expression of activated CaMKIV or activated CREB in the brain were compared side by side, showed that both proteins increased LTP and generated "silent synapses", supporting the idea that they are components of the the same signaling pathway (Marie et al., 2005).

However, the data discussed above are only correlative, and whether or not the primary function of CaMKK/CaMKIV is to phosphorylate and activate CREB has yet to be demonstrated. A recent report showed that there is not always a good correlation between the levels of phosphorylated CREB on S^{133} and transcriptional activation as seen in transgenic mice with a reporter construct consisting of several CRE sequences driving lacZ gene expression; it concluded that the level of phosphorylated CREB is not a reliable indicator of its function (Brodie et al., 2004). That idea fits with the hypothesis that CBP, a binding partner of CREB required for its transcriptional activity, may be limiting in cells where CREB levels are high (Brodie et al., 2004). Since the role of the CaMK cascade has relied on such a correlation, alternative methods to assess downstream consequences of CREB activity, such as changes in mRNA levels of regulated genes (such as c-fos), might be required to better interpret previous results. In this regard, although few studies have analyzed c-fos mRNA levels after stimulation of the CaMK cascade, one study showed that there was a good correlation between inhibiting CaMKIV using the dn-CaMKIV construct and inhibition of c-fos mRNA levels after a stimulus that increased intracellular Ca^{2+} (Kang et al., 2001), confirming that CaMKIV activates CREB-dependent transcription in the neurons.

On the other hand, there are a few observations that raise concerns that are worth mentioning. First, the effects of CREB and the CaMK cascade are not always identical. For example, after stereotaxic injection of viruses expressing a constitutively active CaMKIV (CA-CaMKIV) and a constitutively active form of CREB (CA-CREB), several parameters were compared in the CA area of the hippocampus.

CA-CREB had some effects similar to CA-CaMKIV such as a dramatic increase in the magnitude and maintenance of LTP (late phase), as well as increased excitatory currents mediated by the NMDAR. However, there were some effects that were different including increased excitatory currents initiated by the AMPAR, which were observed in CA-CaMKIV treated animals only (Marie et al., 2005). Therefore, it was concluded that the effects of CaMKIV on LTP mediated by NMDARs correlated with CREB phosphorylation, whereas the effects of CaMKIV mediated by AMPARs must involve some target of CaMKIV other than CREB. Also, some defects in the early phase of LTP in Camk4$^{-/-}$ mice were seen as early as 5 min after stimulation which is much too early for any new gene transcription to be causally relevant (Ho et al., 2000).

Second, as pointed out above, there are differences between the phenotypes of Camk4$^{-/-}$ mice and Camkk2$^{-/-}$ mice. One explanation is that CaMKKα, instead of CaMKKβ, may mediate some of the functions of CaMKIV, involved in fear conditioning, (Peters et al., 2003; Blaeser et al., 2006; Mizuno et al., 2006). Moreover, CaMKI is another target of the CaMKKs which is ubiquitously expressed in brain and its role has not yet been addressed by loss of function studies conducted in animals, so CaMKI might mediate some of the functions of CaMKKβ as well. Indeed, there is a report showing that stimulation of LTP in hippocampus slices results in activation of CaMKIα and ERK, as monitored by their phosphorylation status, and that these effects are probably mediated by a CaMKK, since STO-609 can inhibit phosphorylation of both CaMKI and ERK, as well as LTP (Schmitt et al., 2005). This study suggested that the CaMKK/CaMKIα pathway may mediate the early phase of LTP through ERK activation, based on the inhibition of LTP by STO-609, dn-CaMKKα and dn-CaMKIα (Schmitt et al., 2005).

Third, spatial memory formation is clearly dependent on CREB activation, but PKA and ERK, which also activate CREB, are also important regulators of spatial memory formation (Abel et al., 1997; Blum et al., 1999; Selcher et al., 1999). Since these kinases can phosphorylate CREB at S^{133}, it is not clear why the CaMK cascade would be required in response to the same stimulus to activate the same pathway, unless the CaMK cascade phosphorylates a different target. For example, the CaMK cascade might be required to phosphorylate CBP on S^{301}, to allow its binding to CREB (Impey et al., 2002). In support of this, the ability of the CaMKK cascade to induce CREB/CBP-mediated transcription requires the autonomous activity of CaMKIV, which is only generated upon phosphorylation of T^{200} in the activation loop of CaMKIV by CaMKK (Chow et al., 2005). On the other hand, CaMKIV can stimulate the activity of additional transcriptional activators such as RORα (Kane CD, 2000). Indeed, some phenotypes of the Camk4$^{-/-}$ mice are very similar to those produced by deletion of the RORα gene (Hamilton et al., 1996; Dussault et al., 1998; Steinmayr et al., 1998), inactivation of which produces the staggerer mouse (Hamilton et al., 1996; Dussault et al., 1998). Another transcription factor important for LTP formation is Zif268 (Jones et al., 2001). Zif268 is a zinc finger transcription factor required for LTP and long-term memory, as is CREB. Thus, a number of effects resulting from the inactivation of CaMKIV may be independent of CREB.

5.7. The CaMK Cascade in Neuronal Growth

Some pathologies that result in mental retardation, such as Down's and Fragile X syndromes correlate with dendritic abnormalities of neurons, resulting in abnormal development of neuronal circuits and activity – dependent plasticity. The growth of neurites (which includes axons and dendrites) during neuronal development has also been reported to involve the CaMK cascade. The evidence for such involvement comes from inhibiting the CaMK cascade using STO-609, dn-CaMKK or dn-CaMKIα, which results in abrogation of overall neurite growth in both hippocampal and cerebellar granule neurons. However, expression of dn-Akt or dn-CaMKIVnuc, that carries a nuclear localization signal to restrict it to the nucleus, did not result in alteration of neurite growth, indicating that CaMKK and CaMKI are the relevant members of the CaMK cascade involved in this process (Wayman et al., 2004). The STO-609-mediated inhibition of growth was rescued by expression of an STO-609-insensitive mutant of CaMKK ($CaMKK^{L233F}$) or a constitutively active form of CaMKIα, indicating the specificity of STO-609 for the CaMKK/CaMKI pathway in this case (Wayman et al., 2004).

A subsequent study focused more specifically on dendritic arborization stimulated by neuronal activity. In this study neurons were cultured *in vitro* and stimulated with KCl or bicuculline. Such stimulation resulted in a 70–100% increase in dendritic arborization within 2 days, and was dependent on NMDA receptors (which raise cytosolic Ca^{2+} levels). Again, STO-609 inhibited the arborization and the inhibitory effects of the drug were rescued by overexpression of the STO-609-resistant $CaMKK^{L233F}$, indicating involvement of a CaMKK. Using reagents such as dn-CaMKIα, dn-CaMKIVnuc, and siRNAs that specifically target each one of the 4 isoforms of CaMKI it was concluded that only CaMKIγ is required for dendritic arborization. These experiments suggest that a CaMKK/CaMKIγ pathway is involved in stimulus-dependent dendrite arborization (Wayman et al., 2006), which, in turn, activates the MEK/ERK pathway. It was also concluded that CREB was downstream of the CaMKK/CaMKIγ/MEK/ERK cascade and serial analysis of chromatin occupancy identifed Wnt-2 as a downstream target of the kinase pathway, which when added by itself to hippocampal slices is sufficient to stimulate neurite growth. Based on these results, the authors proposed a Ca^{2+}-dependent pathway which involves CaMKK/CaMKIγ/ERK/CREB/Wnt-2 to be important in the regulation of neurite growth in response to neuronal stimulation (Wayman et al., 2006).

5.8. Concluding Remarks on the CaMK Cascade and Neuronal Growth

The role of CaMKIγ in neurite growth is one of the few physiologically important roles attributed to the CaMKI isoforms. Besides possible roles in cell cycle for CaMKIα and neutrophil activation for CaMKIδ (see below), the functions of the other isoforms of CaMKI remain to be determined. Based on the involvement of CaMKIγ in neurite growth, it would be interesting to learn whether growth and

cell mobility in other cell types is also regulated by CaMKIγ (or other isoforms of CaMKI). It also remains a mystery why many members of the CaMK cascade are expressed in neurons and how neurons discriminate between different stimuli that raise intracellular Ca^{2+} to activate one or more specific members of the CaMK cascade. In this regard, the fact that CaMKIγ is the only CaMKI isoform that is localized to the cell membrane may provide a useful clue to function and specificity.

6. THE CAMK CASCADE REGULATES THE IMMUNE SYSTEM

One member of the CaMK cascade, CaMKIV, is particularly abundant in T lymphocytes. T cells are critical to the mammalian immune system because they recognize antigens on the surface of invading pathogens, viruses and tumor cells. Three major types of T cells have been described: helper T (TH), cytotoxic T (TC), and suppressor T (TS) cells. TH cells recognize and are activated by a sub-set of antigens, resulting in the secretion of cytokines that promote growth and responsiveness of B cells and TC cells. TC cells recognize and are activated by tumor cells or pathogens, resulting in their maturation into active cytotoxic T lymphocytes, which destroy the altered cells. And TS cells inhibit both T cell- and B cell-mediated immunity, limiting what might otherwise be an uncontrolled response (Moffet et al., 1993).

T cells recognize an antigen through a complex mechanism that includes three different receptors: T cell receptor (TCR), CD4 or CD8 and IL-1 receptor. In the case of TH cells, the antigen must be presented in the context of a type II major histocompatibility complex (MHC II), which is present only on macrophages, some B cells and a few other cell types. The TCR on a TH cell will recognize the antigen presented; the CD4 surface receptor on the TH cell will recognize the MHC II from the presenter cell; and finally, the IL-1 receptor on the TH cell will recognize the IL-1 factor secreted by the presenter cell. Only the combination of these stimuli will activate the TH cell, which then responds by releasing additional cytokines that will stimulate growth of other T cells (Moffet et al., 1993).

6.1. The CaMK Cascade and Activation of T Cells

The complex mechanism by which these receptors mediate the T cell response involves Ca^{2+}, among many other signaling molecules. Indeed, stimulation of the TCR leads to a rapid and transient Ca^{2+} peak in the cytoplasm that reaches a maximal level at one min. before being decreased in a manner that results in a lower sustained plateau. This new sustained plateau of Ca^{2+} lasts for 2 h and seems to be required for induction of IL-2 expression (reviewed in Crabtree and Clipstone, 1994). One of the effectors of the Ca^{2+} signaling pathway is the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (also known as serine/threonine phosphatase 2B). Calcineurin dephosphorylates and induces the nuclear translocation of the nuclear factor of activated T-cells (NFAT), which activates transcription of numerous genes encoding growth factors including IL-2 (reviewed in Crabtree

and Clipstone, 1994). However, in addition to NFAT other transcription factors such as CREB, c-fos and c-jun are required for T cell activation. Indeed, CREB phosphorylation on S^{133} was found to occur as early as 1 min. after T cell stimulation, and seemed to be independent of PKA, since the PKA inhibitor H89 failed to prevent CREB phosphorylation. Moreover, the T cell-specific expression of a dominant/negative form of CREB (with S^{133} mutated to A, so it cannot be phosphorylated) in mice revealed a critical role for CREB in T cell activation, as these mice were unable to produce IL-2, induce the expression of immediate early genes such as c-jun, c-fos and fra-2 and subsequently proliferate (Barton et al., 1996). At the time, the activator of CREB in T cells was unknown.

One candidate for the CREB kinase involved in the activation of T cells is CaMKIV, since it is not only expressed in T cells but becomes activated after T cell stimulation (Hanissian et al., 1993). Moreover, CaMKIV is phosphorylated on T^{200} of the activation loop by a CaMKK in the T cell-derived Jurkat cell line, suggesting that the CaMK cascade could be important for T cell function after TCR stimulation (Park and Soderling, 1995; Chatila et al., 1996). Subsequently, the role of CaMKIV has been addressed in mice that express either dn-CaMKIV or are null for the Camk4 gene. Both approaches resulted in defects in T cell activation and IL-2 synthesis, and suggested that CaMKIV is probably required at more than one point during T cell activation. For instance, transgenic mice expressing dn-CaMKIV specifically in thymocytes driven by the proximal promoter of the lck gene, which is active during thymocyte development but inactivated as mature T cells leave the thymus, showed a profound thymocyte activation defect (Anderson et al., 1997). On the other hand, $Camk4^{-/-}$ mice created in the Means' laboratory failed to show decreased activation of thymocytes but were defective in activation of memory T cells (Anderson and Means, 2002). A third study, using $Camk4^{-/-}$ mice created in the Chatila laboratory, did not identify a defect in thymocyte activation but showed a role for CaMKIV in the positive selection of T cells (Raman et al., 2001).

The inhibition of CaMKIV function by the expression of the dn-CaMKIV in thymocytes in living mice results in a smaller thymus that contains only 16% the number of thymocytes characteristic of control mice. One reason for the decreased number of thymocytes is increased apoptosis. This effect is specific to expression of dn-CaMKIV since T cells isolated from spleen are normal and contain control levels of CaMKIV due to the inactivation of the lck-promoter used to express this kinase mutant (Anderson et al., 1997). Consistent with the ability of CaMKIV to phosphorylate CREB, upon stimulation dn-CaMKIV thymocytes are deficient in CREB phosphorylation at S^{133} as well as expression of Fos-B, which is a downstream gene target of CREB (Anderson et al., 1997). In agreement with the hypothesis that Ca^{2+}, CaM, CaMKIV, CREB and Fos-B are components of the signaling pathway that leads to activation of the IL-2 gene, IL-2 production was decreased in dn-CaMIV thymocytes as was the IL-2 receptor component CD25 (Anderson et al., 1997). Because IL-2 is a growth/survival factor for thymocytes, these results were interpreted to indicate that CaMKIV might be required for

thymocyte survival and normal thymus development through the activation of the CREB/Fos-B/IL-2 pathway.

Based on the results obtained in the dn-CaMKIV transgenic mice, it came as a surprise that the thymic phenotype was not recapitulated in mice null for Camk4 (Camk4$^{-/-}$) (Raman et al., 2001; Anderson and Means, 2002). One possibility for the difference is that developmental compensatory mechanisms occurred that normalized thymocyte production and activation in Camk4$^{-/-}$mice. Alternatively, dn-CaMKIV may have sequestered one or both CaMKKs resulting in the inhibition of another pathway(s) necessary for proper thymocyte development not involving CaMKIV. However, one Camk4$^{-/-}$ strain does show a slightly altered maturation of thymocytes, based on the ratio of single-positive CD^{4+} and CD^{8+} T cells present in the thymus. Even in this case, these differences are not found in T cells present in the spleen and a reason suggested to explain this apparent discrepancy was that the expression of CaMKIV is decreased in peripheral T cells relative to thymocytes even in wild type mice (Raman et al., 2001).

One important step that occurs during thymocyte maturation is the process of T cell selection, which can be either positive or negative. It is of critical importance that T cells rapidly and strongly respond to a stimulus so the infection or invading agent can be contained as soon as possible; this response is called positive selection. Of equal importance is the ability of the T cells to recognize normal proteins and molecules as self, therefore avoiding a reaction against normal cells and inducing autoimmune diseases; this mechanism is called negative selection. Both positive and negative selection occur in thymocytes that are double positive (CD^{4+} and CD^{8+}) and are on their way to becoming single positive for either CD^{4+} or CD^{8+} (Sebzda et al., 1999). The role of CaMKIV in positive and negative selection was studied by crossing Camk4$^{-/-}$ mice with 3.L2, 3A9 or H-Y transgenic mice, 3.L2, 3A9 and H-Y being types of TCR that recognize defined peptides. 3.L2, 3A9 and H-Y mice all demonstrate increased positive selection, seen as a high number of single CD^{4+} (or CD^{8+}) T cells that express this particular TCR in the mouse. However, the cross of Camk4$^{-/-}$with either 3.L2, 3A9 or H-Y mice resulted in a low number of single CD^{4+} (and CD^{8+}) cells in each case, indicating that positive selection was impaired in Camk4$^{-/-}$ mice (Raman et al., 2001). The role of CaMKIV appeared to be specific for positive selection, since using the H-Y and 3.L2 strains in the presence of the antigen in the mouse (which is recognized as an endogenous protein and therefore induces negative selection in wild type mice), Camk4$^{-/-}$ mice showed normal negative selection. The mechanism by which CaMKIV regulates positive selection was not determined but correlated with the expression of several transcription factors, such as CD40L (a member of the TNF family), TNF-α and Nurr77, since the expression of these three factors was diminished in Camk4$^{-/-}$ cells after stimulation *in vitro*, compared to control cells (Raman et al., 2001). Surprisingly, however, phosphorylation of CREB on S^{133} was not diminished after stimulation (Raman et al., 2001), which is in stark contrast to the response of thymocytes expressing the dn-CaMKIV. Whether the thymocytes have developed a compensatory pathway to regulate CREB phosphorylation in response

to TCR stimulation during development, or whether CREB phosphorylation only represents an obligatory step for the primary mechanism by which CaMKIV activates transcription (such as CBP phosphorylation, for example), remains to be determined.

Interestingly, whereas thymocyte development is reasonably normal, Camk4$^{-/-}$mice do show an anomaly in T cell function at later stages of maturation: a subset of TH cells, called memory TH cells, but not naïve TH cells, are deficient in IL-2 production after stimulation. Memory T cells are cells that were previously exposed to the antigen they recognize, and consequently express surface makers such as L-selectin^{low} and CD44^{high}. In contrast, naïve cells have never encountered an antigen previously and are characterized by L-selectin^{high} and CD44^{low}. Both cell populations are found in the spleen, are CD^{4+} and express CaMKIV (Anderson and Means, 2002). Separation of these two types of cells can be accomplished by staining of the cell surface markers followed by cell sorting. Analysis of these cells showed that after TCR stimulation, memory T cells from Camk4$^{-/-}$ mice, but not naïve T cells, produced 5–8 fold less of three cytokines important for their activation: IL-2, IL-4 and interferon γ (Anderson and Means, 2002). Similar to thymocytes, both memory and naïve T cells respond to TCR stimulation by activation of CaMKIV and CREB leading to induction of immediate early and cytokine gene expression, but only memory T cells require this pathway for cytokine production. By contrast the Ca^{2+}-dependent pathway required for cytokine production in naïve T cells requires p90rsk rather than CaMKIV to phosphorylate CREB, transcribe immediate early genes and produce cytokines based on this study of cells isolated from Camk4$^{-/-}$ mice (Anderson and Means, 2002).

As naïve T cells also express CaMKIV, a different approach was taken to address the role of CaMKIV in these cells. In human naïve T cells CaMKIV was inhibited acutely using either a specific siRNA or the CaMK inhibitor KN-62 to preclude development of a compensatory mechanism that might result from chronic inhibiton. The acute decrease of CaMKIV resulted in a concomitant decrease in IL-2 expression indication that, similar to memory T cells and thymocytes, naïve human T cells also require CaMKIV for IL production (Pan et al., 2005). However, the mechanism by which CaMKIV regulates IL-2 production in human naïve T cells is independent of CREB, which is consistent with the lack of difference in CREB phosphorylation observed between mouse Camk4$^{-/-}$ and wild type naïve T cells (Anderson and Means, 2002). The role of CaMKIV in human naïve T cells is to phosphorylate Cabin1, which is a CaM-binding protein that functions as a repressor of the transcription factor MEF-2. In resting T cells, Cabin1 is bound to the IL-2 promoter in complex with MEF-2. Upon T cell stimulation, Ca^{2+} is increased which activates CaMKIV and CaMKIV phosphorylates Cabin1 on S^{2126} which promotes its binding to 14-3-3, leading to its nuclear export (Pan et al., 2005). It is important to note that there is a difference in the sequence surrounding S^{2126} between human and mouse/rat Cabin1, in that only the human Cabin1 has a consensus sequence for 14-3-3 docking. This difference may explain why naïve T cells from Camk4$^{-/-}$ mice do not show an IL-2 production phenotype whereas the human T cells do

(Pan et al., 2005). Regardless of the specific mechanism involved, the dn-CaMKIV and Camk4$^{-/-}$ mouse models together with the human naïve T cell results clearly show that CaMKIV is required to activate T cell cytokine synthesis in response to TCR stimulation. The point at which CaMKIV acts in the different types of T cells is summarized in Figure 4.

6.2. The CaMK Cascade in Hematopoietic Stem Cells

There is another aspect of immune function that is affected by the CaMK cascade. This aspect is the maintenance of hematopoietic stem cells that reside in bone marrow and give rise to blood. These cells are clinically important in bone marrow transplants. CaMKIV is expressed in the hematopoietic progenitor population that contains stem cells and are called KLS cells (based on the status of three cell surface markers c-Kit^{+}, Sca-1^{+}, Lin$^{-/low}$). Bone marrow from Camk4$^{-/-}$mice was found to contain a decreased number of KLS cells, as well as a decreased number of leukocytes in the blood (Kitsos et al., 2005). Moreover, the KLS cells from Camk4$^{-/-}$ mice are functionally impaired and unable to reconstitute blood upon bone marrow

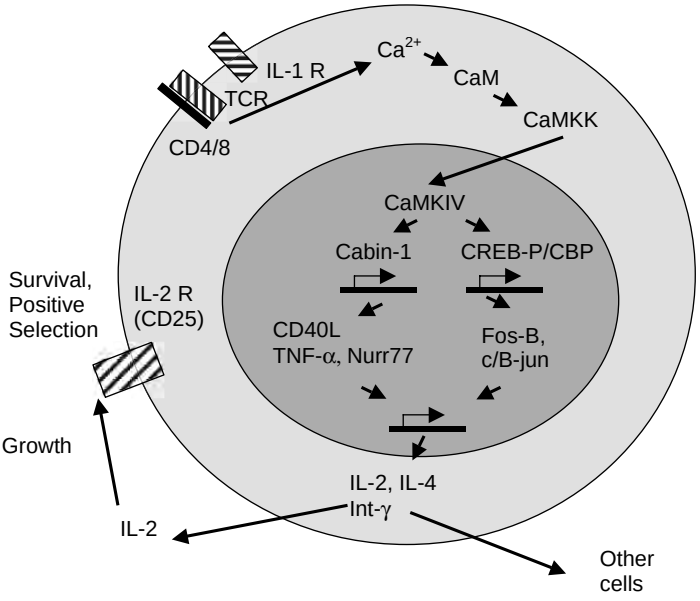

Figure 4. Schematic representation of the Ca^{2+}/CaM/CaMKK/CaMKIV/CREB/immediate early genes/IL pathway in T cells. Activation only takes place after the triple stimulation of the TCR, IL-1R and CD4/8 receptors. The end point effect of CaMKIV, which is the synthesis of interleukins and other growth factors, is common to T cells and thymocytes. Interleukins and other growth factors may have an autocrine or a paracrine effect. The different pathways included here have been addressed using Camk4-/- mouse models. Since the role of the CaMK cascade in hematopoietic stem cells and neutrophils is a little different, they are not included here

transplantation into lethally irradiated mice. Following transplantation there was an abnormally early burst of reconstitution at 3 weeks after the transplant, which was followed by complete exhaustion 9–12 weeks after transplantation. This stem cell exhaustion was confirmed by showing that serial transplantation of cells isolated from the primary transplanted mice had lost their long term repopulation ability.

The decrease in KLS cell number in Camk4$^{-/-}$ mice correlated with an increase in the number of apoptotic KLS cells compared to controls together with a three-fold increase in the number of proliferating KLS cells, suggesting that CaMKIV is required for maintenance of quiescence in the bone marrow stem cells that is critical for self-renewal and thus, for reconstitution of blood. The absence of CaMKIV from KLS cells correlated with decreased CREB phosphorylation at S^{133} in both basal and stimulated conditions in these cells (similar to the results found in T cells and neurons mentioned earlier). The Camk4$^{-/-}$ KLS cells also contain a two-fold decrease in the amount of the CREB binding protein CBP, suggesting that CaMKIV is important for activation of the CREB/CBP pathway in KLS cells (Kitsos et al., 2005). The observation that CBP levels are decreased is significant because mice haplo-insufficient for CBP show a similar hematopoietic phenotype (Kung et al., 2000; Rebel et al., 2002), supporting the view that CBP is a mediator in the CaMKIV pathway (Chawla et al., 1998). Bcl-2 was evaluated as a possible downstream target of CREB/CBP for three reasons. First, the Bcl-2 gene is regulated by Ca^{2+} (Apati et al., 2003) and CREB (Wilson et al., 1996). Second, Bcl-2 both inhibits apoptosis (Domen et al., 2000) and is required to maintain cell quiescence (Greider et al., 2002; Janumyan et al., 2003). Third, overexpression of Bcl-2 increases hematopoietic stem cell number and the ability of these cells to reconstitute blood (Domen et al., 2000). Indeed, Bcl-2 mRNA and protein are decreased in KLS cells from Camk4$^{-/-}$ mice compared to control KLS cells, establishing a clear correlation between CaMKIV, phospho-CREB, CBP and Bcl-2. Re-expression of CaMKIV (but not a kinase-inactive CaMKIV mutant) in Camk4$^{-/-}$ KLS cells resulted in restoration of phopho-CREB as well as CBP and Bcl-2 levels to those characteristic of wild type KLS cells. Similarly, re-expression of wild type but not inactive CaMKIV in Camk4$^{-/-}$ KLS cells rescued their hyperproliferation and apoptotic abnormalities (Kitsos et al., 2005). Collectively, these studies reveal an important role for CaMKIV in the maintenance of hematopoietic stem cells and suggest that inhibition of this enzyme in mammals might have immunosuppressive effects.

6.3. Concluding Remarks on the CaMK Cascade Relating to the Immune System

The presence of CaMKIV is positively correlated with the phosphorylation of CREB on S^{133}, and with the expression of several immediate early genes in response to TCR-mediated activation of thymocytes and T cells, or with the expression of anti-apoptotic genes such as Bcl-2 in hematopoietic stem cells. Whether this correlation is a cause-effect relationship or just parallel events that occur during the relevant activation process triggered by CaMKIV needs to be clarified. For example, since

CaMKIV has been primarily implicated in gene regulation, gene expression arrays using using mRNA isolated from wild type and Camk4$^{-/-}$ cells would provide information about the nature of genes whose expression is affected by CaMKIV. Moreover, the DNA elements in the promoters of genes affected by CaMKIV could be analyzed by chromatin immunoprecipitation using CaMKIV antibodies. If elements with which CaMKIV is associated other than CREs are identified, this could lead to a better appreciation of the transcription factors that might represent additional targets for CaMKIV.

The role of the CaMKIV kinases, CaMKKα and CaMKβ, has not yet been addressed in T cell activation and hematopoiesis. However, preliminary results from our laboratory indicate that there is a defect in hematopoiesis in Camkk2$^{-/-}$ mice as well (K.A. Anderson, unpublished results). The extent to which phenotypes of Camk4$^{-/-}$ and Camkk2$^{-/-}$ mice are similar or divergent, will point to the pathways that may utilize a CaMKKβ/CaMKIV cascade or depend on cascades involving CaMKKβ/AMPK or CaMKKα/CaMKIV. The availability of mice null for CaMKKα (Blaeser et al., 2006; Mizuno et al., 2006) will considerably facilitate obtaining answers to these interesting questions.

Finally, it has been suggested that a specific isoform of CaMKI, CaMKIδ, may be involved in the activation of granulocytes in response to agents such as fMLP that bind to G-protein coupled cell surface recptors or oportunistic pathogenic fungi such as *Aspergillus fumigatus* (Verploegen et al., 2005). In various types of granulocytic cells in culture a specific peptide inhibitor of CaMKIδ inhibited the generation of ROS and motility in response to fMLP as well as phagocytosis in response to *A. fumigatus*. These roles of CaMKIδ could be quite important in immune survelience although neither the cascade of events upstream or downstream of CaMKI that lead to these responses have been elucidated. Future studies investigating these mechanisms could be of consisiderable interest.

7. A CAMK CASCADE REGULATES ENERGY BALANCE

As mentioned in the introduction, CaMKKs can phosphorylate proteins in addition to CaMKI and CaMKIV. One such alternate target is the AMP-dependent protein kinase (AMPK) (Hawley et al., 1995; Hamilton et al., 2002), and recent studies present compelling evidence that CaMKKs are physiologically relevant activators of AMPK in cultured cells. These studies are of particular interest as AMPK is a kinase intimately involved in the regulation of metabolism, both at the cellular and organismal levels (Kemp et al., 1999). In mammals, AMPK has been implicated in diabetes, obesity and cardiovascular disease (Arad et al., 2002; Kemp et al., 2003) prompting a flurry of studies to identify the relevant AMPKK involved in each tissue that participates in the etiology of these diseases.

The AMPK is a heterotrimeric complex containing α catalytic, β scaffolding and γ regulatory subunits. The β subunit binds both α and γ subunits through its ASC domain as well as glycogen through its KIS domain whereas the γ subunit binds two AMP or ATP molecules via its two Bateman domains (Jiang and Carlson, 1997; Cheung et al., 2000; Hudson et al., 2003; Kemp, 2004). In mammals, each subunit is encoded by multiple genes (α1, α2, β1, β2, γ1, γ2 and γ3) which can

express several splice variants and/or be regulated by more than one promoter resulting in a higher number of possible heterotrimeric complexes (Hardie et al., 2003; Carling, 2004). The kinase activity of heterotrimic AMPK is regulated in response to a change in the cellular ratio of AMP:ATP, which occurs as cells become ATP-depleted and is sensed by AMP binding to the AMPKα subunit. The increase in the AMP:ATP ratio also facilitates AMPKK-dependent phosphorylation of T^{172}, which is present in the activation loop of the AMPKα subunit, presumably via a conformational change that exposes $T^{172}$to the activity of the upstream kinase. These events conspire to activate the kinase activity of AMPK. In other cases, however, AMPK can become phosphorylated at T^{172} without a rise in the AMP:ATP ratio, which is sufficient to activate the enzyme (reviewed by Carling (Carling, 2004)). In either case, activation of AMPK results in phosphorylation of downstream substrates leading to decreased energy expenditure and synthesis of ATP. One of the primary AMPK substrates is acetyl CoA carboxylase (ACC). Phosphorylation of ACC on S^{79} inhibits its activity leading to a decrease in conversion of acetyl CoA to malonyl-CoA. The decreased level of malonyl-CoA results in activation of the mitochondrial long chain acyl-CoA transporter CPT1, which in turn results in increased oxidation of acyl-CoA and generation of ATP by the mitochondria (Kahn et al., 2005). The activation of AMPK can also lead to negative or positive changes in gene expression. For example, in pancreatic β cells activation leads to the inhibition of insulin mRNA (Rutter et al., 2003) whereas in NPY neurons present in the arculate nucleus of the hypothalamus, activated AMPK results in an increase of NPY mRNA (Minokoshi et al., 2004).

The establishment of energy balance in the whole organism occurs by activating or inhibiting different pathways in different tissues. For example, in cardiac and skeletal muscle, AMPK activation results in increased fatty acid oxidation and glucose uptake, while in liver and adipose tissue AMPK activation results in decreased fatty acid synthesis; and in pancreatic cells, AMPK inhibits insulin secretion (Carling, 2004). AMPK activation by energy depletion and, in particular, in response to low glucose levels is not peculiar to mammals. Indeed, in unicellular organisms, such as the yeast *S. cerevisiae*, the AMPK ortholog, Snf1p, is also activated and regulates the expression of several genes whose protein products are required for survival under glucose depletion (Hardie et al., 1998). Moreover, there is another parallel between the mammalian AMPK pathway and the yeast Snf1p pathway as both catalytic proteins are subunits of a heterotrimeric enzyme complex and must be phosphorylated by an upstream kinase for full activation of the complex (Hardie et al., 1998). Indeed, studies in *S. cerevisiae* have contributed much to our understanding of the regulation of mammalian AMPK.

7.1. CaMKKs Activate the AMPK Pathway in Cultured Cells

The upstream activators of AMPKα have been intensively studied recently. Initially, three yeast kinases were identified using genetic and biochemical approaches and each was shown to phosphorylate Snf1p on T^{210} leading to its activation: Sak1

(formerly Pak1), Tos3 and Elm1 (Hong et al., 2003). Deletion of the three activators simultaneously (but not individually) reproduces the phenotype of the Snf1 deletion, which is the inability of cells to utilize non-fermentable carbon sources such as raffinose or glycerol/ethanol (Hong et al., 2003; Elbing et al., 2006). Sequence homology between the three yeast activators and mammalian genes was used to search for mammalian activators of AMPK, and three candidate genes were identified: CaMKKα, CaMKKβ and LKB1 (Nath et al., 2003). Recently, a fourth AMPK kinase called TAK1 has been identified (Momcilovic et al., 2006). Each of the four mammalian enzymes can rescue the $Snf1^-$ phenotype resulting from deletion of all three Snf1p kinases in yeast and can function as Snf1p kinases (Hong et al., 2005; Woods et al., 2005; Momcilovic et al., 2006). However, LKB1 was the first mammalian enzyme shown to function as a *bona fide* AMPK kinase based on the ability of purified LKB1 to phosporylate T^{172} of AMPK *in vitro* (Hawley et al., 2003; Hong et al., 2003). In addition, LKB1 is required for metformin-dependent activation of AMPK as HeLa cells (which do not express LKB1) and MEFs from $LKB1^{-/-}$ mice fail to respond to metformin unless LKB1 is re-expressed (Hawley et al., 2003).

The first clue that a CaMKK might serve as a AMPKK in mammals was that a pig brain extract known to contain an enzyme capable of phosphorylating CaMKI could phosphorylate AMPK *in vitro*, although at a lower rate than its known substrates CaMKI and CaMKIV (Hawley et al., 1995). The second clue came after the mammalian CaMKKs had been cloned and expressed and was the observation that purified CaMKKβ could stoichiometrically phosphorylate AMPKα on T^{172}(Hamilton et al., 2002). Additional evidence for the existence of an AMPK other than LKB1 was provided by studies on cardiac ischemia (Altarejos et al., 2005). Finally, three groups published papers almost at the same time which showed that cells lacking LKB1 contained an additional activity capable of phosphorylating AMPK at T^{172} leading to AMPK activation and identified the additional AMPKK to be CaMKKβ (Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005). One approach common to these three studies was the use of STO-609, which was found to inhibit phosphorylation of AMPKα in several mammalian cell lines (CCL3, HeLa and A549 cells) that occurred in response to stimulation with agents that increased intracellular Ca^{2+} (Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005). Since STO-609 is selective but not absolutely specific for the CaMKKs (Hawley et al., 2005), all three groups used siRNA to deplete CaMKKβ and found a marked inhibition of Ca^{2+}-induced AMPK phosphorylation and activation (assayed by examining phosphorylation of ACC). Thus, these studies identified CaMKKβ as a physiologically relevant activator of the AMPK pathway in mammalian cells (Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005). On the other hand, depleting CaMKKα in mammalian cells with siRNA produced mixed results. In one case it significantly inhibited AMPKα phosphorylation and activity in response to 2-deoxyglucose or ionomycin, although depletion of CaMKKα was less effective than depletion of CaMKKβ in reducing the levels of phosphorylated ACC (Hurley et al., 2005). In the other cases, depletion of CaMKKα resulted

in only marginal inhibition of AMPKα phosphorylation and activity in cells in response to increased Ca^{2+} (Woods et al., 2005) or failed to have an effect (Hawley et al., 2005), respectively. Purified CaMKKα can phosphorylate AMPKα *in vitro*, although 7 times less effectively than CaMKKβ (Hawley et al., 2005). Thus, the role for CaMKKα as an AMPK kinase has yet to be conclusively established in mammalian cells and should be the subject of further investigation.

AMPK is also important in the regulation food intake in rodents which is mediated by neurons in the arcuate and paraventricular nuclei of the hypothalamus. In these specific neurons, AMPK is activated by fasting and inhibited by feeding, resulting in increased or decreased food intake, respectively (Minokoshi et al., 2004). The mechanism by which AMPK activity is regulated in the paraventricular nucleus is not well understood, but anorexigenic hormones such as insulin and leptin result in the inhibition of AMPK (Minokoshi et al., 2004) whereas orexigenic hormones such as ghrelin and the agouti-related peptide stimulate hypothalamic AMPK activity (Andersson et al., 2004; Minokoshi et al., 2004), further supporting a central role for AMPK in the hypothalamic signaling pathways that regulate food intake. At this point, the downstream effectors of AMPK in the hypothalamus relevant for food intake regulation are unknown but is the subject of current investigation.

7.2. CaMKKβ Activates AMPK in the Hypothalamus

AMPK is expressed in many cell types including NPY and POMC neurons that are located in the arcuate nucleus of the hypothalamus. These two types of neurons are especially relevant to the regulation of food intake and energy balance because they are responsive to metabolic hormones leptin and ghrelin. Leptin is produced in adipose tissue. This hormone inhibits the expression NPY in NPY neurons and induces the expression of POMC in POMC neurons through activation of the ObRb receptor and consequent decreased phosphorylation of AMPK (Vaisse et al., 1996; Andersson et al., 2004; Minokoshi et al., 2004). Since NPY is a potent mammalian appetite stimulator while POMC is an appetite suppressor, leptin suppresses appetite in a manner that correlates with decreased activity of AMPK. Ghrelin, synthesized by the stomach in response to fasting, stimulates the expression of NPY in NPY neurons through activation of the Gq-coupled receptor GHSR, which results in an increase in intracellular Ca^{2+} as well as AMPK phosphorylation and activation (Andersson et al., 2004; Carreira et al., 2004). Ghrelin, therefore, results in the stimulation of appetite in a manner that is positively correlated with the activity of AMPK.

Recent results from our laboratory establish CaMKKβ as a physiologically relevant AMPK in mice and suggest that it mediates the ghrelin-induced activation of AMPK and NPY in hypothalamic NPY neurons. *In situ* hybridization revealed that CaMKKβ mRNA is expressed in the arcuate nucleus of the hypothalamus. Mice null for the CaMKKβ gene show decreased hypothalamic AMPK phosphorylation and activity as well as NPY/AgRP mRNAs. The CaMKKβ null mice fed

regular mouse chow share many phenotypes common to mice null for NPY and/or one of the NPY receptors. These phenotypes include: 1) normal food intake; 2) slightly increased body weight; 3) increased percent body fat; 4) decreased re-feeding after a fast; 5) increased thermogenic response; 6) increased serum leptin; 7) decreased energy expenditure; and 8) normal fasting blood glucose and free fatty acids (Marsh et al., 1998; Pedrazzini et al., 1998; Segal-Lieberman et al., 2003). We conclude that the primary pathway altered in mice null for CaMKKβ affecting energy balance includes AMPK and regulates NPY gene expression in the NPY hypothalamic neurons. Previous published studies have shown that ablation of the NPY/AgRP neurons in neonatal mice has no effect on feeding behavior whereas ablation of these neurons in adult mice leads to profound appetite and body weight loss (Bewick et al., 2005). To test the possibility that the CaMKKβ null mice may have compensated for the loss of NPY during development in order to maintain body weight, we infused the selective CaMKKβ inhibitor, STO-609, into the 3^{rd} ventricle of living wild-type mice over a 7 day period. The drug-treated mice showed a marked decrease in food intake accompanied by decreased body weight. Analysis of hypothalamic mRNA at the end of the experiment revealed 75% decreases in NPY mRNA in samples obtained from STO-609-treated mice. We postulate that CaMKKβ serves as a primary AMPKK in NPY neurons and that acute inhibition of this enzyme leads to decreased production of NPY which, in turn, causes decreased food intake and weight loss (see model in Figure 5).

7.3. Concluding Remarks on CaMKKβ Regulation of Energy Balance

The Camkk2$^{-/-}$ mouse model clearly shows that CaMKKβ regulates the production of NPY and therefore can regulate fat deposition and feeding under certain conditions. The decrease in NPY due to the absence of CaMKKβ correlates with a decrease in AMPK activity, although it remains to be determined whether it is the change in AMPK activity that mediates the effects of CaMKKβ in NPY neurons. In fact, although these preliminary studies predict the discovery of a new regulatory mechanism many details remain to be elucidated. For example, the specific hormones or receptors that activate CaMKKβ in the hypothalamus are unknown, as is the specific mechanism by which CaMKKβ regulates the levels of NPY in the NPY neurons. It also remains to be determined whether the acute deletion of the CaMKKβ gene in NPY neurons of adult mice will produce the same effects on food intake and body weight we observed due to the infusion of STO-609 into the 3rd ventricle of the brain. Moreover, whether depletion or inhibiton of CaMKKβ will protect mice against diet-induced hyperglycemia and insulin resistance must be explored. At this time it is also unknown whether CaMKKα plays a role in the regulation of energy balance although our *in situ* experiments also suggest that this enzyme is expressed in the arcuate nucleus as well as in other hypothalamic neurons. The availability of mice null for CaMKKα should enable this question to be addressed in the near future. Another unresolved question is the mechanism by which anorexic hormones such as leptin negatively regulate AMPK

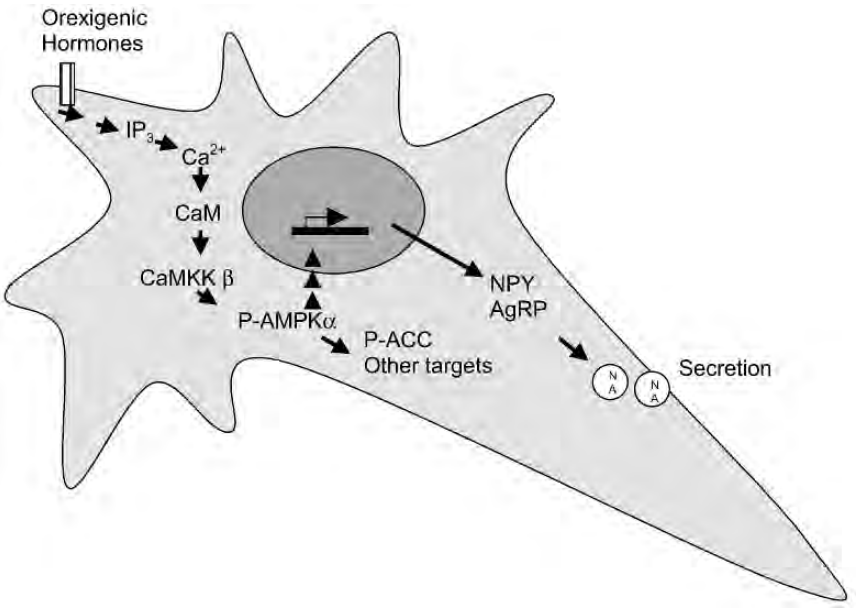

Figure 5. Arcuate nucleus neuron representing a CaMKKβ/AMPK cascade pathway that might be activated after hormonal stimulation. Stimulation by hormones such as ghrelin induce a surge in cytoplasmic Ca^{2+}, which binds to calmodulin and activates CaMKKβ, which in turn activates AMPK α by phosphorylation. Active AMPK can either promote transcription of some orexygenic factors such as neuropeptide Y (NPY) and agouti-related peptide (AgRP), or can phosphorylate metabolic enzymes such as acetyl-CoA carboxylase (ACC). Both NPY (N) and AgRP (A) are paracrine factors that will target other neurons of the central nervous system. This pathway is based on observations in Camkk2$^{-/-}$ mice as well as on several cell lines. Intermediate steps that are currently unknown are represented by several arrows in a row

activity and NPY expression in the arcuate nucleus. Lastly, whether CaMKKα and/or CaMKKβ play any role in the regulation of AMPK and glucose metabolism in other tissues such as muscle, adipose tissue and liver remains an open question. This will be especially interesting to explore because LKB1 has been shown to play an important role in liver and skeletal muscle (Sakamoto et al., 2005; Shaw et al., 2005). In the heart, however, ablation of LKB1 has effects only on AMPKα2 but not in AMPKα1, even though AMPKα1 becomes phosphorylated and activated in response to ischemia and anoxia, which could suggest roles for an additional AMPKK such as one of the CaMKKs in this organ (Sakamoto et al., 2006).

8. CONCLUDING REMARKS

In the recent years, an increasing number of studies show widespread relevance of the CaMK cascade. From the recent description of new isoforms, such as those of the CaMKI subfamily, to the analysis of mouse models null or transgenic

for a dominant/negative form of a specific member of the cascade, the CaMK cascade has been implicated to be involved in pathological processes as diverse as cancer (cell division and apoptosis), memory formation and maintenance, dendrite growth, autoimmune disease (T cell selection), immune system development (T cell maturation and hematopoiesis) and obesity (via NPY regulation in the hypothalamus).

Several of these roles are just emerging, and the implications may be larger than they currently appear. For example, if regulation of the cell cycle by the CaMK cascade is indeed more prominent in cancerous cells than in normal cells, then regulation of this pathway may be therapeutically more relevant than it was previously thought to be. Another role for the cascade that needs further research is the regulation of stem cell maintenance and expansion, in particular of hematopoietic stem cells, as these cells are vitally important for bone marrow transplantion and gene therapy. And certainly the regulation of obesity and diabetes through inhibition of the CaMKKβ/AMPK/NPY pathway is particularly interesting since it might be possible to identify drugs that specifically inhibit the ability of CaMKKβ to phosphorylate AMPKα without altering the ability of the CaMKKs to activate CaMKI or IV or for LKB1 to activate AMPK. Without question, much more research is needed on each member of the CaMK cascade to gain a better understanding of their physiological roles and how intervention of these pathways may lead to or be used to treat human disease.

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CHAPTER 8

CALCIUM CHANNELOPATHIES: VOLTAGE-GATED CALCIUM CHANNELS

P.J. ADAMS[1] AND T.P. SNUTCH[1,2,*]

[1]*Michael Smith Laboratorie, University of British Columbia, Vancouver, BC, Canada V6T1Z4*
[2]*Neuromed Pharmaceuticals Inc., 2389 Health Sciences Mall Vancouver, BC, Canada V6T1Z4*

Abstract: Since the initial identification of native calcium currents, significant progress has been made towards our understanding of the molecular and cellular contributions of voltage-gated calcium channels in multiple physiological processes. Moreover, we are beginning to comprehend their pathophysiological roles through both naturally occurring channelopathies in humans and mice and through targeted gene deletions. The data illustrate that small perturbations in voltage-gated calcium channel function induced by genetic alterations can affect a wide variety of mammalian developmental, physiological and behavioral functions. At least in those instances wherein the channelopathies can be attributed to gain-of-function mechanisms, the data point towards new therapeutic strategies for developing highly selective calcium channel antagonists

Keywords: calcium channel, L-type, P/Q-type, T-type, α_1 subunit, β subunit, $\alpha_2\delta$ subunit, γ subunit, familial hemiplegic migraine, episodic ataxia type 2, spinocerebellar ataxia type 6, Lambert-Eaton myasthenic syndrome, incomplete X-linked congenital stationary night blindness, X-linked cone-rod dystrophy, hypokalemic periodic paralysis, malignant hyperthermia susceptibility, Timothy syndrome, idiopathic generalized epilepsy, autism spectrum disorders, lethargic, ducky, stargazin

1. INTRODUCTION

Voltage-gated calcium channels are found in all excitable and many non-excitable cells where they contribute to numerous physiological processes including triggering muscle contraction and neurotransmitter release, regulating calcium-dependent enzymes and gene expression, mediating repetitive firing patterns and pacemaker

*Dr. Terrance P. Snutch, Michael Smith Laboratories, University of British Columbia, 2185 East Mall, Vancouver, British Columbia, Canada V6T 1Z4 E-mail: snutch@msl.ubc.ca

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activities, and developmentally controlling both neurite outgrowth and growth cone migration (reviewed in (Catterall, 2000)). As intracellular calcium concentrations must be finely controlled temporally and spatially, disruption of normal calcium channel activity can be highly detrimental physiologically. Biophysical analyses have generally categorized voltage-gated calcium channels into low-voltage activated (LVA) and high-voltage activated (HVA) subtypes, depending upon the membrane potentials at which they first open; LVA (also known as T-type) calcium channels open in response to small changes from the resting membrane potential whereas HVA channels are activated by stronger depolarizations. LVA channels have other distinguishing properties including a small single channel conductance (~5–12 picosiemens, pS), overlapping activation and inactivation ranges, rapid activation and inactivation kinetics, slow deactivation (closing) although a poorly defined pharmacology. Contrastingly, HVA calcium channels generally possess larger conductances (~15–25 pS), variable inactivation kinetics, faster deactivation, and have a well-defined pharmacology. Multiple types of distinct native HVA calcium currents have been categorized on the basis of single channel conductance, voltage-dependent, kinetic and pharmacological characteristics (called (L-, N-, P/Q- and R-types).

Biochemical studies have established that HVA calcium channels are multi-subunit protein complexes. The major α_1 subunit (~175–260 kDa) forms the channel proper and contains both the voltage-sensing mechanism and the calcium-selective pore, and is also the target for most pharmacological agents and second-messenger-dependent modulatory interactions. Mammalian genomes contain seven α_1 subunit genes encoding the HVA calcium channel family; $Ca_v1.1(\alpha_{1S})$, $Ca_v1.2$ (α_{1C}), $Ca_v1.3(\alpha_{1D})$ and $Ca_v1.4(\alpha_{1F})$ all encode distinct L-type calcium channels, $Ca_v2.1(\alpha_{1A})$ encodes P/Q-type channels, $Ca_v2.2(\alpha_{1B})$ encodes the N-type channel, while $Ca_v2.3(\alpha_{1E})$ encodes the R-type channel (reviewed in (Snutch et al., 2005)). There are also three genes encoding distinct T-type channel α_1 subunits; $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$. The various HVA α_1 subunits interact with single β and $\alpha2\delta$ subunit proteins which serve to modulate a variety of calcium channel properties including regulation by second messenger pathways, channel processing and trafficking, and biophysical characteristics. In mammals there are four known genes encoding β subunits ($\beta1$–$\beta4$) and four genes encoding $\alpha_2\delta$ subunits ($\alpha_2\delta1 - \alpha_2\delta4$). In the skeletal muscle L-type calcium channel complex there is also an associated forth subunit, γ, whose various members have been shown to also regulate calcium channel properties (Wei et al., 1991; Letts et al., 1998; Klugbauer et al., 2002). The γ subunit family of proteins is also known to be part of a group of AMPA receptor regulatory proteins (called TARPs) and thus they may not be specific to calcium channel complexes *in vivo* (Tomita et al., 2003). While the auxiliary β, $\alpha2\delta$ and γ subunits can also modulate T-type α_1 subunit properties in some exogenous systems, there is as of yet no biochemical data supporting the notion that T-type calcium channels are a multi-subunit complex.

To date, human channelopathies have been associated with five of the ten calcium channel $\alpha_1$subunit genes and two of the auxiliary subunit genes. Given

their widespread expression and contributions in both neuronal and muscle physiologies, mutations in calcium channel genes tend to manifest as severe phenotypes. Also described have been a number of mouse mutations in both α_1 subunit and the auxiliary β, α2δ and γ subunits that are deserving of mention as they provide further important clues as to physiological contributions of calcium channel complexes in whole animals.

2. P/Q-TYPE ($Ca_V2.1/\alpha_{1A}$) CALCIUM CHANNELS; CACNA1A GENE

P/Q-type calcium channels are one of the most abundantly expressed calcium channel subtypes in the mammalian nervous system (Bourinet et al., 1999; Jun et al., 1999). As well as being expressed on many cell bodies and dendrites, they are highly localized at presynaptic terminals throughout the brain and spinal cord where they mediate calcium influx essential for neurotransmitter release (Takahashi and Momiyama, 1993). They also contribute to the precise modulation of intracellular calcium levels important in second messenger signaling and calcium-dependent gene transcription (Sutton et al., 1999). Considering the widespread expression and essential neurophysiological roles, it is perhaps not unexpected that mutations in the CACNA1A gene encoding the P/Q-type channel α_1 subunit (Cav2.1) cause several severe channelopathies. CACNA1A mutations are associated with human neurological disorders such as Familial Hemiplegic Migraine (FHM1), Episodic Ataxia type 2 (EA2) and Spinocerebellar Ataxia type 6 (SCA6), and mice disorders tottering, leaner, rolling nagoya and rocker (Table 1 human, Table 2 mice). In addition to the defined mutations in the $Ca_v2.1$ calcium channel causing disease, an autoimmune attack on $Ca_v2.1$ channels is associated with Lambert-Eaton Myasthenic Syndrome (LEMS).

2.1. Familial Hemiplegic Migraine (FHM1)

Migraine is a severe neurological condition that affects approximately 11% of the North American and Western European populations. Migraine headaches can occur in isolation or, in approximately 20% of migraine sufferers, the migraine headache can be preceded by, or concurrent with, an aura (a subjective sensation; reviewed in (Goadsby et al., 2002)) FHM1 is a rare autosomal dominant subtype of migraine with aura, and other than its characteristic hemiplegia, has similar clinical features to typical migraine with aura. Based on neuroimaging and animal studies, it appears the aura phase of migraine is due to cortical spreading depression (CSD); CSD is a transient wave of neuronal hyperexcitability that begins at a focal point and slowly progresses over the cortex, followed by a long neuronal depression (Lauritzen, 1994). In FHM1, the aura manifests as a motor aura in combination with one or more visual, sensory and/or aphasic auras; the characteristic motor aura most frequently manifests as hemiplegia in both the upper and lower extremities and may or may not be associated with transient or permanent cerebellar

Table 1. CACNA1A ($Ca_v2.1$, α_{1A}): Functional results for mutations associated with FHM = familial hemiplegic migraine, EA2 = episodic ataxia type 2, SCA6 = spinocerebellar ataxia type 6, PCA = progressive cerebellar ataxia (although addition mutations have been associated with these disorders, only those with reported functional data are listed)

Mutation and Disease	Functional Analyses
R192Q- Pure FHM	• hyperpolarized shift in V_{50act} (Melliti et al., 2003) • hyperpolarized shift in $V_{50inact}$, reduced excitatory and inhibitory neurotransmission, reduced Ca^{2+} influx during action potential waveform (APW) (Cao and Tsien, 2005) • no change in current voltage relationship (Kraus et al., 1998; Cao and Tsien, 2005) • increased open probability (Hans et al., 1999) • increased current density (Hans et al., 1999; Tottene et al., 2002; van den Maagdenberg et al., 2004) • reduced current density (Tottene et al., 2002; Cao and Tsien, 2005) • increased single channel conductance (Tottene et al., 2002) • reduces G-protein inhibition (Melliti et al., 2003) • reduced threshold and increased velocity of cortical spreading depression (CSD) in knock-in mice. (van den Maagdenberg et al., 2004) • increased transmitter release at neuromuscular junction of knock-in mouse (Kaja et al., 2005)
T666M- FHM with cerebellar signs	• hyperpolarized shift in V_{50act}, increased rate of inactivation, increased current decay in response to 1Hz train pulse, reduced rate of recovery from inactivation (Kraus et al., 1998; Hans et al., 1999); no change in current voltage relationship, reduced Ca^{2+} influx during action potential waveform (APW), reduced excitatory and inhibitory neurotransmission (Cao and Tsien, 2005) • increased open probability (Tottene et al., 2002) • no change in open probability, reduced unitary conductance (Hans et al., 1999) • reduced current density (Hans et al., 1999; Tottene et al., 2002; Barrett et al., 2005; Cao and Tsien, 2005) • increased single channel conductance (Tottene et al., 2002) • reduction in gating current (Barrett et al., 2005)
V714A- Pure FHM	• hyperpolarized shift in V_{50act} (Kraus et al., 1998; Hans et al., 1999) • increased rates of inactivation and recovery from inactivation (Kraus et al., 1998; Hans et al., 1999) • increased open probability, decreased single channel conductance (Hans et al., 1999) • reduced current density (Hans et al., 1999; Tottene et al., 2002) • increased single channel conductance (Tottene et al., 2002) • decreased current decay in response to 1Hz train pulse (Kraus et al., 1998)
I1811L- FHM with cerebellar signs	• hyperpolarized shift in V_{50act} (Kraus et al., 1998; Hans et al., 1999) • no change in current voltage relationship (Cao and Tsien, 2005) • increase rate of recovery from inactivation (Kraus et al., 1998; Hans et al., 1999) • increased open probability (Hans et al., 1999)

	• reduced current density (Hans et al., 1999; Tottene et al., 2002) • increased single channel conductance (Tottene et al., 2002) • decreased current decay in response to 1Hz train pulse (Kraus et al., 1998)
K1336E- Pure FHM	• hyperpolarized shift in V_{50act} and V_{50inac}, increased current decay in response to 1Hz train pulse, decreased inactivation time constant (Mullner et al., 2004) (most effects are β subunit dependent)
V1457L- Mild FHM	• hyperpolarized shift in V_{50act} (Kraus et al., 2000; Tottene et al., 2002) • slowed rate of inactivation and reduce rate of recovery from inactivation (Kraus et al., 2000) • increased open probability, decreased unitary conductance, reduced current density (Tottene et al., 2002)
V1696I- Pure FHM	• slowed rate of inactivation, reduced rate of recovery from inactivation, increased current decay in response to 1Hz train pulse (Mullner et al., 2004) (most effects are β subunit dependent)
W1684R- FHM with cerebellar signs	• hyperpolarized shift in V_{50act}, hyperpolarized shift in $V_{50inact}$, reduced recovery from inactivation, increased current decay in response to 1Hz train pulse (Mullner et al., 2004) (most effects are β subunit dependent)
R583Q- FHM/Ataxia	• hyperpolarized shift in V_{50act} and $V_{50inact}$, reduced rate of recovery from inactivation, decreased current decay in response to 1Hz train pulse (Kraus et al., 2000)
D715E- FHM/PCA	• hyperpolarized shift in V_{50act}, hyperpolarized shift in $V_{50inact}$, increased rate of inactivation, decreased current decay in response to 1Hz train pulse (Kraus et al., 2000)
S218L- FHM with coma, following minor head injury	• hyperpolarized shift in V_{50act}, increased rate of inactivation initially, followed by a large reduction in the rate of inactivation, increased rate of recovery from inactivation, increased open probability, increased current density at low voltages, and decreased current density at high voltages, increased current decay in response to short train pulses but a decrease in current decay in response to long train pulses (Tottene et al., 2005a)
E147K- EA2	• reduced current density, possibly due to less channels reaching the membrane, reduced rate of inactivation (Imbrici et al., 2004)
F1406C- EA2	• reduction in Ba^{2+} current (Jen et al., 2001) • dominant negative effect – dramatic reduction in Ba^{2+} current (more pronounced effect in 47 than Δ47) (Jeng et al., 2006)
F1491S- EA2	• channel activity completely abolished (Guida et al., 2001)
R1547X , R1549X- EA2	• dramatic reduction in Ba^{2+} currents (Jen et al., 2001) • dominant negative effect – dramatic reduction in Ba^{2+} current (more pronounced effect in 47 than Δ47) (Jeng et al., 2006)

(*Continued*)

Table 1. (*Continued*)

Mutation and Disease	Functional Analyses
E1761K- EA2	• dominant negative effect - dramatic reduction in Ba^{2+} current (more pronounced effect in 47 than Δ47) (Jeng et al., 2006)
G293R- EA2	• no significant shift in V_{50act}, hyperpolarized shift in $V_{50inact}$, reduced recovery rate from inactivation (Wan et al., 2005) • depolarized shift in V_{50act}, no change in recovery rate from inactivation, increased rate of inactivation, decreased mean open time, decreased current density (Wappl et al., 2002); likely due to abnormalities in protein folding and trafficking to the plasma membrane (temperature dependent) (Wan et al., 2005) • increased current decay in response to 1Hz train pulse (Wappl et al., 2002; Wan et al., 2005)
C287Y- EA2	• depolarized shift in V_{50act}, hyperpolarized shift in $V_{50inact}$, decreased current density, likely due to protein abnormalities in protein folding and trafficking to the plasma membrane (temperature dependent; occurs at 37°C) (Wan et al., 2005)
H1736L- EA2	• depolarized shift in V_{50act}, increased rate of inactivation, decreased current decay in response to 1Hz train pulse, increased recovery rate from inactivation, decreased current density (Spacey et al., 2004)
R1279X, R1281X- EA2	• reduction in Ba^{2+} current, dominant negative effect (more pronounced effect in 47 than Δ47) (Jeng et al., 2006)
R1820X Generalized epilepsy and episodic and progressive ataxia	• dominant negative effect – dramatic reduction in Ba^{2+} current (Jouvenceau et al., 2001)
nt.4778-4780 : ctt deletion, deletion of A1593 and Y1594- EA2	• complete loss of channel activity in tsA-201 cells; in Xenopus oocytes depolarized shift in V_{50act}, increased rate of inactivation, increased current decay in response to 1Hz train pulse, reduced recovery rate from inactivation (Wappl et al., 2002)
polyQ expansion, 23 repeats -SCA6	• increased channel density (Piedras-Renteria et al., 2001) • no change in voltage-dependent or time-dependent properties (Piedras-Renteria et al., 2001)
polyQ expansion, 24 repeats (-NP) - SCA6	• hyperpolarized shift in $V_{50inact}$ (Toru et al., 2000) • no change in the $V_{50inact}$ (Matsuyama et al., 1999)
polyQ expansion, 27 repeats - SCA6	• increased channel density, no change in voltage-dependent or time-dependent properties (Piedras-Renteria et al., 2001)
polyQ expansion, 28 repeats (-NP)- SCA6	• hyperpolarized shift in $V_{50inact}$ (Toru et al., 2000)

polyQ expansion, 28 repeats (+NP) - SCA6	• depolarized shift in $V_{50inact}$ (Toru et al., 2000)
polyQ expansion, 30 repeats- SCA6	• hyperpolarized shift in $V_{50inact}$ (Matsuyama et al., 1999); hyperpolarized shift in $V_{50inact}$ when expressed with beta 4 subunit, but not with beta 2 or beta 3, increased inactivation time constant when expressed with beta 4 subunit, but not beta 2, and impaired G-protein regulation others (Restituito et al., 2000)
polyQ expansion, 33 repeats - SCA6	• cytotoxic (Kordasiewicz et al., 2006)
PolyQ expansion, 40 repeats - SCA6	• hyperpolarized shift in $V_{50inact}$ (Matsuyama et al., 1999)
polyQ expansion, 28 repeats - SCA6	• perinuclear aggregates of P/Q-type calcium channel in Purkinje cells of SCA6 patients (Ishikawa et al., 1999) • P/Q-type channels with CAG expansion transfected in HEK293 cells results in perinuclear aggregates and cell death (Ishikawa et al., 1999)
polyQ expansion, 72 repeats - SCA6	• increased channel density, no change in voltage-dependent or time-dependent properties (Piedras-Renteria et al., 2001)

signs such as ataxia or nystagmus. In the majority of cases, the headache pain directly follows the aura phase of the migraine attack and is thought to involve the trigeminovascular system (reviewed in (Pietrobon, 2005a)). The headache pain can last from less than 30 minutes to greater than 72 hours, with the mean duration being approximately 24 hours (for an extensive review of FHM1 features and statistics see (Thomsen et al., 2002)). About 50% of FHM1 patients have a mutation in the CACNA1A calcium channel gene (Ophoff et al., 1996) (FHM1), whereas the other approximately 50% have mutations in either the Na(+)/K(+)-ATPase gene (De Fusco et al., 2003) (ATP1A2;FHM2) or the sodium channel gene (Dichgans et al., 2005) (SCN1A; FHM3).

FHM1 is the most extensively studied calcium channelopathy. Since the first mutations in the CACNA1A gene were discovered in 1996 (Ophoff et al., 1996), 17 in total have now been detected (Figure 1). The 17 mutations appear to be localized primarily to the S4 voltage sensors and flanking regions. Over the past ten years, several groups have investigated the pathophysiological effects of FHM1 mutations on biophysical properties of the $Ca_v2.1$ calcium channel using electrophysiological analysis in *Xenopus* oocytes and mammalian expression systems (Table 1). Overall, the results vary considerably, some even contradictory, leaving our understanding of the precise correlation between channel function and disease mechanism unresolved. For example, whereas some results indicate the R192Q mutation increases $Ca_v2.1$ whole cell current density (Hans et al., 1999; van den Maagdenberg et al., 2004), results obtained by other groups indicate the same mutation decreases whole cell current density (Tottene et al., 2002; Cao and Tsien, 2005). In addition, one group found that the most prevalent FHM1 mutation T666M shifts the voltage dependence

Table 2. cacna1a ($Ca_v2.1$, α_{1A}): Functional results for mutations associated with tottering, leaner, rolling nagoya and rocker

Mutation and Phenotype	Functional Analyses
P601L- Tottering (*tg*)	• reduced current density (Wakamori et al., 1998) • no change in current density in dissociated Purkinje neurons (Dove et al., 1998) • decreased P/Q-type channel contribution at hippocampal Schaffer collateral synapses of *tg* mice (Qian and Noebels, 2000; Zhou et al., 2003) • decreased neurotransmitter release at parallel fiber-Purkinje cell synapse of *tg* mice (Matsushita et al., 2002) • no change in neurotransmitter release at parallel fiber-Purkinje cell synapse of tg mice (Qian and Noebels, 2000; Zhou et al., 2003) • decreased glutamate and GABA release in neocortex (Ayata et al., 2000) • increased threshold and decreased duration for CSD (Ayata et al., 2000) • alteration in gene transcription (Cicale et al., 2002) • increased G-protein-dependent modulation at the hippocampal Schaffer collateral synapse of *tg* mice (Zhou et al., 2003)
nt. 5901: 98bp, Intron insertion and frame shift or nt. 5763–5901: 139bp, exon deletion- Leaner (tg^{la})	• reduced current density (Dove et al., 1998; Lorenzon et al., 1998; Wakamori et al., 1998) • depolarized shifts in V_{50act} and $V_{50inact}$ (Wakamori et al., 1998) • no change in voltage dependent properties but reduced open probability (Dove et al., 1998) • decreased glutamate release in neocortex and increased threshold, decreased duration and reduced rate of CSD (Ayata et al., 2000) • increased apoptotic cell death in cerebellar granule cells in P20 leaner mice (Lau et al., 2004)
R1262G- Rolling Nagoya (tg^{rol})	• depolarized shift in V_{50act}, reduced current density, decreased neurotransmitter release at parallel fiber-Purkinje cell synapse of tg^{rol} mice (Matsushita et al., 2002); increased apoptotic cell death in cerebellar granule cells leaner mice (Suh et al., 2002)
T1310K- Rocker	• no electrophysiology data

of activation to more hyperpolarized potentials (Kraus et al., 1998), while another group found no change in the voltage dependent properties of the T666M mutant $Ca_v2.1$ channels relative to wild-type (Cao and Tsien, 2005). Mutations found in patients with similar clinical phenotypes often have reported opposing effects on the biophysical properties of $Ca_v2.1$ channels; e.g., K1336E and V714A have both been associated with pure FHM1 (without cerebellar signs) but cause reported increased (Mullner et al., 2004) or decreased (Kraus et al., 1998) current decay in response to 1Hz square pulse repetitive stimulations. Although different expression systems (Tottene et al., 2002), auxiliary subunits (Mullner et al., 2004), α_1 subunit splice variants (Adams et al., 2006) and experimental conditions likely account for a portion of the disparity

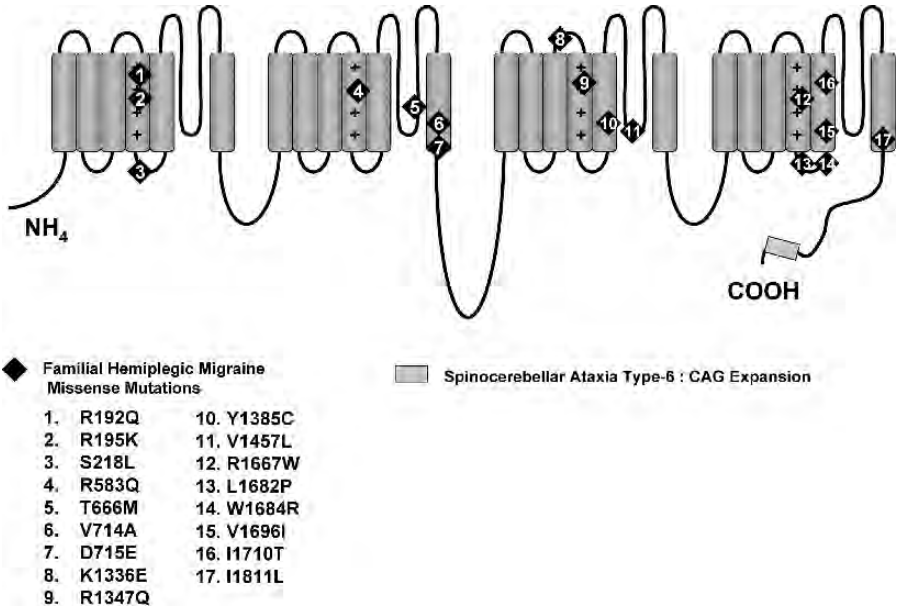

Figure 1. Mutations in the human $Ca_v2.1$ (P/Q-type) voltage-gated calcium channel associated with Familial Hemiplegic Migraine (FHM) and Spinocerebellar Ataxia Type-6 (SCA6)

in results between research groups, it is clear that FHM1 mutation pathophysiology is multivariable and complex.

Interestingly, data from recently generated knock-in mice carrying the R192Q (van den Maagdenberg et al., 2004) and S218L (Gherardini et al., 2006) FHM1 mutations, in conjunction with heterologous expression system analyses, are beginning to show trends in FHM1 mutation effects that may begin to explain the pathology behind the aura phase of migraine. In the heterologous system, several mutations associated with both pure FHM1 as well as FHM1 with cerebellar signs show a hyperpolarizing shift in V_{50act} (Kraus et al., 1998; Hans et al., 1999; Melliti et al., 2003; Mullner et al., 2004) (Table 1), indicating the majority of mutant channels in the expression system are available for opening at lower membrane potentials. Similarly, several mutations studied using single channel recordings show increases in open probability and single channel conductance (Hans et al., 1999; Tottene et al., 2002). All of these affects are predicted gain-of-function phenotypes with the potential to increase calcium influx at lower membrane potentials. *In vivo* work on R192Q and S218L knock-in mice shows a lower threshold for stimulation of CSD depression and increased velocity of propagation across the cortex in intact animals, consistent with gain-of-function effects (van den Maagdenberg et al., 2004; Gherardini et al., 2006). In neuromuscular junction preparations from R192Q knock-in mice, there is increased neurotransmitter release suggesting that FHM1 mutations may directly alter neurotransmitter release at

critical synapses and could therefore contribute to CSD susceptibility (Kaja et al., 2005). Although some data suggests that cultured neurons from Cav2.1 gene knock-out mice and transfected with the R192Q mutation reduce P/Q-type channel contribution to neurotransmitter release without changing overall synaptic strength due to a compensation by N-type calcium channels, *in vivo* evidence from R192Q knock-in mice confirms the neuromuscular junction result in that $Ca_v2.1$ channel-dependent glutamate release at cortical synapses is significantly increased (Tottene et al., 2005b).

Overall, evidence for the pathology of the aura phase of migraine from both the heterologous systems and knock-in mice largely supports the hypothesis that FHM1 mutations increase channel availability and that calcium influx at lower membrane potentials results in increased neurotransmitter release and susceptibility to neuronal firing resulting in CSD causing aura. However, little data has been presented to explain the headache pain phase of migraine. Although one study did show that CSD can activate trigeminovascular afferents and evoke meningeal and brainstem activity consistent with headache pain (Bolay et al., 2002), little is known about the direct involvement, if any, of $Ca_v2.1$ channels in the trigeminal pain pathway itself in the 80% of migraines where aura and CSD are not involved. Some evidence supports the notion that $Ca_v2.1$ P/Q-type channels are important in pain modulation of the trigeminal pain pathway via the periaquaductal gray (Knight et al., 2002; Knight et al., 2003), however further research is required to determine the exact roles of P/Q-type channels and FHM1 mutations in the trigeminal pain pathway relevant to migraine headache.

2.2. Episodic Ataxia Type 2 (EA2)

EA2, similar to FHM1, is an autosomal dominant disorder associated with mutations in the CACNA1A gene, but is clinically quite distinct. EA2 patients experience spontaneous episodes of ataxia (poor muscle coordination) that last for hours to days. In between attacks, patients often experience gaze-evoked or down-beat nystagmus (rapid, involuntary eye oscillations). Approximately 50% of patients experience migraine-like symptoms, and cerebellar atrophy is common (Lorenzon and Beam, 2000). Attacks are often initiated by emotional stress, exercise, or alcohol. Most patients respond well to treatment with acetazolamide (reviewed in (Jen et al., 2004)). EA2 is genetically variable and has been associated with missense, truncation and alternative splice site mutations.

To date, more than 40 individual mutations in the CACNA1A gene have been found to be associated with EA2, although compared to FHM1, significantly less is known concerning the biophysical implications of EA2 mutations on $Ca_v2.1$ properties. EA2 genetic alterations are distributed throughout the channel, with a large number of missense and premature truncations identified within the pore forming p-loops (Figure 2). Generally, the structure-function findings are relatively consistent across experimental conditions (Table 1). Both truncations and missense mutations consistently show a reduction in current density, proposed to be a result

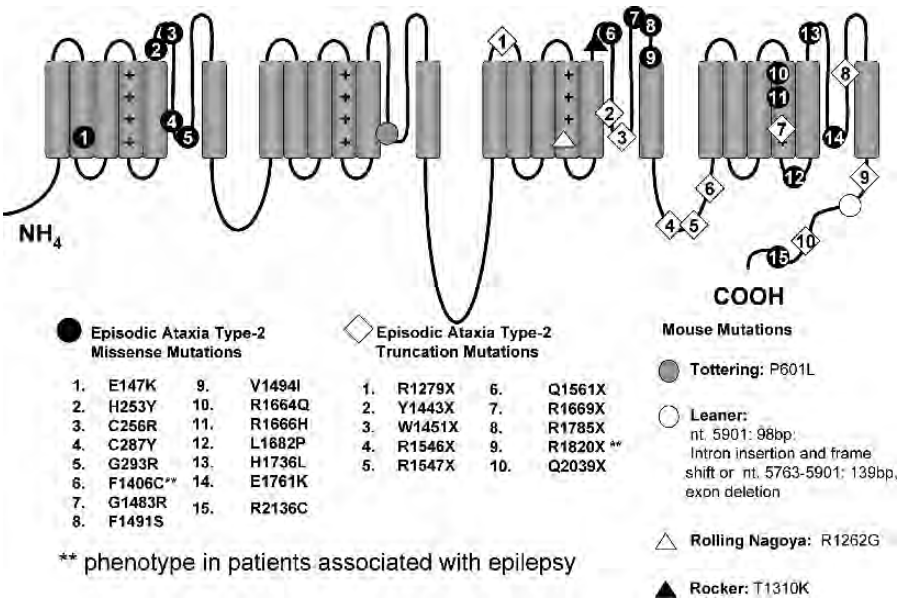

Figure 2. Mutations in the human $Ca_v2.1$ (P/Q-type) voltage-gated calcium channel associated with Episodic Ataxia Type-2 (EA2); mutations in the $Ca_v2.1$ mouse homolog associated with tottering (*tg*), leaner (tg^{la}), rolling nagoya (tg^{rol}) and rocker phenotypes

of fewer channels being properly folded and reaching the membrane (Imbrici et al., 2004; Wan et al., 2005; Jeng et al., 2006). The three EA2 missense mutations G293R, C287Y, H1736L and the deletion mutation nt 4778–4780 are consistent in demonstrating a net reduction of available channels due to a depolarizing shift in V_{50act}, increased rate of inactivation, and a reduced rate of recovery from inactivation (Wappl et al., 2002; Spacey et al., 2004; Wan et al., 2005) (Table 1). Whether it is through reduced trafficking of channels to the membrane or changes in gating properties, all EA2 mutations investigated thus far show a net reduction in $Ca_v2.1$-mediated currents, likely resulting in reduced transmitter release at critical synapses. Overall, unlike the general gain-of-function mutation effects observed for FHM1 mutations, EA2 mutations appear by and large to cause loss-of-channel function phenotypes.

The EA2 data is largely in agreement with results obtained from the epilepsy and ataxia mouse models tottering (*tg*), leaner (tg^{la}), rolling nagoya (tg^{rol}), and rocker (Figure 2). *tg*, tg^{la}, tg^{rol} and rocker all contain $Ca_v2.1$ gene mutations and show varying degrees of epilepsy and ataxia. Heterologous expression of mutations associated with *tg*, tg^{la}, tg^{rol} show similar biophysical properties to those for EA2; decreased current densities and depolarizing shifts in V_{50act} (Dove et al., 1998; Lorenzon et al., 1998; Wakamori et al., 1998; Mori et al., 2000). *In vivo* studies in the animal models confirms that the reduced channel function seen in the heterologous systems translates to decreased neurotransmitter release in neocortex, dissociated

cerebellar Purkinje cells and parallel fibre – Purkinje synapses (Ayata et al., 2000; Matsushita et al., 2002) (for review on $Ca_v2.1$ models see (Pietrobon, 2005b)). Collectively, these data suggest that EA2 mutations in $Ca_v2.1$ channels likely alter neurotransmitter release in whole animals; although definitive *in vivo* analyses remain to be described.

2.3. Spinocerebellar Ataxia Type 6 (SCA6)

A third autosomal dominant disorder associated with mutations in the $Ca_v2.1$ calcium channel is SCA6. SCA6 is characterized by progressive cerebellar atrophy resulting in progressive gait ataxia, incoordination, nystagmus, proprioceptive sensory loss and dysarthia (Zhuchenko et al., 1997). Zhuchenko and coworkers found that patients with SCA6 have a polyglutamine (CAG) expansion in exon 47 (Figure 1); making SCA6 a member in the group of neurodegenerative disorders containing CAG repeats, which includes Huntington's disease amongst others. Whereas unaffected people tend to have CAG repeats numbering between 4 and 16 in CACNA1A, patients with SCA6 have expansions of greater than 21 CAG repeats. The length of the expansion appears directly correlated with age of onset, e.g., greater CAG expansion is associated with early age of disease onset (Ishikawa et al., 1997). The CAG repeat expansion is associated with severe cerebellar Purkinje cell loss, moderate granule cell and dentate nucleus neuronal loss, and mild neuronal loss in the inferior olive (Zhuchenko et al., 1997). The mechanisms involved between SCA6 mutations in the $Ca_v2.1$ channel and neuronal death have not been completely resolved. Biophysical analyses of polyglutamine expansions in the $Ca_v2.1$ calcium channel show a range of effects on voltage and time dependent properties with a strong dependence on both auxiliary subunit and α_1 subunit splice variant composition when expressed in heterologous systems (Table 1) (Restituito et al., 2000; Toru et al., 2000). It has been speculated that the alterations in channel function may result in changes in intracellular calcium concentrations and consequently induced cell death. However, analysis of cerebellar tissue from SCA6 patients reveals perinuclear aggregates in Purkinje cells, and transfection of polyglutamine expanded $Ca_v2.1$ channel cDNA in HEK293 cells suggests that cell death is likely due to the perinuclear aggregates (Ishikawa et al., 1999). A interesting recent report showed that the carboxyl terminus of the $Ca_v2.1$ channel is cleaved and translocated to the nucleus under wild-type conditions, but when the polyglutamine expansion is extended to greater than 33 repeats the nuclear translocated channel somehow induces cell death (Kordasiewicz et al., 2006). Overall, it appears that neuronal loss observed in SCA6 brains is likely due to a combination of altered channel properties resulting in abnormal intracellular calcium concentrations and perinuclear and/or nuclear channel protein aggregates ultimately resulting in cell death.

Although substantial progress has been made towards understanding the cellular mechanisms behind the channelopathies associated with the $Ca_v2.1$ channel, important questions remain. First, all three disorders described above exhibit temporal-related phenotypes, e.g., FHM1 and EA2 are episodic and SCA6 is

progressive; what is responsible for these phenotypes being periodic or progressive? Second, the $Ca_v2.1$ channel is ubiquitously expressed in the mammalian brain (Bourinet et al., 1999; Jun et al., 1999), yet mutations result in highly localized phenotypes. Are these phenomena due to changes in the isoform expression of the α_1 subunit, auxiliary subunit composition or perhaps due to other protein interactions or environmental conditions? Some initial studies are beginning to address these questions (Toru et al., 2000; Mullner et al., 2004; Adams et al., 2006; Jeng et al., 2006)).

2.4. Lambert-Eaton Myasthenic Syndrome (LEMS)

LEMS is a neuromuscular transmission disorder characterized by reduced acetylcholine quantal release and is associated with small-cell lung carcinoma (SCLC) in approximately 60% of patients (Lang et al., 1983). P/Q-type channels are implicated in LEMS, although unlike FHM1, EA2 and SCA6, LEMS is not a true channelopathy as it does not result from defined mutations in the channel. Rather the sera from LEMS patients contains auto-antibodies against voltage-dependent calcium channels, with an apparent preferential targeting of $Ca_v2.1$ calcium channels at the neuromuscular junction (Lennon et al., 1995; Pinto et al., 2002). Clinical features of LEMS includes skeletal muscle weakness in proximal and trunk muscles, with the most severe effects observed in lower limbs (for review see (Flink and Atchison, 2003)). Auto-antibodies are thought to be initiated in response to the SCLC tumor (O'Neill et al., 1988) and via targeting $Ca_v2.1$ channels at the neuromuscular junctions reduce channel availability for neurotransmission (Lennon et al., 1995). It has been shown that the LEMS auto-antibodies do not alter channel voltage or kinetic properties, but instead act in an all or none fashion, likely eliminating available channels from the population (Grassi et al., 1994; Magnelli et al., 1996). Drugs that prolong the duration of action potentials and enhance intracellular calcium levels, such as 4-aminopyridine and 3,4-diaminopyridine, offer symptomatic relief in some LEMS patients; however, side effects can often be severe (Flink and Atchison, 2003).

3. L-TYPE ($Ca_V1.4/\alpha_{1F}$) CALCIUM CHANNELS; CACNA1F GENE

L-type calcium channels are the primary trigger for excitation-contraction (EC) coupling in cardiac, skeletal, and smooth muscles (Bean, 1989). They are also found in most central and peripheral neurons where they in part control calcium-dependent gene expression, as well as in endocrine cells and many types of non-excitable cells where they contribute to a variety of processes including exocytotic release. Unlike most synapses in the brain and spinal cord that rely on P/Q- and N-type calcium channels for neurotransmitter release, (Wheeler et al., 1994), the presynaptic terminals in photoreceptor cells rely on the $Ca_v1.4$ (α_{1F}) L-type calcium channel for mediating glutamate release (Tachibana et al., 1993; Nachman-Clewner et al., 1999). Photoreceptor neurotransmission is atypical; first,

photoreceptor cells do not fire action potentials, but rather have continuous graded membrane potentials; and secondly, most photoreceptors are tonically depolarized in the absence of a light stimulus resulting in continuous glutamate release and subsequently hyperpolarize in response to light stimuli (Wu, 1994). Considering its critical role in vision, it is perhaps not surprising that mutations in the CACNA1F gene are implicated in the vision related channelopathies incomplete X-linked congenital stationary night blindness (IXLCSNB) and X-linked cone-rod dystrophy (CORDX).

3.1. Incomplete X-linked Congenital Stationary Night Blindness (IXLCSNB)

Congenital stationary night blindness begins in childhood and includes a series of non-progressive disorders of varying hereditary patterns including an autosomal dominant (adCSNB), autosomal recessive (arCSNB) and the X-linked (XLCSNB). Based on electroretinogram (ERG) readings, XLCSNB can be subdivided into complete and incomplete forms based on either no rod function or low level rod-mediated function, respectively (Miyake et al., 1986). More than 60 mutations have been identified in the CACNA1F gene in patients with incomplete X-linked congenital stationary night blindness (IXLCSNB). Missense and truncation mutations have been identified in nearly all structural domains of the channel without predominance of any particular region (Figure 3). Unlike the autosomal dominantly inherited channelopathies associated with the P/Q-type channel, XLCSNB follows an X-linked recessive pattern of inheritance (Berger et al., 1995). IXLCSNB is characterized by variable degrees of night blindness, reduced visual acuity, myopia or hyperopia (short and long sightedness, respectively), and nystagmus. Physiologically, the eye fundus is normal, however, ERG recordings indicate rod-related function is significantly reduced and cone function is below normal (Tremblay et al., 1995). Although it is beginning to gain acceptance that the visual impairments are a result of reduced neurotransmission efficiency between photoreceptors and second-order neurons as a result of mutations in the $Ca_v1.4$ channel, experimental results are not yet conclusive as to the precise molecular mechanisms involved.

Similar to that for the analyses of FHM1 mutations in heterologous systems, biophysical investigation of mutations associated with IXLCSNB in heterologous systems have led to variable results; ranging from no reported effects on biophysical properties to contradicting gain-of-function and loss-of-function effects (Table 3). McRory and coworkers found that three IXLCSNB mutations, G674D, L1364H and A928D, did not alter measurable L-type channel biophysical properties when expressed in HEK293 cells (McRory et al., 2004). Hoda and colleagues found a complete loss of channel expression for IXLCSNB mutation S229P, a loss-of-function effect for mutation L1068P due to reduced channel opening, an increased rate of inactivation and reduced rate of recovery from inactivation, and a gain-of-function effect for the G369D mutation due to a hyperpolarizing shift in

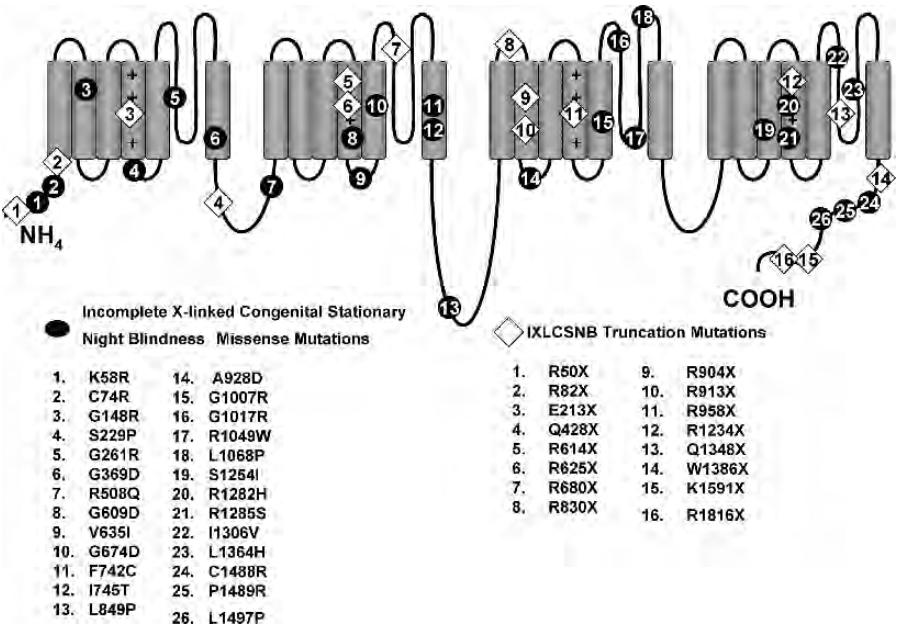

Figure 3. Mutations in the human $Ca_v1.4$ (L-type) voltage-gated calcium channel associated with Incomplete X-linked Congenital Stationary Night Blindness (IXLCSNB)

V_{50act}, reduced rate of inactivation, and a loss of calcium dependent inactivation (Hoda et al., 2005). Contrastingly, McRory and coworkers found a loss-of-function effect due to a depolarizing shift in the V_{50act} for the G369D mutation (McRory et al., 2004). Furthermore, Hemara-Wahanui et al found IXLCSNB mutation I745T caused a gain of function effect by shifting the V_{50act} to more hyperpolarized potentials and reducing the rate of inactivation (Hemara-Wahanui et al., 2005). Collectively, the biophysical data from IXLCSNB mutations thus far does not give a clear indication as to how the distinct point mutations might result in abnormal neurotransmission in photoreceptors. However, a recent investigation by Hoda et al. revealed that mutations R508Q and L1364H affect expression density in a temperature dependent manner (similar to EA2 mutation analysis (Wan et al., 2005)); showing no change at subphysiological temperatures of 29–30 °C but exhibiting reduced protein expression at physiological temperatures (Hoda et al., 2006). The authors speculate that the temperature dependence may be a critical factor in accurately determining the mutation effects in the physiologically relevant environment. Although a plausible hypothesis, similar to that for the FHM1 mutational analyses, the discrepancies in results for IXLCSNB may also be due to channel subunit composition, exogenous expression system, α_1 subunit splice variation and/or experimental conditions including temperature.

Table 3. CACNA1F ($Ca_v1.4, \alpha_{1F}$): Functional results for mutations associated with IXLCSNB = incomplete X-linked congenital stationary night blindness and X-linked cone-rode dystrophy (although addition mutations have been associated with IXLCSNB, only those with functional data completed are listed)

Mutation and Disease	Functional Analyses
S229P- IXLCSNB	• complete loss of channel activity (Hoda et al., 2005)
G369D- IXLCSNB	• hyperpolarized shift in V_{50act}, decreased rate of inactivation, removed calcium-dependent inactivation (Hoda et al., 2005); increased rate of voltage-dependent activation and depolarized shift in V_{50act} (McRory et al., 2004)
R508Q- IXLCSNB	• reduced channel expression (temperature dependent; occurs at 37°C) (Hoda et al., 2006)
G674D- IXLCSNB	• no change in channel properties (McRory et al., 2004)
I745T- IXLCSNB	• hyperpolarized shift in V_{50act} and reduced rate of inactivation (Hemara-Wahanui et al., 2005)
A928D- IXLCSNB	• no change in channel properties (McRory et al., 2004)
L1068P- IXLCSNB	• decreased channel opening, increased rate of inactivation and reduced rate of recovery from inactivation (Hoda et al., 2005)
L1364H- IXLCSNB	• reduced current density, reduced channel expression (temperature dependent), increased rate of inactivation, increased rate of recovery from inactivation (Hoda et al., 2006)
W1386X,	• no change in channel properties (McRory et al., 2004)
W1451X,	• complete loss of protein expression (Hoda et al., 2005)
W1459X, W1440X- IXLCSNB	
IVS28-1,	• no electrophysiology data (Jalkanen et al., 2006)
gcgtc to **tgg** conversion, aberrant splicing- CORDX	

3.2. X-linked Cone-Rod Dystrophy (CORDX)

CORDX usually begins within the first two decades of life and is progressive. Clinical features of the disorder include: reduced visual acuity, poor colour vision, fundus abnormalities, central scotomas in the visual field, photophobia, myopia and low b-waves in ERG recordings (Jacobson et al., 1989; Brown et al., 2000). A single

mutation has been recently found in a CORDX family (Table 3). The mutation is in the splice acceptor site of intron 28 in the CACNA1F gene and is predicted to cause either a premature stop codon in the mRNA transcript, or variable size deletions of the transcript. The mutation co-segregated completely with the disease phenotype in the CORDX family studied (Jalkanen et al., 2006). As of yet, no biophysical analysis has been reported for this mutation.

4. L-TYPE ($Ca_V1.1/\alpha_{1S}$) CALCIUM CHANNELS; CACNA1S GENE

$Ca_v1.1$ (α_{1S}) L-type calcium channels are important in striated muscle cells for coupling membrane depolarization to the release of calcium from cytoplasmic stores and triggering EC coupling (Rios and Brum, 1987). In this instance, depolarizing changes in membrane potential cause a conformational change in $Ca_v1.1$ and without a requirement for calcium influx, induce an allosteric interaction with the sarcoplasmic reticulum (SR) ryanodine receptor (RyR1) ultimately inducing calcium release and muscle contraction (Tanabe et al., 1990; Flucher and Franzini-Armstrong, 1996; Kugler et al., 2004). Related to this role as a voltage sensor for the RyR1, $Ca_v1.1$ has been implicated in two muscle disorders: hypokalemic periodic paralysis (HypoPP) and malignant hyperthermia susceptibility (MHS).

4.1. Hypokalemic Periodic Paralysis (HypoPP)

HypoPP is an autosomal dominant disorder characterized by periodic muscle weakness in association with low serum potassium levels. Disease progression is variable, however, typical progression includes onset during adolescence and symptoms decreasing by fifty or sixty years of age (Morrill et al., 1998). Experiments performed on biopsied intercostal muscle fibres from three patients with HypoPP have revealed that muscle fibres have a reduced excitability and increased sodium conductance, both of which are exacerbated in reduced extracellular potassium concentrations (Rudel et al., 1984). The effects are due to long-lasting depolarizations leading to sodium channel inactivation and loss of membrane excitability (Jurkat-Rott et al., 2000b; Ruff, 2000). Although mutations in both voltage-gated sodium and potassium channels contribute to HypoPP, five missense mutations in the CACNA1S gene encoding for the $Ca_v1.1$ channel have been associated with HypoPP. Four of the five mutations are located in the voltage S4 voltage sensor domains of the $Ca_v1.1$ L-type channel (Figure 4).

Biophysical investigations of mutant $Ca_v1.1$ channels in a variety of heterologous and native systems have also given varying results, some contradictory (Table 4). Although contested, a dominant theme appears to be a reduced current density and reduced rate of activation in mutant channels relative to wild-type (Sipos et al., 1995; Morrill and Cannon, 1999). Both effects are loss of function and could result in less calcium influx into the muscle cells during depolarizations. In

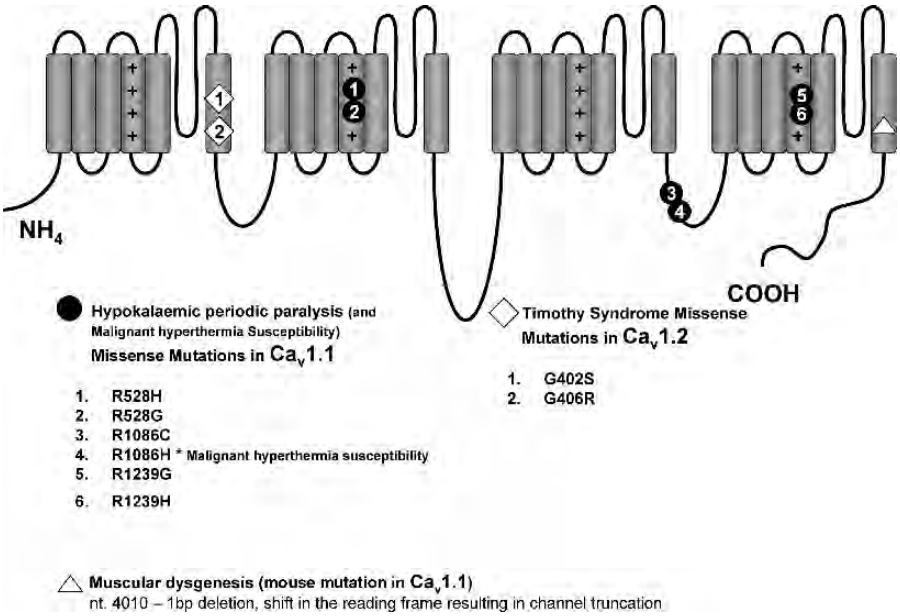

Figure 4. Mutations in the human $Ca_v1.1$ and $Ca_v1.2$ (L-type) voltage-gated calcium channels associated with Hypokalaemic Periodic Paralysis (HypoPP) and Timothy Syndrome (TS), respectively. Also shown is the nucleotide deletion in the mouse $Ca_v1.1$ homolog associated with Muscular Dysgenesis

attempts to correlate the biophysical data from mutant $Ca_v1.1$ with the pathophysiological data, Morill and Cannon hypothesize the reduced calcium influx through $Ca_v1.1$ affects membrane potential through impairment in coupling to a calcium sensitive channel, such as the calcium activated potassium channel that has direct control over the resting potential of the cell. Alternatively, they suggest reduced calcium influx through mutant $Ca_v1.1$ channels throughout development may alter expression and trafficking of channels important in establishing and maintaining resting membrane potential; a hypothesis substantiated by reports that the dominant potassium channel in muscle cells (ATP-sensitive potassium channel) has reduced function in fibres from HypoPP patients and shows irreversible changes in gating properties in response to alterations in calcium concentrations (Morrill and Cannon, 1999; Tricarico et al., 1999). It is equally likely, however, that the pathophysiology of HypoPP is independent of calcium flow through the $Ca_v1.1$ channel, but is instead associated with an uncoupling of EC between the $Ca_v1.1$ channel and the RyR1, resulting in a reduced calcium release from internal stores as seen in the similar muscular dysgenesis disorder in mice (Table 5). To test the possibility of a $Ca_v1.1$ mutation effect on EC, Jurkat-Rott and coworkers compared calcium release from the SR in myotubes from HypoPP patients with the R528H mutation and myotubes from healthy individuals using fura-2 microfluorimetry. Results indicate no detectable difference in the time course or the magnitude of

Table 4. CACNA1S ($Ca_v1.1$, α_{1S}): Functional results for mutations associated with HypoPP = Hypokalemic periodic paralysis and MHS = Malignant hyperthermia susceptibility (only those HypoPP mutations with functional data are listed)

Mutation and Disease	Functional Analyses
R528H- HypoPP	• hyperpolarized shift in V_{50act} (Lerche et al., 1996; Morrill and Cannon, 1999) • no change in V_{50act} (Sipos et al., 1995; Lapie et al., 1996; Morrill et al., 1998) • hyperpolarized shift in $V_{50inact}$ (Sipos et al., 1995; Lerche et al., 1996) • no change in $V_{50inact}$ (Lapie et al., 1996; Jurkat-Rott et al., 1998; Morrill et al., 1998; Morrill and Cannon, 1999) • reduced rate of activation (Morrill et al., 1998; Morrill and Cannon, 1999) • decreased current density (Lapie et al., 1996; Lerche et al., 1996; Morrill et al., 1998; Morrill and Cannon, 1999) • no change in current density (Sipos et al., 1995; Jurkat-Rott et al., 1998) • no change in calcium release from internal stores (Jurkat-Rott et al., 1998)
R1239G-HypoPP	• depolarized shift in V_{50act}, reduced rate of activation, decreased current density (Morrill and Cannon, 1999)
R1239H-HypoPP	• depolarized shift in V_{50act}, reduced rate of activation (Morrill and Cannon, 1999) • decreased current density (Sipos et al., 1995; Morrill and Cannon, 1999) • no change in gating properties (Sipos et al., 1995)

calcium release from SR in response to membrane depolarizations between HypoPP patients and controls, thus it is likely that the pathological effects are too subtle to detect under the experimental conditions used (Jurkat-Rott et al., 1998). Overall, the mechanism by which mutations found in HypoPP patients leads to reduced muscle excitability is yet to be determined.

Table 5. cacna1s ($Ca_v1.1$, α_{1S}): Functional results for mutations associated with muscular dysgenesis

Mutation and Disease	Functional Analyses
nt. 4010 – 1bp deletion, shift in the reading frame resulting in channel truncation Muscular dysgenesis	• membrane depolarization uncoupled from intracellular calcium release (Powell and Fambrough, 1973; Klaus et al., 1983)

4.2. Malignant Hyperthermia Susceptibility (MHS)

MHS is an autosomal dominantly inherited disorder characterized by a predisposition in otherwise healthy individuals for muscle hypermetabolism in response to exposure to volatile anesthetics or depolarizing muscle relaxants. Upon exposure to the precipitating agents, calcium is release from SR in myotubules via the RyR1, resulting in elevated skeletal muscle contraction, glycogenolysis, cell metabolism and subsequent increased heat and lactic acid acidosis. Varying numbers and degrees of symptoms can follow, including: tachycardia, rhabdomyolysis with subsequent creatine kinase elevation, potential cardiac arrhythmia or arrest and possible renal failure. Approximately 70% of patients would die without immediate treatment; early administration of the SR calcium release inhibitor dantrolene drastically reduces mortality (for review see (Striessnig et al., 2004) and (Jurkat-Rott et al., 2000a)). Although many mutations in the RyR1 gene have been associated with MHS (Jurkat-Rott et al., 2000a), a R1086H mutation in the CACNA1S gene has been found in two families with MHS (Figure 4). No biophysical data is available yet and the connection between the mutation and the pathophysiology is still elusive. However, if common CACNA1S mutations are found to be associated with MHS, routine screening prior to exposure to volatile anesthetics and depolarizing muscle relaxants could reduce risks of attacks.

5. L-TYPE ($Ca_V1.2/\alpha_{1C}$) CALCIUM CHANNELS; CACNA1C GENE

The $Ca_v1.2$ L-type channel is widely expressed in heart, brain, smooth muscle, pituitary, gastrointestinal systems lungs, immune system and testis (Ertel et al., 2000; Splawski et al., 2004). In cardiac tissue and lung smooth muscle, the $Ca_v1.2$ channel is involved in EC coupling (Du et al., 2006). However, unlike EC coupling between $Ca_v1.1$ and RyR1 receptors in skeletal muscle, EC coupling in cardiac tissue requires calcium influx through $Ca_v1.2$ in order to activate the RyR2 in the SR and release calcium from internal calcium stores to initiate muscle contraction (Meissner, 1994). Two mutations in the sixth transmembrane segment of domain I of the $Ca_v1.2$ channel have recently been associated with a severe arrhythmic disorder, Timothy syndrome (TS) (Figure 4).

5.1. Timothy Syndrome (TS)

TS is a multisystem disorder, with characteristic functional and developmental abnormalities in several organ systems including heart, skin, eyes, teeth, immune system and the brain; consistent with the ubiquitous expression of the $Ca_v1.2$ channel. In a study of seventeen children with TS, it was established that while arrhythmias are the most serious element of the disorder, patients have additional elements including congenital heart disease, dysmorphic facial features, developmental and cognitive delays, immune deficiency, intermittent hypoglycemia and hypothermia; and four of the seventeen children studied with TS met the criteria

Table 6. CACNA1C ($Ca_v1.2, \alpha_{1C}$): Functional results for mutations associated with TS = Timothy Syndrome and implicated in ASD = autism spectrum disorder

Mutation and Disease	Functional Analyses
G402S- TS	• near complete loss of voltage-dependent channel inactivation (Splawski et al., 2005)
G406R, G436R-TS and implicated in ASD	• near complete loss of voltage-dependent channel inactivation (Splawski et al., 2005) • increased mode 2 gating (Erxleben et al., 2006)

for autism. Greater than 50% of TS patients die between two and three years of age (Splawski et al., 2004). In a study of 13 patients with TS, mutation analysis of a specific $Ca_v1.2$ splice variant (found in ~20% of cardiac $Ca_v1.2$ transcripts) revealed a *de novo* missense mutation in exon 8A in all 13 patients (Splawski et al., 2004). A subsequent study on two patients with severe TS (TS2) revealed two *de novo* missense mutations in exon 8 of the dominant $Ca_v1.2$ splice variant found in heart and brain (Splawski et al., 2005). All patients had a heterozygous genotype suggesting the mutations are dominant.

Functional analysis of all mutations shows a near complete loss of voltage-dependent inactivation (Splawski et al., 2004; Splawski et al., 2005) and increased mode 2 gating (CaMKII dependent channel gating characterized by prolonged channel open time) (Erxleben et al., 2006), both effects predicted to prolong calcium influx (Table 6). Simulation analysis indicates that the prolonged calcium current can cause significant cardiac action potential prolongation and prolonged QT intervals (Q-T interval is the time for electrical activation and inactivation of the ventricles). The prolonged QT interval can result in abnormal secondary depolarizations, arrhythmia and sudden death (Splawski et al., 2004; Splawski et al., 2005). It is conceivable that prolonged calcium influx through the mutant $Ca_v1.2$ channels in TS patients could result in the additional functional and developmental abnormalities considering the requirement for precise regulation of internal calcium concentrations for normal cell signaling and gene transcription during development; however substantiating evidence is lacking.

6. T-TYPE ($CA_V3.2/\alpha_{1H}$) CALCIUM CHANNELS; CACNA1H GENE

T-type calcium channels play critical roles in shaping the electrical and plastic properties of neurons and are also implicated in hormone secretion, differentiation and muscle development (Huguenard, 1996; Perez-Reyes, 2003). In thalamic reticular and relay neurons, T-type channels contribute to rhythmic rebound burst firing and spindle waves associated with slow-wave sleep. T-type channels also play crucial roles in dendritic integration and calcium-mediated spiking in hippocampal pyramidal cells, and in synaptic release at olfactory dendrodendritic

synapses (Tsakiridou et al., 1995; Kim et al., 2001; Anderson et al., 2005). Of the three genes encoding low voltage-activated calcium channels ($Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$), the $Ca_v3.2$ isoform is thus far the only T-type channel known associated with channelopathies. $Ca_v3.2$ channels are widely expressed in the juvenile and adult hippocampus, cerebellum, pons/medulla, striatum, thalamus/hypothalamus, olfactory bulb, and cortex (Talley et al., 1999; McRory et al., 2001; Perez-Reyes, 2003; McKay et al., 2006). Due to their unique biophysical properties, $Ca_v3.2$ channels are predicted to be important in generating short burst firing potentials in the cortex and thalamus during slow wave sleep, however their specific contributions are only beginning to be revealed (Chemin et al., 2002). In the periphery, $Ca_v3.2$ channels are found in the primary nociceptor pathway and have been shown to contribute to both acute and chronic nociceptive behaviors (Snutch and David, 2006).

6.1. Idiopathic Generalized Epilepsy (IGE)

The incidence of epilepsy is variable between age and gender, but occurrence is approximately 1.6% in the general population. IGE is a classification of general epilepsies including juvenile myoclonic and juvenile absence epilepsies (JME and JAE, respectively) and childhood absence epilepsy (CAE) which represent approximately 40% of all epilepsies. Absence seizures are the most characteristic general epilepsies and include short lasting seizures accompanied by sudden impairment of consciousness and a sudden synchronous 3 to 6 Hz spike-wave discharge (SWD) that propagates bilaterally over many cortical areas (Sander, 1996). Based on experiments on the rat genetic model for absence epilepsy, (WAG/Rij), initiation of the neuronal synchronization is thought to occur in the cortex with subsequent generation of an oscillatory cortico-thalamocortical loop network which sustains the synchronous SWD for the duration of the seizure (Meeren et al., 2002). In the last few years researchers have uncovered several point mutations in the CACNA1H gene in patients with CAE and IGE, implicating a pathogenic role of the $Ca_v3.2$ channel in these disorders. The majority of mutations in the CACNA1H channel for both CAE and IGE are densely localized in the $Ca_v3.2$ intracellular loop between domain I and II (Figure 5).

The IGEs are non-Mendelian "complex" idiopathic genetic disorders (Robinson and Gardiner, 2000), therefore, mutations in a single gene, such as the CACNA1H, may not always contribute substantially to the pathophysiology of IGE patients. However, biophysical analyses of CACNA1H mutations associated with IGEs indicate channel properties are in fact altered in many cases (Table 7), suggesting at least a contribution to the pathophysiology. Similar to that for some of the other channelopathy mutations described previously, biophysical analysis of some CAE and IGE mutations in the $Ca_v3.2$ have produced a variety of results, some contradictory. The discrepancies may be due to the various expression systems and conditions utilized by different groups, however even mutations from patients with similar clinical phenotypes have very different, even opposite, affects on

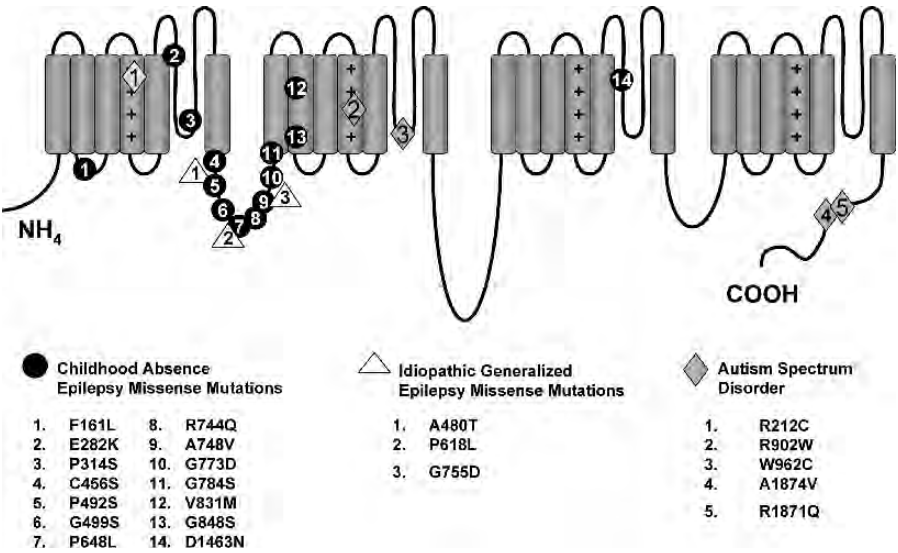

Figure 5. Mutations in the human $Ca_v3.2$ (T-type) voltage-gated calcium channel associated with Childhood Absence Epilepsy (CAE), Idiopathic Generalized Epilepsy (IGE) and Autism Spectrum Disorder (ASD)

Table 7. CACNA1H ($Ca_v3.2$, α_{1H}): Functional results for mutations associated with CAE = childhood absence epilepsy, IGE = Idiopathic generalized epilepsy and ASD = Autism Spectrum Disorder (only those mutations with functional data are listed)

Mutation and Disease	Functional Analyses
F161L- CAE	• hyperpolarized shift in V_{50act} (Khosravani et al., 2004); no change in V_{50act}, depolarized shift in $V_{50inact}$, increased rate of inactivation (Vitko et al., 2005)
E282K- CAE	• hyperpolarized shift in V_{50act} (Khosravani et al., 2004) • no change in channel properties (Vitko et al., 2005)
C456S- CAE	• no change in channel properties (Khosravani et al., 2004); hyperpolarized shift in V_{50act} and increased rate of activation (Vitko et al., 2005)
G499S- CAE	• reduced rate of inactivation (Vitko et al., 2005) • no change in channel properties (Peloquin et al., 2006)
P648L- CAE	• depolarized shift in $V_{50inact}$, reduced rate of inactivation and reduced rate of closed state inactivation (Vitko et al., 2005); no change in channel properties (Peloquin et al., 2006)

(Continued)

Table 7. (Continued)

Mutation and Disease	Functional Analyses
R744Q- CAE	• no change in channel properties (Vitko et al., 2005; Peloquin et al., 2006)
A748V- CAE	• increased rate of activation (Vitko et al., 2005); no change in channel properties (Peloquin et al., 2006)
G773D- CAE	• increased current density (Wang et al., 2006); depolarized shift in V_{50act} and depolarized shift in $V_{50inact}$, reduced rates of activation and deactivation (Vitko et al., 2005); no change in channel properties (Peloquin et al., 2006)
G784S- CAE	• decreased rate of activation and increased rate of closed state inactivation (Vitko et al., 2005); no change in channel properties (Peloquin et al., 2006)
V831M- CAE	• depolarized shift in $V_{50inact}$, reduced rates of activation and inactivation (Khosravani et al., 2004; Vitko et al., 2005); reduced rate of deactivation and increased recovery rate from inactivation (Vitko et al., 2005); no change in recovery rate from inactivation (Khosravani et al., 2004)
G848S- CAE	• reduced rate of deactivation (Vitko et al., 2005); reduced rates of activation and inactivation (Peloquin et al., 2006)
D1463N- CAE	• no change in channel properties (Khosravani et al., 2004) • increased rate of activation (Vitko et al., 2005)
A480T- IGE	• No change in biophysical properties (Khosravani et al., 2005)
P618L- IGE	• increased rates of activation and inactivation (Khosravani et al., 2005)
G755D- IGE	• increased rate of inactivation (Khosravani et al., 2005)
R212C- ASD	• depolarized shift in V_{50act}, reduced rate of inactivation, increased rate of deactivation, reduced current density (Splawski et al., 2006),
R902W- ASD	• depolarized shift in V_{50act}, depolarized shift in $V_{50inact}$, reduced rate of activation, reduced rate of inactivation, increased rate of deactivation, reduced current density (Splawski et al., 2006)
W962C- ASD	• reduced current density (Splawski et al., 2006)
A1874V/R1871Q- ASD	• depolarized shift in V_{50act}, depolarized shift in $V_{50inact}$, reduced rate of activation, reduced rate of inactivation (Splawski et al., 2006)

biophysical properties when analyzed under the same experimental conditions; see analysis data from C456S and G773D (Table 7) (Vitko et al., 2005). However, in many mutations gain-of-function effects are demonstrated by hyperpolarizing shifts in V_{50act}, depolarizing shifts in $V_{50inact}$, increased rates of activation, decreased rates of inactivation, decreased rates of deactivation and increased current densities (Table 7). The gain-of-function effects are predicted to result in more available channels at lower membrane potentials, increased calcium influx, and likely to result in neuronal hyperexcitability and increased spike and wave discharge under certain conditions. Additional studies using native systems in combination with the heterologous data will likely contribute significantly to our understanding of the roles of $Ca_v3.2$ channels in epilepsy. The somewhat mild and various effects of $Ca_v3.2$ mutations are consistent with the polygenic, non-Mendelian inheritance of the IGEs and suggests that interactions with other proteins may come into consideration.

6.2. Autism Spectrum Disorders (ASDs)

The prevalence of ASDs has risen drastically over the last twenty years to a staggering 1 in 166. ASDs include prototypic autistic disorder, Asperger syndrome and pervasive developmental disorder-not otherwise specified (PDD-NOS). Autistic disorder is characterized by deficits in communication, abnormal social interactions and restrictive and/or repetitive behaviours and/or interests with considerable variation in the severity of each phenotype; Asperger syndrome does not typically include intelligence or communication deficits, and PDD-NOS does not include repetitive behaviours or communication deficits. All three disorders often occur within the same family, suggesting they are not genetically distinct (for review see (DiCicco-Bloom et al., 2006)). As diverse as the phenotypes associated with ASDs is the associated diversity in the pathological findings in brains of patients with ASD. Some consistent findings indicate histological abnormalities in limbic structures including the hippocampus and amygdale, abnormal brain size including enlargement in cortex and cerebellum, and reduced tissue volume in cerebellar vermis (Courchesne et al., 2001; Kaufmann et al., 2003; Matsuo et al., 2003; Hazlett et al., 2005; Sadamatsu et al., 2006). Postmortem neuropathological data indicate a dramatic loss of cerebellar Purkinje cells (Ritvo et al., 1986). fMRI imaging studies indicate that skill deficits in ASD patients are accompanied by reduced function in brain regions attributed to the specific skills (for a full review of pathology of ASD see (DiCicco-Bloom et al., 2006). Family studies show a strong polygenic genetic dependence for ASD with complex gene-gene and gene-environmental interactions (Szatmari, 1999).

Four missense mutations have been identified in the CACNA1H gene encoding the $Ca_v3.2$ T-type calcium channel in six families from a sample of 461 individuals with ASD (none present in 480 ethically matched controls; (Splawski et al., 2006)). Two of the mutations are in the S4 voltage sensor regions, one is in the pore forming p-loop and the other is found in the carboxyl terminus (Figure 5). Biophysical analysis of the four missense mutations predicts an overall decrease in calcium

entry (Table 7). Although unconfirmed by experimental data at this time, it is possible that the biophysical effects of the mutations translate into altered neuronal development and communication considering the tight regulation of internal calcium levels necessary for normal cell signaling and gene expression (Splawski et al., 2006). It is important to note that of the 461 ASD patients screened, only 6 carried the CACNA1H gene missense mutations. In addition, three families with a child having a CACNA1H missense mutation had an additional affected child without a CACNA1H mutation and one sibling and several parents contained one of the mutations but were not obviously affected. These data are consistent with the polygenic inheritance pattern of ASD, and indicate a potential, but likely small, role for the $Ca_v3.2$ channel in overall ASD susceptibility.

7. CALCIUM CHANNEL AUXILIARY SUBUNITS

In most instances, HVA calcium channel α_1 subunits appear to be associated with auxiliary β and $\alpha_2\delta$-subunits in order to fully reconstitute most native biophysical and modulatory characteristics (De Waard et al., 1996). The skeletal muscle $Ca_v1.1$ L-type channel also co-purifies with a γ1 subunit although it remains unknown how generally this particular subunit is associated with voltage-gated calcium channel complexes. In addition to the ten genes coding for α_1 subunits, mammals possess four genes encoding for β-subunits (β_1-β_4), four $\alpha_2\delta$-subunit genes ($\alpha_2\delta1$-$\alpha_2\delta4$) and eight γ subunits genes (γ1-γ8; (Snutch et al, 2005)). To date, two human calcium channel auxiliary subunits have been associated with disease; 1) linkage analysis has indicated mutations in the $\alpha_2\delta1$ auxiliary subunit may play a role in malignant hyperthermia susceptibility (discussed previously in association with the $Ca_v1.1$ L-type channel), although no mutations have yet been described in the CACNL2A gene that encodes for the $\alpha_2\delta1$ subunit (Iles et al., 1994) and 2) truncation and missense mutations in the CACNB4 gene (encoding the β_4 subunit) are associated with families with juvenile myoclonic epilepsy, generalized epilepsy/praxis-induced seizures and episodic ataxia (Escayg et al., 2000). As the β_4 subunit is known to interact directly with the $Ca_v2.1$ channel α_1 subunit (Walker and De Waard, 1998; Walker et al., 1999) Escayg and coworkers co-transfected the mutant β_4 subunit with $Ca_v2.1$ into *Xenopus* oocytes and examined channel biophysical properties. The results indicate that both truncation and missense mutations in the β_4 subunit increase current density while the truncation mutation selectively alters channel gating (Table 8). This association between mutations in the β modulatory subunit and $Ca_v2.1$ channel functional alterations and epilepsy and ataxia, supports the evidence described previously indicating a substantial role for the $Ca_v2.1$ P/Q-type channel in these disorders.

Additional substantiating evidence that calcium channel auxiliary subunits might play pivotal roles in disease processes comes from the study of mouse models. Five murine diseases with similar phenotypes to $Ca_v2.1$ P/Q-type channel mutant-associated diseases have been linked to calcium channel auxiliary subunits (Tables 9–11).

Table 8. CACNB4 (β_4): Functional results for mutations associated with JME = juvenile myoclonic epilepsy, GE = generalized epilepsy and EA = episodic ataxia

Mutation and Disease	Functional Analyses
R482X- JME	• increased current density and rate of inactivation (Escayg et al., 2000)
C104F- GE and EA	• increased current density (Escayg et al., 2000)

Table 9. Cchb4 (β_4): Functional results for mutations associated with lh = lethargic

Mutation and Phenotype	Functional Analyses
4 bp insertion, aberrant splicing:deletion of α_1 subunit binding site	• no affect on calcium channel ratio or Ca^{2+} dependent neurotransmitter release in the hippocampal Schaffer collateral synapse in *lh* mice (Qian and Noebels, 2000). • complete loss of β_4 expression in forebrain and cerebellum, associated with decreased $Ca_v2.2$ expression and increased β_{1b} expression; no change in β_2 or β_3 in lethargic brain (McEnery et al., 1998) • increased $Ca_v2.1$ and $Ca_v2.2$ association with β_{1-3}, without any change to protein expression in immunolocalization assay in lethargic brain (Burgess et al., 1999)

Table 10. Cacna2D2 ($\alpha_2\delta2$): Functional results for mutations associated with *du/du*^{2J}= ducky and ent = entla.

Mutation and Phenotype	Functional Analyses
Aberrant splicing resulting in altered mRNA sequence and protein truncation-*du*	• reduced current density, no change in single channel conductance (Barclay et al., 2001; Brodbeck et al., 2002); abnormal cytoarchitecture in Purkinje cells (Brodbeck et al., 2002)
tg – deletion in exon 9, resulting in protein truncation-*du*^{2J}	• no electrophysiological data
39 aa duplication near the N terminus, no protein truncation-*ent*	• reduced current density and decreased gabapentin binding (Brill et al., 2004)

7.1. Lethargic Mouse (Cchb4: β_4)

A deletion in the mouse β_4 gene (Cchb4) has been associated with the lethargic (*lh*) phenotype (Burgess et al., 1997). Lethargic mice display ataxia, lethargic behaviours and focal motor seizures; a similar phenotype to that associated with

Table 11. Cacng2 (γ_2): Functional results for mutations associated with stargazer

Mutation and Disease	Functional Analyses
Transposon insertion in intron 2: loss of transcription	• presence of wt stargazin (γ_2) causes a hyperpolarized shift in $V_{50inact}$ and enhanced current amplitude for $Ca_v2.1$ channels, in mutant stargazin the lack of transcription ultimately would result in a depolarizing shift in $V_{50inact}$(Letts et al., 1998; Kang et al., 2001) • no change in stimulating CSD (Ayata et al., 2000)

human β_4 mutations. Functional studies on hippocampal Schaffer collateral synapses in *lh* mice, although not in complete agreement, indicate that the β_4 deletion results in a shift in β subunit expression and association with $Ca_v2.1$ P/Q-type and $Ca_v2.2$ N-type channels from predominantly β_4 to the β_1, β_2 and β_3 subunits (Table 9). It is likely that this change in β subunit isoform interactions with α_1 subunits causes an overall reduction in channel function (Patil et al., 1998), leading to the observed ataxic and epileptic phenotypes.

7.2. Ducky/Entla Mice (Cacna2d2: $\alpha_2\delta2$)

Several genetic mutations, an aberrant splice, a deletion mutation and an amino terminal duplication in the calcium channel auxiliary subunit Cacna2d2 gene (encoding the $\alpha_2\delta2$ subunit) result in protein truncations associated with the allelic mutants ducky (*du*) and ducky2 (du^{2J}) and an elongated protein associated with *entla* (*ent*), respectively (Barclay et al., 2001; Brill et al., 2004). The ducky phenotypes are characterized by an ataxic gait, paroxysmal dyskinesia and pathological findings of abnormal cerebellar, medulla, and spinal cord development (Meier, 1968; Barclay et al., 2001). The *ent* phenotype is characterized by ataxia, paroxysmal dyskinesia and absence epilepsy without any gross anatomical abnormalities (Brill et al., 2004). The $\alpha_2\delta1$, $\alpha_2\delta2$, $\alpha_2\delta3$ and $\alpha_2\delta4$ subunits have previously been shown to be important modulators of several α_1/β-subunit combinations (Walker and De Waard, 1998; Barclay et al., 2001). *In vitro* analysis using whole cell and single cell recordings of heterozygous, homozygous and wild-type *du* mice in acutely dissociated Purkinje cells demonstrates a significant reduction in $Ca_v2.1$ channel current density in homozygous *du/du* mice, without changing single channel conductance (Table 10) (Barclay et al., 2001; Brodbeck et al., 2002). *In vitro* analysis of acutely dissociated Purkinje cells from heterozygous, homozygous and wild-type *ent* mice demonstrates a significant reduction in current density of $Ca_v2.1$ channels in homozygous *ent/ent* mice and also reduced gabapentin binding. An overall loss-of-function effect on $Ca_v2.1$ P/Q-type channels appears to be a consistent effect of the *du/du* and *ent/ent* mutations, possibly due to interference of the mutant $\alpha_2\delta2$ in contributing to channel function, trafficking and/or assembly.

7.3. Stargazin Mouse (Cacng2: γ_2)

An insertion in the mouse Cacng2 gene, which encodes the γ_2 auxiliary subunit stargazin, has been implicated in the stargazer phenotype. Stargazer mice are characterized by head-tossing, ataxic gait and electrocorticograph readings typical of absence seizures. The γ_2 subunit was found to be ubiquitously expressed in brain synaptic membranes, with altered expression in the stargazer mouse due to an intronic insertion (Letts et al., 1998). Functional studies in BHK cells and *Xenopus* oocytes indicates that co-expression of the wild-type γ_2 with the $Ca_v2.1$ α_1 subunit (and β_{1a}/ or β_3 and $\alpha_2\delta$) demonstrates a modulatory role of γ_2 on P/Q-type channels by shifting the V_{50inac} in the hyperpolarized direction and in reducing current density (Letts et al., 1998) (Table 11). It has been therefore suggested that the altered expression of the stargazin γ_2 may functionally affect P/Q-type channels, potentially leading to the observed seizure susceptibility (Letts et al., 1998). However, studies on stargazin mice examined for the threshold for initiating cortical spreading depression found no significant change in mutant mice relative to wild-type and substantiating evidence for the exact contribution of stargazin concerning the epileptic phenotype remains to be defined (Ayata et al., 2000).

8. CONCLUSIONS

Structure-function analyses involving the introduction of genetic abnormalities into cloned voltage-gated calcium channels has provided important clues concerning gain-of-function and loss-of-function properties and may help lead to new therapeutic pathways. It should however be noted, that the data from exogenous expression studies can sometimes be contradictory and the more labor intensive approach of genetic knock-in will likely be required to resolve some of the mechanistic issues related to disease phenotype. Additionally, we have just touched the surface concerning the more obvious and robust phenotypes and there likely remains much to discover at multiple levels. For example, critical to the phenotypic aspects of human channelopathies, the various calcium channel α_1 and auxiliary subunits exhibit distinct temporal and spatial expression patterns, yet we know little concerning the exact correlation between channel expression-related processes and disease progression and phenotype. Additionally, there is a demonstrated preferential association between individual calcium channel α_1 subunit isoforms expressed in particular tissues and specific subtypes of β subunits (e.g., in skeletal muscle $Ca_v1.1$ with β_{1a}, in cardiac muscle $Ca_v1.2$ with β_{2c}, and in the cerebellum the cell type-specific expression of $Ca_v2.1$ with either β_4 or β_{2a}), yet we have few clues as to the physiological significance as this relates to channelopathies. Also of further importance, and this issues likely relates to all channelopathies, is to resolve the apparent disconnect between widespread ion channel expression patterns and the often highly localized disease phenotypes (e.g., pan-neuronal $Ca_v2.1$ expression yet mutations manifesting as cerebellar ataxias and congenital migraine headache). As alternative splicing can greatly diversify ion channel function and also provide for

the precise localization of unique molecular isoforms, the contribution of splicing has the potential of greatly affecting the phenotypic expression of channelopathies in general. To date, this area has largely been understudied and the analysis of both the effects of individual splice-variants on specific genetic mutations, together with the developmental and spatial correlation of splice variant expression patterns may provide new and important insights into the clinical manifestation of channelopathies.

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CHAPTER 9

TRP CHANNELS IN DISEASE

S.E. JORDT AND B.E. EHRLICH

Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520-8066

Abstract: The transient receptor potential (TRP) channels are a large family of proteins with six main subfamilies termed the TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and TRPA (ankyrin) groups. The sheer number of different TRPs with distinct functions supports the statement that these channels are involved in a wide range of processes ranging from sensing of thermal and chemical signals to reloading intracellular stores after responding to an extracellular stimulus. Mutations in TRPs are linked to pathophysiology and specific diseases. An understanding of the role of TRPs in normal physiology is just beginning; the progression from mutations in TRPs to pathophysiology and disease will follow. In this review, we focus on two distinct aspects of TRP channel physiology, the role of TRP channels in intracellular Ca^{2+} homeostasis, and their role in the transduction of painful stimuli in sensory neurons

Keywords: transient receptor potential, intracellular calcium, polycystic kidney disease, pain

1. INTRODUCTION

The TRP channels were first identified in insects (Montell and Rubin 1989). Interest in the study of these channels increased greatly after the publication of several key findings: that the TRP channels are in mammalian cells, that these channels are used in many cell types for sensing thermal and chemical changes and for reloading intracellular calcium (Ca^{2+}) stores, and that mutations and alterations in these channels are responsible for specific human diseases (Nilius, Voets et al. 2005). There have been a number of excellent reviews describing the history of these discoveries and of the current state of knowledge. In this brief review we will describe how TRP channels are involved in Ca^{2+} homeostasis and signaling and we will focus on aspects of the channels that may be altered in the generation of pathophysiological states.

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We will discuss two well established examples for the involvement of TRP channels in disease. First, we will address the role of TRP channels in polycystic kidney disease, a Mendelian kidney disorder with high prevalence in caucasian populations. Two genes have been found to be mutated in this disorder, polycystin 1, a very large membrane protein, and polycystin 2, a TRP channel interacting with polycystin 1. In the second part of our review we will discuss the role of TRP ion channels in pain and somatosensation. Several members of the TRP family are specifically expressed in peripheral sensory neurons, including the nociceptors, the neurons involved in pain transduction. These channels are involved in the sensation of hot and cold temperature and are targets of inflammatory chemical mediators and signaling pathways.

2. TRP CHANNELS IN POLYCYSTIC KIDNEY DISEASE (PKD) AND INTRACELLULAR CA^{2+}HOMEOSTASIS

Autosomal dominant polycystic kidney disease (ADPKD) is a systemic hereditary disease that is characterized by renal and hepatic cysts, and results in end-stage renal failure in approximately 50% of affected individuals (Wu, Kamimura et al. 2000). Most cases (>95%) are caused by genetic mutations in either the *PKD1* or the *PKD2* gene, which encode polycystin-1 (PC1) and polycystin-2 (PC2), respectively (Mahata, O'Connor et al. 1997). Although disease-associated mutations have been identified in these two proteins, the sequence of molecular events leading up to clinical symptoms is still unknown. There is no known treatment or preventative therapy for this disease. Elucidation of the signaling pathway involved in normal PC1/PC2 function, the functional consequences of PC1/PC2 mutation, and the role of Ca^{2+} signaling will all help to unravel the molecular mechanisms of cystogenesis in PKD.

2.1. Polycystin-1

PKD1 encodes a ~462-kDa protein (PC1), and it is expressed in many tissues, including the kidney, heart, brain, muscle, and bone (Hughes, Ward et al. 1995; Geng, Segal et al. 1997). Although PC1 is not a member of the TRP family, it is associated with PC2, which is a TRP, and is important for some functions of PC2. PC1 is localized to the plasma membrane and primary cilia (Hanaoka, Qian et al. 2000; Newby, Streets et al. 2002; Yoder, Hou et al. 2002). It has 11 transmembrane spanning domains, a long extracellular N-terminus, and a short intracellular C-terminus. The N-terminus contains several subdomains that have significant homology with defined functional units (Hughes, Ward et al. 1995). Domains in the extracellular amino terminus of the protein are predicted to support protein-protein interactions (Huan and van Adelsberg 1999; Boletta, Qian et al. 2000; Scheffers, van der Bent et al. 2000; Xu, Sikaneta et al. 2001; Dackowski, Luderer et al. 2002) and to constitute a G-protein-coupled receptor-type proteolytic site (GPS) (Ponting, Hofmann et al. 1999; Mengerink, Moy et al. 2002). The coiled-coil

and heterotrimeric G-protein binding and activation domains were identified in the cytoplasmic carboxy terminus (Sandford, Sgotto et al. 1997; Parnell, Magenheimer et al. 1998). Although PC1 has all these putative signaling motifs, its function is still poorly understood compared to PC2.

2.2. Polycystin-2

PKD2 gene product, PC2, encodes a 110-kDa protein (Mochizuki, Wu et al. 1996), and it is expressed in most adult and fetal tissues (Luo, Vassilev et al. 2003). PC2 has 6 transmembrane spanning domains and both the C- and N-terminus are intracellular. The C-terminus contains a coiled-coil domain, an EF hand motif, four putative phosphorylation sites and an ER retention signal. PC2 has N-glycosylation sites on its first and second cytoplasmic loops and three SH3 domains on its N-terminus (Mochizuki, Wu et al. 1996). The second through sixth transmembrane domains share significant sequence homology with the voltage-activated- Ca^{2+} and sodium-channels and TRPC1 (Mochizuki, Wu et al. 1996; Tsiokas, Arnould et al. 1999). PC2 can form homodimers and, it co-assembles with PC1 both *in vitro* and *in vivo* through its coiled-coil domains (Qian, Germino et al. 1997; Tsiokas, Kim et al. 1997; Hanaoka, Qian et al. 2000; Xu and Arnaout 2002).

It became more important to understand the channel properties of PC2 after it was found that a pathogenic missense mutation of PC2 (D511V), where a single amino acid in the third membrane-spanning domain is mutated, results in loss of PC2 channel activity (Koulen, Cai et al. 2002). This missense mutant retains its localization and C-terminal-mediated protein interaction and regulatory domains of the wild type protein, thus providing evidence that the loss of channel function alone is sufficient to cause PKD.

Recent studies indicate that the channel properties of PC2 are similar regardless of whether it is expressed exogenously in *Xenopus* oocytes, Sf9 insect cells, or mammalian cell lines, or it is reconstituted into lipid bilayer membranes from native vesicles or affinity-purified protein (Hanaoka, Qian et al. 2000; Gonzalez-Perrett, Kim et al. 2001; Vassilev, Guo et al. 2001; Koulen, Cai et al. 2002). PC2 is a nonselective cation channel with multiple subconductance states, and a high permeability to Ca^{2+}(Gonzalez-Perrett, Kim et al. 2001). PC2 is permeable to monovalent cations such as Na^+, Cs^+, and K^+ and to divalent cations such as $Ba^{2+}$and Mg^{2+} (Gonzalez-Perrett, Kim et al. 2001; Koulen, Cai et al. 2002), but it is more permeable to divalent than monovalent cations (Koulen, Cai et al. 2002). PC2 channels are inhibited by high concentrations of Ca^{2+} (Cai, Anyatonwu et al. 2004). Other known inhibitors include La^{3+}, Gd^{3+} (Figure 1), amiloride, and a reduction in pH (Gonzalez-Perrett, Kim et al. 2001). It is interesting to note that PC2 currents were insensitive to the regulators of the $InsP_3R$ and the RyR, but PC2 was inhibited by gadolinium (Gd^{3+}) (Figure 1) in the same concentration range as other TRP's (Trebak, Bird et al. 2002).

It is unknown how many subunits make up the pore of the PC2 channel. With an understanding of the permeation properties of the PC2 channel, an estimate

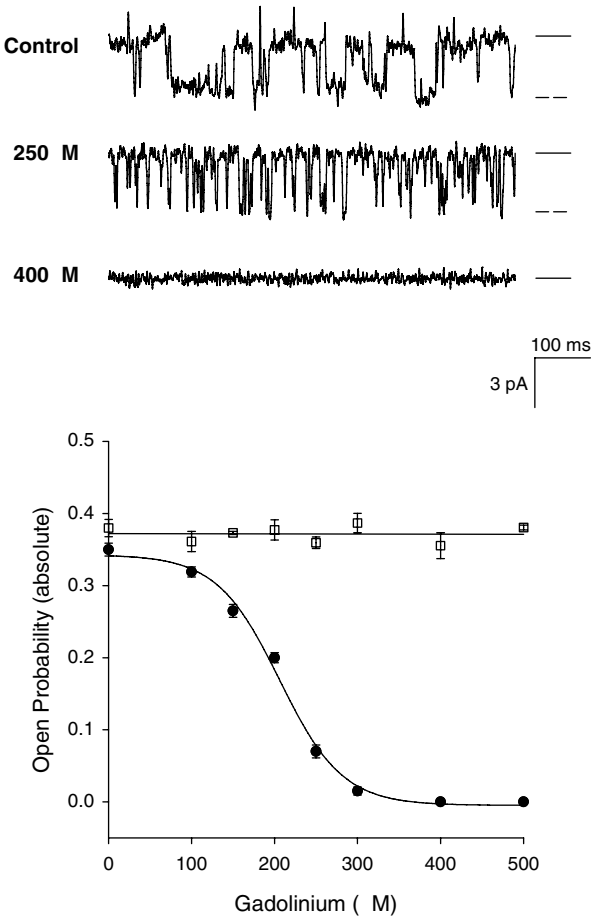

Figure 1. PC2 channels inhibited by Gd^{3+}. The top panel shows currents obtained from PC2 incorporated into a bilayer. This is a control trace showing Cs^{+} current measured at –45 mV transmembrane potential. The solid and broken lines to the right of the traces indicate the closed and open states, respectively. The second and third traces show currents after the addition of Gd^{3+}. The bottom panel shows the concentration dependence of the block by Gd^{3+}, circles when added to the cytoplasmic side, squares when added to the lumenal side. The Kd is 206 μM; Gd^{3+} only blocks when added to the cytoplasmic side. Modified from (Anyatonwu and Ehrlich 2005)

of the channel composition can be made. As with the RyR (Tinker and Williams 1993) we estimated the pore size of PC2 by using organic cations of increasing size as current carriers through the PC2 channel after PC2 was incorporated into lipid bilayers. We found that dimethylamine, triethylamine, tetraethylammonium, tetrabutylammonium, tetrapropylammonium, and tetrapentylammonium were permeable through the PC2 channel. The slope conductance of the PC2 channel decreased as

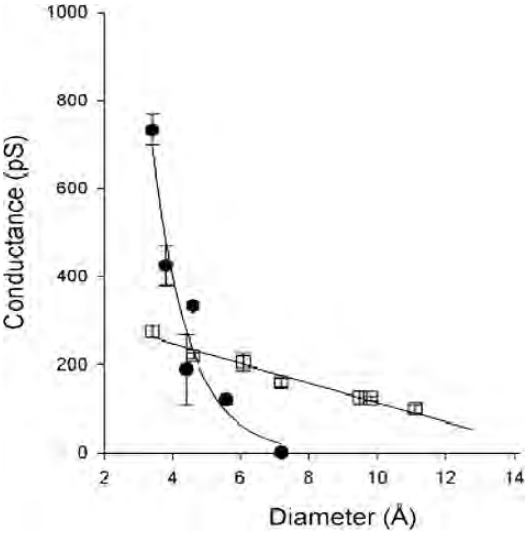

Figure 2. Comparison of the conductance of RyR and PC2. Modified from (Anyatonwu and Ehrlich 2005)

the ionic diameter of the organic cation increased (Figure 2). These results suggest that the PC2 channel has a minimum pore diameter of at least 11 Å.

The large pore suggests that the primary state found *in vivo* is closed to avoid rundown of cation gradients across the plasma membrane and excessive Ca^{2+} leak from the endoplasmic reticulum stores.

One of the big challenges in understanding the function of PC2 is finding the physiological activator of the channel. A knowledge of these activators is needed to investigate the mechanism of action of PC2 in normal cells and how alterations in this protein leads to disease. At the present time, only mechanical linkage to PC1 and alterations in intracellular Ca^{2+} are known to activate PC2.

2.3. PC1/PC2 Linkage

Because mutations in PC1 and PC2 result in similar kidney disease in humans and PC2 is able to interact with PC1 through its coiled-coil domain *in vitro*, it has been suggested that PC1 and PC2 form a functional complex. In cells containing both PC1 and PC2, deletion of the C-terminus of either PC1 or PC2 can alter intracellular Ca^{2+} signals (Hanaoka, Qian et al. 2000). These intracellular Ca^{2+} changes can be induced by fluid flow along the apical surface of kidney (Praetorius and Spring 2001) and bile duct cells (Masyuk, Masyuk et al. 2006). It is important to note that fluid-flow induced signaling requires the presence of primary cilia (Praetorius and Spring 2001). Reduction in the protein levels of either PC1 or

PC2 by small interfering RNAs abolishes the flow-induced Ca^{2+} signal (Masyuk, Masyuk et al. 2006), providing further evidence that the ciliary signaling complex utilizes both PC1 and PC2.

However, in cultured cells which lack the primary cilia, the intracellular Ca^{2+} signals can still be altered by PC2. Addition of the extracellular agonist, vasopressin, stimulated cells overexpressing full-length PC2 to generate a Ca^{2+} transient that is approximately two-fold larger in magnitude and approximately ten-fold longer in duration when compared to cells expressing native levels of wild type PC2 (Koulen, Cai et al. 2002). The increased duration and amplitude of transients persisted in the absence of extracellular Ca^{2+}, suggesting that the increase in cytosolic Ca^{2+}results from release from intracellular stores (Koulen, Cai et al. 2002). In addition, $InsP_3R$ activation was required for PC2-mediated Ca^{2+} release (Koulen, Cai et al. 2002), providing further support for the role of intracellular stores as the major source of the released Ca^{2+}. These studies show that PC2 can function as a channel when expressed without PC1.

2.4. Alterations in Intracellular Ca^{2+}

Channel activity was increased by elevating the free Ca^{2+} concentrations on the cytoplasmic side, but increases in the Ca^{2+} concentration above 1 μM decreased the open probability of PC2 channels. This bell-shaped dependence is similar to that found for the inositol 1,4,5 trisphosphate receptor ($InsP_3R$) and the ryanodine receptor (RyR), but PC2 is more sensitive to Ca^{2+}-dependent inhibition than the RyR, another intracellular channels (Figure 3).

This Ca^{2+} dependence can be altered by phosphorylation of PC2 (Cai, Anyatonwu et al. 2004), in particular, phosphorylation of serine 812. When the point mutation S812A of PC2 was tested in bilayer reconstitution experiments, it had channel activity similar to wild type PC2, except it was less sensitive to Ca^{2+}. When wild type PC2 channels were treated with alkaline phosphatase to remove the phosphate, the sensitivity to Ca^{2+} also declined, implying that this site could be important for Ca^{2+}-dependent regulation of PC2 (Cai, Anyatonwu et al. 2004). One caveat with using this type of regulation to explain changes in PC2 function in intact cells is that mutations of S812 have not been identified as a pathologenic mutation.

2.5. PC2 Channels can Regulate Intracellular Ca^{2+} Channels

The downstream effects of PC2 have been hypothesized to depend upon an interaction between PC2 and RyR (Nauli, Alenghat et al. 2003). Although previous work suggested that the RyR is important for propagating the intracellular Ca^{2+}signals, the major intracellular Ca^{2+} channel in epithelial cells is the $InsP_3R$. Recently a functional interaction between the $InsP_3R$ type 1 and PC2 was described (Li, Wright et al. 2005). The C terminal portion of PC2 was shown to bind directly to the $InsP_3R$ and the duration of intracellular signals was prolonged after overexpression

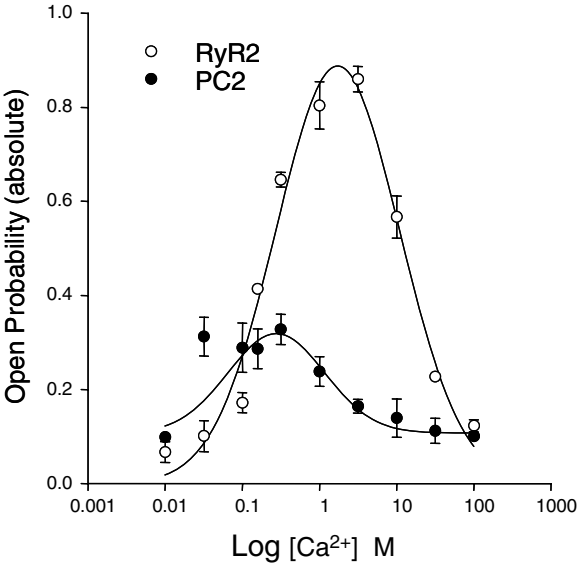

Figure 3. The bell-shaped Ca^{2+} dependence of PC2 and RyR. Note that the PC2 channels are inhibited at a Ca^{2+} concentration lower than that needed to fully inhibit RyRs. Data from (Cai, Anyatonwu et al. 2004)

of either the C terminus or full length PC2 (Li, Wright et al. 2005). In this case, mutations in PC2, especially those that occur in the C terminal portion of the protein will lead to alterations in intracellular signals.

PC2 may also function as an intracellular Ca^{2+} channel itself. A large population of PC2 channels can be detected in the endoplasmatic reticulum (Vassilev, Guo et al. 2001; Koulen, Cai et al. 2002; Giamarchi, Padilla et al. 2006). Until recently, it was thought there were only two major classes of intracellular Ca^{2+} channels: the RyRs and the $InsP_3Rs$. It is now agreed that there are three classes of intracellular channels. Many cell types contain all three classes of channels, but the relative densities vary dramatically. Cells also can have multiple isoforms of these channels. The co-existence of a variety of intracellular channels is not surprising as cells need to respond to diverse stimuli with specific responses. Whether the intracellular function of PC2 channels is relevant for the disease mechanism of PKD remains to be established.

2.6. The Implications of Ca^{2+} Signaling by PC2 in PKD

PKD involves abnormal proliferation and differentiation of kidney epithelial cells, resulting in the formation of cysts that eventually destroy the kidneys in affected individuals. Together, PC1 and 2 appear to form a complex that senses the state of the kidney epithelium as an early step in a pathway that controls epithelial

proliferation, differentiation, and/or apoptosis (Igarashi and Somlo 2002). The molecular steps that are altered when there is a mutation in PC1 or PC2 are still poorly understood.

It is likely that the ciliary polycystins provide a Ca^{2+} signal in response to mechanosensory stimulation, and then this Ca^{2+} and other signals are integrated into an appropriate cellular response. The transformation of a renal epithelial cell into a hyperproliferating epithelial cell which divides and becomes a cyst will probably involve changes in gene expression that reflects aberrant Ca^{2+} mobilization from intracellular stores (Calvet 2003). Identifying the affected signaling pathways and their gene targets is crucial if one hopes to discover how the system interacts and ultimately the role they play in cyst formation in polycystic kidney disease.

Clearly, the disease begins with a defect in the polycystins, signaling proteins which are located on the kidney primary cilia. These defects render the PC complex incapable of transmitting their normal response to downstream signals which maintain cell function. The sensing function of PC1, the channel properties of PC2, the ability to hand off the signal are examples of steps that could be disrupted. Once the cascade is broken there are changes in the cell's ability to regulate proliferation, migration, and differentiation. Dissection of these questions and issues will be a challenge. An integration of these pathways, involving G proteins, phosphorylation of serine kinases and protein kinases, and intracellular second messengers including Ca^{2+} and cAMP, will be critical for understanding cell function in this disease. Studies of mice with targeted mutations of PKD1 and PKD2 (Lu, Peissel et al. 1997; Wu, D'Agati et al. 1998) or transgenic animals created in which either gene can be inactivated during adulthood could provide answers to these questions.

3. TRP CHANNELS, CALCIUM AND PAIN

Noxious and innocuous physical and chemical stimuli are sensed by peripheral sensory neurons (Wall 1999). Action potentials elicited by peripheral stimuli travel along sensory nerve fibers until they reach synapses in the spinal cord. These incoming signals are integrated within the CNS, resulting in the sensation of pain, warmth, cool or pressure. Intracellular Ca^{2+} levels in sensory neurons are tightly regulated. Ca^{2+} ions play an important role in sensory neural function, both by promoting depolarization and by activating the local release of pro-inflammatory mediators from sensory nerve endings, causing neurogenic inflammation (Julius and Basbaum 2001). These mediators, including neuropeptides such as Substance P and CGRP or chemical transmitters such as ATP, are stored within vesicles in the sensory nerve endings. Vesicles fuse with the plasma membrane upon Ca^{2+}-influx, triggering the release of their cargo. Substance P and CGRP cause local vasodilation, vascular permeabilization and edema formation. In addition, these sensory neuropeptides modulate the function of immune cells, thereby acting as a link between the sensory neural system and the immune system. Neurogenic inflammation is an important inflammatory mechanism contributing to chronic inflammation in arthritis, asthma and other conditions.

Until recently, the major stimulus-dependent Ca^{2+} influx pathways in sensory neurons were unknown. Although sensory neurons express voltage-gated Ca^{2+} channels, neuronal Ca^{2+} dynamics and signaling can not be explained solely through the properties of these channels. The study of the mechanism of action of pain-inducing plant-derived natural products finally led to a breakthrough in our understanding of sensory neural excitability and Ca^{2+}-dynamics. Natural products such as capsaicin, the pungent ingredient in chili peppers, or mustard oil (allyl isothiocyanate) are strong activators of nociceptors, the sensory neurons involved in pain transduction (Wall 1999). Both capsaicin and mustard oil were used as important tools to define neural cellular subpopulations and to study the mechanism of neurogenic inflammation. Capsaicin also activates the sensation of warmth or heat. Other natural products can activate the sensation of cooling. These include menthol, the active ingredient in peppermint, and eucalyptol, a compound found in eucalyptus. Both compounds are thought to cause the sensation of cooling through the activation of cold-sensitive sensory neurons.

Pharmacological and functional studies of capsaicin, mustard oil and menthol activity found that all three compounds activate Ca^{2+} influx into specific neural subpopulations through the activation of non-selective cation conductances with properties similar to TRP channels. In 1997 the receptor for capsaicin was cloned through an expression cloning strategy based on its capsaicin-sensitive Ca^{2+}-influx activity (Caterina, Schumacher et al. 1997). The receptor, TRPV1, is the founding member of a novel branch of the TRP gene family, the TRPV channels. Subsequent studies using similar approaches led to the discovery of the receptor for mustard oil, TRPA1, and TRPM8, the receptor for menthol (McKemy, Neuhausser et al. 2002; Jordt, Bautista et al. 2004). In the following paragraphs we will discuss the physiological properties and the biological functions of these TRP ion channels.

3.1. TRPV1, the Capsaicin Receptor

TRPV1 is very likely the most intensively studied mammalian TRP channel to date. Since the cloning of TRPV1 was reported in 1997, more than 1000 studies have been published describing the physiology, structure and function of TRPV1. Because a comprehensive review of this literature would go way beyond the limitations of this review, we will focus on some novel aspects of TRPV1 structure and function, and its involvement in inflammatory regulation in diabetes and asthma.

TRPV1 is a non-selective cation channel, permeable for sodium, potassium, calcium and magnesium (Caterina, Schumacher et al. 1997; Caterina and Julius 2001). It is expressed in C-fibers, the capsaicin-sensitive sensory neurons that mediate pain, thermal stimuli and inflammation. Many C-fibers contain inflammatory neuropeptides such as CGRP and Substance P. TRPV1 can fulfil multiple functions in the sensory neuron. TRPV1 is a polymodal detector of physical and chemical stimuli, activated by hot temperature, by extracellular acidity and capsaicin-like endogenous agonists. Activation of TRPV1 can lead to depolarization

of the neural membrane, triggering action potentials that signal pain. In addition, Ca^{2+}-influx through TRPV1 can trigger the release of inflammatory neuropeptides into the surrounding tissue, causing neurogenic inflammation. In addition, TRPV1 is sensitized by inflammatory signaling pathways. For example, bradykinin, a peptide released in injured tissue, sensitizes TRPV1 through activation of the bradykinin receptor, a G-protein coupled receptor signaling through phospholipase C (Chuang, Prescott et al. 2001). Sensitization results in thermal hyperalgesia, the painful hypersensitivity to hot temperature. In addition to the bradykinin receptor, activation of many other G-protein coupled receptors in sensory neurons leads to sensitization of TRPV1. These include protease activated receptors (PARs), receptors for ATP and ADP, and for chemokines. Tyrosine kinase receptors such as the nerve growth factor TrkA also sensitize TRPV1 through PLC and PI3-Kinase mediated pathways. Thus, TRPV1 is a central integrator of many inflammatory signaling pathways in neurons, translating inflammatory input into pain signaling and neurogenic release of inflammatory mediators.

3.2. TRPV1: Molecular Basis of Ligand Interaction

TRPV1 is a major target for the development of novel analgesic and anti-inflammatory agents. Most major pharmaceutical companies established TRPV1 research programs. Some of the newly developed inhibitors show promising analgesic and anti-inflammatory activity in animal models and have progressed into clinical trials. Analgesics targeting TRPV1 may have advantages over classical analgesics, with high specificity for pain-transducing peripheral neurons, and less side effects on the cardiovascular or digestive system, when compared to non-steroidal anti-inflammatory drugs (NSAIDs).

Intensive structure function studies identified a single region in TRPV1 that is essential for interaction with agonists and antagonists (Jordt and Julius 2002). This region is localized to putative transmembrane domains 2–4 in the channel and can be transferred to capsaicin-insensitive ion channels such as TRPV2, establishing capsaicin-sensitivity in this channel. Initially, this region was identified as the binding site for capsaicin and resiniferatoxin, a highly potent capsaicin analog. Capsaicin is likely to bind to the channel from the intracellular side, and several amino acid residues in the binding domain are essential for capsaicin interaction. Antagonists such as capsazepine also bind to this region in the channel (Jordt and Julius 2002). It is likely that the ligand binding region is also involved in the gating of TRPV1 by temperature.

3.3. TRPV1 in Diabetes and Asthma

The more we learn about the function of sensory neurons and their receptors and signaling mechanisms, the more it becomes clear that sensory neurons are not only passive mediators of painful or inflammatory signals. Recently it has been shown that sensory neurons play an active role in the inflammatory process in

chronic diseases such as asthma and also in diabetes. Sensory neurons measure the inflammatory state of tissue, and provide active feedback by neurogenic release of mediators in their target tissues.

A recent study by Razawi et al. provides convincing evidence that normal sensory neural function is required to prevent autoimmune (type I) diabetes, in which the insulin-secreting beta cells in the pancreas are destroyed (Razavi, Chan et al. 2006). C-fibers are known to express receptors for insulin, and TRPV1 channels have been shown to be sensitized by insulin (Van Buren, Bhat et al. 2005). In addition, C-fiber nerve endings have been identified in close proximity to beta cells in the pancreas. Beta cells express receptors for the neuropeptide substance P that has been identified in sensory nerve endings in the vicinity (Razavi, Chan et al. 2006). Razawi et al. investigated the NOD mouse strain that spontaneously develops type I diabetes. Strikingly, the authors mapped the NOD locus to the mouse TRPV1 gene. Sequencing of the NOD TRPV1 gene revealed two mutations that are likely to affect the function of the ion channel. The authors hypothesize that a neuroendocrine feedback loop exists between beta cells and sensory nerve endings, requiring normal function of TRPV1. If TRPV1 is mutated, insulin is unable to elicit normal neural activity such as a tonic release of substance P. The authors could show that application of exogenous substance P to the pancreas delays the onset of diabetes in NOD mice, proving that substance P is a crucial mediator in this feedback mechanism. When substance P is withdrawn, or sensory neurons are eliminated by injections of large doses of capsaicin, the autoimmune response proceeds, resulting in beta cell damage and diabetes. While additional studies are required to elucidate the exact mechanism of this protective feedback, the authors provide strong evidence that in the case of type I diabetes sensory neurons play an active role in anti-inflammatory regulation. These data suggest that pharmacological modulation of sensory neurons by TRPV1 antagonists or agonists may be used to delay the onset of type I diabetes by suppressing the inflammatory process.

While the involvement of TRPV1 in other inflammatory diseases is not as clear as in diabetes, close contact between sensory nerve endings and specific immune cells have been observed in many different organs. For example, Langerhans cells in the skin are innervated by neurons expressing the neuropeptide CGRP (Hosoi, Murphy et al. 1993). Sensory nerve endings have also been identified in the thymus and lymph nodes, where sensory neural activity affects the maturation of different T-cell populations (Shepherd, Beresford et al. 2005). Sensory nerve fibers may also affect the etiology of a variety of inflammatory airway diseases such as asthma or chronic obstructive pulmonary disorder. The lower and upper airways are innervated by several types of mechano- and chemo-sensory nerve fibers that regulate the cough reflex. In certain forms of asthma, capsaicin induces cough at much lower concentrations than in normal subjects (Geppetti, Materazzi et al. 2006). Thus, TRPV1 is likely to be sensitized in asthma. Whether this is a consequence of immunogenic inflammation or a neurogenic phenomenon remains to be established. As in type I diabetes TRPV1 may represent a useful target to suppress inflammatory feedback in airway disease.

3.4. TRPA1, the Receptor for Mustard Oil and Environmental Irritants

TRPA1 is the only member of the TRPA subbranch of the TRP gene family in mammals. This ion channel is characterized by a large number of ankyrin repeats (~17) in its cytosolic N-terminus, a TRP channel membrane domain, and a short cytosolic C-terminal domain. The transcript of TRPA1 was initially identified in a cell line derived from a lung tumor (Jaquemar, Schenker et al. 1999). However, its functional role in these cells has not been studied further, and expression in lung tissue could not be confirmed.

Because of its multiple ankyrin repeats TRPA1 shows significant homology to TRPN (nompC-like) channels, representing a different branch of the TRP channel family essential for certain aspects of sensory mechanotransduction in fruit flies and zebrafish (Walker, Willingham et al. 2000; Sidi, Friedrich et al. 2003). TRPN channels also have a large number of N-terminal ankyrin repeats, but show low homology to TRPA channels in their channel transmembrane moiety. While TRPN channels are present in most animals, mammals lost this branch of the TRP gene family during evolution. Because mammals retained all major mechanosensory modalities, it is therefore unclear whether TRPN channels play a role as primary mechanosensors, or if they have auxiliary roles in more species-specific specialized structures. Based on its partial similarity to TRPN channels, its biophysical and pharmacological properties and its localization it was hypothesized that TRPA1 represents the mechanotransduction channel in the hair cell of the inner ear (Corey, Garcia-Anoveros et al. 2004). This channel is involved in converting mechanical stimuli induced by sound waves into electrical signals, thereby allowing the hearing process to happen. However, recent studies of mice deficient in TRPA1 indicate that TRPA1 is not essential for hearing (Bautista, Jordt et al. 2006; Kwan, Allchorne et al. 2006).

The role of TRPA1 in pain transduction and inflammatory sensitization is much more firmly established. Initially, TRPA1 expression was identified in a small subset of peptidergic sensory nerve fibers, representing <4% of all sensory neurons (Story, Peier et al. 2003). Because TRPA1 can be activated by cold stimuli in some heterologous expression systems, it was hypothesized that TRPA1 is a sensor for noxious cold temperature (Story, Peier et al. 2003). However, later studies showed that TRPA1 is more broadly expressed (in 20–35% of all neurons) (Jordt, Bautista et al. 2004). Because cold-sensitive neurons represent a much smaller population of neurons, this observation implicated alternative or additional roles for TRPA1 in sensory transduction.

Indeed, it was found that TRPA1 is sensitive to a broad range of reactive noxious chemicals, establishing a role of TRPA1 in chemosensation (Jordt, Bautista et al. 2004). Evidence for this role came from a line of research investigating the effects of noxious and pungent chemicals on sensory neurons. This research focused on the effects of the noxious chemical mustard oil (allyl isothiocyanate), the pungent ingredient in mustard. Similar to capsaicin, mustard oil has been used for decades as a chemical probe to study the function of different subsets of sensory neurons in

vitro and in vivo. Mustard oil activates a subpopulation of sensory nerve fibers that is included with the C-fibers that are sensitive to capsaicin. Capsaicin can effectively desensitize these fibers, rendering them insensitive to mustard oil (Jancso, Jancso-Gabor et al. 1967). Thus, a potential receptor for mustard oil is likely to be expressed in fibers co-expressing the capsaicin receptor, TRPV1. Because initial reports about TRPA1 expression indicated exactly that, TRPA1 was tested for sensitivity to mustard oil. Mustard oil strongly activated TRPA1 channels expressed in *Xenopus* oocytes and mammalian culture cells, and also activated Ca^{2+}-influx into TRPA1-expressing cultured sensory neurons (Jordt, Bautista et al. 2004). These results indicated that, in addition to TRPV1, another TRP channel, TRPA1, functions as a sensor for noxious chemicals in inflammatory C-fibers.

Subsequent studies identified other chemical agonists of TRPA1. These included pungent chemicals derived from other plants, including cinnamon, garlic and onion (Bandell, Story et al. 2004; Bautista, Movahed et al. 2005; Macpherson, Geierstanger et al. 2005). In addition, TRPA1 was found to be activated by acrolein, a noxious environmental toxicant produced during combustion and present in tobacco smoke, automobile exhaust and chemical smog (Bautista, Jordt et al. 2006). Sensory nerve endings in the upper and lower airways are highly sensitive to acrolein, and acrolein has been shown to promote cough hypersensitivity and asthma (Morris, Stanek et al. 1999; Morris, Symanowicz et al. 2003). How can such broad sensitivity to such a diverse array of chemical structures be achieved? Most chemical activators of TRPA1 are reactive electrophiles capable of forming covalent bonds with cysteine and other amino acid residues such as lysine. Indeed, it could be found that TRPA1 channels mutated within a cluster of three N-terminal cysteine residues were unresponsive to mustard oil and other reactive activators, indicating that these residues are the targets for chemical modification (Hinman, Chuang et al. 2006). These data imply that TRPA1 activation does not occur through "classical" receptor-ligand interaction, but through covalent modification, explaining the broad sensitivity of TRPA1 to reactive chemicals. TRPA1 may have evolved to induce a flight response in animals exposed to reactive chemicals in fires or toxic environments.

TRPA1 is also affected by inflammatory signaling pathways in sensory neurons. For example, TRPA1 is activated through the receptor for bradykinin, a peptide produced during tissue injury and inflammation (Bandell, Story et al. 2004; Bautista, Jordt et al. 2006). The bradykinin receptor, a G-protein coupled receptor, activates phospholipase C (PLC), promoting phospholipid hydrolysis, followed by intracellular Ca^{2+}-release and signaling through lipid metabolites. Both elevated intracellular Ca^{2+} and lipid signaling molecules may promote TRPA1 activity. Lowering intracellular Ca^{2+}-concentrations reduces TRPA1-activity (Jordt, Bautista et al. 2004). How does Ca^{2+} affect TRPA1 activity? TRPA1 contains a predicted rudimentary EF-finger sequence in its N-terminus that may serve as an interaction site for Ca^{2+}-ions. This domain is situated within the ankyrin repeat domain-containing part of the channel. Because of this unusual location, its partial overlap

with an ankyrin repeat domain, and its weak resemblance to classical EF-hand sequences, further studies are required to elucidate the role of this domain.

The recent analysis of mice deficient in TRPA1 confirmed its role as the sole pain-inducing receptor for mustard oil in sensory neurons (Bautista, Jordt et al. 2006; Kwan, Allchorne et al. 2006). Acute mustard oil-induced pain behavior is absent in these mice. In addition, mustard-oil induced thermal sensitization and mustard-oil dependent mechanical sensitization were eliminated. More interestingly, bradykinin-induced thermal hyperalgesia, a mechanism previously ascribed to TRPV1, was dramatically reduced (Bautista, Jordt et al. 2006). This was a surprising finding that sheds light on a potential regulatory interaction between the bradykinin receptor, TRPA1 and TRPV1. The two independent studies of the TRPA1 knockout mouse differed in their results addressing mechanosensitivity and cold sensitivity. While the first study found no significant differences in either modality (Bautista, Jordt et al. 2006), the second study showed a reduction in certain aspects of mechanosensitivity and a moderate reduction in cold sensitivity in female knockout mice (Kwan, Allchorne et al. 2006). Further studies, involving larger cohorts of backcrossed animals, are likely to be required to prove significance of these differences.

3.5. TRPM8, the Cold/Menthol Receptor

Initially only TRPV channels were thought to have specific functions in sensory transduction in sensory neurons. This view changed when TRPM8, a channel of the TRPM (melastatin-like) branch of the TRP gene family, was identified as a cold-sensitive ion channel and receptor for the cooling agent menthol (McKemy, Neuhausser et al. 2002). Subsequently, other TRPM channels were identified as mediators of sensory input. For example, TRPM5 channels were found to be expressed in taste buds in the tongue, where they are thought to mediate gustatory signals activated through primary receptors for tastants (Zhang, Hoon et al. 2003).

TRPM8 is expressed in a small (~5–15%) population of sensory neurons that is activated by cold and by menthol (McKemy, Neuhausser et al. 2002; Peier, Moqrich et al. 2002). These neurons have small soma diameters, but share only a few other aspects with inflammatory C-fibers expressing TRPA1 and TRPV1. Sensitivity to cold is increased in certain inflammatory conditions, resulting in cold allodynia, the painful hypersensitivity to cold. It is currently unknown whether TRPM8 is involved in this process, or if other cold-sensitive signaling mechanisms are required. Further studies are necessary to characterize the TRPM8-expressing cellular population and its role in cold transduction and, potentially, inflammatory pain.

Extensive biophysical and molecular studies identified several regions in TRPM8 essential for ligand-dependent activation and channel modulation. Similar to TRPA1, some aspects of channel function depend on the concentration of intracellular Ca^{2+}. For example, the activation of TRPM8 by icilin, a cooling agent more potent than menthol, is potently enhanced when intracellular Ca^{2+}-levels are

elevated, either through TRPM8 activity itself, or through release of Ca^{2+} from intracellular stores (Chuang, Neuhausser et al. 2004). Interestingly, a single amino acid change accounts for sensitivity to icilin in mammalian channels when compared to icilin-insensitive avian channels such as chicken TRPM8 (Chuang, Neuhausser et al. 2004). In contrast, activation by menthol is independent of Ca^{2+} and requires different structural determinants within the channel molecule (Bandell, Dubin et al. 2006). Sites essential for menthol interaction were identified in the second putative transmembrane domain, and in the C-terminal TRP domain, a short consensus sequence found in many TRP channels.

3.6. Outlook: TRP Channels in Disease

In our review we presented the more established roles of TRP channels in disease, including their involvement in PKD, pain and inflammation. Recently, several studies were published that indicated that TRP channels are important in other pathological conditions. For example, mutations in TRPM6 are responsible for hypomagnesemia and hypocalcemia in humans (Chubanov, Waldegger et al. 2004). TRPM6 is a magnesium uptake channel in the intestine and is essential for resorption of magnesium from the diet. TRPC channels have been implied in the regulation of vascular endothelial tone and permeability and in neural development and lung permeability (Freichel, Vennekens et al. 2005). These findings indicate that TRP channels may play additional important and diverse roles in normal physiology and pathophysiological processes.

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CHAPTER 10

DISEASES ASSOCIATED WITH ALTERED RYANODINE RECEPTOR ACTIVITY

W.J. DURHAM[1], X.H.T. WEHRENS[1,2], S. SOOD[1] AND S.L. HAMILTON[1]

[1]*Department of Molecular Physiology and Biophysics*
[2]*Department of Medicine (Division of Cardiology), Baylor College of Medicine, Houston, TX 77030*

Abstract: Mutations in two intracellular Ca^{2+} release channels or ryanodine receptors (RyR1 and RyR2) are associated with a number of human skeletal and cardiac diseases. This chapter discusses these diseases in terms of known mechanisms, controversies, and unanswered questions. We also compare the cardiac and skeletal muscle diseases to explore common mechanisms

Keywords: calcium homeostasis, excitation-contraction coupling, malignant hyperthermia, central core disease, multi-minicore disease, and catecholaminergic polymorphic ventricular tachycardia arrhythmogenic right ventricular dysplasia type 2, heart failure, cardiomyopathy, sudden death, and atrial fibrillation

1. RYR MUTATIONS AND HUMAN DISEASE OVERVIEW

Mutations in two intracellular Ca^{2+}-release channels (also known as ryanodine receptors), RyR1 and RyR2, are associated with several human diseases (Table 1). These two channels regulate the release of Ca^{2+} from intracellular stores in skeletal and cardiac muscle, respectively, and control muscle contraction. Mutations in RyR1 are associated with malignant hyperthermia (MH), exertional heat stroke (EHS), central core disease (CCD), and multi-minicore disease (MmD), while mutations in RyR2 have been linked to catecholaminergic polymorphic ventricular tachycardia (CPVT), sudden infant death syndrome (SIDS), and arrhythmogenic right ventricular dsyplasia type 2 (ARVD2, although this is now disputed, see below). Acquired defects in RyR2 also appear to contribute to human disease in heart failure (HF), diabetic cardiomyopathy and atrial fibrillation (AF). Before describing these diseases in more detail, the role of RyRs in striated muscle function is briefly reviewed.

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Table 1. RyR Diseases

	RyR1	RyR2
Inherited mutations	• Malignant Hyperthermia • Exertional heat stroke • Central Core Disease • Multi-Mini Core Disease	• Catecholaminergic Polymorphic Ventricular Tachycardia • Arrhythmogenic RightVentricularDysplasia/Cardiomyopathy • Sudden Infant Death Syndrome
Acquired defects	• MH after Exertional Heat Stroke? • Muscle Weakness in Heart Failure	• Heart Failure • Diabetic Cardiomyopathy • Atrial Fibrillation

2. CA^{2+} HOMEOSTASIS IN STRIATED MUSCLE AND THE ROLE OF RYRS

In striated muscle, Ca^{2+} release occurs primarily through a very large, homotetrameric protein assembly known as the ryanodine receptor (named for its ability to bind the alkaloid ryanodine). When the RyR is open, Ca^{2+} travels down its concentration gradient from the lumen of the sarcoplasmic reticulum (SR, ~1 mM) to the cytosol (~100 nM) (Lyfenko 2004), where it binds to troponin C, initiating movement of tropomyosin away from the myosin-binding sites on the actin filament and triggering contraction. Excitation-contraction (E-C) coupling refers to the process by which electrical depolarization of the muscle cell membrane produces Ca^{2+} release and, ultimately, contraction. In skeletal muscle, depolarization of the T-tubule leads to a conformational change in the dihydropyridine receptor (DHPR), which is an L-type voltage dependent Ca^{2+} channel (Cav1.1), leading to opening of RyR1 via mechanical coupling. Depolarization also causes Ca^{2+} influx via Cav1.1 but this Ca^{2+} is not required for skeletal muscle E-C coupling and the role of this entering Ca^{2+} in skeletal muscle function is currently unknown. The mechanically gated opening of RyR1 releases Ca^{2+} from the sarcoplasmic reticulum to initiate contraction. Since RyR1 is also activated by Ca^{2+}, the Ca^{2+} released from mechanically gated RyRs is likely to activate neighboring RyRs not coupled to DHPRs. This latter process is called calcium-induced calcium release (CICR). Complementing orthograde coupling (DHPR→RyR), retrograde coupling (RyR→DHPR) between RYR1 and the DHPR increases the conductance of the DHPR and the duration of the mechanical activation of RyR1 by the DHPR (Dirksen 2002a).

To prevent continuous Ca^{2+} influx and prolonged contraction, SR Ca^{2+} release must be terminated and cytosolic Ca^{2+} concentrations reduced back to the resting level. Termination of release is brought about by several mechanisms. Repolarization of

the t-tubule causes the DHPR to return to its original conformation, which prevents the activation of RyR1 via orthograde mechanical coupling and also terminates Ca^{2+} influx into the cell. In addition, high [Ca^{2+}] may directly inhibit Ca^{2+} release via channel inactivation, providing a feedback mechanism to prevent a vicious cycle of Ca^{2+} release inducing CICR. Finally, Ca^{2+} reuptake into the SR removes Ca^{2+} from the cytosol, thereby lowering the cytosolic concentration (see below).

In contrast to skeletal muscle, in which the electrical initiation of contraction occurs at the neuromuscular junction, the electrical signal in cardiac muscle is in the form of a depolarization wave originating in the pacemaker cells of the right atrium and transmitted from cell to cell by means of gap junctions (Bers 2002a; Apkon 2003). As in skeletal muscle, however, the depolarization changes the conformation of the cardiac DHPR (Cav1.2), and leads to Ca^{2+} influx through Cav1.2. During the plateau phase of the action potential, this small influx of Ca^{2+} triggers a much greater Ca^{2+} release from the SR via RyR2 through CICR (Fabiato 1983), leading to contraction of the cardiac myocyte. In contrast to the situation in skeletal muscle, this CICR is the pivotal mechanism that controls E-C coupling in cardiac myocytes (Fabiato 1983), where there is no direct physical coupling between the L-type Ca^{2+} channel and RyR2. However, the Cav1.2 channels are apparently closely positioned to the RyR2 to form functional calcium release units (Franzini-Armstrong 1999).

Ca^{2+} removal from the cytosol is accomplished primarily by the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), which pumps cytosolic Ca^{2+} back into the SR (Koss 1997; Apkon 2003). Several isoforms of SERCA exist in striated muscles: SERCA1a and SERCA1b are found in fast adult and neonatal skeletal muscles (Brandl 1987; Zhang 2003; Rossi 2006), respectively, whereas SERCA 2b is found in adult and neonatal cardiac and slow skeletal muscles (Rossi 2006). A Ca^{2+}-ATPase and a Na^{+}-Ca^{2+} exchanger at the sarcolemma are of minor importance for reducing cytosolic Ca^{2+} in the setting of normal SERCA function, primarily acting to balance the small amount of Ca^{2+} influx through the DHPR in response to depolarization (Bers 1989; Apkon 2003). Mitochondria are also able to take up Ca^{2+} from the cytosol; however, their capacity for Ca^{2+} storage is much smaller than that of the SR (Sembrowich 1985). Although not a major route for Ca^{2+} removal from the cytosol, elevations in mitochondrial Ca^{2+} likely contribute to the increased production of ATP and reactive oxygen species (ROS) that occur in response to elevations of cytosolic Ca^{2+}, such as during contraction (Rudolf 2004; Rossi 2006).

Both RyR1 and RyR2 are homotetramers of four >500 kDa subunits that are inserted in the sarcoplasmic reticulum membrane (Smith 1988; Liu 2002). Roughly 90% of both RyRs is cytosolic, with the remainder forming the transmembrane and pore regions in the SR membrane (Shah 2001; Zhou H 2006). The carboxy terminus (spanning the carboxyterminal 600 amino acids) is the pore-forming region, which is thought to be composed of 6–8 transmembrane segments (Du 2002; Ma 2004). A model of the membrane spanning regions of RyR1 is shown in Figure 1a and an analysis of the regions of the protein that

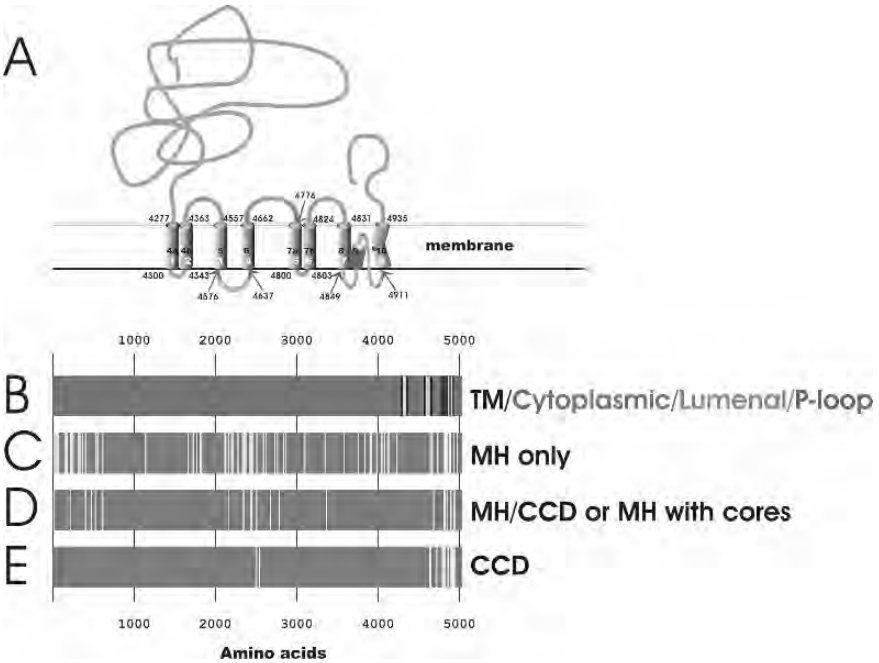

Figure 1. RyR1 Sequences and Disease Mutations. A) Arrangement of RyR1 in the SR membrane, indicating the transmembrane domains. B) Location of transmembrane, cytoplasmic, P-loop, and lumenal regions in the primary sequence of RyR1. C-E) Location of MH, MH + CCD, and CCD mutations in the primary amino acid sequence of RyR1 (See Colour Plate 16)

are likely to be cytoplasmic and lumenal is shown in Figure 1b. Also shown in this figure are the location of mutations in RyR1 known to be associated with human disease and which will be discussed later. A 9Å structure of RyR1 with some of the transmembrane helices identified and demonstrating the much greater size of the cytoplasmic portion versus the lumenal portion is shown in Figure 2.

Given its massive size, the RyR represents an enormous target for binding and modification. Accordingly, RyRs are thought to form large macromolecular complexes with a number of modulators which influence activity. Junctin, triadin, and calsequestrin interact with RyRs in the SR lumen. The transmembrane proteins junctin and triadin appear to bind directly to both RyRs (Zhang 1997; Gyorke 2004) and calsequestrin. Calsequestrin, the major Ca^{2+}-binding protein in the SR, provides a high capacity intra-SR Ca^{2+} buffer (Terentyev 2003) and is important for maintenance of SR Ca^{2+} stores. The larger cytoplasmic portion of the RyR binds to a number of important modulators, including the Cav1.1 (discussed above), calmodulin (Chu 1990; Tripathy 1996; Zhang 1999; Hamilton 2000), protein phosphatases (PP1, PP2A, and PP2B (calcineurin)), immunophilins such as the FK506 binding proteins (FKBP12 and FKBP12.6), (Marx 2001; Chelu 2004), sorcin

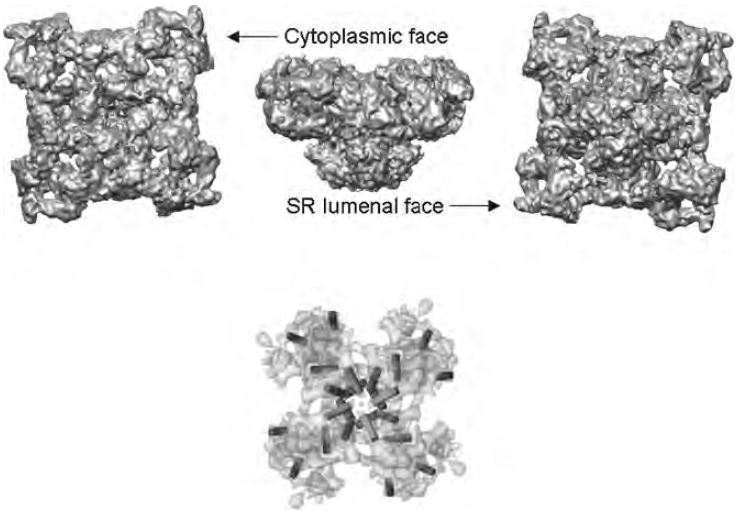

Figure 2. RYR1 structure at 9.5 Å. The color ribbons represent a helices, the inner helix is labeled red, the pore helix green, and other helices are colored blue or purple (See Colour Plate 17)

(Meyers 1995), protein kinase A (PKA) (Marx 2000), Ca^{2+}/CaM-dependent kinase II (CaMKII); (Wehrens 2003a; Currie 2004), and phosphodiesterase 4D3 (PDE4D3 (Lehnart 2005). These kinases and phosphatases are targeted to RyR by scaffolding and anchoring proteins (Marx 2001; Lyfenko 2004; Treves 2005; Rossi 2006; Yano 2006). A variety of cellular mediators and modifications can also modulate the activity of RyR, including Ca^{2+}, Mg^{2+}, ATP, phosphorylation (Fill 2002), oxidation, S-nitrosylation, and S-glutathionlyation (Hidalgo 2005). Regulation by Ca^{2+} varies according to RyR isoform. Both RyR1 and RyR2 are activated at $[Ca^{2+}]$ of 0.1–10μM (Smith 1986; Bull 1993; Chu 1993; Coronado 1994; Tripathy 1996; Copello 1997; Fill 2002); but RyR1 is more sensitive to inhibition by higher concentrations of Ca^{2+}. There is evidence to suggest that the different Ca^{2+} dependencies may be dictated at least partly by the redox environment of each of the channels (Marengo 1998). Lumenal Ca^{2+} may also regulate the activity of RyRs (Jiang 2004). Finally, environmental factors such as pO_2 (Eu 2000; Cheong and Salama 2005) and temperature (Konishi 1985) are known to modulate RyR activity as well.

3. RYR DISEASES: INTRODUCTION

To define the molecular events by which mutations in RyRs cause disease, it is essential to first consider the possible ways that the mutations in RyRs could alter intracellular Ca^{2+} handling and muscle function. We know that RyRs control the release of Ca^{2+} from internal stores and the mutations could alter this process by altering either the probability of channel opening or the magnitude of the Ca^{2+} efflux

via the channel. These effects could alter excitation-contraction coupling, resting Ca^{2+} concentrations, the magnitude of the Ca^{2+} transient and/or a large variety of Ca^{2+}-dependent signal transduction events. Changes in the contractile properties of the muscle could reflect the immediate effects of altered Ca^{2+} homeostasis and/or adaptive or developmental changes in muscle properties arising from earlier changes in Ca^{2+} handling. Although important information about inherent changes in the activity of RyRs can be obtained with purified proteins or by studying single channels, it is highly unlikely that these types of studies can alone define the consequences of the mutations to muscle function that produce the human disease.

4. RYR1 DISEASE: MALIGNANT HYPERTHERMIA

MH is a clinical syndrome in which genetically susceptible individuals respond to inhalation anesthetics (e.g. halothane) and muscle relaxants (e.g. succinylcholine) with attacks of skeletal muscle rigidity, extreme elevations in body temperature, rhabdomyolysis, hypermetabolism, lactic acidosis, hypoxia and tachycardia (Mickelson 1996; Jurkat-Rott 2000). There are some data to suggest that MH episodes can be triggered by other stimuli such as 3,4-methylenedioxymethamphetamine ("ecstasy"), psychological stress, environmental heat, or re-warming following surgery (Fiege 2003; Lyfenko 2004; Treves 2005; Crean 2006). *In vitro* contracture tests (IVCTs), in which the force produced by excised muscle samples in response to triggering stimuli, are used to detect MH susceptibility (McCarthy 2000), but recently genetic screens and analysis of Ca^{2+} homeostasis in lymphoblasts from patients with RyR1 diseases are being used more frequently. The incidence of MH has been estimated to be from ~1 in 15,000 anesthetized children and ~1 in 50,000–100,000 anesthetized adults, (Britt 1991), but the incidence of MH is thought to be grossly underestimated. For example, the incidence in the general population has been estimated to be 1 in 10, 000 in Japan (Ibarra 2006) and 1 in 2–3,000 in the French population (Monnier 2003). There also appears to be greater severity and higher probability of a response in males than in females (Strazis 1993).

As the name implies, during an MH episode a hypermetabolic state develops, characterized by increases in body temperature, metabolic rate, blood and muscle acidity, and blood CO_2 (Litman 2005). The rate of contracture development is very slow (minutes) relative to a normal, volitional contraction (milliseconds). Moreover, the maximal force developed, based on *in vitro* experiments in a mouse model, is ~40–50% of that produced during a maximal, electrically-stimulated contraction (Chelu 2006). This suggests that it is the unrelenting nature of the Ca^{2+} release, rather than an initial gross mismatch between release and uptake, that eventually manifests in a fulminant MH episode. These episodes also result in leakage of proteins and electrolytes from skeletal muscle which can lead to kidney failure and cardiac arrhythmias (Litman 2005). Left untreated, malignant hyperthermia is fatal in ~90% of cases (Lyfenko 2004; Litman 2005; Treves 2005) Treatment with dantrolene, an inhibitor of Ca^{2+} release through RyR, as well as suspension of the

triggering agent, and hyperventilation with 100% O_2 has reduced mortality from MH to ~5% (Lyfenko 2004; Litman 2005), (for review see, Parness, Chapter 24 in ryanodine receptors: structure, function and dysfunction in clinical disease 2005; Eds Wehrens and Marks).

Contained within the original description of MH by Denborough and colleagues (Denborough 1962) was the first mention of another notable characteristic of MH, namely, the temperature dependence of the response. These investigators related the case of a young man with a broken leg who was more apprehensive about receiving anesthesia than about his injury, due to the fact that several of his relatives had died after anesthetic treatment (Denborough 1962). His fear was well-founded, as he developed symptoms of MH (hyperthermia, tachycardia) following halothane treatment. Fortunately, whole body cooling rescued this patient, demonstrating that an interaction exists between development of an MH episode and temperature. Subsequent studies in pigs (Nelson 1990) and mice (Chelu 2006) have supported this notion, demonstrating that MH does not develop, despite exposure to a triggering stimulus, when core body temperature is reduced by as little as 1–2°C.

MH mutations are localized to human chromosome 19q13.1 (Table 2), which includes the locus of RyR1 (Loke 1998; Jurkat-Rott 2000; McCarthy 2000). Mutations in the RyR1 gene account for more than one-half of all MH families. At least 143 different point mutations or deletions in RyR1 (Figure 1c) are linked to MH (Robinson 2006). Although linkage to the RyR1 gene is shown for more than 50% of all MH kindred, other MH-susceptible gene loci exist on chromosomes 17 (17q11.2-q24), 7 (7q21-q22), 3 (3q13.1), and 1 (1q31) (Jurkat-Rott 2000; McCarthy 2000). Two different point mutations of a highly conserved arginine residue in the III-IV linker of the skeletal muscle DHPR a_1-subunit (R1086H and R1086C) are confirmed for the 1q31 locus (Monnier 1997; Jurkat-Rott 2000). The finding that MH manifests from point mutations in both the skeletal muscle DHPR and RyR1, two key proteins of muscle EC coupling, reinforces the notion that MH is a disease of muscle EC coupling.

In accordance with the pharmacogenetic nature of MH, pharmacological activators of RyR1 Ca^{2+} release such as caffeine, halothane, or 4-chloro-m-cresol induce contractures at lower concentrations in MH muscle than in normal muscle when studied *in vitro*. This differential susceptibility forms the basis of the *in vitro* contracture test (IVCT) used in the determination of MH susceptibility (1984; Larach 1989; Rosenberg 2002).

Research into the causes and manifestations of malignant hyperthermia was greatly aided by the serendipitous observation that pigs bred to produce leaner meat sometimes experienced episodes of malignant hyperthermia (Gronert 1994). Subsequent genetic analysis revealed that this condition was due to an R615C point mutation in RyR1 (Lyfenko 2004; Treves 2005). Pigs with this mutation were found to be susceptible to episodes of malignant hyperthermia in response to stress (i.e., the 'porcine stress syndrome'), halogenated anesthetics, and heat (Ording 1985; Gronert 1994). The response to stress was initially thought to be initiated by increased sympathetic activity; however, subsequent studies support a

Table 2. RyR1 Mutations

RyR1 Mutation	Exon	Disease	ref	RyR1 Mutation	Exon	Disease	ref
L13R	1	MH	(Ibarra 2006)	R2458H	46	MH	(Manning 1998a)
ΔD17	2	MH	(Ibarra 2006)	R2508C	47	MH/CCD	(Ibarra 2006)
C35R	2	MH	(Lynch 1997)	R2508G	47	CCD	(Ibarra 2006)
R44C	2	MH	(Tammaro 2003)	R2508H	47	MH/CCD	(Galli 2006)
R44H	2	MH	(Robinson 2006)	Y2510H	48	MH	(Robinson 2006)
D60N	3	MH	(Robinson 2006)	E2545D	48	CCD	(Ibarra 2006)
S71Y	3	MH	(Galli 2006)	R2591G	48	MH	(Galli 2006)
Q155K	6	MH	(Ibarra 2006)	V2627L	49	MH	(Galli 2006)
E160G	6	MH/CCD	(Shepherd 2004)	R2676W	50	MH/CCD/MmD	(Guis 2004)
R163C	6	MH/CCD/MmD	(Quane 1993)	G2733D	51	MH	(Sambuughin 2005)
R163L	6	MH	(Monnier 2005)	E2764K	52	MH	(Galli 2006)
G165R	6	MH	(Monnier 2005)	T2787S	53	MH with cores	(Guis 2004)
D166N	6	MH	(Rueffert 2002)	R2840W	54	MH	(Ibarra 2006)
R177C	6	MH	(Monnier 2005)	L2867G	55	MH	(Galli 2006)
Y178C	6	MH	(Monnier 2005)	E3104K	63	MH	(Robinson 2006)
G215E	8	MH/CCD	(Romero 2003)	R3350H	67	MH	(Sambuughin 2005)
V218I	8	MH	(Ibarra 2006)	K3367R	67	MH/CCD	(Wu 2006)
D227V	8	MH	(Monnier 2005)	P3527S	71	MmD	(Ferreiro 2002a)
G248R	9	MH	(Gillard 1992)				
R316L	10	MH	(Ibarra 2006)	Q3756E	79	MH	(Oyamada 2002)
R328W	11	MH	(Loke 2003)	V3840I	82	MH	(Ibarra 2006)
G341R	11	MH	(Quane 1994)	R3903Q	85	MH	(Galli 2006)
R367Q	11	MH	(Galli 2006)	I3916M	85	MH	(Monnier 2002)
R401C	12	MH/CCD/EHS	(Davis and Pollock 2002)	D3986E	87	MH	(Robinson 2006)
R401G	12	MH	(Robinson 2006)	G3990V	87	MH	(Robinson 2006)
R401H	12	MH	(Rueffert 2002)	R4041Y	89	MH	(Galli 2006)
I403M	12	MH/CCD	(Quane 1993)	S4050Y	89	MH	(Robinson 2006)
Q474H	13	MH/CCD	(Ibarra 2006)	T4081	89	MH	(Ibarra 2006)
Y522S	14	MH/CCD	(Quane 1994)	N4119Y	90	MH	(Sambuughin 2005)

Y522C	14	MH	(Yeh 2005)	R4136S	90	MH	(Galli 2002)
R533C	15	MH	(Tammaro 2003)	V4234L	91	MH	(Galli 2002)
R533H	15	MH	(Brandt 1999)	T4637A	95	CCD	(Scacheri 2000)
R552W	15	MH	(Keating 1997)	T4637I	95	CCD	(Davis 2003)
R614C	17	MH/CCD/EHS	(Gillard 1991)	G4638D	95	CCD	(Davis 2003)
R614L	17	MH	(Quane 1997)	R4645Q	95	MH	(Ibarra 2006)
R1140C	26	MH	(Robinson 2006)	L4650P	95	CCD	(Romero 2003)
R1667C	34	MH	(Ibarra 2006)	H4651P	95	CCD	(Davis 2003)
S1728P	34	MH	(Sambuughin 2005)	P4668S	96	MH	(Oyamada 2002)
S1728F	34	MH	(Robinson 2006)	F4684S	96	MH/MmD	(Monnier 2005)
M1729R	34	MH	(Robinson 2006)	K4724Q	96	CCD	(Romero 2003)
P1773S	34	MH	(Ibarra 2006)	Y4733E	98	MH	(Sambuughin 2005)
M1814K	34	MH	(Robinson 2006)	G4734E	98	MH	(Robinson 2006)
A1832G	34	MH	(Monnier 2000)	R4737W	98	MH	(Galli 2002)
V2117L	39	MH	(Tammaro 2003)	R4737Q	98	MH	(Monnier 2005)
D2129E	39	MH	(Rueffert 2001)	L4793P	100	CCD	(Monnier 2001)
R2163C	39	MH	(Manning 1998a)	Y4796C	100	CCD	(Monnier 2000)
R2163H	39	MH/CCD	(Manning 1998a)	F4808I	100	CCD	(Davis 2003)
R2163P	39	MH	(Fortunato 2000)	L4814F	100	MH/CCD	(Shepherd 2004)
V2168M	39	MH	(Manning 1998b)	I4817F	100	MH	(Robinson 2006)
A2200V	40	MH	(Sambuughin 2005)	G4820W	100	MH	(Robinson 2006)
T2206M	40	MH	(Manning 1998a)	L4824P	100	MH/CCD	(Sei 2004)
V2210F	40	MH	(Sambuughin 2005)	R4825C	100	CCD	(Monnier 2001)
V2212D	40	MH	(Galli 2006)	R4825P	100	CCD	(Robinson 2006)
V2214I	40	MH	(Sambuughin 2001)	T4826I	100	MH	(Brown 2000)
V2280I	42	MH	(Galli 2002)	L4838V	101	MH	(Oyamada 2002)
R2336H	43	MH	(Robinson 2006)	V4849I	101	MH/CCD	(Jungbluth 2002)
R2336Q	43	MH	(Galli 2006)	A4856G	101	MH	(Robinson 2006)
N2342S	43	MH	(Wehner 2003)	F4860V	101	CCD	(Robinson 2006)

(Continued)

Table 2. (Continued)

RyR1 Mutation	Exon	Disease	ref	RyR1 Mutation	Exon	Disease	ref
V2346M	44	MH/CCD	(Shepherd 2004)	R4861H	101	CCD	(Monnier 2001)
E2344D	44	MH	(Monnier 2005)	R4861C	101	CCD	(Davis 2003)
E2348G	44	MH/CCD	(Shepherd 2004)	Y4864C	101	CCD	(Quinlivan 2003)
A2350T	44	MH	(Sambuughin 2001)	K4876R	101	MH	(Monnier 2005)
R2355W	44	MH	(McWilliams 2002)	M4880T	101	MH	(Sambuughin 2005)
E2362G	44	MH	(Galli 2006)	G4891R	102	CCD	(Tilgen 2001)
F2364V	44	MH	(Robinson 2006)	R4893W	102	CCD	(Monnier 2001)
P2366R	44	MH	(Ibarra 2006)	R4893Q	102	CCD	(Davis 2003)
A2367T	44	MH	(Sambuughin 2001)	A4894V	102	CCD	(Robinson 2006)
G2375A	44	MH	(Wehner 2004)	A4894T	102	MH	(Ibarra 2006)
A2428T	45	MH	(Rueffert 2002)	I4898T	102	MH/CCD	(Lynch 1999)
D2431N	45	MH	(Sambuughin 2001)	G4899R	102	MH/CCD	(Tilgen 2001)
D2431Y	45	MH	(Robinson 2006)	G4899E	102	CCD	(Monnier 2001)
G2434R	45	MH	(Keating 1997)	A4906V	102	CCD	(Tilgen 2001)
R2435H	45	MH/CCD	(Zhang 1993)	R4914G	102	CCD	(Monnier 2001)
R2435L	45	MH/CCD	(Barone 1999)	R4914T	102	CCD	(Davis 2003)
A2437V	45	MH	(Sei 2004)	F4921S	102	CCD	(Davis 2003)
E2439D	45	MH	(Galli 2006)	V4927F	102	CCD	(Robinson 2006)
R2452W	46	MH	(Chamley 2000)	I4938M	103	MH	(Shepherd 2004)
R2452Q	46	MH	(Ibarra 2006)	D4939E	103	MH/CCD	(Shepherd 2004)
I2453T	46	MH/CCD	(Rueffert 2004)	A4940T	103	CCD	(Quinlivan 2003)
R2454C	46	MH	(Brandt 1999)	G4942V	103	MH/CCD	(Galli 2002)
R2454H	46	MH	(Barone 1999)	P4973L	104	MH	(Monnier 2002)
R2458C	46	MH	(Manning 1998a)				

possible contributory, but secondary, role of sympathetic activation in the development of an episode (Haggendal 1990; Gronert 1994; Maccani 1996). More recently, mice with a Y522S mutation of RyR1 have been reported to be the first mouse model for malignant hyperthermia (Chelu 2006). Initial studies in these mice support the findings observed in pigs bearing the R615C mutation, including increased sensitivity to halogenated anesthetics (isoflurane) and temperature (Chelu 2006).

Although the disease-causing mutations in RyR1 were originally thought to cluster into three regions: the N-terminal (cytosolic) region ("region 1", amino acids 35–614), the central (cytosolic) region ("region 2", amino acids 2129–2458), and the C-terminal pore-forming region ("region 3", amino acids 3916–4973)) (Lyfenko 2004; Treves 2005; Yano 2006), it is now becoming apparent that, at least for MH, the mutations are more widely distributed throughout the RyR1 primary sequence (Figure 1c).

A number of studies have addressed the effects of the MH mutations on RyR1 activity and Ca^{2+} homeostasis (Table 3). Studies of the effects of the disease causing mutations have been studied by expression of recombinant RyR1 channels in HEK cells or RyR1 deficient myotubes. The "pure" MH mutations that do not appear to be associated with a core myopathy (see below) appear to make the RyR1 open more readily in response to activators but, for the most part, are not associated with Ca^{2+} leak to produce store depletion and/or increased resting Ca^{2+}. One possible explanation for these findings has been put forward by Ikemoto and Yamamoto (Ikemoto 2002), who proposed that an intra-molecular interaction between regions 1 and 2 regulates channel activation by stabilizing a closed state of the channel. They suggested that the MH mutations weaken this interaction, allowing the channel to open more readily in response to activators. This explanation is not mutually exclusive of others, such as the possibility that mutations in cytoplasmic domains could also alter the binding of modulators of RyR function.

There are number of unanswered questions about MH. These include: 1. Is the enhanced sensitivity of RyR1 to MH-producing triggers due to an inherent change in the sensitivity of RyR1 to these agents, or does altered Ca^{2+} homeostasis in the muscle drive other changes that enhance RyR1 sensitivity to activation? 2. Why are MH episodes more prevalent in children? 3. Why are males more likely to have an MH response? 4. Do mutations in proteins other than RyR1 and the DHPR produce MH?

5. RYR1 DISEASE: EXERTIONAL HEAT STROKE (EHS)

Exertional heat stroke (EHS), a severe form of heat illness, occurs during exercise in the heat. Patients with the RyR1 mutations associated with MH may be more susceptible to exertional heat stroke and, conversely, patients who have had EH episodes may be more susceptible to an MH response (Sei 1999; Wappler 2001; Davis and Pollock 2002). The two disorders appear to have many common features

Table 3. Functional Consequences of RyR1 Mutations

Mutation (human seq #)	Expression system	Human Disease	Rest Ca^{2+}	Maximal Caffeine induced release	Caffeine sensitivity	Maximal Voltage gated release	Sensitivity to voltage	Ca^{2+} stores	ref
C35R	HEK	MH	nc	↓	↑			↓	(Tong 1997; Tong 1999)
R163L	HEK	MH/CCD			↑				(Monnier 2005)
R163L	HEK	MH/CCD	nc	↓					(Lynch 1999)
R163C	RyR1 deficient myotubes	MH		↓	↑	↑	↑		(Yang 2003)
R163C	RyR1 deficient myotubes	MH	↑	↓	↑	↓	↑	↓	(Avila 2001a)
R163C	HEK	MH	nc	↓	↑			↓	(Tong 1997; Tong 1999)
G248R	HEK	MH	nc	↓	↑			nc	(Tong 1997; Tong 1999)
G341R	RyR1 deficient myotubes	MH	nc	↓	↑			Uncertain (↓ caffeine response, ↑ K^+ response)	(Yang 2003)

G341R	HEK	MH	nc	↓	↑			↓	(Tong 1997; Tong 1999)
I403M	RyR1 deficient myotubes	MH/CCD	↑	↓				nc	(Avila 2001a)
I403M	HEK	MH/CCD	nc	↓	↑			nc	(Tong 1997; Tong 1999)
Y522S	HEK	MH/CCD	↑	nc				↓	(Brini 2004)
Y522S	HEK	MH/CCD		↓				↓	(Lynch 1999)
Y522S	HEK	MH/CCD	↑	↓	↑			↓	(Tong 1997; Tong 1999)
Y522S-het	rat muscle myotubes	MH/CCD		nc		nc		↓ (terminal SR only)	(Brini 2004)
Y522S	RyR1 deficient myotubes	MH/CCD	↑	↓		↓	↑	↓	(Avila 2001a; Avila 2003)
Y522S Heterozygous	knockin mouse myotubes rm T	MH/CCD	nc	nc	↑	↑	↑		(Chelu 2006)
Y522S-	knockin mouse	MH/CCD	↑	↓	↑		↑		Durham et al, in preparation

(Continued)

Table 3. (Continued)

Mutation (human seq #)	Expression system	Human Disease	Rest Ca^{2+}	Maximal Caffeine induced release	Caffeine sensitivity	Maximal Voltage gated release	Sensitivity to voltage	Ca^{2+} stores	ref
Homozygous Y522S-Het	myotubes rm T knockin mouse myotubes 37°C	MH/CCD	↑	↓	↑				Durham et al, in preparation
R552W	HEK	MH	nc	↓	↑			↓	(Tong 1997; Tong 1999)
R614C	HEK	MH	nc	↓	↑			nc	(Tong 1997; Tong 1999)
R614C	MH pig	MH			↑				(Hawkes 1992)
R614C	RyR1 deficient myotubes	MH	nc	nc		nc	↑	nc	(Dirksen 2004)
R614C	RyR1 deficient myotubes	MH		↓	↑	↑	↑		(Yang 2003)
R614L	HEK	MH	nc	↓	↑			↓	(Tong 1999)
R999H	Lympho-blastoid	MmD	nc		nc				(Ducreux 2006)

P1787L	HEK				nc				(Monnier 2005)
R2163H	RyR1 deficient myotubes	MH/CCD	↑	↓		↓	↑	↓	(Avila 2001a; Dirksen 2004)
R2163H	HEK	MH/CCD	↑	↓	↑	↓		↓	(Tong 1997; Tong 1999)
R2163C	RyR1 deficient myotubes	MH	nc	nc		nc	↑	nc	(Avila 2001a; Dirksen 2004)
R2163C	RyR1 deficient myotubes	MH		↓	↑	↑	↑		(Yang 2003)
R2163C	HEK	MH	nc	↓	↑			↓	(Tong 1997; Tong 1999)
V2168M	HEK	MH			↑				(Monnier 2005)
V2168M	RyR1 deficient myotubes	MH		↓	↑	↑	↑		(Yang 2003)
T2206M	HEK	MH	nc	↓	↑				(Monnier 2005)
ΔE2347	RyR1 deficient myotubes	MH	nc	nc		nc	↑	nc	(Dirksen 2004)

(Continued)

Table 3. (Continued)

Mutation (human seq #)	Expression system	Human Disease	RestCa^{2+}	Maximal Caffeine induced release	Caffeine sensitivity	Maximal Voltage gated release	Sensitivity to voltage	Ca^{2+} stores	ref
T2206R	HEK	MH			↑				(Monnier 2005)
A2428T	HEK	MH			↑				(Monnier 2005)
R2435H	RyR1 deficient myotubes	MH/CCD	↑				↑		(Avila 2001a)
R2435L	RyR1 deficient myotubes	MH/CCD	↑	↓		↓	↑	↓	(Dirksen 2004)
R2435H	HEK	MH/CCD	nc	↓	↑	↓		nc	(Tong 1997; Tong 1999)
R2454C	HEK	MH	nc		↑				(Monnier 2005)
R2454H	HEK	MH		nc	↑				(Monnier 2005)
R2458H	RyR1 deficient myotubes	MH		↓	↑	↑	↑		(Yang 2003)
R2458H	HEK	MH	nc	↓	↑			↓	(Tong 1997; Tong 1999)

R2458C	HEK	MH	nc	nc	↑			nc	(Tong 1997; Tong 1999)
P3527S heterozygous	Lympho-blastoid	MmD	↑	nc	nc				(Ducreux 2006)
P3527 homozygous	Lympho-blastoid	CCD/MmD	↑	↓	↑				(Ducreux 2006)
Δ4214–4216	RyR1 deficient myotubes, HEK Lympho-blastoid	CCD		↓	nc	↓	↑		(Lyfenko 2006)
T4637A	RyR1 deficient myotubes	CCD	↑	↓		↓	↑	↓	(Dirksen 2004)
Y4796C	HEK	CCD	↑	↓	↑				(Monnier 2005)
Y4796C	RyR1 deficient myotubes	CCD	↑	↓		↓	↑	↓	(Dirksen 2004)
T4826I	RyR1 deficient myotubes	MH	nc	nc	nc	nc	nc	nc	(Dirksen 2004)
T4826I	RyR1 deficient myotubes	MH		↓	↑	↑	↑		(Yang 2003)
V4849I heterozygous	Lympho-blastoid	MH/CCD	↑	nc	nc				(Ducreux 2006)

(Continued)

Table 3. (Continued)

Mutation (human seq #)	Expression system	Human Disease	RestCa^{2+}	Maximal Caffeine induced release	Caffeine sensitivity	Maximal Voltage gated release	Sensitivity to voltage	Ca^{2+} stores	ref
V4849I homozygous	Lympho-blastoid	MH/CCD	↑		nc				(Ducreux 2006)
Δ4863-4869	Lympho-blastoid HEK	CCD							(Zorzato 2003)
G4891R	RyR1 deficient myotubes	CCD	nc	↓		↓		nc	(Avila 2003)
R4893W	RyR1 deficient myotubes	CCD	nc	↓ (small effect)		↓		nc	(Avila 2003)
R4893W	HEK	CCD		↓					(Du 2004)
I4898T	RyR1 deficient myotubes	CCD	nc	↓		↓	nc	nc	(Avila 2001b; Avila 2003)
I4898T	HEK	CCD	↑	↓				↓	(Lynch 1999)
I4898T	HEK	CCD	nc	↓	nc				(Brini 2004)
I4898T-Het	Rat myotubes	CCD	↑	↓		nc		↓	(Brini 2004)
G4899E	RyR1 deficient myotubes	CCD	nc	↓		↓		nc	(Avila 2003)

G4899R	RyR1 deficient myotubes	MH/CCD	nc	↓	↓		nc	(Avila 2003)
A4906V	RyR1 deficient myotubes	CCD	nc	↓	↓		nc	(Avila 2003)
R4914G	RyR1 deficient myotubes	CCD	nc	↓	↓		nc	(Avila 2003)
Δ4927–4928	RyR1 deficient myotubes Lympho-blastoid HEK	CCD		↓	↓	↓		(Lyfenko 2006)

including: rhabdomyolysis, increases in serum creatine kinase, hyperkalemia, tachycardia, metabolic acidosis and increased production of inflammatory cytokines (IL-6, IL1β, aand IL-10) by skeletal muscle (Bouchama 2002; Ducreux 2004). Wappler et al (Wappler 2001) performed *in vitro* contracture tests on patients who had had episodes of EHS. Out of 12 patients, 10 showed IVCTs that suggested MH susceptibility with three having RyR1 mutations associated with MH. There also appears to be a correlation in the military with soldiers who have environmental heat illness with positive IVCT tests (Hackl 1991; Hopkins 2000; Bendahan 2001).

Two RyR1 mutations (R401C and R614C) are now thought to correlate with MH, exertional heat stroke and exercise-induced rhabdomyolysis (Wappler 2001; Davis 2002). These findings raise questions as to how exercise increases the probability of an MH response. Intense or prolonged exercise increases oxidative stress (Cooper 2002), core body temperature, and sympathetic activity (Seals 1991). Any of these changes could trigger SR Ca^{2+} release if the mutated RyR1 is more sensitive to these stimuli. The relationship between exertional heat illness and MH is currently a topic of great interest. A particularly intriguing question is whether MHS can be acquired as the result of an EHS episode. Interested readers are referred to an insightful and comprehensive review of the relationship between MH and environmental heat illness by Muldoon et al (Muldoon 2004).

6. RYR1 DISEASE: CENTRAL CORE DISEASE (CCD)

CCD (Shy 1956) is the most frequently observed congenital myopathy in humans (Shuaib 1987; Dirksen 2002b; Lueck 2004). Muscle weakness of the lower extremities leading to delayed attainment of motor skill milestones is the most common manifestation. In individuals exhibiting such characteristics, the diagnosis of CCD is based on histochemical identification in type 1 muscle fibers with clearly circumscribed amorphous areas (cores) that lack mitochondria and oxidative enzyme activity (Shuaib 1987). CCD was first described in 1956 (Shy 1956), making it the first reported human congenital myopathy (Lyfenko 2004; Treves 2005). The clinical presentation of CCD is variable, with the functional spectrum ranging from individuals with no noticeable limitations of activities of daily living throughout their lives to those displaying profound muscle weakness from an early age, manifested as infantile hypotonia (floppy infant syndrome) and delayed achievement of motor milestones (Lyfenko 2004; Treves 2005). In the latter cases, hip dislocation (Gonatas 1965; Bethlem 1971), kyphoscoliosis, and flat feet are common (Shuaib 1987; Lyfenko 2004). Weakness is usually more pronounced in proximal muscle groups and is non-progressive or slowly progressive (Patterson 1979; Lamont 1998; Lyfenko 2004; Treves 2005). Until recently, CCD was thought to be inherited solely in an autosomal dominant fashion. However, it is now known that in rare instances inheritance can be autosomal recessive (Jungbluth 2002; Romero 2003).

The eponymous characteristic of this disease is a lack of oxidative staining in regions (“cores”) of skeletal muscle fibers. These cores can either be centrally

located or at the periphery of the fiber (referred to as eccentric cores) and in longitudinal sections are observed to encompass numerous sarcomeres along the long axis of the fiber (Treves 2005). In addition to lack of mitochondrial or oxidative enzyme staining, cores lack glycogen granules and exhibit variable degrees of myofibrillar disorganization, such as Z-line streaming (Lyfenko 2004). Type I fiber predominance is typically observed (Lyfenko 2004; Treves 2005).

The factor(s) responsible for core development are not known. Currently proposed explanations center on responses to aberrant calcium signaling. In these scenarios, the core represents an area of the fiber in which Ca^{2+} homeostasis cannot be properly regulated. One possibility is that mitochondrial Ca^{2+} levels are either increased or reduced in CCD skeletal muscle, leading to pathological mitochondrial dysfunction. Calcium is an activator of mitochondrial ATP production (Lyfenko 2004) but Ca^{2+} overload can lead to destruction of mitochondria. It has also been proposed that core formation represents a protective response to calcium dysregulation in some parts of the cell (Loke 1998). In this case, the core isolates the dysfunctional region of the muscle fiber from the areas in which calcium homeostasis is better maintained. Notably, the presence of cores alone is not pathognomic for CCD, as ~40% of individuals exhibiting this characteristic may be clinically normal (Shuaib 1987). In addition, the prevalence of cores does not correlate with disease severity (Fardeau 1994; Sewry 2002).

Although primarily identified by the presence of central cores in the muscle fiber, RyR1 mutations can also be associated with eccentric or peripheral cores, multiple minicores, uniformity of type 1 fibers with or without cores, mild unevenness of oxidative enzyme staining, or marked muscle replacement by fatty tissue in association with cores (Muntoni 2003). Most disease-causing mutations in RyR1 are single point mutations that are likely to directly alter RyR1 function. Recently, however, Monnier *et al* (Monnier 2003) reported an out-of-frame mutation that leads to reduction of RyR1 in the muscle, suggesting the amount of RyR1 expressed is also crucial for function. In addition, as mentioned above, CCD may present as a multi-minicore myopathy and can show a recessive inheritance. Ferreiro *et al.* (Ferreiro 2002b) identified a homozygous mutation in the RYR1 gene in an Algerian family. The skeletal muscles from adult affected members of this family showed typical central cores, but muscle of the affected children presented as a minicore myopathy. These studies emphasize the need to assess the effects of aging in CCD and that the presentation of CCD can be highly variable even within a single family, with both severely affected and asymptomatic family members all carrying the same mutation.

One family with CCD arising from an RyR1 mutation also had nemaline rods similar to those found with nemaline myopathies (NM) (Scacheri 2000). Muscle fibers from these patients show frequent clusters of rod-like structures. The autosomal dominant form of NM has been linked to mutations in the genes for α-tropomyosin and skeletal α-actin. A recessive form is associated with mutations in nebulin and α-tropomyosin. A major, unanswered question is whether and/or how a mutation in RyR1 can produce a similar pathology.

For certain RyR1 mutations, individuals exhibit both MH and CCD, whereas others appear to result in either a pure MH phenotype or CCD in the absence of enhanced MH-susceptibility. Importantly, because MH and CCD are predominantly inherited in an autosomal dominant manner, the majority of these mutations are thought to produce "gain-of-function" effects on SR Ca^{2+} release channel activity, a prediction supported by the functional studies discussed below. As summarized in Table 3, most *in vitro* studies have found that mutations association with both MH and CCD are characterized as "leaky" in that Ca^{2+} stores are depleted and cytosolic resting Ca^{2+} concentrations may be elevated. A dichotomy exists in the functional effects of CCD mutations in region 3 of RyR. Some mutations in this region increase channel activity (Lyfenko 2006; Yano 2006), similar to the effects of the cytoplasmic, aminoterminal mutations. In contrast, a subset of mutations in region 3 is thought to inhibit permeation of Ca^{2+} through the pore (Lyfenko 2004; Lyfenko 2006). When expressed *in vitro*, such channels are insensitive to activation by voltage. Due to this insensitivity, such channels are sometimes referred to as "EC uncoupled" channels (Lyfenko 2006). It should be noted, however, that there is no evidence to suggest that there is an alteration in the interactions between the DHPR and RyR1. Instead, the lack of voltage-gated release is likely to reflect the altered conductance of the RyR1 channel itself rather than the coupling. We would like to suggest that this type of mutation be termed a "pore or conductance altering" mutation rather than an "EC uncoupling" mutation. The term "EC uncoupling" should be reserved for a condition in which the interactions between the DHPR and RyR can be clearly demonstrated to be altered.

To date, there are no convenient animal models of central core disease, although cores have been reported in horses (Paciello 2006). Chelu et al. (Chelu 2006) introduced a point mutation (Y522S) in RYR1 of mice that had previously been associated with central cores in a French kindred (Quane 1994). However, these mice did not exhibit central cores (Chelu 2006), but do develop muscle weakness with age (unpublished observation). For additional information on MH and CCD readers are referred to the recent excellent review/update by Robinson et al (Robinson 2006).

As with MH, there are a number of important questions that need to be answered to elucidate the molecular events altered in CCD: What causes cores to form? Why don't cores form in mice with mutations that produce cores in humans? How can a single mutation produce widely variable phenotypic outcomes?

7. RYR1 DISEASE: MULTI-MINICORE DISEASE (MMD)

Some patients exhibit cores that are smaller and shorter (longitudinally) than the cores observed in central core disease and that occur both in slow and fast type muscle fibers (Ferreiro 2002b; Robinson 2006). These cores are referred to as *multicores* or *minicores* and patients with myopathic symptoms (e.g. proximal muscle weakness, delayed motor development, difficulty rising, etc.) along with these core-like structures are said to have *multiminicore disease (MmD)*. Considerable overlap

exists between CCD and MmD, making it difficult to determine whether MmD is a disorder distinct from CCD or a subset of CCD which manifests unique histological characteristics (Lyfenko 2004; Robinson 2006). As for CCD, the presence of minicores alone is not sufficient for diagnosis and the disease follows a slowly or non-progressive course (Fardeau 1994).

Multiminicore disease may also result from mutations in another protein found in the lumen of the ER/SR, selenoprotein N (Ferreiro 2002b). The function of selenoprotein N is currently unknown but may be to participate in regulation of redox homeostasis. An intriguing question is the relationship between RyR1 and selenoprotein N such that mutations in either protein can produce similar changes in muscle structure and function.

8. DEFECTS IN RYR2 ASSOCIATED WITH CARDIAC DISEASE

Autosomal-dominant mutations in the *RyR2* gene have been identified in families and in sporadic (*de novo*) patients affected by two inherited cardiac arrhythmia syndromes, 'catecholaminergic polymorphic ventricular tachycardia' (CPVT) and 'arrhythogenic right ventricular dysplasia type 2' (ARVD2) (see Table 1) (Laitinen 2001; Priori 2001; Tiso and Thiene 2001). Unlike ARVD2, which was originally reported to be associated with progressive degeneration of the right ventricular myocardium, CPVT is not typically characterized by structural heart degeneration (Tiso and Thiene 2001).

It was originally reported that the *RyR2* mutations linked to CPVT and ARVD2 cluster in three distinct 'hotspot' domains of the channel, corresponding to similar mutation domains in the type 1 ryanodine receptor (RyR1) linked to malignant hyperthermia and central core disease (see Table 3 and Table 4). However, the recent identification of *RyR1* mutations linked to MH between the first and second mutation domains suggests that disease-linked mutations may be found in additional RyR regions. Since the initial screening for mutations concentrated on the three known hotspot domains, it will not come as a surprise if many mutations in other regions of the channel have been overlooked during initial sequencing efforts.

9. RYR2 DISEASE: CATECHOLAMINERGIC POLYMORPHIC VENTRICULAR TACHYCARDIA (CPVT)

In most cases, CPVT is inherited as an autosomal-dominant disease. Genetic analysis has demonstrated that approximately 40% of these patients carry a mutation on the *RyR2* gene (Priori 2002). In addition, mutations in the calsequestrin gene (*CASQ2*) have been linked to a rare, autosomal-recessive form of CPVT (Lahat 2001). Patients with CPVT characteristically develop ventricular arrhythmias during strenuous physical exercise or emotional stress (Priori 2001; Priori 2002). Studies in patients with *RyR2* mutations have revealed that CPVT can be induced by catecholamine infusion, but typically not by programmed electrical stimulation

Table 4. Inherited RyR2 mutations associated with cardiac arrhythmias and sudden cardiac death

RyR2 mutations	Disease	References	RyR2 mutations	Disease	References
Mutation domain 1			**Mutation domain 3**		
A77V	ARVD2/CPVT	(d'Amati 2005; Tiziana di Gioia 2006)	L3778F	CPVT	(Priori 2002)
P164S	CPVT	(Tester 2006)	C3800F	CPVT	(Tester 2005)
R169Q*	CPVT	(Hsueh 2006)	S3938R	CPVT	(Tester 2005)
R176Q*	ARVD2	(Tiso and Thiene 2001)	G3946S*	CPVT	(Priori 2002)
V186M	CPVT	(Tester 2006)	F4020L	CPVT	(Postma 2005)
R414C/L	CPVT	(Tester 2005; Creighton 2006)	E4076K	CPVT	(Postma 2005)
I419F*	CPVT	(Tester 2005)	N4097S	CPVT	(Tester 2004)
R420W	ARVD2	(Bauce 2002; Tester 2004)	N4104K/I	CPVT	(Priori 2001)
L433P	ARVD2	(Tiso and Thiene 2001)	H4108N/Q	CPVT	(Postma 2005)
P466A	CPVT	(Tester 2005)	S4124T	CPVT	(Tester 2005)
Mutation domain 2			E4146K	CPVT	(Tester 2004)
E1724K	CPVT	(Postma 2005)	T4158P	CPVT	(Tester 2004)
S2246L	CPVT	(Prestle 2001; Priori 2002; Tester 2004)	T4196A	CPVT	(Tester 2006)
A2254V	CPVT	(Postma 2005)	Q4201R	CPVT	(Laitinen 2001)
R2267H	CPVT	(Tester 2006)	R4497C	CPVT	(Priori 2001)
V2306I	CPVT	(Laitinen 2003)	F4499C	CPVT	(Tester 2005)
E2311D*	CPVT	(Priori 2002)	M4504I	CPVT	(Bagattin 2004)
P2328S	CPVT	(Laitinen 2001)		CCD	(Wu 2006)
F2331S*	CPVT	(Creighton 2006)	A4510T	CPVT	(Tester 2005)
N2386I	ARVD2	(Tiso and Thiene 2001; Postma 2005)	A4556T	CPVT	(Tester 2005)
A2387T/P	CPVT	(Tiso and Thiene 2001; Bagattin 2004)	S4565R*	CPVT	(Tester 2006)
Y2392C	ARVD2	(Bauce 2002)	A4607P	CPVT	(Bagattin 2004)
A23946	CPVT	(Postma 2005)			
R2401H/L*	CPVT	(Aizawa and Takahashi 2005; Creighton 2006)	V4653F	CPVT	(Laitinen 2001)
A2403T*	CPVT	(Tester 2005)	INS EY4657-4658	CPVT	(Tester 2005)

Mutation domain 2			Mutation domain 3		
R2474S*	CPVT	(Priori 2001)	G4662S*	CPVT	(Postma 2005)
T2504M	ARVD2	(Tiso 2001)	G4671R	CPVT	(Tester 2005)
L2534V	CPVT	(Hasdemir 2004)	H4762P	CPVT	(Postma 2005)
			V4771I	CPVT	(Priori 2002)
			I4848V	CPVT	(Tester 2005)
			A4860G	CPVT	(Priori 2002)
			I4867M*	CPVT	(Priori 2002)
			V4880A	CPVT	(Bagattin 2004)
			N4895D	CPVT	(Priori 2002)
			P4902L/S*	CPVT	(Laitinen 2003)
			E4950K	CPVT	(Priori 2002)
			R4959Q	CPVT	(Laitinen 2003)

(Priori 2002; Sumitomo 2003). In addition, since the morphology of ventricular tachycardia observed in CPVT (bidirectional, broad complex tachycardia with alternating QRS complex polarity) resembles that of digitalis induced ventricular tachycardia (Wehrens 2005a; Liu 2006), it has been suggested that arrhythmogenesis in CPVT originates from delayed afterdepolarizations (DADs) and triggered activity.

Important insights into the electrophysiological and cellular mechanisms of CPVT were obtained in a knock-in mouse model of the R4496C mutation, which is the mouse equivalent of the R4497C mutation identified in patients with CPVT (Cerrone 2005). After exercise stress testing followed by epinephrine administration, heterozygous R4496C knockin mice developed bidirectional ventricular tachycardia (Cerrone 2005). Interestingly, pretreatment with β-adrenergic receptor blockers did not prevent the occurrence of these arrhythmias in this mouse model of CPVT, which is consistent with the observation in CPVT patients that β-blockers provide incomplete protection against arrhythmias and sudden cardiac death (Priori 2002). In cardiomyocytes isolated from R4496C mice, DADs were observed following rapid electrical stimulation (Liu 2006). These DADs most likely result from spontaneous SR Ca^{2+} release (Wehrens 2003b; Kannankeril 2006), which leads to the activation of the Na^{+}/Ca^{2+}-exchanger associated with a transient inward current (I_{ti}) (Scoote 2002; Baartscheer 2005). If the amplitude of this inward current exceeds the threshold membrane potential, depolarization may occur and cardiac arrhythmia may be propagated (Wehrens 2005a; Lehnart 2006). Factors that increase the likelihood that the depolarization threshold will be reached include a faster heart rate and intracellular Ca^{2+} overload (Santana 1996). The association with faster heart rates may explain why arrhythmias in CPVT almost exclusively occur during exercise or stress (Priori 2002).

Experimental studies of CPVT-linked mutant RyR2 channel have revealed features consistent with the clinical phenotype in mutation carriers (Wehrens 2003b). Most studies have shown that under resting conditions, CPVT-mutant channels are indistinguishable from normal (wild-type) channels, in agreement with the observation that CPVT mutation carriers do not develop arrhythmias at rest (see Table 4) (George 2003; Wehrens 2003b). In contrast, mutant RyR2 typically show gain-of-function defects in response to channel activation by PKA phosphorylation (Wehrens 2003b; Lehnart 2004) or caffeine (George 2006; Kannankeril 2006). Although the molecular mechanisms underlying increased activity of mutant RyR2 are somewhat controversial (Wehrens 2004a; Liu 2006), most studies have demonstrated that CPVT-linked mutant RyR2 become hyperactive following stimulation leading to SR Ca^{2+} leak (Table 5). For example, using a planar lipid bilayer assay to characterize single channel gating features, Wehrens et al. (Wehrens 2003b; Lehnart 2004) demonstrated that CPVT-mutant RyR2 channels fail to remain fully closed under conditions that mimic diastole in the heart. Consistent with these studies, intracellular Ca^{2+} leak was observed after β-adrenergic stimulation in HL-1 atrial tumor cells expressing the same CPVT mutant RyR2 channels (George 2003). Jiang et al. (Jiang 2004) also demonstrated enhanced Ca^{2+} release through

Table 5. Functional consequences of RyR2 mutations

RyR2 Mutation(s)	Experimental Methods	Basal RyR2 Act.	Stimulated RyR2 Act.	FKBP12.6 Affinity	Other Mechanisms	Ca Release Events	Reference
R4497C	[3H]ryanodine; bilayer; Ca release in HEK cells	↑	↑ (w/ caffeine)	N/A		↑	(Jiang 2002)
ARVD vs. CPVT	Yeast two-hybrid system	N/A	N/A	↑ CPVT, ↓ ARVD		N/A	(Tiso and Thiene 2001)
S2246L R2474S R4497C	Bilayer; FKBP12.6 binding assay	=	↑ (w/ PKA)	↓ affinity		N/A	(Wehrens 2003b)
S2246L N4104K R4497C	Ca release in HL-1 cells	N/A	N/A	= binding		↑ (w/ caffeine, 4-CMC, iso)	(George 2003)
P2328S Q4201R V4653F	Bilayer; FKBP12.6 binding assay	=	↑ (w/ PKA)	↓ affinity	↓ inhibition by Mg	N/A	(Lehnart 2004)
N4104K R4496C N4895D	[3H]ryanodine; bilayer; Ca release in HEK cells	=	↑ (at high luminal [Ca])	N/A		↑ (w/ caffeine)	(Jiang 2004)

(Continued)

Table 5. (Continued)

RyR2 Mutation(s)	Experimental Methods	Basal RyR2 Act.	Stimulated RyR2 Act.	FKBP12.6 Affinity	Other Mechanisms	Ca Release Events	Reference
L433P N2386I R176Q/T2504M	Ca release in HEK cells	N/A	N/A	N/A		↑ (w/ caffeine)	(Thomas 2004)
L433P N2386I R176Q/T2504M	Ca release in HEK cells	N/A	N/A	N/A	loss of Ca-dependent inactivation	altered (w/caffeine)	(Thomas 2005)
R176Q/T2504M L433P S2246L Q4201R I4867M	[3H]ryanodine; bilayer; Ca release in HEK/HL-1	=	↑ (at high luminal [Ca])	= (at 4 °C)		↑ (w/ caffeine)	(Jiang 2005)
S2246L N4104K R4497C	FRET in CHO/HL1 cells	N/A	N/A	N/A	abnormal interdomain interactions	↑ (w/ caffeine)	(George 2006)
S2246L	Ca sparks and release; peptides in rat myocytes	N/A	N/A	N/A	abnormal interdomain interactions	↑ (w/ caffeine)	(Yang 2006)

mutant RyR2 expressed in HEK293 cells. Together, these studies revealed that mutant RyR2 channels exhibit diastolic SR Ca^{2+} leak and a reduced threshold for Ca^{2+} spilling from the SR, which under certain conditions may lead to delayed afterdepolarizations and cardiac arrhythmias.

Several mechanisms have been proposed to explain decreased stability of the closed conformational state of mutant RyR2 channels. Wehrens et al. (Wehrens 2003b) reported that CPVT mutant RyR2 channels have a decreased binding affinity for the channel-stabilizing subunit FKBP12.6 (calstabin2). Additional evidence for the link between FKBP12.6 binding to RyR2 and arrhythmias has come from studies in FKBP12.6 knockout mice, in which FKBP12.6 deficiency has been linked to the development of catecholaminergic polymorphic VTs that resemble those occurring in CPVT patients (Wehrens 2003a; Wehrens 2004b). Moreover, increasing FKBP12.6 binding to RyR2 either using the experimental drug JTV519 (which enhances FKBP12.6 affinity for RyR2) (Wehrens 2004a; Wehrens 2004b) or transgenic overexpression of FKBP12.6 (Huang 2006) suppresses the vulnerability to ventricular arrhythmias and sudden cardiac death in mice. Nevertheless, decreased binding affinity of FKBP12.6 to CPVT-mutant RyR2 was not observed by George et al. (George 2003). These authors determined FKBP12.6 protein levels in microsomes isolated from HL-1 cell transfected with WT and CPVT-mutant RyR2 channels. However, loading controls and the expression levels of RyR2 were not provided for each sample, making it difficult to assess the implications of these findings.

As for RyR1 (see above discussion of MH), it has been proposed that inherited mutations in RyR2 may alter interdomain interactions and autoregulation of RyR2. George et al. (George 2006) co-expressed fluorescent RyR2 fusion proteins corresponding to the C terminus and cytoplasmic N-terminal domain containing either WT sequences or CPVT-linked mutations. Although biochemical evidence for binding of the C terminal and N terminal fusion proteins is not provided, the authors propose that CPVT mutations alter fluorescence resonance energy transfer (FRET) between the two RyR2 domains, which might represent greater conformational changes in RyR2 following activation by caffeine (George 2006). Yang et al. (Yang 2006) also described potential changes in interdomain interactions associated with CPVT mutations. They perfused rat cardiomyocytes with a peptide homologous to the central domain of the RyR2 channel (DPc10; amino acids 2460–2496). Although the overall results show that the DPc10 peptide mimics the functional effects of the R2474S mutation (i.e. impaired interdomain interactions and autoregulation), most effects were transient and not consistent with diastolic SR Ca^{2+} leak, observed in many other studies. Thus, future studies will be necessary to determine the physiological significance of potential changes in interdomain interactions within the RyR2 channel.

Another mechanism has been proposed to contribute to diastolic SR Ca^{2+} leak through CPVT-mutant RyR2. Jiang et al. (Jiang 2004) expressed wildtype and mutant RyR2 in HEK293 cells and found altered luminal Ca^{2+} activation in cells expressing mutant RyR2. They proposed that a reduced threshold for store

overload-induced Ca^{2+} release (SOICR) in CPVT linked RyR2 mutations triggers the arrhythmias (Jiang 2004; Jiang 2005). However, it should be noted that these studies were conduced in HEK293 cells which are non-muscular in origin and lack junctional SR which is crucial for proper Ca^{2+} handling in cardiomyocytes. Moreover, these authors have reported that CPVT mutant channels are defective under baseline conditions, which is at odds with the clinical disease phenotype (i.e., exercise-induced cardiac arrest).

Nevertheless, most functional characterizations of RyR2 mutants performed so far agree on the presence of a diastolic SR Ca^{2+} leak upon β-adrenergic stimulation and on a lower threshold for Ca^{2+} spilling from the SR. These dysfunctions are likely to promote the development of DADs and triggered arrhythmias in CPVT.

Finally, a less common type of CPVT is caused by mutations in the calsequestrin gene (*CASQ2*). Although the molecular mechanisms of CASQ2-linked CPVT are beyond the scope of this chapter, it is thought that CASQ2 mutations reduce effective Ca^{2+} buffering inside the SR (Terentyev 2003), or cause altered interactions with the RyR2 channel complex leading to impaired RyR2 regulation by luminal Ca^{2+} (Terentyev 2006).

Unanswered questions: Are there disease-associated mutations in RyR2 that alter the pore and/or channel conductance, as have been described for RyR1 (see above)? To date, similar mechanisms have not been described for arrhythmia-linked RyR2 mutations. It has been suggested that the incidence of arrhythmias and syncope is higher in male patients with mutations in RyR2; however, the molecular mechanisms for such an occurrence remain to be studied (Priori 2002).

10. RYR2 DISEASE: ARRHYTHMOGENIC RIGHT VENTRICULAR DYSPLASIA (ARVD)

ARVD is an inherited disorder characterized by progressive replacement of right ventricular free wall with fibrous and fatty tissue (Corrado 1997). The clinical presentation of ARVD is characterized by arrhythmias of right ventricular origin, ranging from premature beats to sustained ventricular fibrillation, often associated with sudden cardiac death. To date, at least 10 genes have been linked to ARVD (Awad 2006). Like other forms of ARVD, ARVD type 2 (ARVD2) is characterized by fibrofatty substitution of myocardial tissue, but to a much lesser extent (Tiso and Thiene 2001). In addition, ARVD2 is distinctive in causing polymorphic effort-induced ventricular arrhythmias, similar to CPVT (Danieli 2002).

Missense mutations in *RyR2* have also been linked to arrhythmogenic right ventricular cardiomyopathy/dysplasia type 2 (ARVD2) (Tiso and Thiene 2001). This report was subject of recent debate, as ARVD was generally believed to be a disease of adhesion molecules (Corrado 2006). Only one research group has claimed an association of RyR2 mutation with ARVD (Bauce 2002), whereas several other cardiogenetics groups have failed to find evidence for such linkage (Priori 2005). To resolve this controversy, we have recently generated knockin mice carrying the ARVD2-associated R176Q mutation in RyR2 (Kannankeril 2006). This mutation

was originally identified in patients that also carry a second RyR2 mutation on the same allele, T2504M (Tiso and Thiene 2001). Although *in vitro* experiments revealed that the R176Q and T2504M mutations alone resulted in altered RyR2 function (Thomas 2004; Jiang 2005; Thomas 2005), it was unknown whether the R176Q mutation would be sufficient to cause ventricular arrhythmias and ventricular dysfunction in intact animals. Indeed, heterozygous R176Q mice develop catecholamine-induced ventricular tachycardia and sudden cardiac death following isoproterenol administration (Kannankeril 2006). In addition, we observed mild right ventricular contractile defects consistent with restrictive ventricular filling, although structural abnormalities such as fibrosis or fibrofatty replacement were not observed (Kannankeril 2006). Based on recent clinical and experimental data, it is currently believed that the presence of some minor structural abnormalities of the ventricle may be part of the CPVT phenotype that nonetheless remains a condition clinically and physiologically distinct from ARVD.

11. RYR2 DISEASE: SUDDEN INFANT DEATH SYNDROME (SIDS)

Recent molecular autopsy studies have suggested that mutations in *RyR2* gene may cause sudden infant death syndrome (SIDS), the leading cause of death in infants under one year of age (Tester 2006a; Tester 2006b). Although the etiology of SIDS is largely unknown, several theories including fatal cardiac arrhythmias have been suggested (Schwartz 1998). Molecular autopsy studies have revealed that 10–20% of sudden deaths in young children can be attributed to mutations in the calcium release channel gene *RyR2* (Tester 2004; Wehrens 2004b). These findings suggest that a CPVT-like arrhythmia syndrome may cause fatal arrhythmias in these infants. However, mechanistic studies of SIDS-linked RyR2 mutations are currently not available.

12. CARDIAC DISEASE ASSOCIATED WITH ACQUIRED RYR2 DEFECT

In addition to inherited mutations in RyR2, several cardiac disorders have been associated with acquired defects in RyR2 (Vest 2005; Wehrens 2005b; Phrommintikul 2006). Changes in the subunit composition or the regulation of the RyR2 channel complex have been reported in chronic heart failure, cardiomyopathy, and atrial fibrillation.

13. RYR2 DISEASE: HEART FAILURE

The pathogenesis of heart failure is characterized by depressed cardiac contractility, which is normally controlled by the rhythmic release and reuptake of Ca^{2+} from the SR. A reduction in SR Ca^{2+} content has consistently been described in various forms of heart failure. Several mechanisms have been proposed to explain the decrease in SR Ca^{2+} loading in failing hearts, including 1) increased diastolic SR Ca^{2+} release,

2) decreased Ca^{2+} reuptake into the SR, and 3) decreased Ca^{2+} buffering within the SR (Bers 2005). Since these mechanisms have been reviewed in great detail elsewhere (Eisner 2002; Trafford 2002; Bers 2003; Wehrens 2005b; Chu 2006), we will only focus on the potential contribution of RyR2 dysfunction to abnormal Ca^{2+} handling in heart failure.

Increased diastolic SR Ca^{2+} leak via RyR2 has been proposed as an important contributor to a reduction in SR Ca^{2+} load and, thus, to a reduced intracellular Ca^{2+} transient (Marx 2000; Bers 2003). Marks et al. (Marx 2000; Marks 2001) have proposed that chronic activation of the β-adrenergic pathways in heart failure results in maladaptive changes in the heart, including chronic PKA hyperphosphorylation of RyR2. Although several other groups have confirmed these findings, some have not found evidence for increased PKA phosphorylation of RyR2 in failing heart (reviewed in (Wehrens 2005b)). Indeed, the finding of chronically enhanced PKA phosphorylation of RyR2 seems counterintuitive in view of the downregulation and desensitization of beta-adrenergic receptors in heart failure (Rockman 2002). However, recent studies suggest that alterations in the local signaling modules associated with the RyR2 macromolecular channel complex may indeed create a local environment in which enhanced phosphorylation of RyR2 is favored. For example, reduced levels of the protein phosphatases PP1 and PP2A can prevent dephosphorylation of RyR2 (Marx 2000; Reiken 2003). Moreover, Lehnart et al. (Lehnart 2005) recently demonstrated that downregulation of the phosphodiesterase 4D3 isoform (PDE4D3) in the RyR2 complex in failing hearts leads to increased levels of 3',5'-cyclic adenosine monophosphate (cAMP) in the RyR2 microdomain, associated with enhanced activity of the cAMP-dependent enzyme PKA (Dodge 2001). Consistent with these findings in human heart biopsies, PDE4D-deficient mice develop defective RyR2 channel function associated with heart failure (Lehnart 2005). Thus, diastolic SR Ca^{2+} leak through defective and remodeling RyR2 channels may lead to depletions of the SR Ca^{2+} stores, associated with depressed contractility in the failing heart. Moreover, abnormal diastolic Ca^{2+} leak may provide the trigger for ventricular arrhythmias, which are frequently observed in patients with heart failure (Pogwizd 2004; Wehrens 2005a).

14. RYR2 DISEASE: DIABETIC CARDIOMYOPATHY

Decrease in the ability of the heart to effectively contract is one of the major causes for the increased incidence of morbidity and mortality in diabetic patients (Rubler 1972). Both electrical and mechanical properties of the myocardium from diabetic patients may be significantly impaired, which has been attributed to alterations in intracellular Ca^{2+} homeostasis (Pierce 1983; Bouchard 1991; Yaras 2005). Weakened contractility of myocytes isolated from streptozotocin (STZ)-induced diabetic rats correlated with a reduced rate of the rise and decline of intracellular Ca^{2+} transients elicited by electrical stimulation (Choi 2002), which has been attributed to abnormal SR pump activity (Ganguly 1983), decreased SR Ca^{2+} storage (Bouchard 1991), and reduced Na^{+}/Ca^{2+} exchanger activity (Yaras 2005). However,

abnormal Ca^{2+} handling in diabetic cardiomyopathy may also result from enhanced diastolic SR Ca^{2+} release through RyR2.

Yu and McNeill (Yu 1991) were the first to implicate RyR2 dysfunction in the etiology of diabetic cardiomyopathy when they noticed significantly reduced post-rest potentiation in heart from diabetic rats. They also showed that membrane vesicles prepared from diabetic rat hearts bound less [^{3}H]-ryanodine compared with age-matched, control rats (Yu 1994), suggesting decreased RyR2 expression or activity during diabetes. Guner et al. (Guner 2004) subsequently showed that RyR2 expression is decreased in the hearts of chronic diabetic patients, as well as in hearts of STZ-induced diabetic rats (Teshima 2000; Bidasee 2001; Netticadan 2001; Zhong 2001). However, since critical Ca^{2+} cycling proteins are usually expressed in amounts that exceed that required for minimal physiologic functioning, it seems unlikely that decreased expression of RyR2 by itself could be solely responsible for the depressed post-rest potentiation associated with diabetes. Moreover, other studies have demonstrated that the expression of RyR2 may be unaltered in the STZ-induced diabetic rat model (Zhong 2001). Thus, it seems likely that mechanisms other than changes in RyR2 expression are responsible for the loss in ventricular contractility associated with diabetes.

Diabetes-induced dysfunction of RyR2 could be, in part, due to the formation of disulfide bonds between adjacent sulfhydryl groups, while chronic diabetes increases advance glycation end (AGE) products (Bidasee 2003a; Bidasee 2003b; Bidasee 2005). Moreover, a reduced amount of FKBP12.6 binding and increased protein kinase A (PKA) phosphorylation of RyR2 has been demonstrated in rats with diabetic cardiomyopathy (Yaras 2005). Consistent with the findings, Yaras et al. (Yaras 2005) demonstrated decreased Ca^{2+} transient amplitudes and SR Ca^{2+} loads as well as increased diastolic Ca^{2+} levels in cardiomyocytes from rats with diabetes. Therefore, it is likely that the decrease in SR Ca^{2+} load is, at least in part, caused by anomalous RyR2 activity resulting from abnormal posttranslational modifications and changes in the binding affinity of regulatory subunits.

15. RYR2 DISEASE: ATRIAL FIBRILLATION

Atrial fibrillation (AF) is the most important cardiac rhythm disorder that commonly occurs in the context of structural heart disease, in particular congestive heart failure. Coordinated atrial activity is replaced by disorganized rapid atrial depolarizations, which may be of short duration in paroxysmal AF, or chronically present in permanent AF (Dobrev 2006). It is generally accepted that alterations in intracellular Ca^{2+} handling are associated with arrhythmias in patients with AF, although the underlying cellular mechanisms are not fully understood (Nattel 2002; Dobrev 2006). For example, it has been proposed that atrial myocytes alter L-type Ca^{2+} channel (LTCC) function as a compensatory response, contributing to action potential duration shortening and a reduction of the refractory period (Lai 1999; Van Wagoner 1999; Yue 1999). Data by Dobrev *et al*. (Christ 2004) suggest that changes

in LTCC function are caused by abnormal channel phosphorylation. Thus, electrical remodeling of the atrial myocardium may create a substrate for the initiation and maintenance of AF (Sun 1998; Nattel 2002).

In addition to changes in sarcolemmal ion channels, functional changes in proteins involved in intracellular Ca^{2+} handling have been reported in AF (Brundel 1999; Ohkusa 1999; El-Armouche 2006). Altered intracellular Ca^{2+} handling in AF may be associated with enhanced PKA and CaMKII phosphorylation of phospholamban, increasing SR-ATPase (SERCA2a) Ca^{2+} reuptake function (El-Armouche 2006). On the other hand, Vest et al. (Vest 2005) have shown that the activity of the SR Ca^{2+} release channel RyR2 is increased in patients with AF, which may be due to enhanced PKA phosphorylation of RyR2 and reduced binding of the channel-stabilizing subunit FKBP12.6 (Wehrens 2003b). Consistent with these findings, Hove-Madsen *et al*. (Hove-Madsen 2004) provided direct evidence for increased SR Ca^{2+} leak in single myocytes isolated from patients with chronic AF. The abnormal release of SR Ca^{2+} may activate the Na^{+}/Ca^{2+}-exchanger, resulting in a transient inward current (I_{TI}) that depolarizes the cell membrane, causing after depolarizations and cardiac arrhythmias (Schlotthauer 2000; Bers 2002b; Wehrens 2003b). Thus, abnormal intracellular Ca^{2+} release can act as a local trigger generator, leading to a small reentry circuit or ectopic focal activity (Mandapati 2000; Mansour 2001). If these Ca^{2+} release events and subsequent I_{TI} reach the threshold membrane potential, spontaneous action potentials may result, producing ectopic firing (if they arise in tissue outside the sinus node) (Bers 2002; Nattel 2002). This theory is supported by the finding that left atrial sources of ectopic activity are of particular importance in a subset of patients with paroxysmal AF (Haissaguerre 1998). Thus, there is emerging evidence that defects in the regulation of RyR2 may contribute to arrhythmogenesis in atrial fibrillation.

Table 6. Mutations found in both RyR1 and RyR2

RyR1 mutations	RyR2 mutations	Disease
R156K	R169Q	MH*/CPVT#
R163C/L	R176Q	MH, CCD*/ARVD2#
I403M	I419F	MH/CCD*/CPVT#
E2344D	E2311D	MH/CPVT
F2364V	F2331S	MH/CPVT
R2435H/L	R2401H/L	MH, CCD/CPVT
A2437V	A2403T	MH/CPVT
R2508C/G/H	R2474S	
G3990V	G3946S	MH*/CPVT#
T4637I/A	S4565R	CCD*/CPVT#
G4734E	G4662S	MH*/CPVT#
I4938M	I4867M	MH*/CPVT#
P4973L	P4902L/S	MH*/CPVT#

16. SIMILARITIES IN RYR DYSFUNCTION: SAME DISEASE DIFFERENT TISSUE?

RyR1 and RyR2 are similar in the structure, subunit composition, gating behavior and response to most modulators. Mutations in the genes encoding RyR1 and RyR2 are associated with diseases of skeletal and cardiac muscle, respectively. Several inherited mutations in RyR1 have also been identified in the corresponding residue in RyR2 (Table 6). Moreover, studies at the molecular and cellular level have revealed that mutations in both RyR1 and RyR2 may lead to very similar mechanisms of abnormal calcium release.

Future studies will hopefully enlighten our understanding of the extent to which similar alterations in RyR structure and function, such as altered interdomain interactions, phosphorylation, modulator binding, and redox modifications, occur in both RyR1 and RyR2 and how such alterations lead to diseases of skeletal and cardiac muscle.

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CHAPTER 11

INOSITOL 1,4,5-TRIPSHOSPHATE RECEPTOR, CALCIUM SIGNALLING AND HUNTINGTON'S DISEASE

I. BEZPROZVANNY*

Department of Physiology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390, USA

Abstract: Huntington's disease (HD) is an autosomal-dominant neurodegenerative disorder that has no cure. HD primarily affects medium spiny striatal neurons (MSN). HD is caused by polyglutamine (polyQ) expansion (exp) in the amino-terminal region of a protein huntingtin (Htt). The connection between polyQ expansion in Htt^{exp} and MSN neurodegeneration remains elusive. My laboratory discovered that mutant Htt^{exp} protein specifically binds to the carboxy-terminal region of the type 1 inositol 1,4,5-trisphosphate receptor ($InsP_3R1$), an intracellular Ca^{2+} release channel. Moreover, we found that Htt^{exp} association with $InsP_3R1$ causes sensitization of $InsP_3R1$ to activation by $InsP_3$ in planar lipid bilayers and in primary MSN. Mutant Htt^{exp} has also been shown to activate Ca^{2+}-permeable NR2B-containing NMDA receptors. All these results suggested that deranged neuronal Ca^{2+} signaling may play an important role in pathogenesis of HD. In support of this idea, we demonstrated a connection between abnormal Ca^{2+} signaling and apoptosis of MSN cultured from YAC128 HD mouse model. These results indicate that $InsP_3R$ and other Ca^{2+} signaling proteins should be considered as potential therapeutic targets for treatment of HD

Keywords: calcium signaling, huntingtin, neurodegeneration, polyglutamine expansion, inositol 1,4,5-trisphosphate, NMDA receptor, apoptosis, mitochondria, memantine

1. HUNTINGTON'S DISEASE (HD) AND HUNTINGTIN (HTT)

Huntington's disease (HD) is an autosomal-dominant neurodegenerative disorder with the age of onset between 35 and 50 years and inexorable progression to death 15–20 years after onset. The symptoms include motor abnormalities including

*Dr. Ilya Bezprozvanny, Dept. of Physiology, ND12.502, UT Southwestern Medical Center at Dallas, Dallas, TX 75390-9040, E-mail: Ilya.Bezprozvanny@UTSouthwestern.edu

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chorea and psychiatric disturbance with gradual dementia (Vonsattel and DiFiglia, 1998). Neuropathological analysis reveals selective and progressive neuronal loss in the striatum (caudate nucleus, putamen and globus pallidus) (Vonsattel et al., 1985; Vonsattel and DiFiglia, 1998). GABAergic medium spiny striatal neurons (MSN) are the most sensitive to neuronal degeneration in HD (Vonsattel et al., 1985; Vonsattel and DiFiglia, 1998). Positional cloning efforts demonstrated that at the molecular level the cause of HD is polyglutamine (polyQ) expansion in the amino-terminal of a 350 kDa evolutionary conserved cytosolic protein called huntingtin (Htt) (1993). Clinical signs of HD develop if the length of polyQ track in Htt exceeds a pathological threshold of 35Q. The CAG repeat length is inversely correlated with age of onset (Langbehn et al., 2004). Htt is widely expressed in the brain and in non-neuronal tissues and not particularly enriched in the striatum (Li et al., 1993; Strong et al., 1993; Sharp et al., 1995). Htt plays an essential function in development, as deletion of the Htt gene in mice is embryonic lethal (Duyao et al., 1995; Nasir et al., 1995). Analysis of Htt primary sequence suggests that Htt is likely to function as a signaling scaffold (MacDonald, 2003), but the precise function of Htt in cells is not known. In order to elucidate the pathogenesis of HD, a number of transgenic HD mouse models have been generated (Menalled and Chesselet, 2002; Rubinsztein, 2002).

The key question in HD research is how does polyQ-expanded huntingtin (Htt^{exp}) kill MSN? The answer to this question is a prerequisite to development of effective HD therapies. The HD mutation at least in part creates a "gain of function". A number of toxic functions have been assigned to Htt^{exp}, including effects on gene transcription, induction of apoptosis, disruption of key neuronal functions such as proteasomal function, ubiquitination, axonal transport, endocytosis and synaptic transmission. The evidence in favor of these hypotheses are reviewed elsewhere (Tobin and Signer, 2000; Menalled and Chesselet, 2002; Ross, 2002; Rubinsztein, 2002; Harjes and Wanker, 2003; Sugars and Rubinsztein, 2003; Li and Li, 2004). All of these models are consistent with a toxic function of Htt^{exp} in neurons, but none of these models explain the selective vulnerability of MSN in HD. In this review I discuss recently emerging results that support the concept that HD may be a disease of deranged calcium (Ca^{2+}) signaling.

2. HTT^{EXP} SENSITIZES $INSP_3R1$ TO $INSP_3$

The inositol (1,4,5)-triphosphate receptor ($InsP_3R$) is an intracellular calcium (Ca^{2+}) release channel that plays an important role in neuronal Ca^{2+} signaling (Berridge, 1998). Three isoforms of $InsP_3R$ have been identified (Furuichi et al., 1994). The type 1 receptor ($InsP_3R1$) is the predominant neuronal isoform. Mice lacking $InsP_3R1$ display severe ataxic behavior (Matsumoto et al., 1996), and mice with a spontaneous mutation in the $InsP_3R1$ gene experience convulsions and ataxia (Street et al., 1997), suggesting a major role of $InsP_3R1$ in neuronal function. In the search for novel $InsP_3R1$-binding partners we performed a yeast two-hybrid screen with $InsP_3R1$ carboxy-terminal bait and isolated Htt-associated protein 1A

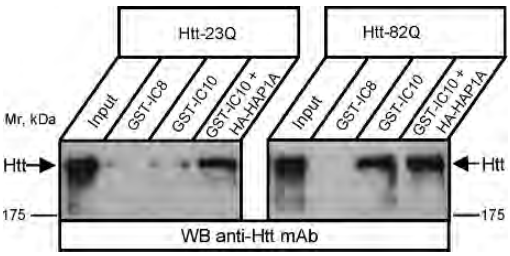

Figure 1. Mutant Htt^{exp} specifically binds to InsP$_3$R1 carboxy-terminal fragment.
IC8 (D2590-F2627) and IC10 (F2627-A2749) fragments of rat InsP$_3$R1 carboxy-terminal tail were expressed as GST fusion proteins in bacteria and utilized in pull-down experiments. Wild type Htt-23Q and mutant Htt-82Q were expressed in HEK293 cells. HA-HAP1A protein was expressed in COS7 cells and included in pull-down reaction as indicated. The precipitate Htt protein was detected by Western blotting with anti-Htt monoclonal antibodies. Adapted from (Tang et al., 2003)

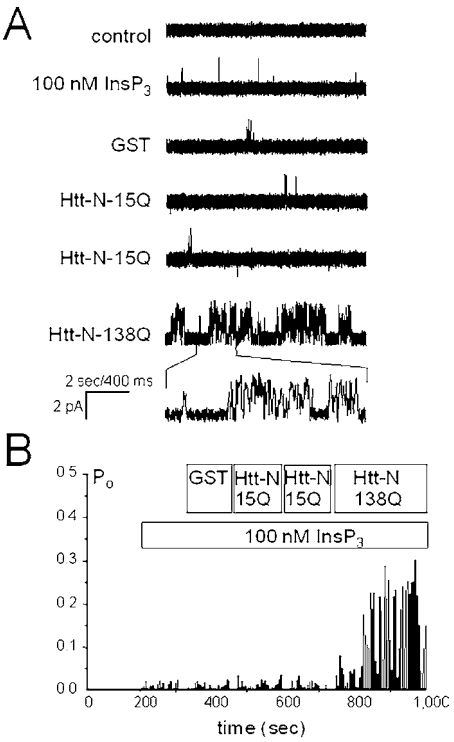

Figure 2. Htt^{exp} amino-terminal fragment sensitizes InsP$_3$R1 to activation by InsP$_3$ in planar lipid bilayers.
(A) Effects of GST, GST-Htt-N-15Q and GST-Htt-N-138Q on activity of recombinant InsP$_3$R1 in planar lipid bilayers at 100 nM InsP$_3$. Each current trace corresponds to 10 sec (2 sec for expanded traces) of current recording from the same experiment. (B) The average InsP$_3$R1 open probability (P_o) in the presence of 100 nM InsP$_3$ is calculated for a 5 sec window of time and plotted for the duration of an experiment. The time of InsP$_3$, GST, GST-Htt-N-15Q, and GST-Htt-N-138Q additions are shown. Adapted from (Tang et al., 2003)

(HAP1A) (Tang et al., 2003). In biochemical experiments, we demonstrated the formation of $InsP_3R1$-HAP1A-Htt ternary complex *in vitro* and *in vivo* (Tang et al., 2003).

What is an effect of Htt^{exp} mutation on ability of Huntingtin to associate with $InsP_3R1$? On $InsP_3R1$ function? In a series of pull-down experiments we discovered that mutant Htt^{exp}, but not wild type Htt, binds directly to the $InsP_3R1$ carboxy-termini (Figure 1). Furthemore, in planar lipid bilayer reconstitution experiments we demonstrated sensitization of $InsP_3R1$ to $InsP_3$ in the presence of Htt-138Q amino-terminal fragment (Figure 2a, 2b) or full-length Htt-82Q

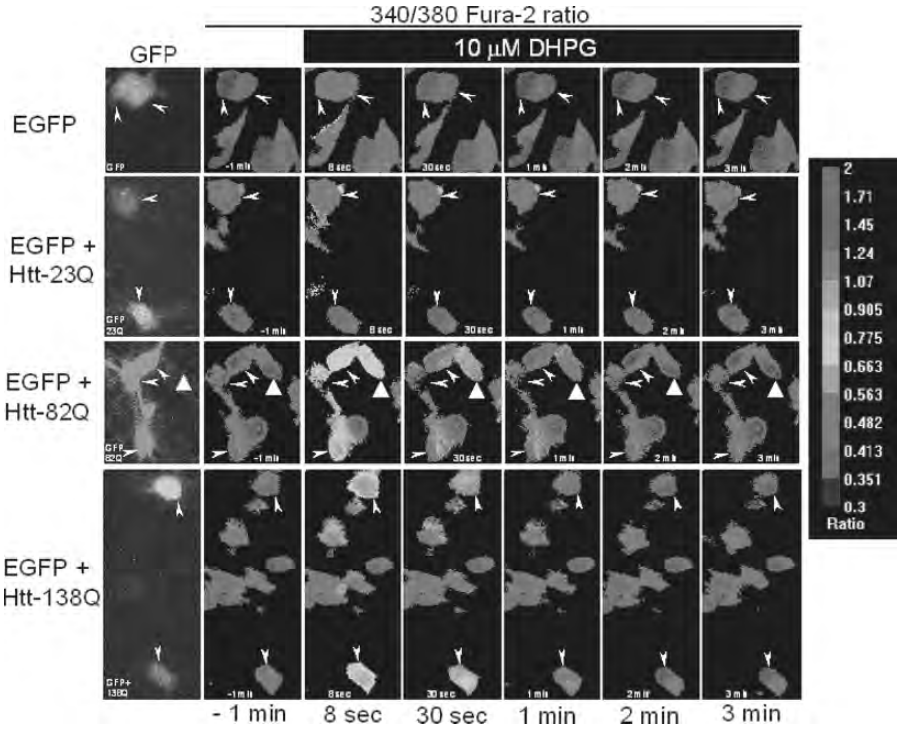

Figure 3. Htt^{exp} facilitates $InsP_3R1$-mediated Ca^{2+} release in cultured MSN.
The images show Fura-2 340/380 ratios in transfected rat MSN. Pseudocolor calibration scale for 340/380 ratios is shown on the right. The recordings were performed in Ca^{2+}-free ACSF containing 100 μM EGTA. GFP images (1st column) were captured before Ca^{2+} imaging to identify transfected cells (arrowheads). $InsP_3R1$-mediated Ca^{2+} release was initiated by addition of 10 μM DHPG, a specific mGluR1/5 agonist. Ratio recordings are shown for DHPG-induced Ca^{2+} transients in MSN neurons transfected with EGFP (first row), EGFP + Htt-23Q (second row), EGFP + Htt-82Q (third row), and EGFP + Htt-138Q (fourth row). The 340/380 ratio images are shown for MSN neurons 1 min before (2nd column), and 8 sec, 30 sec, 1 min, 2 min, and 3 min after application of 10 μM DHPG as indicated. Adapted from (Tang et al., 2003) (See Colour Plate 18)

(Tang et al., 2003). These effects were specific for Htt^{exp}, as the Htt-15Q amino-terminal fragment (Figure 2a, 2b) or full-length Htt-23Q (Tang et al., 2003) had no effect on InsP$_3$R1 sensitivity to InsP$_3$.

In experiments with primary cultures of rat MSN we demonstrated facilitation of InsP$_3$R1-mediated Ca^{2+} release in the presence of Htt-82Q and Htt-138Q proteins, but not in the presence of Htt-23Q protein (Figure 3). The ability of Htt^{exp} to sensitize InsP$_3$R1 to activation by InsP$_3$ correlated with ability of Htt^{exp}, but not Htt, to associate directly with InsP$_3$R1 carboxyl-terminal region (Tang et al., 2003). Thus, we reasoned that potentiating effect of Htt^{exp} on InsP$_3$R1-mediated Ca^{2+} release is due to direct association of Htt^{exp} with InsP$_3$R1 carboxyl-terminus.

From these results we proposed that upregulation of InsP$_3$R1 by Htt^{exp} may be a contributing factor to Ca^{2+} overload and degeneration of MSN in HD (Tang et al., 2003). MSN are highly enriched for mGluR5, a member of the group I mGluRs (Testa et al., 1995; Kerner et al., 1997; Tallaksen-Greene et al., 1998; Mao and Wang, 2001, 2002). Stimulation of group I mGluR in MSN leads to the generation of InsP$_3$ and release of Ca^{2+} (Figure 3). The alterations in ER enzymes that have been observed in HD postmortem brains (Cross et al., 1985) are consistent with malfunction of ER Ca^{2+} handling in HD MSN neurons.

3. HTT^{EXP} ACTIVATES NR2B-CONTAINING NMDA RECEPTORS

MSN abundantly express NR2B subtype of NMDA receptors (Monyer et al., 1994; Landwehrmeyer et al., 1995; Portera-Cailliau et al., 1996). In contrast to NMDA receptors containing NR2A subtype, NR2B-containing NMDA receptors have significant permeability for Ca^{2+} and activation of these receptors may have a dramatic effect on intracellular Ca^{2+} signals in MSN. Importantly, studies from Lynn Raymond's and Michael Hayden's laboratories suggested that expression of mutant Htt^{exp} protein facilitates activity of NR2B subtype of NMDAR receptors in a heterologous HEK293 cells expression system (Chen et al., 1999). Interestingly, the potentiating effect of Htt^{exp} was specific for the NR1/NR2B NMDAR subtype and not for the NR1/NR2A NMDAR subtype (Figures 4a, 4b). Using the same HEK293 cells expression system it was also demonstrated that cells co-transfected with NMDAR and Htt-138Q plasmids were more sensitive to NMDA-induced apoptosis than the cells co-transfected with NMDAR and Htt-15Q or GFP (control) plasmids (Zeron et al., 2001). Similar to effects on NMDAR currents (Figures 4a, 4b), potentiating effects of Htt-138Q on excitotoxic cell death were more pronounced in the presence of the NR1/NR2B NMDAR subunit combination than in the presence of the NR1/NR2A subunit combination (Zeron et al., 2001).

Further support for the potentiating effects of Htt^{exp} on NMDAR activity was obtained by Lynn Raymond's and Michael Hayden's laboratories in the analysis of YAC72 HD mouse model (Hodgson et al., 1999). NMDA-evoked currents (Figure 4c, 4d) (Zeron et al., 2002) and NMDA-mediated Ca^{2+} transients (Zeron et al., 2004) were significantly increased in striatal neurons from YAC72 mouse when compared to wild type controls. Consistent with the HEK293 cells expression

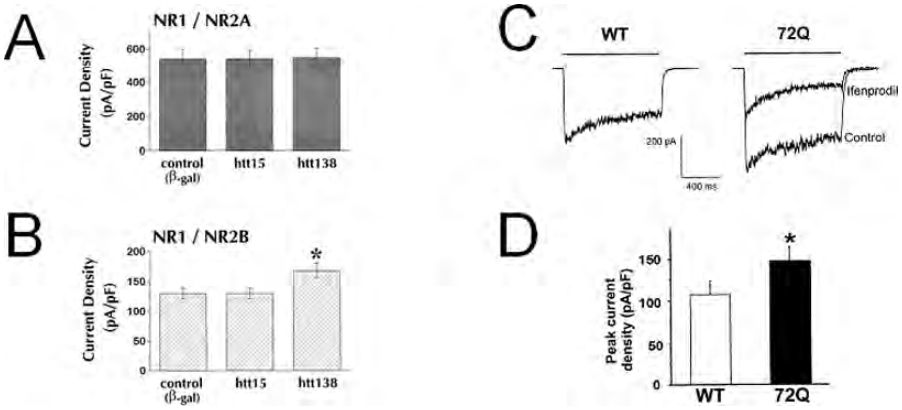

Figure 4. NR2B NMDAR are upregulated by Htt^{exp}.
(A). A combination of NR1 and NR2A NMDAR subunits was co-transfected into HEK293 cells together with β-gal plasmid (control), Htt-15Q, or Htt-138Q plasmids. The NMDA-induced currents in transfected cells were measured by whole-cell recordings and normalized to cell capacity. No significant differences in the size of NMDA-evoked currents were observed between 3 groups of cells. (B). A combination of NR1 and NRB NMDAR subunits was co-transfected into HEK293 cells together with β-gal plasmid (control), Htt-15Q, or Htt-138Q plasmids. The NMDA-induced currents in transfected cells were measured by whole-cell and normalized to cell capacity. The size of NMDA-evoked current is significantly higher in cells co-transfected with Htt-138Q (asterisk) than in other 2 groups of cells. (C) The size of NMDA-evoked current in primary MSN from non-transgenic (WT) and YAC72Q (72Q) mouse. Inhibition of NMDA-evoked current by ifenrpodil is shown for YAC72 mouse MSN.(D). An average NMDA-evoked peak current density in MSN from non-transgenic (WT) and YAC72 (72Q) mouse. Panels A and B are adapted from (Chen et al., 1999). Panels C and D are adapted from (Zeron et al., 2002)

data (Figures 4a, 4b), the NMDAR currents in striatal neurons potentiated in the presence of YAC72 transgene were selectively blocked by NR1/NR2B-specific antagonist ifenprodil (Figure 4c).

How does Htt^{exp} activate NMDAR? Experiments in a heterologous expression system demonstrated that Htt binds to a modular adaptor protein PSD95 and that Htt^{exp} binds to PSD95 less strongly than Htt (Sun et al., 2001). The PDZ domains of PSD95 bind to the carboxy-terminal region of the NMDAR NR2 subunit. The association of PSD95 with the NR2 subunit leads to recruitment of Src tyrosine kinase, tyrosine phosphorylation of NMDAR and an increase in NMDAR currents (Ali and Salter, 2001). It was proposed that weakened association of Htt^{exp} with PSD95 increases the pool of PSD95 available for interactions with NR2 subunits, leading to hyperphosphorylation of NMDAR by Src kinase. Consistent with this hypothesis, tyrosine hyperphosphorylation of NR2B subunits was observed in a heterologous expression system in the presence of a Htt-48Q construct (Song et al., 2003). Moreover, inhibition of NR2B phosphorylation by the Src tyrosine kinase inhibitor SU6656 attenuated Htt-48Q-facilitated apoptotic cells death in rat hippocampal neuronal cell line HN33 (Song et al., 2003). Future experiments will be needed to determine if PSD95 and Src mediated pathway is responsible for

NMDAR potentiation by Htt^{exp} *in vivo*. An alternative hypothesis may involve direct or cytoskeleton-mediated effects of Htt^{exp} on NMDAR gating or changes in NMDAR surface expression and/or localization in the presence of Htt^{exp}.

4. CA^{2+} SIGNALING AND APOPTOSIS OF HD MSN

Several lines of evidence indicate that glutamate-mediated excitotoxicity plays a role in neurodegeneration of HD MSN. Striatal injection of kainic acid induced death of MSN and yielded one of the first animal models of HD (Coyle and Schwarcz, 1976; McGeer and McGeer, 1976). Importantly, effects of kainate required presence of corticostriatal neurons (McGeer et al., 1978), suggesting that glutamate release is required for kainate-induced MSN cell death. More direct evidence for an involvement of NMDAR was obtained when HD-like lesions were observed following striatal injection of the NMDAR agonist quinolinic acid (Beal et al., 1986; Hantraye et al., 1990; Beal et al., 1991). Consistent with the excitotoxicity hypothesis, striatal neurons from YAC72 mouse were sensitized to neuronal death induced by quinolinic acid and NMDA (Zeron et al., 2002). Moreover, excitotoxic cell death of YAC72 MSN was blocked by ifenprodil (Zeron et al., 2002), supporting a direct involvement of NR1/NR2B NMDAR subtypes in HD.

Based on the results described above (sections 2 and 3) we previously suggested that overactivation of $InsP_3R1$-mediated Ca^{2+} release and NR2B-mediated Ca^{2+} influx in HD MSN may lead to Ca^{2+} overload and apoptosis of these neurons (Bezprozvanny and Hayden, 2004). To test this "Ca^{2+} hypothesis of HD" my laboratory recently used TUNEL assay to compare glutamate-induced apoptosis of MSN cultured from wild type mice and mice expressing mutant human Htt-128Q gene (YAC128 mouse (Slow et al., 2003)). The mice expressing normal human Htt-18Q gene (YAC18 (Hodgson et al., 1999)) was used as a control in these experiments. At 14 DIV all 3 groups of MSN were challenged by an 8 h application of glutamate (from 0 to 250 μM) to mimic physiological stimulation. Following exposure to glutamate, MSN were fixed, permeabilized and scored for apoptotic cell death using TUNEL staining. We determined that in basal conditions (no glutamate added) approximately 10% of MSN in all 3 experimental groups were apoptotic (TUNEL-positive) (Figures 5a, 5b). Addition of 25 μM or 50 μM glutamate increased the number of apoptotic cells to 15–20% in all 3 experimental groups (Figures 5a, 5b). Addition of 100 μM or 250 μM glutamate increased apoptotic death to 60–70% for YAC128 MSN (Figures 5a, 5b), but only to 25–30% for wild type and YAC18 MSN (Figures 5a, 5b). Thus we reasoned that exposure to glutamate concentrations in 100 – 250 μM range leads to selective apoptosis of YAC128 MSN (Tang et al., 2005).

The "*in vitro* HD" model described above (Figure 5) enabled us to test a connection between abnormal Ca^{2+} signaling and apoptosis of HD MSN. We found that inhibition of mGluR1/5 receptors (by a mixture of MPEP and CPCCOEt) reduced the glutamate-induced apoptosis of YAC128 MSN to WT MSN levels (Figure 6). NMDAR-inhibitor (+)MK801 or NR2B-specific antagonist ifenprodil

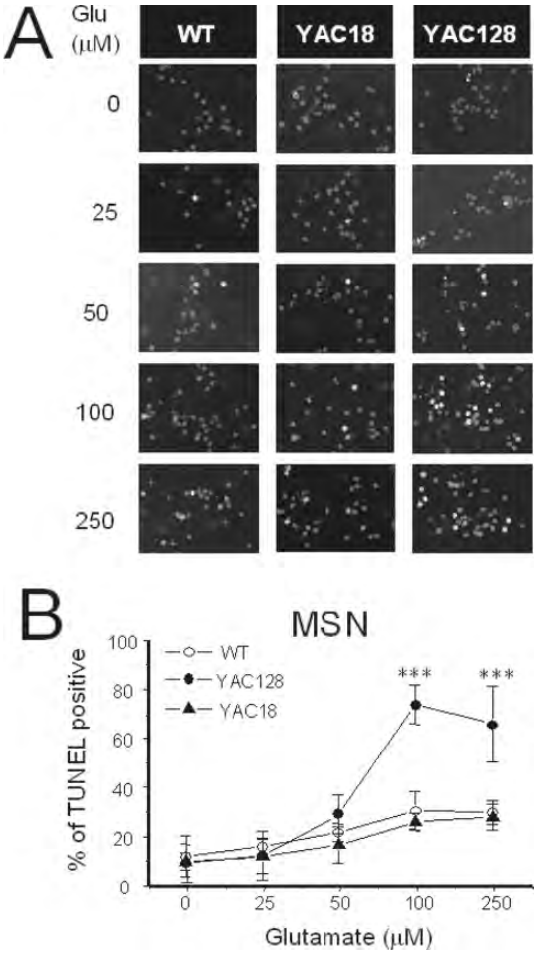

Figure 5. In vitro HD assay.
(A) 14 DIV MSN from wild type (WT), YAC18 and YAC128 mice were exposed to a range of glutamate concentrations for 8 h, fixed, permeabilized and analyzed by TUNEL staining (green) and propidium iodide counterstaining (PI). (B) The fraction of TUNEL-positive MSN nuclei was determined as shown on panel A and plotted against glutamate concentration for wild type (WT) (open circles), YAC128 (filled circles), and YAC18 (filled triangles) mice. At each glutamate concentration the data are shown as mean ± SD (n = 4–6 microscopic fields, 200–300 MSN per field). At 100 μM and 250 μM glutamate the fraction of TUNEL-positive MSN is significantly ($p < 0.05$) higher for YAC128 than for WT or YAC18. Similar results were obtained with 10 independent MSN preparations. Adapted from (Tang et al. 2005) (See Colour Plate 19)

had similar neuroprotective effects (Figure 6). Consistent with direct involvement of $InsP_3R1$, preincubation of the MSN cultures with a membrane-permeable $InsP_3R$ blocker 2-APB (Maruyama et al., 1997) protected YAC128 MSN from glutamate-induced apoptosis (Figure 6). All these results supported an idea that

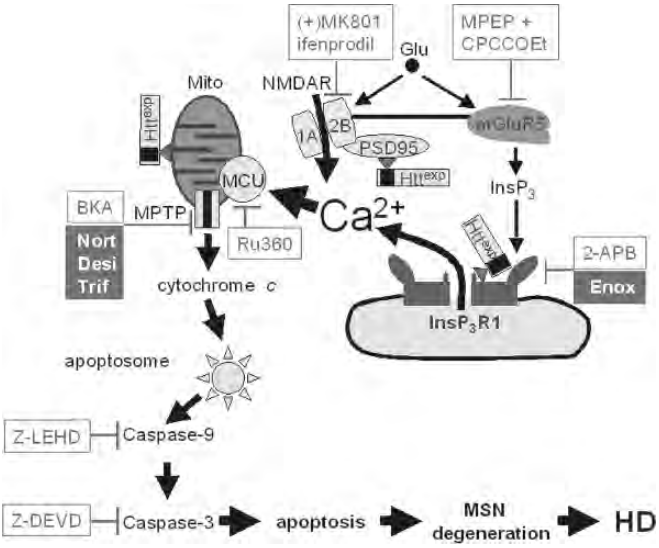

Figure 6. Ca^{2+} hypothesis of HD.
Glutamate released from corticostriatal projection neurons stimulates NR1A/NR2B NMDAR and mGluR5 receptors abundantly expressed in striatal MSN (Landwehrmeyer et al., 1995; Testa et al., 1995). Htt^{exp} affects Ca^{2+} signaling in HD MSN by sensitizing InsP$_3$R1 to activation by InsP$_3$ (Tang et al., 2003), stimulating NR2B/NR1 NMDAR activity (Chen et al., 1999; Sun et al., 2001; Zeron et al., 2002), and destabilizing mitochondrial Ca^{2+} handling (Panov et al., 2002; Choo et al., 2004). As a result, stimulation of glutamate receptors leads to supranormal Ca^{2+} responses in HD MSN and mitochondrial Ca^{2+} overload. Once mitochondrial Ca^{2+} storage capacity is exceeded, mitochondrial permeability transition pore (MPTP) opens, leading to release of cytochrome c into the cytosol and activation of caspases 9 and 3. Activation of caspase-3 leads to progression of apoptosis, MSN degeneration and HD. The model is supported by ability of blockers (shown in red) to reduce glutamate-induced apoptosis of YAC128 MSN to wild type levels in our experiments. The blockers which were effective in our experiments are: NMDAR blocker (+)MK801 and NR2B-specific blocker ifenprodil; mGluR1/5-specific blockers MPEP and CPCCOEt; membrane-permeable InsP$_3$R1 blockers 2-APB and Enoxaparin; MCU blocker Ru360, MPTP blockers BKA, Nortriptyline, Desipramine and Trifluoperazine, membrane-permeable caspase-9 blocker Z-LEHD-FMK and caspase-3 blocker Z-DEVD-FMK. Adapted from (Tang et al., 2005) (See Colour Plate 20)

glutamate-induced Ca^{2+} overload plays a key role in induction of apoptotic cell death of HD MSN.

How do supranormal Ca^{2+} signals induce apoptosis of HD MSN? The best known link between Ca^{2+} overload and apoptosis involves mitochondrial Ca^{2+} overload and activation of intrinsic apoptotic pathway (Choi, 1995; Hajnoczky et al., 2003; Orrenius et al., 2003; Rizzuto et al., 2003). Consistent with this idea, we found that glutamate-induced apoptosis of YAC128 MSN in our experiments can be prevented by Ruthenium 360 (Ru360), an inhibitor of mitochondrial Ca^{2+} uniporter/channel (MCU) (Figure 6).

The observation of dysfunctional mitochondria in HD mouse models and in HD patients (Panov et al., 2002; Choo et al., 2004) provides further support

to mitochondrial involvement in HD pathogenesis. We further found that the glutamate-induced apoptosis of YAC128 MSN was prevented by mitochondrial permeability transition pore (MPTP) inhibitor bongkrekic acid (BKA) and by memrbane permeable inhibitors of caspases-9 and 3 (Figure 6). These data support a model that links Htt^{exp} mutation, abnormal Ca^{2+} signaling and apoptosis of HD MSN (Figure 6) (Tang et al., 2005). Many similar conclusions have been reached in the studies of NMDA-induced apoptosis of YAC72 and YAC128 MSN performed recently by Lynn Raymond's laboratory (Zeron et al., 2004; Shehadeh et al., 2005, 2006).

5. CA^{2+}-RELATED TARGETS AND TREATMENT OF HD

Despite efforts by many laboratories and cloning of Huntingtin in 1993, there is still no cure for HD. The proposed model (Figure 6) suggests that Ca^{2+} signaling blockers, such as NR2B-specific inhibitors of NMDAR and blockers of mGluR5 and $InsP_3R1$, may be beneficial for the treatment of HD. Inhibitors of Htt^{exp} association with $InsP_3R1$ may potentially be used as a more specific HD therapeutic. These concepts are currently being tested in my laboratory. When compared to inhibitors of apoptosis an advantage of using Ca^{2+} signaling blockers and inhibitors of $InsP_3R1$-Htt^{exp} association is that they may stop pathological process at its earliest point, before severe neuronal dysfunction triggers apoptotic cell death. In our recent study we demonstrated that clinically relevant NMDA receptor inhibitor memantine protected YAC128 MSN from glutamate-induced apoptosis in "in vitro HD" model (Wu et al., 2006). Interestingly, a 2-year-long human clinical study suggests that memantine also has an ability to retard the progression of HD based on observed UHDRS scores (Beister et al., 2004). Further evaluation of memantine and other clinically-relevant Ca^{2+} inhibitors will be required to establish if Ca^{2+} pathway constitute a useful target for treatment of HD.

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CHAPTER 12

SERCA PUMPS AND HUMAN DISEASES

A. HOVNANIAN

INSERM, U 563, Toulouse, F-31300 France; Université Toulouse III Paul-Sabatier, UMR-S 563, Toulouse, F-31400 France;
E-mail: alain.hovnanian@toulouse.inserm.fr

Abstract: Sarco(endo)plasmic reticulum (SER) Ca^{2+} ATPases represent a highly conserved family of Ca^{2+} pumps which actively transport Ca^{2+} from the cytosol to the SER against a large concentration gradient. In humans, 3 genes (ATP2A1-3) generate multiple isoforms (SERCA1a,b, SERCA2a–c, SECA3a–f) by developmental or tissue-specific alternative splicing. These pumps differ by their regulatory and kinetic properties, allowing for optimized function in the tissue where they are expressed. They play a central role in calcium signalling through regenerating SER Ca^{2+} stores, maintaining appropriate Ca^{2+} levels in this organelle and shaping cytosolic and nuclear Ca^{2+} variations which govern cell response. Defects in ATP2A1 encoding SERCA1 cause recessive Brody myopathy, mutations in ATP2A2 coding for SERCA2 underlie a dominant skin disease, Darier disease and its clinical variants. SERCA2a expression is reduced in heart failure in human and in mice models. Gene-targeting studies in mouse confirmed the expected function of these isoforms in some cases, but also resulted in unexpected phenotypes: SERCA1 null mutants die from respiratory failure, SERCA2 heterozygous mutant mice develop skin cancer with age and SERCA3 null mice display no diabetes. These unique phenotypes have provided invaluable information on the role of these pumps in specific tissues and species, and have improved our understanding of Ca^{2+} regulated processes in muscles, the heart and the skin in human and in mice. Although the understanding of the pathogenesis of these diseases is still incomplete, these recent advances hold the promise of improved knowledge on the disease processes and the identification of new targets for therapeutic interventions

Keywords: Sarco(endo)plasmic reticulum (SER) Ca^{2+}-ATPases (SERCA), ATP2A1, ATP2A2, ATP2A3, SERCA1, SERCA2, SERCA3, Brody disease, Darier disease (Darier -White disease), Segmental Darier, Acantholytic dyskeratotic epidermal nevi, Acrokeratosis verruciformis of Hopf (Hopf disease), muscle, heart, skin, Ca^{2+} pump

1. SERCA PUMPS: STRUCTURE AND FUNCTION

The SERCA pumps belong to the P-type ATPase family, which actively transport cations across membranes at the expense of ATP hydrolysis. They show a high degree of conservation among species and their structure has recently been

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determined by X-crystallography. They play a key role in Ca^{2+} signaling by bringing the cytosolic free Ca^{2+} concentration back to its resting level after cell activation, and by replenishing the SER stores, which is indispensable for cell function. They act in concert with other Ca^{2+} regulatory proteins involved in Ca^{2+} clearance mechanism, including plasma membrane Ca^{2+}-ATPase (PMCAs) and Na^{+}/Ca^{2+} exchangers (NCXs) (Brini and Carafoli, 2000; Carafoli *et al*., 2001; Missiaen *et al*., 2000; Strehler and Treiman, 2004; Wuytack *et al*., 2002).

1.1. *ATP2A1-3* Genes and SERCA1-3 Isoforms

In humans, SERCA pumps are encoded by three genes, *ATP2A1-3*, each encoding several protein isoforms (SERCA1a,b, SERCA2a-c, SERCA3a-f, respectively) as a result of developmental or tissue-specific alternative splicing (Table 1).

1.1.1. ATP2A1 and SERCA1a and b Isoforms

ATP2A1 maps to chromosomal region 16p12.1 (Callen *et al*., 1991), spans 12.29 kb, contains 23 exons and encodes 3.3 kb transcripts. Exon 22 can be alternatively spliced to generate 2 isoforms: SERCA1a in adults and SERCA1b in neonates (Brandl *et al*., 1986; Zhang *et al*., 1995). SERCA1a (994 amino acids in human) and SERCA1b (1001 amino acids in human) differ by their carboxy-terminal end (Figure 1). In the SERCA1b form, a highly charged octapeptide (993 to 1001) replaces residue Glu994 of SERCA1a. Both isoforms are almost exclusively

Table 1. Human ATP2A1-3 genes, SERCA isoforms, tissue expression and disease

Gene	Locus	Isoforms	Tissue specificity	Disease
ATP2A1	*16p12.1*	SERCA1a (adult) SERCA1b (neonate)	Fast twitch skeletal muscle (type 2) fibers	Brody's myopathy (recessive)
ATP2A2	*12q23-q24.1*	SERCA2a	Heart, slow twitch skeletal muscle, brain	Darier disease (dominant)
		SERCA2b	All tissues (*e.g*.epidermis)	
		SERCA2c	Epithelial, mesenchymal and hematopoietic cell lines, monocytes	
ATP2A3	*17p13.3*	SERCA3a, b, c, e,	Fetal tissue and most adult tissues, most abundant in hematopoietic cell lineages (bone marrow, leukocytes, platelets, lymph nodes, thymus, spleen), salivary glands trachea, lung, pancreas, kidney and colon	Type II diabetes?
		SERCA3d, 3f	All tissues	

expressed in fast-twitch skeletal-muscle fibers in adults (SERCA1a) or in neonates (SERCA1b) where they represent abundant proteins of the SR.

1.1.2. ATP2A2 and SERCA2a–c isoforms

ATP2A2 is located on chromosomal region 12q23-q24, spans 65.69 kb, and is organized in 22 exons, exon 21 being a newly described coding sequence located within intron 22 (Gelebart *et al*., 2003). It is transcribed into three alternatively-spliced mRNAs of 4.4 kb, encoding the SERCA2a, SERCA2b and SERCA2c isoforms, which differ in their carboxy termini and show different expressions patterns (Gelebart *et al*., 2003; Verboomen *et al*., 1992; Verboomen *et al*., 1994). Alternative splicing of exon 20 gives rise to SERCA2a (997 amino acids) and SERCA2b (1042 amino acids), the latter having an eleventh transmembrane domain and a tail extending into the ER lumen. SERCA2c is a recently identified splice variant resulting from the inclusion of a short coding sequence (exon 21) located in intron 20 and containing an in-frame stop codon (Gelebart *et al*., 2003) (Figure 1).

SERCA2a is expressed predominantly in the heart, in slow twitch skeletal muscle and in the brain, where it represents the main SERCA isoform. In contrast, SERCA2b is a "house-keeping" isoform, ubiquitously expressed in smooth muscle and non-muscle tissue (Wuytack *et al*., 2002). Although both isoforms are detectable in keratinocytes and dermal fibroblasts in culture, SERCA2b is the major isoform expressed in the epidermis from adult skin sections. SERCA2c is expressed in epithelial, mesenchymal and hematopoietic cell lines, and in monocytes (Table 1).

1.1.3. ATP2A3 and SERCA3a–f isoforms

ATP2A3 is the most recently identified member of the *ATP2A* family. The gene spans over 40.59 kb, was assigned on chromosomal region 17p13.3 and contains 23 exons encoding 4.7 kb transcripts. Alternative splicing of exons 21, 22 or 23 give rise to 6 different isoforms (SERCA3a–f) (Figure 1). SERCA3a (human 999 amino acids (aa)), SERCA3b (1043 aa), or SERCA3c (1024 aa) isoforms have different C termini and result from exclusion of exons 21 and 22 (SERCA3a), partial inclusion (SERCA3b) or total inclusion (SERCA3c) of exon 21 with exclusion of exon 22 (Dode *et al*., 1998). SERCA3d (1044 aa) and SERCA3e (1052 aa) retain partial or total exon 21, respectively, with inclusion of exon 22 (Martin *et al*., 2002). SERCA3f (1033 aa) is a recently identified isoform which lacks exon 21 but retains exon 22 (Bobe *et al*., 2004). However, at the protein level, there is at present evidence for expression of SERCA3a–c isoforms only. SERCA3 isoforms are most abundant in hematopoietic cell lineages (bone marrow, lymph nodes, thymus, spleen), salivary glands, trachea, colon and pancreas (endocrine pancreatic beta cells) (Wuytack *et al*., 1995) (Table 1). SERCA3 appears to be almost always co-expressed with the house-keeping SERCA2b.

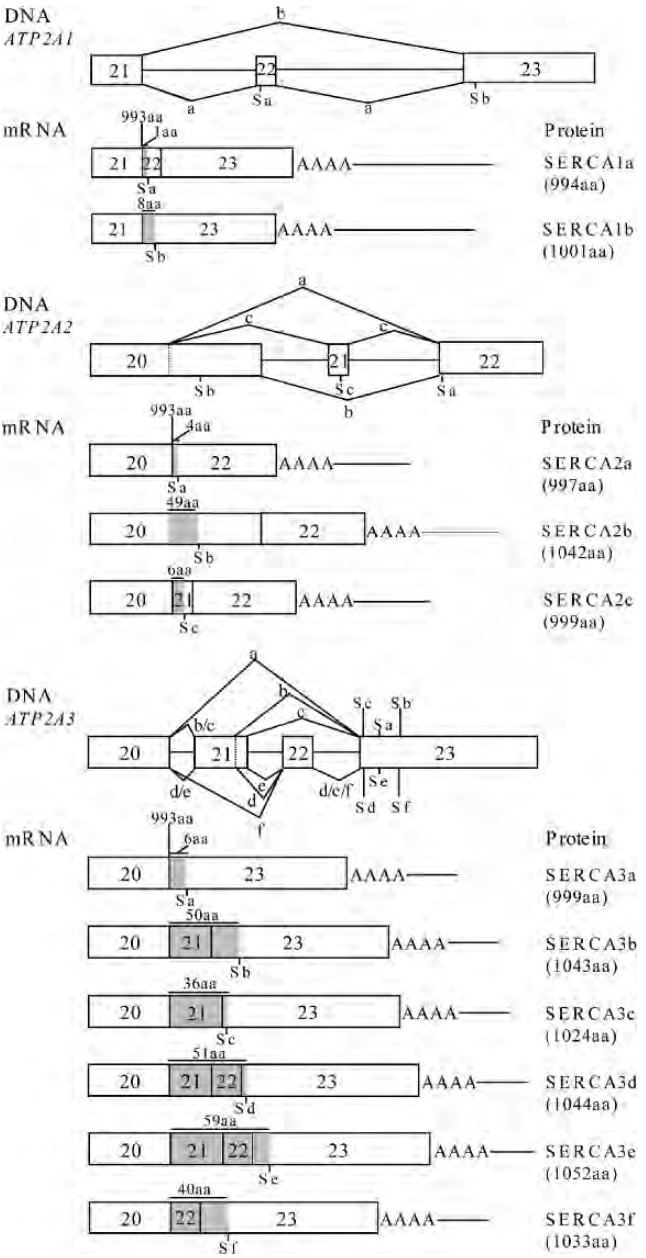

Figure 1. Alternatively splicing of human ATP2A1-3 genes generates multiple SERCA isoforms. Exons are represented by open boxes, introns by horizontal lines. The different splicings are indicated by lines and letters and are described in the text. Sa-Se indicate the position of stop codons of the different isoforms. Boxes in grey represent coding sequences specific for the isoforms

1.2. SERCA1-3 Isoforms Structure

SERCA pumps are members of the P-type ATPase family, which use the energy derived from ATP hydrolysis to actively transport specific ions across biological membranes against a concentration gradient. The P-type Ca^{2+}-ATPase family also includes the plasma membrane Ca^{2+} pumps PMCA1-4 (Shull *et al.*, 2003) and the secretory pathway Ca^{2+} ATPase SPCA1 of the Golgi complex (Hu *et al.*, 2000; Sudbrak *et al.*, 2000). Other P-type ATPase family members include Na^+/K^+-ATPAse and H^+/K^+-ATPAses. The term "P-type" refers to the formation of an energy-rich covalent aspartyl-phosphorylated intermediate after ATP binding to an aspartate residue in a highly conserved phosphorylation sequence DKTGT (Kuhlbrandt, 2004).

SERCA pumps are polypeptides with a molecular mass of 110 kDa. They are localized in the SR or the ER membrane where they play a pivotal role in intracellular Ca^{2+} signalling. They catalyse the hydrolysis of ATP coupled with the translocation of 2 Ca^{2+} ions from the cytosol (100 nanoM) to the endoplasmic reticulum lumen where Ca^{2+} is stored at high concentrations (500 microM) (MacLennan *et al.*, 1997). SERCA1 was the first member of the P-type Ca^{2+}-ATPase family to be cloned. It has been extensively studied by site-directed mutagenesis, which allowed to understand its structure-function relationship. The crystal structure of SERCA1a has recently been determined in different conformations and provides detailed insights into the molecular mechanism of ATP-driven Ca^{2+} translocation (Toyoshima and Inesi, 2004; Toyoshima *et al.*, 2000; Toyoshima *et al.*, 2003). SERCA1 comprises 3 cytoplasmic domains (the actuator, the phosphorylation and the ATP binding domains) linked by 5 stalk domains to 10 transmembrane domains anchored in the ER membrane. Four of these domains (M4, M5, M6 and M8) form the two Ca^{2+} binding sites of the molecule (Figure 2). The predicted secondary structure of the SERCA2 and SERCA3 pumps is based on the crystallization and functional studies of SERCA1, which serves as an archetype for SERCA pumps (Carafoli and Brini, 2000; MacLennan *et al.*, 2002). However, the primary sequence of SERCA2b and SERCA3b-f isoforms predicts the presence of an eleventh transmembrane domain.

1.3. SERCA1-3 Isoforms Function

1.3.1. Common cycle/function

To transport Ca^{2+} from the cytosol to the ER lumen, the SERCA pump goes through a complex cycle involving major conformational states. Upon binding of two Ca^{2+} ions, transphosphorylation from ATP leads to conformational modifications changing the high-Ca^{2+}-affinity SERCA protein into a low-Ca^{2+}-affinity phosphoenzyme intermediate (Figure 3). These steps result from critical interactions between the cytoplasmic domains (ATP binding domain, phosphorylation and actuator domains). These conformational changes are transmitted through stalks 4 and 5 to the Ca^{2+} binding sites and the translocation domain, to result

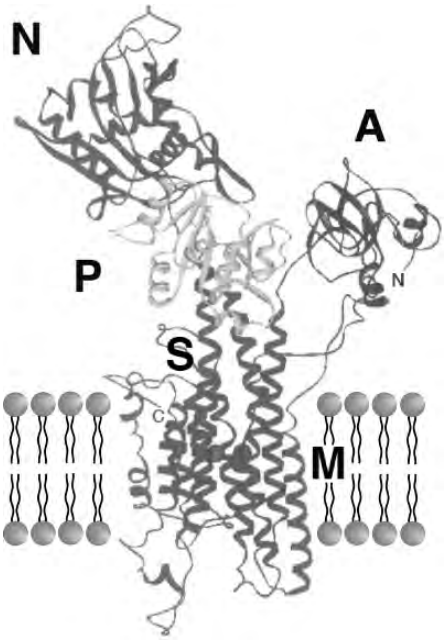

Figure 2. Ribbon diagram showing the different domains in the crystal structure of the rabbit skeletal muscle Ca^{2+}-ATPase (SERCA1) at 2.6 A resolution. The cytoplasmic region of the molecule is organized in three interacting domains: the nucleotide-binding (N) domain in red, which binds ATP; the phosphorylation (P) domain in yellow encompassing the phosphorylation site at Asp351, and the actuator (A) domain in blue. The transmembrane domain (M) in green comprises ten SER-transmembrane domains (M1–M10), four of which (M4, M5, M6 and 8) form the Ca^{2+} (red spheres) binding pocket. Stalk domains (S) link cytoplasmic domains to transmembrane domains. Major conformational changes occur during ATP-energized Ca^{2+} transport (Reprinted from (MacLennan et al., 2002) with permission from Elsevier) (See Colour Plate 21)

in the release of two Ca^{2+} ions into the ER lumen (Carafoli and Brini, 2000; MacLennan *et al*., 2002).

1.3.2. Comparison of SERCA1, 2 and 3 isoform properties

The SERCA isoforms show a high degree of conservation in their primary structure, SERCA2a being 84% identical to SERCA1a, and SERCA3 sharing 75% identity to SERCA1 and SERCA2. Their primary structure being highly conserved, all SERCA isoforms are predicted to have similar transmembrane topology and protein conformation. However, differences in their C-terminal portion account for slighltly different functional properties between SERCA isoforms. SERCA1 and SERCA2 isoforms, but not SERCA3 isoforms, are inhibited by thapsigargin.

1.3.2.1. SERCA1 and SERCA2a The enzymatic properties of SERCA isoforms have been studied in heterogous cell systems by overexpressing recombinant

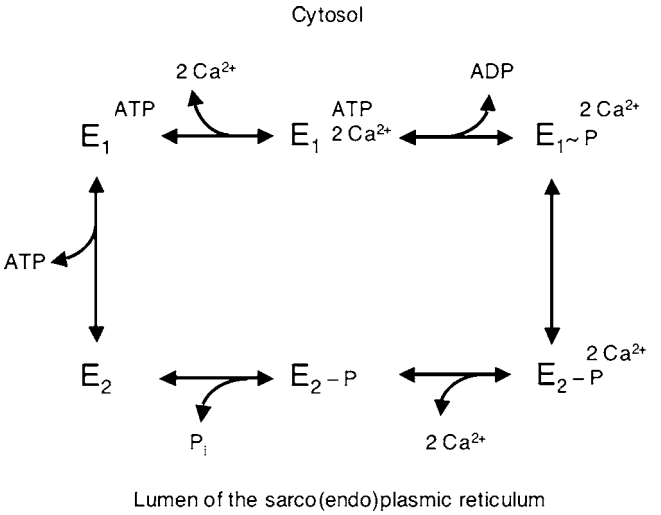

Figure 3. Schematic representation of the reaction cycle of SERCA pumps. The SERCA pumps exist in two conformational state: E1 binds Ca^{2+} with high affinity at the cytoplasmic site of the SER membrane, while E2 has low affinity for Ca^{2+} and thus releases it on the opposite site of the membrane. ATP phosphorylates a highly conserved aspartic acid residue allowing for the translocation of Ca^{2+} in the SER lumen

SERCA pumps in cultured COS cells. Their functional properties were studied using isolated microsomes. The initial *in vitro* studies reported that SERCA1 and SERCA2a isoforms shared similar Ca^{2+} affinity and velocity of Ca^{2+} uptake (Vmax). Subsequently, a higher kinetic turnover was demonstrated for the SERCA1 compared with the SERCA2a isoform (5.0- versus 2.6-fold increase in calcium uptake rate) (Sumbilla *et al*., 1999).

1.3.2.2. SERCA2a–c The three SERCA2 isoforms display functional differences: SERCA2b has a two-fold higher apparent affinity for Ca^{2+} but a two-fold lower catalytic turnover rate resulting in a lower rate of transport compared to 2a (Verboomen *et al*., 1992). SERCA2a and SERCA2b display the same sensitivity towards phospholamban and thapsigargin (Lytton *et al*., 1991). SERCA2c was recently shown to have a lower affinity for Ca^{2+} than SERCA2a and SERCA2b, and it is speculated that SERCA2c could work in a high local Ca^{2+} environment (Dally *et al*., 2006).

1.3.2.3. SERCA1a, SERCA2a and SERCA3a–c SERCA3 isoforms show a 5-fold lower apparent affinity for $Ca2^{+}$, which suggests that SERCA3 becomes active when Ca^{2+} cytosolic reaches high levels during cell stimulation (Dode *et al*., 2002; Martin *et al*., 2002). SERCA3 isoforms have a 10-fold higher apparent affinity for vanadate inhibition compared to other SERCA pumps and are not sensitive to phospholamban.

1.3.3. SERCA pumps in Ca^{2+} signaling

SERCA pumps are essential actors of the Ca^{2+} signaling pathway in excitable and non excitable cells. Following cell stimulation, they restore low Ca^{2+} cytosolic concentrations and contribute to generate intracellular Ca^{2+}signals leading to specific cell responses. They reconstitute dynamic Ca^{2+} stores in the SR/ER required for post-translational modifications, for quality control in the SR/ER and for subsequent Ca^{2+} release.

1.3.3.1. In muscle and heart cells, SERCA pumps Ca^{2+} induce muscle relaxation SERCA1 (fast-twitch skeletal muscles) and SERCA2a (heart and slow-twitch skeletal muscles) maintain low cytoplasmic Ca^{2+} levels during muscle inactivity and generate calcium stores (Gommans *et al*., 2002; Rossi and Dirksen, 2006). Upon excitation, membrane depolarization activates the voltage-sensing dishydropyridine receptors (DHPR) located in the transverse tubular system (T-tubules). These receptors directly interact with the ryanodine recpetor (RyR) located in the SR membrane, resulting in Ca^{2+} release from the SR into the cytosol. Ca^{2+} binding to troponin C induces changes in the troponin and tropomyosin complex, allowing mysosin to interact with actin and contraction to be generated. Thus, in both cardiac and skeletal muscle, the force-generating molecular motors (cross-bridges) are turned on by increasing the intracellular free calcium level that regulates the troponin-tropomyosin system. Subsequent calcium reuptake by SERCA1 and SERCA2 initiates muscle relaxation. Other clearance mechanisms exist, including plasma membrane Ca^{2+}-ATPase (PMCAs), and Na^{+}/Ca^{2+} exchangers, but SERCA pumps are the most important contributor in removing Ca^{2+} during and after contraction.

1.3.3.2. SERCA pumps generate Ca^{2+} fluctuations leading to specific cell responses In non excitable cells, Ca^{2+} signaling involves binding of an extracellular agonist to a plasma membrane receptor which activates phospholipase C and generates inositol 1,4,5-tris-phosphate (IP3) and diacylglycerol from phosphatidyl inositol bis phosphate (PIP2). IP3 in turn acts as a second messenger, binds to its receptors onto the ER to trigger release of Ca^{2+} from the intracellular stores into the cytoplasm. Emptying of ER Ca^{2+} stores activates influx of extra-cellular Ca^{2+} through plasma membrane Ca^{2+} channels which function like store-operated channels (SOC) (capacitive entry mechanism) (Parekh and Putney, 2005; Prakriya *et al*., 2006). The SERCA pumps play a key role in Ca^{2+} signaling by refilling the internal reservoir of Ca^{2+} of the ER, which is considered as the major intracellular Ca^{2+} store. SERCA pumps also participate in the spatio-temporal distribution of Ca^{2+}. They modulate the spatial profile of Ca^{2+} signals locally generated at the periphery of cells by the activation of individual cluster of Ca^{2+} channels. They prevent the globalization of Ca^{2+} induced responses, and thus contribute to the shaping of Ca^{2+} signals in space, time and amplitude in the cell (Mackenzie *et al*., 2004).

1.3.3.3. SERCA pumps sequester Ca^{2+} in the ER lumen By maintaining appropriate Ca^{2+} concentrations in the ER lumen, SERCA pumps also play an essential role in protein synthesis, folding and transport of membrane and secreted proteins. This involves in particular chaperone-dependent processing and post-translational modifications which require a unique calcium rich environment. Chaperone molecules such as calreticulin and calnexin are involved in the quality control pathway in the ER (Berridge, 2002; Ellgaard and Helenius, 2003; Michalak *et al*., 2002).

1.3.4. Regulation of SERCA pumps activity

SERCA pump activity is tightly regulated to respond to cellular stimuli and stress signals. Regulation is achieved at the level of gene transcription and by protein-protein interactions with small regulatory molecules such as phospholamban (SERCA2) and sarcolipin (SERCA1 and SERCA2), or with other SER resident or associated molecules in a tissue-specific manner (Vangheluwe *et al*., 2005).

In the heart and slow twich skeletal-muscle

Phospholamban (PLB) is a small-molecular-weight reversible endogenous inhibitor of SERCA which has been extensively studied (MacLennan *et al*., 2003; MacLennan and Kranias, 2003). PLB is found in the SR or ER of mainly muscle cells including the heart, but is absent from fast-twitch skeletal-muscle fibers (where SERCA1 is the major Ca^{2+} pump isoform). Dephosphorylated PLB interacts *in vitro* with SERCA1, SERCA2a and SERCA2b, but not with SERCA3. Binding of PLP to SERCA2a results in lower affinity for Ca^{2+} and inhibition of the SERCA pump activity. PLB phosphorylation by the cyclic adenosine monophosphate (cAMP) or the Ca^{2+}/calmodulin-dependent protein kinases, relieves SERCA inhibition. Together with sarcolipin, PLB represents the main regulator of SERCA2 pumps.

In fast- and slow twich skeletal muscle and in the heart

Sarcolipin (SLN) is a shorter homologue of PLB (Hellstern *et al*., 2001). SLN is highly expressed in fast-twitch skeletal muscle where it is a key regulator of SERCA1. SLN is also expressed in slow-twitch and in cardiac muscle. Unlike PLB, the interaction of SLN with SERCA depends mainly on SLN's expression level, but not on its phosphorylation. At low Ca^{2+} concentrations, SLN inhibits SERCA1 activity by decreasing Ca^{2+} affinity, whereas it activates SERCA1 activity at high Ca^{2+} concentrations by increasing Vmax (Asahi *et al*., 2003).

Other interactions described with SERCA2

Calnexin and **calreticulin** are Ca^{2+} binding proteins which act as chaperone molecules in the ER by binding to unfolded and newly synthesized glycoproteins. It has been proposed that **calnexin** and **calreticulin** could modulate the affinity/turnover state of SERCA2b, by interacting with a consensus site for N-linked glycosylation (Asn1036) located in the SERCA2b lumenal carboxy-terminal sequence (John *et al*., 1998). However, recent evidence suggest that Asn1036 may not be glycosylated and that the interaction with calnexin would require the phosphorylation of Ser562 of SERCAb (Roderick *et al*., 2000).

The ER oxydoreductase **Erp57** promotes disulphide bond formation during protein folding. Erp57 participates, together with calreticulin and calnexin, to the quality control and folding of newly synthesized (glyco)proteins. Erp57 was recently shown to interact with two conserved cysteine residues (C875 and C887) located in the intraluminal loop 4 of SERCA2b. This interaction is Ca^{2+} dependent, promotes disulphide bond formation and results in the inhibition of the pump. ER Ca^{2+} depletion would subsequently displace Erp57 from SERCA2b, resulting in relieve of the inhibition of SERCA2b activity. This suggests that Erp57 could modulate SERCA2b activity by modifying the redox state of ER facing thiols of the pump (Li and Camacho, 2004).

S100 proteins represent a large family of EF-hand Ca^{2+} binding proteins which regulate fundamental biological processes. S100A1 is abundantly expressed in the striated muscle and cardiac muscle, where it is a major regulator of cardiac contractility. S100A1 co-localizes with the SR *in vivo*, interacts with SERCA2a *in vitro* and was shown to stimulate Ca^{2+} uptake by SERCA2a pump (Kiewitz *et al*., 2003). These observations suggest that S100 proteins could enhance SERCA2 activity through a direct protein-protein interaction.

Desmoplakins I and **II** are key plaque proteins which anchor desmosomes to keratin filaments in the epidermis. Desmosomes are critical adhesion complexes between adjacent epithelial cells, that assemble in response to cell–cell contact and raised levels of extracellular Ca^{2+} (Green and Gaudry, 2000). SERCA2 and desmoplakins have been shown to interact in a Ca^{2+} dependent manner on the ER membrane in cultured human primary keratinocytes, suggesting that SERCA2 may have an accessory function in desmosomal assembly (Dhitavat *et al*., 2003a).

2. HUMAN DISEASES AND ANIMAL MODELS

Genetic defects in ATP2A1 encoding SERCA1 have been shown to cause recessive Brody myopathy, while mutations in ATP2A2 underlie a dominant skin disease, Darier disease and its clinical variants. SERCA2a expression is reduced in heart failure in human and in heterozygous Serca2 mutant mice. Gene-targeting studies in mouse have resulted in unexpected phenotypes, often distinct from human diseases, providing evidence for species specificity (Foggia and Hovnanian, 2004; Hovnanian, 2004; Missiaen *et al*., 2000; Periasamy and Kalyanasundaram, 2007; Shull *et al*., 2003).

2.1. Brody's Disease

Brody disease (BD, OMIM 601003) was first decribed by Dr. Irwin A. Brody in 1969 (Brody, 1969). It is a rare genetic muscular disease which can be transmitted in an autosomal recessive or dominant manner. The disease is genetically heterogeneous, and ATP2A1 mutations have been identified in recessive forms of the disease only (Odermatt *et al*., 1996).

2.1.1. Clinical manifestations

This genetic myopathy is characterized by impaired muscle relaxation, painless cramps and stiffness following exercise. The symptoms occur after repetitive, sustained exercise and mainly affect the limb muscles. They are exacerbate in the cold.

2.1.2. Genetics

Early studies have reported reduced Ca^{2+}ATPase activity in the sarcoplasmic reticulum of Brody patients, sometimes associated with diminished SERCA1 expression in fast-twitch skeletal muscles. These observations pointed to a possible involvement of ATP2A1 in the disease (Benders *et al*., 1994; Karpati *et al*., 1986). A first study in 3 Brody patients found no mutation in ATP2A1, although one of these patients was later shown to have an ATP2A1 mutation (Zhang *et al*., 1995). In 1996, MacLennan and his group identified 3 causative mutations (a splice site and 2 mutations leading to premature termination codons (PTC)) in ATP2A1 encoding the SERCA1 isoform in 2 families affected with recessive Brody disease (Odermatt *et al*., 1996). Since then, 3 mutations leading to PTC and a deleterious missense mutations in ATP2A1 were identified in a total of 6 families with the recessive form of the disease (Odermatt *et al*., 2000; Odermatt *et al*., 1997).

2.1.3. Heterogeneity

Sequence analysis of ATP2A1 in other recessive as well as dominant Brody disease could not identify ATP2A1 mutations. Moreover, genetic linkage with ATP2A1 was excluded in a family with recessive Brody disease. These results indicate that Brody disease is genetically heterogeneous, and that defects in a second gene must cause some forms of recessive Brody disease, whereas defects in a third gene underlie dominant forms of the disease. Of the molecules which modulate SERCA1 activity, SLN encoded by the SLN gene (Odermatt *et al*., 1997) was a good candidate for an alternative causative gene. However, no SLN mutation has been identified in 13 Brody families studied (Odermatt *et al*., 1997).

2.1.4. Pathophysiology

In muscle cells, the contraction is induced by Ca^{2+} release from the sarcoplasmic reticulum, as a result of membrane depolarization and activation of RyR1 receptors located at the surface of the SR. The subsequent transport of cytoplasmic Ca^{2+} back into the lumen of the sarcoplasmic reticulum restores low resting calcium levels and allows muscle relaxation. In fast-twitch skeletal muscle fibers, Ca^{2+} uptake is mediated by the sarco(endo)plasmic reticulum Ca^{2+} ATPase SERCA1 which represents more than 99% of SERCA isoforms in these muscle fibers.

In Brody disease, as a result of loss of function mutations in ATP2A1, Ca^{2+} is not transported back into the SR after its release from the stores and accumulates in the myoplasm, resulting in delayed relaxation and muscle cramping. It is of interest that patients with Brody disease are still able to relax their fast-twitch skeletal muscles, even though relaxation is significantly reduced. This suggest that SERCA2

or SERCA3 ectopic expression could compensate in part for defective Ca^{2+} loading in the sarcoplasmic reticulum. Other compensatory mechanisms could also involve myoplasmic Ca^{2+} removal by Ca^{2+}-ATPases and Na+/ Ca^{2+} exchangers of the plasma membrane or uptake in the mitochondria (MacLennan, 2000).

2.1.5. Treatment

Some patients have been treated with dantrolene, which reduces the Ca^{2+} release from the SR by blocking the RyR. However, several patients treated with this molecule complained of muscle weakness, likely to result from a decreased Ca^{2+} release and ineffective muscular contraction.

2.1.6. Atp2a1 knock out mouse model

To gain a better understanding of the pathogenesis of Brody disease, David MacLennan's group developed knock out mice for Serca1 (Pan *et al*., 2003). Serca1-null mice displayed a gasping respiration, became cyanotic and die shortly after birth of respiratory failure. They also exhibited slow limb movement and evidence for cramping. Histological examination of diaphragm muscle revealed hypercontracture injury. Ca^{2+} uptake activity in tissue homogenates from the Serca1 null mice was severely reduced, consistent with defective Ca^{2+} removal from the cytosol. There was no evidence for compensation by Serca2 or Serca3, nor hanges in the levels of sarcolipin or phospholamban., These results confirmed the major role of Serca1 in contraction/relaxation coupling. However, patients with Brody disease do not show respiratory disease. The fact that the respiratory rate in newborn mice is much faster than in human, and that slow-twitch fibres account for less than 10% of diaphram muscle in mice, instead of 40% in human, could explain the species differencies observed (Pan *et al*., 2003; Prasad *et al*., 2004; Shull *et al*., 2003).

2.2. Darier Disease

Darier's disease (DD)(MIM 124200) or Darier-White's disease (also referred as keratosis follicularis) is a rare autosomal dominant disease, first described by Darier and White in 1889. DD affects both sexes and all ethnic groups, with a prevalence estimated between 1 in 26 300 and 1 in 100,000 in different countries (Godic *et al*., 2005), (Tavadia *et al*., 2002), (Wilkinson, 1977) (Cooper and Burge, 2003; Svendsen and Albrecten, 1959). Penetrance of the disease is complete, and expression is highly variable between and within affected families.

2.2.1. Clinical manifestations

Onset of the disease is usually around puberty. The majority of patients will develop the first lesions between 10 and 20 years of age. Typical lesions are greasy, keratotic papules, skin coloured, yellow-brown or brown, which can be isolated or form relatively large, crusted and confluent plaques. The sites of predilection are the seborrhoeic areas of the trunck and face: the upper chest, the back, the sides of

the neck, the forehead, the ears, and the scalp (Figure 4a). The flexures are also frequently involved. The skin lesions can become vegetating in the folds, are often infected, malodorous and responsible for major discomfort. They can be limited or form extensive plaques during acute phases. Careful examination of the palms and soles will frequently reveal small pits or punctuated keratoses which are highly suggestive of DD. Hands and feet can also show discrete flat, skin-coloured papules on the dorsum of the hands and feet, similar to acrokeratosis verruciformis of Hopf (Cooper and Burge, 2003). Nails show almost constant abnormalities which are highly specific of the disease. The nails are fragile and split easily, they show the combination of red and white longitudinal stripes, have a V-shaped nick at the free margin of the nail and subungual hyperkeratosis. The mucous membranes can be affected and may be the site of whitish small papules (Frezzini *et al*., 2006). Intra-familial disease severity has been reported in several studies, indicating that additional genetic and/or environmental factors influence disease expression (Onozuka *et al*., 2004).

2.2.2. Pathology

Histology examination of skin lesions shows hyperkeratosis (thickening of the stratum corneum), focal dyskeratosis (premature and abnormal keratinisation of single keratinocytes) associated with suprabasal acantholysis (cell separation above the basal layer) leading to suprabasal clefts (Figure 4b). Rounded eosinophilic dyskeratotic cells which have lost contact with neighbouring cells are called "corps ronds" or "grains" and are highly suggestive of the disease. They are thought to correspond to apoptotic keratinocytes. Electron-microscopy has revealed separation of the keratin filaments from the desmosomes (rupture of the desmosome-keratin complexes) with perinuclear tonofilaments clumping (Hakuno *et al*., 2000).

2.2.3. Genetics

The gene for Darier's disease was mapped to chromosome 12q23-24 in 1993 (Craddock *et al*., 1993) and was identified by positional cloning as being ATP2A2 in 1999 (Sakuntabhai *et al*., 1999b). The identification of a Ca^{2+} pump as the defective protein in DD was unexpected and disclosed a key role of Ca^{2+} signalling and SERCA2 in the homeostasis of the epidermis (Hovnanian, 2004).

2.2.3.1. Spectrum of ATP2A2 mutations More than 130 ATP2A2 mutations have now been reported in Darier's disease patients, the majority of which being family specific (Dhitavat *et al*., 2003c; Dhitavat *et al*., 2003d; Godic *et al*., 2004; Ikeda *et al*., 2003; Jacobsen *et al*., 1999; Onozuka *et al*., 2004; Racz *et al*., 2005; Racz *et al*., 2004; Ren *et al*., 2006; Ringpfeil *et al*., 2001; Ruiz-Perez *et al*., 1999; Sakuntabhai *et al*., 1999a; Sakuntabhai *et al*., 1999b; Takahashi *et al*., 2001; Wada *et al*., 2003; Yang *et al*., 2001) (HGMD http://www.biobase.de/ggmd/gene.php?gene = ATP2A2). Mutations are distributed across the entire molecule, with no evidence for clustering or mutation hot spots, and disrupt critical functional domains of SERCA2. Spontaneous mutations are frequent. The majority of the ATP2A2

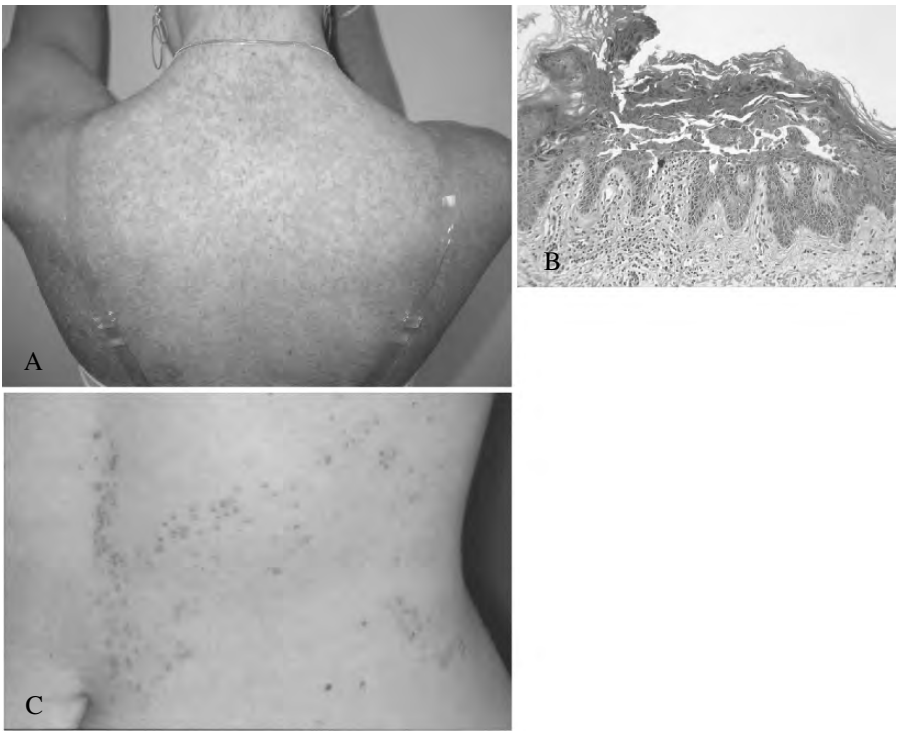

Figure 4. **a**, Clinical aspect of Darier disease showing extensive and confluent keratotic and inflammatory papules on the back of a 27 years old patient; **b**, Histology of affected skin in Darier disease showing suprabasal cleft of the epidermis containing acantholytic cells, associated with hyperkeratosis and rounded dyskeratotic cells (« corps ronds » or « grains »); **c,** Acantholytic epidermal naevi (segmental Darier disease). Limited and unilateral distribution of keratotic papules following Blaschko lines on the lower abdominal region. These lesions correspond to somatic mosaicism for an ATP2A2 mutation. (Courtesy of Dr Susan Burge, Department of Dermatology, Churchill hospital, Oxford, UK) (See Colour Plate 22)

mutations identified are missense mutations (50%) or in-frame deletions or insertions (8%) which predict the synthesis of a structurally abnormal protein. Other mutations include nonsense mutations (12%) and frameshift mutations (23%) which lead to premature termination codon (PTC) and predict loss of protein expression through nonsense mRNA decay. The remaining mutations are splice-site mutations (7%) the consequences of which on the mutated protein remain to be documented. Genotype-phenotype comparison has initially suggested that ATP2A2 missense mutations are more frequent in severe or atypical forms, but this has not been confirmed in other studies (Ringpfeil *et al*., 2001). However, considerable inter- and intra-familial variability in the disease phenotype indicates that additional genetic or environmental factors influence disease expression (Onozuka *et al*., 2004; Ruiz-Perez *et al*., 1999; Sakuntabhai *et al*., 1999b).

Mutations in SERCA2b isoform only can cause DD. While the majority of Darier patients harbour a mutation in a region of ATP2A2 which is common to both isoforms, three families with classical Darier's disease were heterozygous for an ATP2A2 mutation located in a region of exon 20 which is specific for SERCA2b isoform (Dhitavat *et al*., 2003c; Ikeda *et al*., 2001). Each of these mutations leads to a PTC, indicating that loss of SERCA2b expression is sufficient to cause Darier's disease, and that SERCA2a isoform cannot compensate for it. This finding is consistent with the most prominent expression of SERCA2b in the epidermis, although both isoforms are expressed in cultured keratinocytes (Ruiz-Perez *et al*., 1999; Tavadia *et al*., 2004).

2.2.3.2. Functional effects of SERCA2 mutations

On Ca^{2+} transport capacities of the mutated pumps To date, the functional consequences of a total of 68 *ATP2A2* mutations identified in DD patients have been studied by site-directed mutagenesis in three separate studies (Ahn *et al*., 2003; Dode *et al*., 2003; Miyauchi *et al*., 2006). Over-expression and functional analysis of recombinant mutated SERCA2 pumps in COS cells has shown that in most cases, DD missense mutations cause loss of Ca^{2+} transport through defective ATPase activity (Ahn *et al*., 2003; Dode *et al*., 2003; Miyauchi *et al*., 2006). Some mutants cause reduced protein expression due to protein instability. A rare mutant was found to have increased Ca^{2+} transport activity, but higher affinity. Together with *ATP2A2* mutations leading to PTC and loss of expression of the mutant allele, these results support the proposition that haplo-insufficiency is a common mechanism for the dominant inheritance of Darier's disease (Ahn *et al*., 2003; Dode *et al*., 2003; Miyauchi *et al*., 2006). However, some missense mutations partially inhibit the co-expressed wild-type protein and were associated with a more severe phenotype (Ahn *et al*., 2003), suggesting that some missense mutants may act at least in part through a dominant negative mechanism.

On Ca^{2+} homeostasis in keratinocytes In normal keratinocytes, there is an increasing epidermal gradient in the normal skin, from the basal layer to the superficial layers. High extra-cellular concentrations are required for epidermal inter-cellular adhesion, differentiation and cornification. As a consequence of loss of SERCA2 Ca^{2+} transport on Ca^{2+} homeostasis, DD keratinocytes were shown to display depletion of ER Ca^{2+} stores in two studies (Foggia *et al*., 2006; Leinonen *et al*., 2005). Cytosolic Ca^{2+} resting levels were found to be increased in the study by Leinonen and col., but were decreased in the study by Foggia and col. In the latter, reduced cytosolic Ca^{2+} was shown to result from up-regulation of *ATP2C1*, encoding hSPCA1, the Ca^{2+} pump of the Golgi apparatus. The Ca^{2+}-signaling capability of the DD cells was preserved, indicating that the Golgi Ca^{2+} store compensates for defective SERCA2-dependent signaling. In the same study, inhibition of ATPC1 by siRNA diminished DD keratinocyte viability, indicating that up-regulation of *ATP2C1* was a compensatory mechanism against apoptosis. Recently, Pani and col. have shown up-regulation of a member of plasma membrane capacitative entry channels, TRPC1 (Transient receptor Potential

Cationique channel) in DD and SERCA2+/− mouse keratinocytes (Pani *et al*., 2006). These authors also show that up-regulation of TRPC1 enhances cell proliferation and restricts apoptosis, illustrating another compensatory mechanism in response to ER Ca^{2+} store depletion. These results emphasised the interplay between ER, Golgi apparatus and plasma membrane Ca^{2+} channels such as TRPC1 in Ca^{2+} signaling. They indicate that up-regulation of other Ca^{2+} transport ATPase (*ATP2C1*), and channels could at least in part compensate for *ATP2A2* dysfunction at a steady-state, and that blockade of these compensatory responses compromise cell viability.

On desmosome formation Ca^{2+} triggers the switch between keratinocyte proliferation and differentiation. Ca^{2+} is in particular required for the assembly of desmosomes and adherens junctions, and actin polymerisation (Kitajima, 2002). A change in the Ca^{2+} ER luminal content is likely to have a major effect on Ca^{2+}signaling, protein post-translational modifications and trafficking. Although no glycosylation defect of the desmosomal proteins was detected in DD keratinocytes in culture, trafficking of desmoplakin to the membrane was shown to be significantly hindered (Dhitavat *et al*., 2003b). Defective addressing to the desmosomal plaque of this key molecule which links the cytoskeleton to the desmosomal complexes, could contribute to impair cell-to-cell adhesion in the epidermis in DD. Inappropriate Ca^{2+} concentrations in the lumen could cause dysfunction of calcium dependent chaperone molecules leading to impair folding, assembly and/or trafficking of proteins. Whether this effect preferentially affects proteins playing a key role in cell-to-cell adhesion and/or differentiation, or is a general consequence on protein processing remains to be determined. Finally, since Ca^{2+} plays a key role in the transcriptional regulation of a wide range of genes which are essential for epidermal cell-to-cell adhesion and terminal differentiation, it is also possible that abnormal Ca^{2+} signaling have a profound impact on the transcription of these genes.

The role of triggering factors The observation that exposure to UV B irradiation, heat and infection trigger the disease, illustrate the role of external factors unmasking the basic defect in DD. In the absence of stress, SERCA2 deficiency could be compensated by increased expression of the normal allele and/or other regulatory systems. Triggering factors would disrupt this subtle balance by down-regulating ATP2A2 or increasing requirement of SERCA2 to maintain a unique Ca^{2+} content in the lumen. Because only one copy of ATP2A2 is functional, reduction of SERCA2 levels would be excessive, or compensation would not reach adequate levels. Consistent with this possibility, ultraviolet B irradiation and pro-inflammatory cytokines have been shown to down-regulate ATP2A2, as well as ATP2C1, mRNA expression (Mayuzumi *et al*., 2005). Reduced levels of SERCA2 would impair Ca^{2+} signaling.

2.2.4. Course

The skin lesions generally appear between early childhood and the age of 20 years. The condition runs a chronic relapsing course, with exacerbations throughout life. Some patients will have a relatively mild disease, while others will develop a more

severe form, sometimes within the same family. In particular, the skin lesions are exacerbated by exposure to sunlight or artificial 'UV B radiation, heat, sweating, friction and infections. Patients with Darier's disease appear to have an increased susceptibility to herpes simplex and chronic pyogenic. infections. The occurrence of spinous cell carcinoma has been rarely reported.

2.2.5. Associated manifestations

Behavior and learning difficulties have been observed in patients with DD, but they may be, at least in part, secondary to the social handicap caused by the disease. Neuro-psychiatric abnormalities have also been reported in families with Darier's disease (Craddock *et al*., 1994). These include epilepsy, mild mental retardation, bipolar disease and schizophrenia. Familial cosegregation of DD and bipolar disorder has been reported in several families and strongly supports the existence of a bipolar disorder susceptibility gene in the DD region (Jones *et al*., 2002). However, *ATP2A2* has been excluded as a common susceptibility gene for bipolar disease (Jacobsen *et al*., 2001). These observations, together with SERCA2b expression in the brain (Baba-Aissa *et al*., 1998) and the importance of calcium signaling in neurons, raise the question of occurrence of independent skin and neuro-psychiatric conditions, or expression of the same genetic defect affecting skin and brain (Jacobsen *et al*., 1999).

2.2.6. Clinical forms

Erosive or bullous forms, others with malodorous, vegetating lesions in the flexures have been reported. More rarely, cornifying forms of the legs (Katta *et al*., 2000), comedon-like (Hallermann and Bertsch, 2004) and haemorrhagic macules on palms and soles have been described (Foresman *et al*., 1993). Two particular forms of the disease should be distinguished: **segmental and acral Darier disease.**

a. **Segmental Darier disease, or Acantholytic dyskeratotic epidermal naevi (ADEN),** or linear, zosteriform Darier's disease present as localized and unilateral keratotic papules which follow Blaschko's lines (Figure 4c). (Munro and Cox, 1992; Reese *et al*., 2005). Molecular analysis of these patients has shown that they correspond to mosaic forms of Darier's disease, carrying a postzygotic ATP2A2 mutation in affected areas only (Sakuntabhai *et al*., 2000; Wada *et al*., 2003). There has been no report of patients with segmental Darier's disease having a child with generalised Darier's disease, and the risk of transmission of a generalised form remains unknown.
b. **Acral** forms are difficult to distinguish from **Acrokeratosis verruciformis of Hopf**, which could be considered as a clinical variant of Darier's disease, since both diseases are due to ATP2A2 mutations (see below)(Dhitavat *et al*., 2003d; Niedleman and Mc, 1962).

Acrokeratosis verruciformis of Hopf (AKV)(MIM 101900) was originally described by Hopf in 1931 (Hopf, 1931). AKV is inherited in an autosomal dominant fashion. The onset is early in life, often since birth or later in infancy.

The disease affects distal extremities and usually presents with multiple, small flat-topped papules predominantly on the dorsum of the hands and feet (Rallis *et al*., 2005; Schueller, 1972). Punctate keratoses and pits on the palms and soles can be present. Nails can be involved. The rest of the skin is clear, with no involvement of seborrhoeic areas. Lesions tend to persist throughout life, increase in number slowly and become more prominent after sun exposure. Transformation to squamous cell carcinoma has been reported in two cases (Dogliotti and Schmaman, 1971; Panja, 1977; Wang *et al*., 2006).

The histopathology of skin lesions shows hyperkeratosis, hypergranulosis and acanthosis with "church spikes" circumscribed elevations of the epidermis. There is no features of dyskeratosis or acantholysis, which are classical lesions of DD.

A possible relationship with Darier's disease has long been postulated. Although initially viewed as distinct entities, AKV and DD, especially in its acral form, have been considered as related disorders by several authors, even by Hopf and Darier themselves (Panja, 1977). In support of AKV being part of the DD spectrum is the description of the co-occurrence of AKV and DD in the same patient (Hafner and Vakilzadeh, 1997; Piskin *et al*., 2004) or in affected members of the same family (Herndon and Wilson, 1966). In addition, it is notable that 50% of DD patients have acral warty papules and that patients with histological changes of AKV have been reported to develop typical histological features of DD (Penrod *et al*., 1960; Waisman, 1960).

The recent identification of a heterogeneous Pro602Leu mutation in the ATP2A2 gene in a large British pedigree with AKV shows that both diseases can be due to defects in the same gene (i.e. are allelic disorders) (Dhitavat *et al*., 2003d). This mutation is located in the ATP-binding domain of SERCA2 and has not been reported in patients with classical DD. Functional analysis showed that it abolishes Ca^{2+} transport. The mechanism by which this specific missense ATP2A2 mutation gives rise to AKV, and not classical DD, is not understood. It is possible that the variable expression may be determined by the nature and location of the mutation, but it is also likely that modifying genes could play a role in the phenotypic expression of AKV and DD.

In a recent study, no evidence for the implication of ATP2A2 could be found in a large Chinese family, raising the possibility of genetic heterogeneity in AKV (Wang *et al*., 2006). It is thus possible that AKV and acral DD represent distinct entities caused by mutations in the same or another gene in other families (Wang *et al*., 2006). Further identification of specific ATP2A2 mutations in AKV could help addressing this issue.

2.2.7. Why does Darier disease affect skin only?

It is not clear why clinical symptoms of DD are restricted to the epidermis and predominate to certain areas of the skin. A possible explanation could be that non-cutaneous tissues have compensatory mechanisms that are missing in the skin. SERCA1 expression is limited to fast-twitch muscles, but SERCA3 appears to be co-expressed with SERCA2b in a majority of tissues except the epidermis

(Tavadia *et al*., 2004). Thus, SERCA3 might provide some redundancy in non-cutaneous tissues. Another explanation could be that the requirement of epidermal cells in SERCA2 pumps is more important than in other tissues, and that half the normal quantity of SERCA2 pumps is insufficient to achieve correct function in the epidermis (tissue-specific gene dosage effect), whereas it is sufficient in other tissues. This could also explain why the disease is exacerbated by external factors such as heat, UV exposure, friction and infection. Dysfunction of the SERCA2 pump would be compensated by other mechanisms at the basal state, but exposure to stress would require an increase in SERCA2 activity to a level that the cells cannot achieve, owing to the loss of function of one copy of the gene. This would disrupt a subtle balance and lead to clinical symptoms of the disease. The late onset of the disease (after puberty) suggests age-dependent and possibly hormone-induced changes in the regulation of calcium homeostasis and consequently different SERCA2 requirements between adult and child skin.

2.2.8. Darier patients show no heart deficiency

As indicated above, the vast majority of Darier patients carry a mutation in the region of the ATP2A2 gene which is common to both SERCA2a and SERCA2b isoforms. However, there was no report in the literature of increased heart disease in Darier patients. Therefore, detailed cardiac investigation was performed in two independent cohorts of patients with Darier disease. Both studies showed that heterozygous disruption of SERCA2 a and b isoforms was not associated with impaired cardiac performance in DD patients (Mayosi *et al*., 2006; Tavadia *et al*., 2001).

2.2.9. Treatment

Patients with mild Darier disease will benefit from advice about sun protection and avoidance of heat. When skin lesions are limited, emollients containing urea or lactic acid, or topical applications of tretinoïn, isotretinoin or tazarotene can reduce crusting. Antiseptics (topical or in bath), antibiotics and anti-fungus are helpful to prevent or treat pyogenic and fungal infection. Herpes infection are suspected when lesions are painful and exacerbated, which requires antiviral treatment with acyclovir. For those with more severe disease, oral retinoids such as acitretine (Soriatane) is the most effective treatment. Their efficiency lasts only as long as the treatment is continued. The initial dose is usually 0.5 mg/kg/day for 1 to 2 months if ifwell tolerated. Possible side-effects must be monitored carefully and oral contraception is mandatory in females, 1 month before, during and 2 years after treatment. After initial clearing of the lesions, the dose is reduced progressively and stopped (in winter) or maintained as low as possible to avoid relapse (Cooper and Burge, 2003). Recently, topical Tacrolimus, a calcineurin inhibitor, has been successfully used to treat extensive DD (Rubegni *et al*., 2006). The disease can cause considerable social handicap and can require a specific psychological support by a medical psychologist. Genetic counselling should be offered, although prenatal diagnosis of the disease should be restricted to extremely disabling forms.

2.2.10. Mouse models for SERCA2 dysfunction

2.2.10.1. Serca2$^{-/-}$mice To gain a better understanding of the physiological functions of SERCA2 in vivo, Gary Shull's group developed knockout mice by deleting the promoter and first two coding exons of Serca2. Homozygous mutants were not observed, consistent with the idea that Serca2b serves an essential housekeeping function. Serca2 heterozygous mutants appeared healthy but showed reduced cardiac muscle contractility and relaxation. With age, heterozygous mutant mice did not develop Darier-like lesions but unexpectedly, a very high frequency of squamous cell carcinomas (Liu *et al*., 2001; Periasamy *et al*., 1999; Prasad *et al*., 2005; Prasad *et al*., 2004; Shull *et al*., 2003). These tumors resulted from haploinsufficiency rather than loss of heterozygosity. Patients with Darier disease do not show a predisposition to cancer, although some squamous cell carcinoma have been reported in a limited number of patients. These results revealed significant species differences in susceptibility to the disease and disclosed a new mode of cancer susceptibility.

2.2.10.2. Serca2a$^{-/-}$mice To investigate the effect of selective loss of expression of Serca2a isoform, homozygous mutants expressing only Serca2b were developed by Frank Wuytack's group by disrupting the splicing mechanism generating the SERCA2a isoform (Ver Heyen *et al*., 2001). Homozygous mice mutants expressing only SERCA2b were obtained with some embryolethality due to cardiac malformations. The homozygous mutants survived to adulthood and, in contrast to heterozygous mutant for SERCA2 a and b isoforms, adult mice expressing only SERCA2b developed a compensatory concentric cardiac hypertrophy (Ver Heyen *et al*., 2001). This result showed that Serca2b could only in part compensate for SERC2a deficiency in the heart (Liu *et al*., 2001; Periasamy *et al*., 1999; Prasad *et al*., 2005; Prasad *et al*., 2004; Shull *et al*., 2003).

2.3. SERCA2a in Heart Failure

Extensive studies in animal models and in human have reported decreased SR Ca^{2+} transport and SR Ca^{2+}content in heart failure, due to SERCA2a mRNA and protein diminution. Despite considerable heterogeneity in the expression level of SERCA in failing hearts, it is thought that altered Ca^{2+} transport plays a role in the mechanism of heart failure. However, the observation that heterozygous disruption of SERCA2 (a and b isoforms) is not associated with the impairment of cardiac performance in patients with Darier disease suggests that decreased SERCA2a levels in heart failure may not be a critical causal pathway in humans (Mayosi *et al*., 2006; Tavadia *et al*., 2001).

2.4. SERCA3 and Diabetes in Human and in Mice Model

The expression of SERCA3 in pancreatic beta-cells (Arredouani *et al*., 2002), the association of sequence variants of ATP2A3 with Type II diabetes (Varadi *et al*., 1999)

and the description of impaired Ca^{2+}-ATPase activity in diabetic rats model (Levy *et al*., 1998) have suggested that SERCA 3 could be implicated in Ca^{2+} dysregulation that accompanies diabetes. To gain insight into Serca3 role in Ca^{2+} signaling, Gary Shull's group has generated Serca3 null mice (Liu *et al*., 1997). Serca3 null mutants were normal, suggesting that Serca3 plays a non dispensable role in mice. These mice displayed a smooth muscle relaxation defect in vitro, but with no disease phenotype. Blood pressure and cardiovascular performance which were impaired in Serca2 heterozygote mice, were normal. Despite some alterations in Ca^{2+} signaling, Serca3 null mice did not develop diabetes, were normoglycemic and have normal insulinemia, demonstrating that in this model, Serca3 was not essential for glucose homeostasis. However, this result does not exclude the possibility that in another background, reduction of SERCA3 activity could contribute to impaired glucose homeostasis and the development of diabetes. In addition, SERCA3 inhibition by antisense oligonucleotide does not deplete ER Ca^{2+} stores, in contrast to SERCA2b inhibition. These data suggest that the role of SERCA3 in pancreatic beta-cells may be different from the other SERCA pumps, and may be involved in buffering at Ca^{2+} peak levels (Martin *et al*., 2002; Shull *et al*., 2003).

3. CONCLUSION

These unique phenotypes have provided invaluable information on the role of these pumps in specific tissues and species, and have improved our understanding of Ca^{2+} regulated processes in muscles, the heart and the skin in human and in mice. Although the understanding of the pathogenesis of these diseases is still incomplete, these recent advances hold the promise of improved knowledge on the disease processes and the identification of new targets for therapeutic interventions.

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CHAPTER 13

THE PLASMA MEMBRANE CALCIUM ATPase AND DISEASE

B.L TEMPEL AND D.J. SHILLING

The Department of Otolaryngology-Head and Neck Surgery, the Department of Pharmacology, and the V.M. Bloedel Hearing Research Center; University of Washington School of Medicine, Seattle WA 98195-7923, USA

Abstract: The plasma membrane calcium ATPase (PMCA) uses energy to pump calcium (Ca^{2+}) ions out of the cytosol into the extracellular milieu, usually against a strong chemical gradient. This energy expenditure is necessary to maintain a relatively low intracellular net Ca^{2+} load. Mammals have four genes (*ATP2B1-ATP2B4*), encoding the proteins PMCA1 through PMCA4. Transcripts from each of these genes are alternatively spliced to generate several variant proteins that are in turn post-translationally modified in a variety of ways. Expressed ubiquitously and with some level of functional redundancy in most vital tissues, only one of the four genes – *Atp2b2* – has been causally linked through naturally occuring mutations to disease in mammals: specifically to deafness and ataxia in spontaneous mouse mutants. In humans, a missense amino acid substitution in PMCA2 modifies the severity of hearing loss. Targeted null mutations of the *Atp2b1* and *Atp2b4* genes in mouse are embryonic lethal and cause a sperm motility defect, respectively. These phenotypes point to complex human diseases like hearing loss, cardiac function and infertility. Changes in PMCA expression are associated with other diseases including cataract formation, carciniogenesis, diabetes, and cardiac hypertension and hypertrophy. Severity of these diseases may be affected by subtle changes in expression of the PMCA isoforms expressed in those tissues

Keywords: calcium, PMCA, *ATP2B*, deafness, hearing, cochlea, vision, retina, sperm motility, embryonic lethality, cardiac, mouse mutants, human disease

1. INTRODUCTION

In this review, we will focus on the diseases caused by genetic mutations of the plasma membrane calcium (Ca^{2+}) ATPases (PMCA1 – PMCA4), encoded by distinct genes in mouse (*Atp2b1-Atp2b4*) and their orthologs (*ATP2B1 – ATP2B4*) in human. We will also cover diseases that are linked to the PMCA isoforms based on changes in their levels of expression. The reader is referred to several

E. Carafoli and M. Brini (eds.), Calcium Signalling and Disease, 365–383.

other reviews, each of which address various aspects of PMCA alternative splicing, biochemical regulation, and tissue-specific expression in greater detail (Penniston and Enyedi, 1998; Carafoli, 2002; Lehotsky et al., 2002; Shull et al., 2003; Prasad et al., 2004; Strehler and Treiman, 2004; Guerini et al., 2005; Oceandy et al., 2006; Withers et al., 2006).

2. PMCA STRUCTURE AND ALTERNATIVE RNAS

The amino acid sequences of the PMCA isoforms are highly homologous with each other (Strehler et al., 1991) and highly conserved in critical functional regions with other P-type ATPases (Kuhlbrandt, 2004). The recent crystal structure determination of the sarco-endoplasmic reticular Ca^{2+} ATPase (SERCA) in each of four conformational states provides a model on which to predict the consequences of mutations occuring in PMCA (Toyoshima et al., 2004; Ma et al., 2005; Moller et al., 2005). Critical regions defined by the SERCA structure include the membrane-spanning helixes (numbered 1–10, Figure 1), which bind two Ca^{2+} ions at residues in transmembrane domains 4, 5, 6, and 8. The cytosolic portion of the pump can be divided into four functional regions. The large cytosolic loop between transmembrane segments 4 and 5 contains the nucleotide binding (N-)domain, which divides the phosphorylation (P-)domain into two parts. The P-domain is the catalytic core of the pump, containing the signature D*KTGTLT amino acid sequence that includes

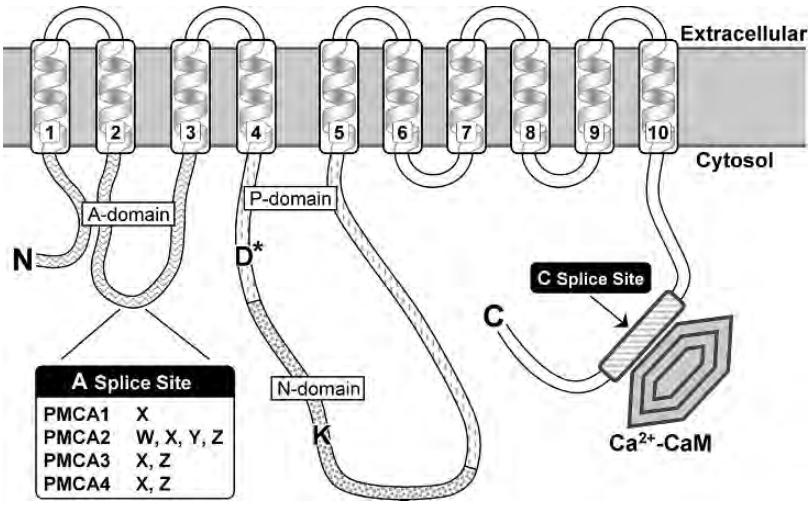

Figure 1. Schematic drawing of PMCA. Transmembrane regions are numbered 1–10. Domains defined by the SERCA structure are indicated by differential stippling. Within the A-domain alternative splicing occurs; predominant variants are indicated for each isoform (Z, X, Y and W indicate 0, 1, 2 and 3 exons included, respectively). Approximate positions of the phosophoryl-aspartate (D*) and the signature lysine (K) of the nucleotide-binding site are shown. In the C-terminal region, the Ca^{2+}-calmodulin binding site is boxed. Shorter "a" variants (not shown) at the C splice site have lower Ca^{2+}-calmodulin affinity

the phosphorylated aspartate (D*). The N-terminus and cytosolic loop between transmembrane domains 2 and 3 together form the actuator (A-)domain, which contains an invariant TGE sequence motif, important in stabilizing the transfer of the phosphate from ATP to the phosphorylatable aspartate. The pumping cycle is quite dynamic. Intramembranous binding of two Ca^{2+} ions and ATP binding at a conserved lysine (K) in the N-domain start the cycle; hydrolosis and formation of the phosphorylated aspartate is accompanied by swinging and twisting of the A-domain and a tethered piston-like movement of the transmembrane helixes results in transport of the two Ca^{2+} ions to the extracellular milieu in exchange for two hydrogen ions. The C-terminus provides regulatory functions including a Ca^{2+}-calmodulin binding site.

Each of the PMCA isoforms undergoes alternative splicing within the protein coding region at two sites (Strehler et al., 1991; Strehler and Zacharias, 2001). The C splice site lies in the C-terminal region and determines the affinity of PMCA for Ca^{2+}-calmodulin and the presence or absence of phosphorylation sites. The longer "b" isoform maintains an intact calmodulin binding site, which, in PMCA4, has been shown to have an intramolecular regulatory interaction with the catalytic core region (Padanyi et al., 2003). The regulatory functions of C-site splice variants have been studied biochemically in detail and recently reviewed (Guerini et al., 2005). Importantly, in an intact cell system expressing Ca^{2+} indicator probes, expression of each of the four PMCA isoforms showed that PMCA2 was the most effective isoform at controlling induced Ca^{2+} influx and that PMCA3 was more effective than either of the ubiquitously expressed isoforms, PMCA1 and PMCA4 (Brini et al., 2003). Surprisingly, the shorter C-splice site variants of PMCA3 and PMCA4, with significantly reduced calmodulin affinity, were equally effective at clearing Ca^{2+} as their corresponding longer variants with intact calmodulin binding sites. These results suggest that in intact cells, regulatory signals other than calmodulin binding may be primary in determining functional PMCA activity levels.

Splice site A lies coincidentally within the A-domain and adjacent to a phospholipid-sensitive basic region of PMCA. Studies in MDCK cells transfected with A-splice site variants of PMCA2 showed that inclusion of 3 alternative exons in the "w" form specifically targeted to the apical membrane (Chicka and Strehler, 2003). This work was extended recently to auditory and vestibular hair cell bundles of the rat where endogenously PMCA2w was the primary A-site variant detected, being sufficient to direct stereociliary (i.e. apical) localization in epitope-tagged transfection studies using variants at both A- and C-splice sites (Grati et al., 2006a; Hill et al., 2006). These studies identify PMCA2wa as the primary splice variant of PMCA2 localized to stereocilia, extending other studies that have shown the abundant and specific localization of PMCA2 to stereocilia in various species including human (Dumont et al., 2001; Wood et al., 2004; Grati et al., 2006b; McCullough et al.,2007).

While splicing of the open reading frame results in alteration of protien structure, differential promoter usage at the 5' end of the transcript can critically determine

tissue specific expression levels and may direct downstream splice site usage. Gene structures including transcriptional start sites have been studied in human *ATP2B1* and in mouse *Atp2b2* (Hilfiker et al., 1993; Silverstein and Tempel, 2006). In *ATP2B1*, a single 5' untranslated first exon was identified approximately 35 kb upstream from the ATG-containing exon. Similar single first exons are predicted for *Atp2b3* and *Atp2b4* based on information in the UCSC Genome Database. In contrast, four independent first exons spanning 200 kb were identified at the *Atp2b2* locus, each splicing into a common ATG-containing coding exon (Silverstein and Tempel, 2006). Two of the unique first exons were expressed in lactating mammary gland while the other two were abundant throughout the central nervous system as well as the cochlea. Using first exon specific probes, the two neuronal transcripts show differential expression in Purkinje and granule cells of the cerebellum, as well as in microdissected hair cell containing fractions of the cochlea. These results suggest that, in addition to the complex pattern of alternative splicing that occurs within the protein coding regions of each of the PMCA isoforms, additional transcriptional regulation is imposed at the level of unique first exons with independent promoter activities. Both forms of regulation could be affected by genetic changes that may contribute in turn to complex diseases discussed below.

3. PATTERNS OF PMCA EXPRESSION

As reviewed by Strehler and Zacharias (2001), PMCA1 and PMCA4 are expressed widely, being considered the "ubiquitous" isoforms. PMCA2 and PMCA3 have more limited expression, being referred to as the "neuronal" isoforms although PMCA2 is also expressed in heart, kidney, uterus, testis, and lactating mammary gland and PMCA3 is expressed in neonatal skeletal muscle (Strehler and Zacharias, 2001). Developmentally, all four PMCAs are detected in embryonic stem cells; PMCA1 is expressed strongly in various tissues during organogenesis while PMCA2 and 3 become prominent starting at embryonic day 12.5 in mouse (Zacharias and Kappen, 1999). In adult rodent brain, PMCA1 is expressed very broadly, but more strongly in olfactory bulb and hippocampus (Stahl et al., 1992). Expression of PMCA3 is especially strong in cerebellar granule cells and in the choriod plexus (Eakin et al., 1995). PMCA2 is expressed strongly in cerebellar Purkinje cells and granule cells although different promoters predominate in driving PMCA2 expression in these cells (Stahl et al., 1992; Silverstein and Tempel, 2006).

PMCAs are strongly and uniquely expressed in the visual and auditory peripheral nervous systems. All four PMCAs are expressed in the retina, but unique distributions between photoreceptor rods, cones and their respective bipolar cells suggest that specific cell types have unique requirements for calcium regulation. PMCA1 is expressed in photoreceptors, cone bipolar cells and horizontal cells; PMCA2 is expressed in inner segments and synaptic terminals of rod photoreceptors, rod bipolar and most retinal neuron types, but not in cones (Krizaj et al., 2002; Duncan et al., 2006). Given faster, stronger calcium clearance by PMCA2 (Brini et al., 2003), rods and their bipolar cells must need tight calcium regulation relative to

cones. In the auditory system, the expression and localization of PMCA has been studied extensively (Dumont et al., 2001; Wood et al., 2004). PMCA1 is expressed strongly in the stria vascularis and in the basolateral membranes of inner hair cells. Recent, rigorous studies showed that in stereocilia of rat vestibular and cochlear outer hair cells, the primary isoform found was PMCA2wa (3 exons included at the A-site; the short, low-affinity calmodulin splice alternative at the C-site) (Hill et al., 2006). The unique and precisely targeted expression of PMCAs suggests that specific isoforms are important to specific cellular functions. It follows that mutation or removal of a specific isoform may lead to functional loss, and in some instances to disease.

4. DISEASES OF THE UBIQUITOUS ISOFORMS, PMCA1 AND PMCA4

Because PMCA1 and 4 are expressed ubiquitously, they are often co-expressed in the same cell types and their roles in disease are studied in parallel. No spontaneous mutations of either gene have been identified in either mouse or human, so insight into the independent functions of these genes has come primarily from work on targeted null mutations generated in mice.

The phenotypes of *Atp2b1* and *Atp2b4* null mice have revealed the role that these proteins play in maintaining calcium homeostasis. Okunade et al. disrupted the genes encoding both PMCA1 and PMCA4 proteins by targeted deletion of the exon containing the catalytic phosphorylation site in each (Okunade et al., 2004). In heterozygous null mutants where one copy of either the PMCA1 or the PMCA4 gene product is lost, no overt phenotype was observed, despite the wide spread expression of both PMCA1 and PMCA4 (Greeb and Shull, 1989; Stauffer et al., 1993). However, loss of both copies of the gene encoding PMCA1 caused embryonic lethality. PMCA1 null embryos were observed before implantation, but did not survive the major period of organogenesis, dying before embryonic day 9.5 (Okunade et al., 2004). In contrast to PMCA1, PMCA4 null mice survived, and appeared normal and healthy. However, males were found to be infertile due to loss of sperm motility (Okunade et al., 2004; Schuh et al., 2004). Studies showed that PMCA4 null sperm contains only 10% of the PMCA compared to their wild-type littermates indicating that PMCA4 is the major isoform expressed in sperm. Additionally, calcium imaging revealed impaired calcium handling (Okunade et al., 2004) and immunostaining showed that PMCA4 is primarily located at the mid-section of the sperm's tail (called the principal piece), where Ca^{2+} flux is strongest (Okunade et al., 2004; Schuh et al., 2004). Ultrastructure analysis revealed that the only structural differences between wild type and PMCA4 null sperm was an increased percentage of condensed mitochondria, indicative of calcium overload (Okunade et al., 2004).

Besides sperm dysfunction, comparison of PMCA4 null 129/SvJ and Black Swiss mixed background mice revealed a strain specific smooth muscle phenotype, impaired phasic contractions and an apoptotic tendency for portal vein smooth

muscles (Okunade et al., 2004). When the PMCA4 null was transferred to a pure Black Swiss background, the phenotype was observed only if the mice were also heterozygous for the PMCA1 null. In summary, these studies on PMCA1 and PMCA4 null mice suggest a critical housekeeping role for PMCA1 during embryogenesis, and the sperm cell specific importance of PMCA4 for male fertility. These data also suggest PMCA4 may provide a target for non-hormonal male contraceptives. Finally, the loss of PMCA4 can lead to calcium overload and activity induced apoptosis in some tissues.

These studies suggest that the ubiquitously expressed forms of PMCA contribute to critcal cellular functions; loss of function causes embyronic lethality or male sterility. Perhaps more subtle changes – such as point mutations or changes in level of expression – would reveal a phenotype in one or more cell types. Here we review several processes in which PMCA expression plays a role. For example, cell proliferation (Lipskaia and Lompre, 2004) and apoptosis (Orrenius et al., 2003) are both sensitive to changes in cellular calcium levels. Aberrant PMCA expression has also been implicated in carcinogenesis, although the direction of the change can depend on the type of cancerous cell. Analysis of primary oral premalignant lesions (OPLs) and of oral squamous cell carcinomas (OSCCs) show a decrease in PMCA1 expression: by 40% in primary OPLs and by 43% in primary OSCCs where it has been shown that the promoter is hypermethylated (Saito et al., 2006). In contrast, groups studying breast cancer cell lines have reported an increase in the expression of PMCA1, 2 and 4. When human breast cancer cell lines were deprived of serum for 72h, PMCA1 expression increased as much as 270% compared to control mammary gland epithelial cells (Lee et al., 2002). Expression of PMCA2 and 4 increased 100-fold and 8-fold, respectively, in the ZR-75-1 breast cell cancer line as compared to the non-tumorigenic 184B5 cell line (Lee et al., 2005a). Yet more striking evidence for the role of PMCA in breast cancer was provided by anti-sense inhibition experiments. Using an inducible PMCA antisense expression construct to down-regulate expression of all PMCA isoforms in the MCF-7 breast cell cancer line, dramatic inhibition in cellular proliferation was observed along with a return to normal breast epithelial cell morphology for a subset of cells (Lee et al., 2005b). These findings suggest that PMCA expression levels may play a role in carcinogenesis. The contradictory changes of PMCA expression found in oral versus breast cancer cells reflect the ability of Ca^{2+} to promote cell cycle progression or apoptosis depending on its concentration within the cell.

In platelets, intracellular calcium is responsible for aggregation. Non-insulin-dependent diabetes mellitus (NIDDM) is a chronic disease that results in severe cardiovascular complications, in part due to platelet hyperactivity (Carr, 2001). Several groups have reported that patients suffering from NIDDM show elevated Ca^{2+} in various cell types including platelets (Balasubramanyam et al., 2001; Li et al., 2001). Indeed, platelets from NIDDM patients exhibited a significantly slower decay to resting calcium levels after Ca^{2+} release from intracellular stores was stimulated. A significant decrease in PMCA protein was found along with an increased level of tyrosine phosphorylation on residue 1176 (Rosado et al., 2004),

a transient phosphorylation event that inhibits the activity of the pump (Wan et al., 2003). These finding suggest that PMCA is involved in platelet activation and may contribute to the severe cardiovascular complications resulting from NIDDM.

Elevated calcium concentrations in the lens are known to cause cataracts, a clouding of the lens that results in severe visual impairment. The abnormal calcium level causes calpain activation, a calcium dependent protease that degrades lens proteins (Paterson et al., 1997). Membranes prepared from cataractous lens epithelium showed 50% of the Ca^{2+} ATPase activity of those prepared from clear lens epithelium, leading to the suggestion that decreased PMCA activity underlay cataract formation (Paterson et al., 1997). Recent studies on the UPL rat, a model for hereditary cataract formation, showed that PMCA expression and Ca^{2+} ATPase activity increased during cataract development, whereas ATP concentrations decreased significantly (Nabekura et al., 2004). Previous reports had shown that nitric oxide is involved in cataract development (Ito et al., 2001) and that nitric oxide is toxic to ATP production (Moncada and Erusalimsky, 2002), leading Nabekura et al. to suggest that increased NO synthase activity was responsible for cataract formation. Excess nitric oxide would decrease the ATP concentration within the cell, diminishing the ability of PMCA to effectively expel calcium. This would lead to the increased calcium levels that cause calpain activation. The observed upregulation of PMCA expression during cataract development may be a compensatory mechanism. Supporting this hypothesis are the observations that inhibitors of inducible nitric oxide synthase prevent opacification of the lens (Ito et al., 2001; Nabekura et al., 2003) and HLE B-3 human lens epithelial cells are capable of upregulating PMCA1 in a time and dose-dependent manner when treated with thapsigargin (Marian et al., 2007). Thus, although PMCA is involved in the process of lens opacification, it does not appear to be the primary cause of the disorder.

In cardiomyocytes, the Na^{+}/Ca^{2+} exchanger is the main mechanism for calcium extrusion. Despite its minor role in calcium homeostasis, important roles for PMCA in cardiac tissue have been suggested, including involvement in both hypertension and hypertrophy. The role of PMCA in hypertension has been primarily based on its ability to interact with nNOS. The importance of nNOS in the excitation contraction cycles of the heart and isolated cardiomyocytes has been demonstrated (Barouch et al., 2002; Sears et al., 2003). The specific inhibitory effect of PMCA4b on nNOS is mediated by PDZ binding. Inhibition was not observed when mutants of nNOS or PMCA4b that lacked their PDZ domains were tested. However, overexpression of PMCA4b containing an Asp672Glu mutation, which reduces activity of the pump by roughly 90%, did not result in nNOS inhibition. This suggests that inhibition of nNOS, a calcium/calmodulin dependent enzyme, is due to PMCA sequestering it in a low calcium environment (Schuh et al., 2001). More recently, another protein has been implicated in the PMCA-nNOS macromolecule. The catalytic loop of human PMCA1 and 4, the area between transmembrane regions four and five, has been reported to interact with α-1 syntrophin in cardiac cells (Williams et al., 2006). In addition, co-overexpression of both PMCA and α-1 syntrophin acted

synergistically to down-regulate nNOS. Clearly, the role of PMCA in hypertension warrants further investigation.

Sustained increases in intracellular calcium levels in cardiac myocytes result in hypertrophy, an increase in myocardial cell size associated with activation of fetal cardiac genes. A prolonged increase in intracellular Ca^{2+}-calmodulin is thought to contribute to cardiac hypertrophy by activating calcineurin, which dephosphorylates, thereby activating, the nuclear factor of activated T-cell (NFAT) transcription factors (Molkentin et al., 1998). Recent experiments show a direct role of PMCA in the Ca^{2+}/calmodulin-NFAT pathway. Buch et al. used NFAT transcriptional activity as a measure for calcineurin activity in HEK 293 cells overexpressing PMCA4b (Buch et al., 2005). The increased PMCA activity caused endogenous calcineurin to move from the cytoplasm to the plasma membrane and resulted in a 60% inhibition in NFAT transcriptional activity. These effects were reversible by co-expression of the catalytic loop of PMCA4b, which contains the calcineurin interacting site. They also reported that the PMCA4b mediated inhibition was lost in the presence of mutant calcineurin A, a Ca^{2+}/calmodulin-independent constitutively active protein. These findings support the hypothesis that PMCA4b recruits calcineurin to the plasma membrane, a low calcium environment, thereby inhibiting activation of NFAT. The role of PMCA in regulating the NFAT signaling pathway is becoming apparent. Further study in this area may provide insight into PMCA as a novel drug target in the treatment of cardiac disorders.

The function of PMCA 1 and 4 have been investigated in a variety of disorders. PMCA null mice revealed the crucial housekeeping function of PMCA1, and the requirement for PMCA4 in sperm motility. Additional studies suggest a role for PMCA4 in maintaining calcium homeostasis in smooth muscle cells. Research has also suggested that aberrant expression and/or activity of these PMCAs may play a role in many complex diseases: oral and breast cancers, cardiovascular complications associated with NIDDM, lens opacification, and cardiac hypertension and hypertrophy. Although improper regulation of PMCA1 and 4 may contribute quantitatively to complex disease phenotypes, to date there have been no reports linking changes in the nucleotide sequence of these genes to any human disease. Therefore, the exact role of these PMCAs in complex phenotypes is difficult to discern. More research will be required to determine if alterations in PMCA1 or PMCA4 expression play a primary role in these diseases, or if observed changes in levels of these PMCAs reflect compensatory responses. Independent of their status in terms of causality, the potential for PMCA1 or PMCA4 as a therapeutic target for drug therapy remains open.

5. DISEASES OF THE "NEURONAL" ISOFORMS, PMCA2 AND PMCA3

While PMCA2 and PMCA3 are expressed in some non-neuronal tissues, these isoforms are found predominantly in the central and peripheral nervous systems (CNS and PNS, respectively) (Strehler and Zacharias, 2001). The functional effects

of PMCA2 expression are best understood in auditory and visual sensory systems and in lactating mammary gland. Mutations in *Atp2b2* have been shown to affect each of these tissues and will be discussed in detail below. No mutations in *Atp2b3* (encoding PMCA3) have been reported, so the role of PMCA3 in disease is difficult to predict. Given the strong expression of *Atp2b3* in the choriod plexus of the brain (Eakin et al., 1995), one might anticipate an effect on the ionic composition of cerebral spinal fluid, affecting in turn either brain function or development. The generation of a null (KO) mutant allele of *Atp2b3* would help to address these questions.

In contrast, for *Atp2b2* (encoding PMCA2) several spontaneous as well as an induced null (KO) allele have been identified and studied extensively (Figure 2). The mutations identified in PMCA2 occur in several different structural regions as defined by alignment with the crystal structure determinations of the SERCA pump. Within the transmembrane regions, deaf waddler^{3J} (*Atp2b2*^{dfw3J}, referred to here as *dfw*^{3J}) causes a frameshift midway through transmembrane region 2 (McCullough and Tempel, 2004). Analogously, the targeted knockout, *Atp2b2*^{tm1Ges} (referred to here as *Atp2b2*-KO or just KO) was constructed to interrupt exon 19, which encodes transmembrane region 8 (Kozel et al., 1998). Both mutations cause there to be a lack of both mRNA and protein for PMCA2, resulting in phenotypes that include profound deafness and severe ataxia. The wriggle mouse Sagami (*Atp2b2*^{wri}, referred to here as *wri*) shows a similar phenotype, the result of a point mutation that causes an amino acid change from acidic glutamate to basic lysine in transmembrane 4 (Takahashi and Kitamura, 1999), at a residue thought to provide a charge balance for Ca^{2+} ions being transported across the membrane by the pump.

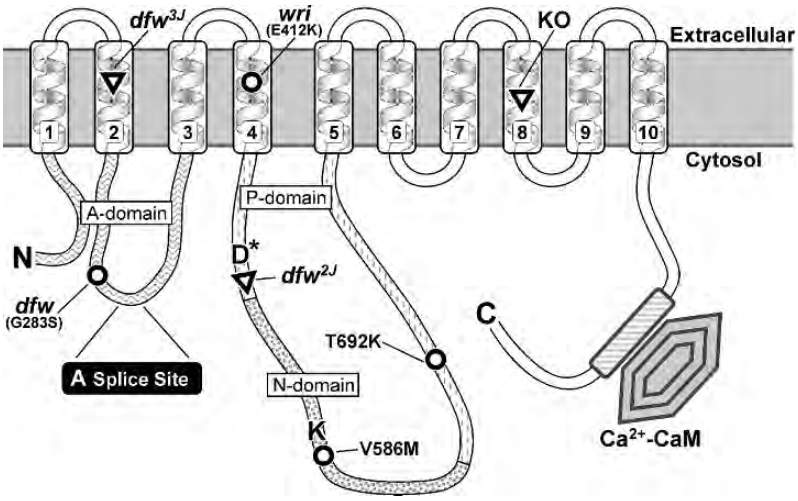

Figure 2. Mutations of PMCA2. Mutations are named as referred to in the text and shown on the structural schematic from Figure 1. Approximate locations of mutations are indicated by circles for point (missense) mutations or triangles for frameshift (truncation) mutations

Two mutations directly affect the phosphorylation (P-)domain, which is divided into two parts. In the first section of the P-domain lies the P-type ATPase signature sequence, D*KTGTLT, which contains the aspartate (D) phosphorylated during ATP-hydrolosis (Kuhlbrandt, 2004). In the deaf waddler^{2J} (*Atp2b2*^{dfw2J}, referred to here as *dfw*^{2J}), a 2-basepair deletion located only two residues beyond the D*, causes a frameshift (Street et al., 1998) that results in the generation of an aberant protein, which is degraded (McCullough and Tempel, 2004). In the second section of the P-domain, *Atp2b2*^{m1Mae} (referred to here as T692K) is a missense mutation that substitutes threonine with lysine (T692K), imposing a sterically larger and charged residue into the alpha-helix that supports the conserved loop 686-TGDN, which is part of the active site that catalyzes aspartyl-phosphorylation (Toyoshima and Mizutani, 2004). This mutation is of particular interest genetically because it arose spontaneously during clonal expansion of a targeted embryonic stem cell line in the production of a mutant allele of a nearby gene, the peroxisome proliferator-activated receptor gamma (*Pparg*) (Tsai et al., 2006). The mutation in *Atp2b2* was suspected based on the phenotypes of ataxia and deafness in the *Pparg* targeted mice. Direct sequence analysis identified a spontaneous mutation in *Atp2b2* hitchhiking with the targeted *Pparg* allele through the process of embryonic stem cell line selection. Each of the five mutations in *Atp2b2* discussed above result in a severe phenotype, presumably the consequence of a non-functional protein being expressed or a truncated protein being degraded, as has been shown directly for *dfw*^{2J}, *dfw*^{3J}, and the targeted KO allele.

In contrast, two mutations have more subtle effects on protein structure, stability and function. The deaf waddler mutant (*Atp2b2*^{dfw}, referred to here as *dfw*) carries a point mutation in the actuator (A-)domain, changing a very highly conserved glycine to a larger, polar serine (G283S) (Street et al., 1998). This substitution presumably disrupts the ß-sheet jelly-roll fold structure that is involved in positioning the invariant TGE sequence motif, found in all P-type pumps and known to move into close proximity of the phosphorylation site during the catalytic cycle (Kuhlbrandt, 2004). Biochemically, the G283S mutation in *dfw* reduces the Ca^{2+} transport activity to approximately 30% of that seen in wildtype PMCA2 (Penheiter et al., 2001). Genetically, the *dfw* mutation gives partial function, making it a hypomorphic allele. The homozygous *dfw*/*dfw* mutant would be expected to provide 30% activity while the heterozygous +/*dfw* mutant would provide approximately 65% activity (Figure 3). Anatomical data at both the light and transmission electron micrographic levels show that in 7 week old *dfw*/*dfw* mice inner hair cells (IHC) are present with intact afferent terminals while outer hair cells (OHC) – especially rows 2 and 3 – appear apoptotic and lack stereocilia (Dodson and Charalabapoulou, 2001). Spiral ganglion neurons are present but appear abnormal. In older (~18 month old) homozygotes OHC and IHCs are lost, many spiral ganglion neurons are also lost while those remaining have very few cytoplasmic organelles. Even second order neurons of the cochlear nucleus are reduced in size. In contrast, heterozygous +/*dfw* appear similar to control +/+ mice at 7 weeks; at 18 months no loss of OHC or IHC is observed although the cytoplasm of spiral ganglion cells is shrunken and

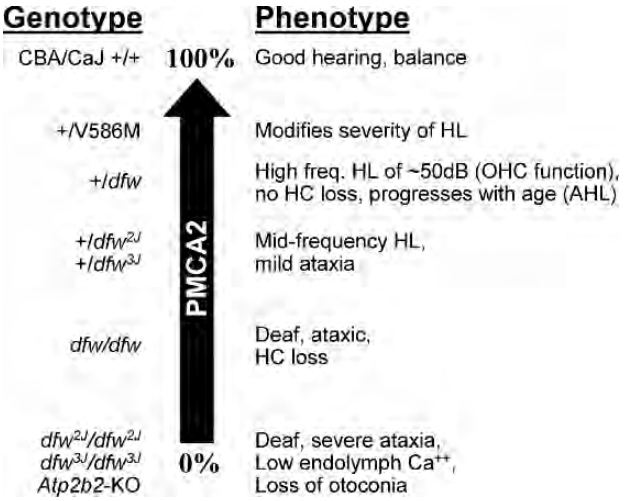

Figure 3. An allelic series of mutations showing differential severity of phenotype caused by different dosages of PMCA2 activity. Homozygous null mutations provide 0% activity; heterozygous null mutations provide about 50% activity; wild type strain CBA/CaJ has 100%

vacuolated. These data suggest that starting with OHC and progressing to IHC and neurons of the central auditory pathway, loss of PMCA2 activity to the 30% level in *dfw*/*dfw* mice leads to progressive cell death or cellular pathology.

A second hypomorphic allele of *ATP2B2* was discovered in a consanguineous family with autosomal recessive sensorineural hearing loss (Schultz et al., 2005). Among five affected siblings, two had hearing loss at high frequencies while the other three had severe to profound hearing loss at all frequencies. Genetic analysis showed linkage to chromosome 10q22.1 in all five affected individuals. The causative mutation was identified as a homozygous point mutation in cadherin 23 (*CDH23*), mutation of which is known to cause deafness in the waltzer mouse mutant (Di Palma et al., 2001) and Usher syndrome in human (Bolz et al., 2001). As for the different severities of hearing loss in the family, a modifier gene interaction was suspected based on previous studies in mice showing that the modifier of deafwaddler (*mdfw*) is *Cdh23* (Noben-Trauth et al., 2003). Schultz et al. examined the *ATP2B2* gene in the affected family and showed that individuals with severe to profound hearing loss at all frequencies had a point mutation, changing a valine to a bulkier methionine at residue 586 (referred to here as V586M). This mutation is only five amino acids away from the signature lysine (K) of the nucleotide-binding site; placement on the SERCA crystal structure suggests that V586M distorts the base of the nucleotide-binding pocket. Biochemically, V586M has approximately 50% of the Ca^{2+} pumping activity of wild type PMCA2 (Schultz et al., 2005). Because each of the severely affected individuals is heterozygous for V586M (of note, no instance of homozygosity was observed), we would predict that they have approximately 75% of wild type PMCA2 activity (Figure 3). This modest reduction

in PMCA2 activity does not cause hearing loss by itself as heterozygous carriers of V586M showed no hearing loss; only in the presence of a causative mutation in another hearing loss gene (e.g. *CDH23*) does V586M cause severe to profound hearing loss to occur.

In support of the idea that mutation of *ATP2B2* is associated with hearing loss in humans, a recent study shows that in hemizygous individuals (i.e. having only one copy of the *ATP2B2* gene due to a chromosomal deletion syndrome) severe hearing loss is observed when one copy of *ATP2B2* is missing (McCullough et al., 2007). Additionally, immunostaining for PMCA2 showed localization in human cochlear hair cell stereocilia similar to that observed in previous rodent studies.

The mutations available in *Atp2b2* provide the opportunity to study an allelic series in which the dosage of functional PMCA2 is reduced incrementally from 100% in littermate control animals to 0% in homozygous null mutants (Figure 3). Given the strong influence of genetic background on auditory function in mice, an allelic series is best studied with all alleles transferred to the same background by serial backcrosses to a good hearing inbred strain such as CBA/CaJ. Under these conditions, heterozygous $+/dfw$, $+/dfw^{2J}$ and $+/dfw^{3J}$ show haploinsufficiency with different ages of onset for both auditory and vestibulomotor dysfunction; homozygous mutants in the same background are deaf and severely ataxic (McCullough and Tempel, 2004). Wood et al. examined the Ca^{2+} concentration in endolymph, the unique extracellular solution with high potassium and low sodium that bathes the stereocilia of auditory and vestibular hair cells, finding Ca^{2+} to be significantly reduced in homozygous null dfw^{2J}/dfw^{2J} mice (6.6 ± 0.6 μM Ca^{2+} in dfw^{2J}/dfw^{2J} vs 22.9 ± 3.5 μM Ca^{2+} in $+/+$ controls). Immunocytochemical studies on dfw^{2J}/dfw^{2J} mice reveal an up-regulation and re-localization of PMCA1 and PMCA4 to the stereocilia, indicating that these compensatory changes are not sufficient to restore hearing or normal Ca^{2+} concentration to the endolymph (Wood et al., 2004). Homozygous null alleles have additional deficits including the loss of otoconia, the calcium carbonate crystals that overlay the hair cells in the vestibular system and provide inertial momentum (Kozel et al., 1998). This loss of otoconia may be secondary to the reduced Ca^{2+} concentration in the endolymph. In sum, the allelic series of deaf waddler mice suggests that slight reductions in PMCA2 function impair high frequency hearing while elimination of PMCA2 causes profound deafness, a loss of Ca^{2+} in the endolymph and potential secondary effects including loss of hair cells (Dodson and Charalabapoulou, 2001) and otoconia.

As discussed earlier, PMCA2 is expressed in the retina, predominating in synaptic terminals of rod photoreceptors and rod bipolar cells, present in most retinal neuron types, but absent from cones (Krizaj et al., 2002; Duncan et al., 2006). Anatomically, at the light microscopic level, the retinas of dfw^{2J}/dfw^{2J} mice are indistiguishable from $+/+$ littermate controls throughout development. Electrophysiological recordings from the outer segment of the retina show no difference in the photosensitivity or amplitude of light responses between dfw^{2J} mutants and controls. However, rod-mediated electroretinogram (ERG) responses are ~45% smaller and

significantly slower in *dfw*^{2J} mutants. Further, recordings from individual rod bipolar cells show that the sensitivity of scotopic (i.e. dim light, rod-mediated) light-evoked synaptic transmission is reduced by ~50% in *dfw*^{2J} mice (Duncan et al., 2006). These data suggest that the synapse between rod terminals and bipolar cells is the primary site of the loss in sensitivity. As PMCA2 is localized to both the presynaptic rod terminal and the postsynaptic rod bipolar cell, it should provide high efficiency Ca^{2+} extrusion, potentially increasing the efficacy of neurotransmission at the synapse and providing a post-transduction mechanism for increasing the gain in the scotopic rod pathway (Duncan et al., 2006). Thus, the absence of PMCA2 from the retina causes no change in cytoarchitecture during development or with aging, but affects the synaptic signaling between rods and rod bipolar cells.

PMCA2 is expressed broadly in the CNS as well as in other tissues, notably in lactating mammary glands where, in rat, the splice variant PMCA2bw is localized on the apical membrane of the secretory cells (Reinhardt et al., 2000). As lactation starts, PMCA2bw mRNA and protein increase dramatically (~100-fold), in parallel with increased milk production. Milk from *Atp2b2*-KO null mutant mice had 60% less calcium than milk from either heterozygotes or wild type controls (Reinhardt et al., 2004). In support of this strong induction of PMCA2 in rodent mammary, rats and mice have evolved unique first exon and promoter regions that drive expression in mammary gland (Silverstein and Tempel, 2006). This strong, inducible mechanism for enriching calcium with lactation may be necessary to provide the relatively high levels of Ca^{2+} found in rodent milk or to accommodate the relatively short gestation time and large litter size typical of rodents. These promoter regions are not well conserved in other mammalian species (Silverstein and Tempel, 2006), although as discussed above, *Atp2b2* mRNA is expressed in multiple human breast cancer cell lines (Lee et al., 2002). Further studies would be necessary to determine if PMCA2 is regulated strongly in primate lactation.

A number of studies have addressed the expression and regulation of specific PMCA isoforms in specific regions of the CNS. For example, PMCA2a is found to be enriched in forebrain synaptosomes and co-localizes with pre-synaptic markers in the CA3 region of the hippocampus (Jensen et al., 2006). Pharmacological inhibition of PMCA causes an increase in the frequency of mini-excitatory post-synatpic currents (mEPSCs), presumably because of a failure to clear calcium rapidly in the presynaptic terminal. The relationship of fast PMCA2a-mediated calcium clearance in the hippocampus to behavior or disease is unclear at present, but may relate to epilepsy or aging in the CNS.

PMCA2 is expressed strongly in Purkinje neurons of the cerebellum (Stahl et al., 1992). In the *wri* mutant, development of the Purkinje cell dentritic trees is impaired; synaptic connections of parallel fibers on dendritic spines of Pukinje cells are decreased at two weeks of age, but by nine weeks malformed synaptic boutons lacking synaptic membrane specializations are significantly increased (Inoue et al., 1993). These developmental abnormalities in synaptic structure of the cerebellum likely contribute to the ataxic behavior observed in *wri* and other *Atp2b2* mutants.

A similar approach has used the *Atp2b2*-KO to study the development of the neocortex in congenitally deaf mice (Hunt et al., 2006). In the absence of auditory input, the size and modality of cortical fields became mixed with other sensory fields. Neurons in the normal "auditory cortex" region responded to somatosensory and/or visual stimulation; primary visual cortex increased in size and contained neurons responding to somatosensory stimulation. These changes correlate with decreased auditory input. However, in light of the synaptic changes induced by changes in PMCA2 function within the CNS just discussed, one wonders how sensory cortex might be altered with normal auditory input, but with a genetically targeted local deficit of *Atp2b2* function in the neocortex. These questions point to the emerging role of PMCAs as important modulators of calcium levels within cells and subcellular regions of cells throughout the organism.

6. SUMMARY AND PERSPECTIVES ON PMCAS AND DISEASE

The PMCA family of pumps export calcium from the cell, being in a sense, a final mediator of net cellular calcium load. Four genes (*Atp2b1 – Atp2b4*) exist in mammals giving rise to four proteins (PMCA1 – PMCA4), each of which is alternatively splicing at two sites. PMCA1, 2 and 4 have each been implicated or directly proven through the occurance of spontaneous mutations to cause disease in mice and man. For PMCA1 and PMCA4 the only extant mutations are targeted null mutations. Male mice lacking PMCA4 are infertile, the result of a failure in sperm motility. The PMCA1 null is embryonic lethal, implicating a critical role in organogenesis, but preventing an analysis of the role of PMCA1 in cellular functions in mature animals. To study the functions PMCA in mature animals, conditional null alleles for PMCA1 and PMCA4 as well as for PMCA3 should be developed, allowing normal development to occur with the ability to turn off the gene in a time and tissue-specific manner.

PMCA2 plays a clear role in hearing loss and ataxia, as displayed in varying degrees of phenotypic severity by mutants with varying degrees of loss of function of the PMCA2 protein (Figure 3). This allelic series includes null alleles producing no functional protein and hypomorphic alleles whose protein product retains partial Ca^{2+} pumping activity. Of the seven different mutations known in the *Atp2b2* gene, three affect transmembrane regions, two affect the phosphorylation (P)-domain and one affects each of the actuator (A)-domain and the nucleotide binding (N)-domain. Interestingly, the two hypomorphic alleles – one discovered in deaf waddler mice and one in human families with hearing loss – are each point mutations, occurring in the A- and N-domains, respectively, and altering slightly the protein structure adjacent to invariant signature sequences involved in catalysis of ATP at the core of the pump. These mutations suggest that even a slight reduction in PMCA2 function results in hearing loss, probably a reflection of the unique localization of PMCA2 in the stereocilia where it provides the primary means for fast calcium clearance after Ca^{2+} ions enter the stereocilia during sound transduction. In contrast, only a

severe mutation of PMCA2 causes decreased sensitivity and slowing of scotopic visual signals in the rod pathway of the retina.

While a clear causitive role has been established for PMCA2 in hearing loss, further studies on hypomorphic alleles could address their effect on age-related or noise-induced hearing loss. Generating a mouse model of the human V586M mutation should provide an even less affected hypomorph for further studies.

The more subtle roles of different PMCAs in cardiac and sperm function, synaptic signaling in retina and hippocampus, etc. suggest that these are not housekeeping genes, rather they provide an important function in many cell types and tissues. It seems likely that changes in each of the PMCA isoforms may contribute quantitatively to a number of complex diseases in ways yet to be discovered.

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CHAPTER 14

DISEASES INVOLVING THE GOLGI CALCIUM PUMP

J. VANOEVELEN, L. DODE, L. RAEYMAEKERS, F. WUYTACK AND L. MISSIAEN

Laboratory of Physiology, KULeuven Campus Gasthuisberg O&N1, Herestraat 49 bus 802, B-3000 Leuven, Belgium

Abstract: Secretory-pathway Ca^{2+}-transport ATPases (SPCA) provide the Golgi apparatus with Ca^{2+} and Mn^{2+} needed for the normal functioning of this organelle. Loss of one functional copy of the human SPCA1 gene (*ATP2C1*) causes Hailey-Hailey disease, a rare skin disorder characterized by recurrent blisters and erosions in the flexural areas. Here, we will review the properties and functional role of the SPCAs. The relationship between Hailey-Hailey disease and its defective gene (*ATP2C1*) will be adressed as well

Keywords: SPCA, *ATP2C1*, *ATP2C2*, Hailey-Hailey disease, Golgi apparatus

1. INTRODUCTION

Intracellular Ca^{2+} stores release Ca^{2+} in the cytosol to control a whole range of physiological processes depending on the amplitude, frequency and subcellular localization of the rise in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) (Berridge, 1993). The Ca^{2+} concentration in the lumen of the store ($[Ca^{2+}]_l$) may however not become too low during this release process, since a sufficiently high $[Ca^{2+}]_l$ is needed for the normal synthesis, chaperone-dependent processing, glycosylation, sorting and eventual breakdown of newly formed proteins (Chanat and Huttner, 1991; Oda, 1992; Carnell and Moore, 1994; Meldolesi and Pozzan, 1998). The endoplasmic reticulum is the major agonist-sensitive intracellular Ca^{2+} store (Berridge, 2002). Experiments using aequorin targeted to the Golgi apparatus however revealed that also the Golgi complex loses Ca^{2+} during agonist stimulation (Pinton *et al*., 1998).

Ca^{2+} uptake in the Golgi apparatus is mediated by two groups of Ca^{2+} pumps: the sarco(endo)plasmic-reticulum Ca^{2+}-transport ATPases (SERCA) and the secretory-pathway Ca^{2+}-transport ATPases (SPCA) consisting of SPCA1 and SPCA2. SPCA1 is expressed in all cells (Vanoevelen *et al*., 2005), whereas SPCA2 is only present in

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a limited number of cell types (Xiang *et al*., 2005; Vanoevelen *et al*., 2005). SPCAs in addition transport Mn^{2+} (Mandal *et al*., 2000a; Van Baelen *et al*., 2001), which is a cofactor in many enzymatic reactions in the lumen of the Golgi apparatus. We will review the properties of the SPCAs and highlight the differences with the SERCAs.

Haploinsufficiency of human SPCA1 results in Hailey-Hailey disease (HHD) (Hu *et al*., 2000; Sudbrak *et al*., 2000), which highlights the critical role of this housekeeping Ca^{2+} and Mn^{2+} pump in cellular ion homeostasis. We will review the properties of this mutated SPCA1 and discuss how SPCA1 contributes to the physiology of the normal skin.

2. SPCAS

SPCAs, like the SERCAs and the plasma-membrane Ca^{2+}-transport ATPases (PMCAs), belong to the family of P-type ATPases. The name "P-type" refers to the transient formation of a high-energy covalent bond between the γ-phosphate of ATP and an aspartyl residue in a highly conserved phosphorylation motif during the catalytic cycle. SPCAs are, together with SERCAs, classified as type P_{2A} enzymes while PMCAs belong to the P_{2B} subfamily (Figure 1). The P_2-subfamily

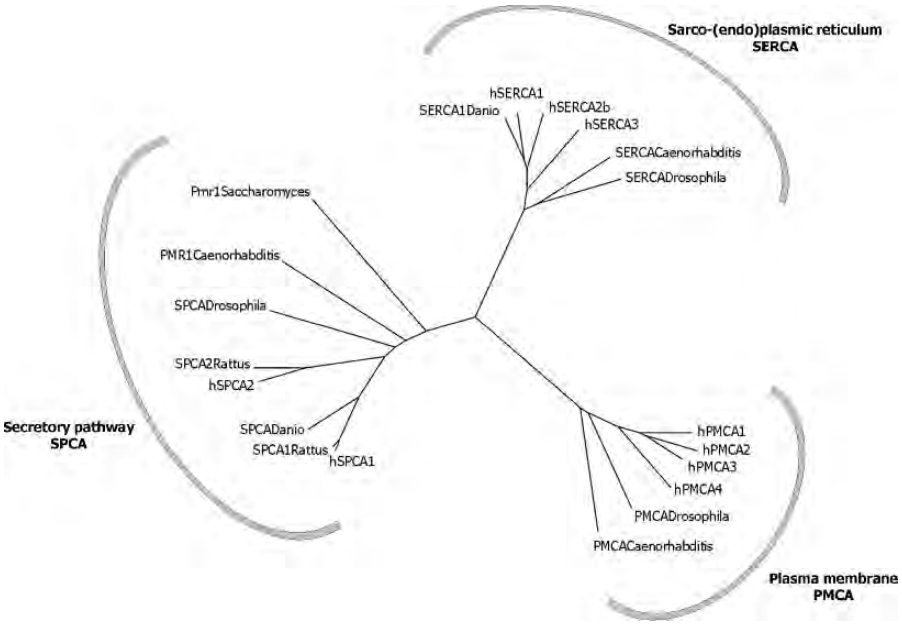

Figure 1. Phylogenetic tree of P_{2A} and P_{2B} subfamilies of Ca^{2+}-transporting ATPases. Protein sequences of P-type ATPases of different species were aligned using ClustalW software and a phylogenetic tree was generated using TreeView. Three different branches representing the three main subtypes of Ca^{2+}-ATPases are apparent: SERCAs, PMCAs and SPCAs

is completed by the Na^+,K^+-ATPases and gastric H^+,K^+-ATPases (for complete classification, see http://www.patbase.kvl.dk/).

2.1. Gene Structure and Alternative Splicing

Pmr1 (for plasma-membrane ATPase-related) of *Saccharomyces cerevisiae* was the first intracellular Ca^{2+}-ATPase that was identified as a member of the SPCA family (Rudolph *et al*., 1989; Antebi and Fink, 1992; Sorin *et al*., 1997). There are no introns in the coding region, like for most genes in *S. cerevisiae*. The Pmr1 protein (GenBank™ accession no. AAA34884) comprises of 950 amino acids and has a molecular weight of 104 kDa.

The SPCA gene in the nematode *Caenorhabditis elegans* consists of 12 exons (Van Baelen *et al*., 2001). Its transcripts are alternatively spliced and, like it is often the case in *C. elegans*, *trans*-spliced at their 5'-end. Despite the presence of three alternative polyadenylation signals within the 3'-terminus, no alternative splicing of the pre-mRNA could be demonstrated at this end. SPCA gene transcription in the worm leads to a single SPCA protein (GenBank™ accession no. CAC19896) with 901 amino acids.

The SPCA gene in *Drosophila melanogaster* (*SPoCk*) (GenBank™ accession no. AC014929) contains only 3 intervening sequences separating the 4 exons indicating that several introns have been lost during evolution. Three alternative splice variants have been described containing 901 (SPoCk-A), 1034 (SPoCk-B) or 924 (SPoCk-C) amino acids (Southall *et al*., 2006).

The first mammalian member of the SPCA family was cloned from rat but could not be characterized functionally at that time (Gunteski-Hamblin *et al*., 1992). The corresponding human gene (*ATP2C1*) was described by Hu *et al*. (2000) and Sudbrak *et al*. (2000). *ATP2C1* is localized on the *q* arm of chromosome 3 at position 24.1. Its exon/intron layout is more complex than in *C.elegans* and *D. melanogaster*. The gene consists of 28 exons. Only five intron/exon boundaries are conserved between the human and worm genes and only two between the human and fly genes. Initiation of *ATP2C1* transcription in human HaCaT cells (a keratinocyte derived cell line) depends on 2 critically important segments, namely the –347 to –311 and +22 to +76 DNA stretches located in a rather GC-rich region (Kawada *et al*., 2005). Computer-assisted sequence analysis of the 5'-flanking region pointed to the absence of a canonical TATA box and the existence of highly conserved *cis*-acting binding sites for several transcription factors. YY1 and Sp1 factors, when overexpressed, transcriptionally activate the *ATP2C1* promoter in normal human keratinocytes.

Alternative processing at the 3'-end of the human *ATP2C1* pre-mRNA results in four transcripts with C-termini differing in length and amino-acid sequence (Fairclough *et al*., 2003) as shown in Figure 2. Isoforms *ATP2C1a* and *ATP2C1c* are generated by the coupling of exon 26 to exon 27 or to exon 28 respectively. Exon 27 contains two 5'-internal splice-donor sites D_1 and D_2. The transcript *ATP2C1b*

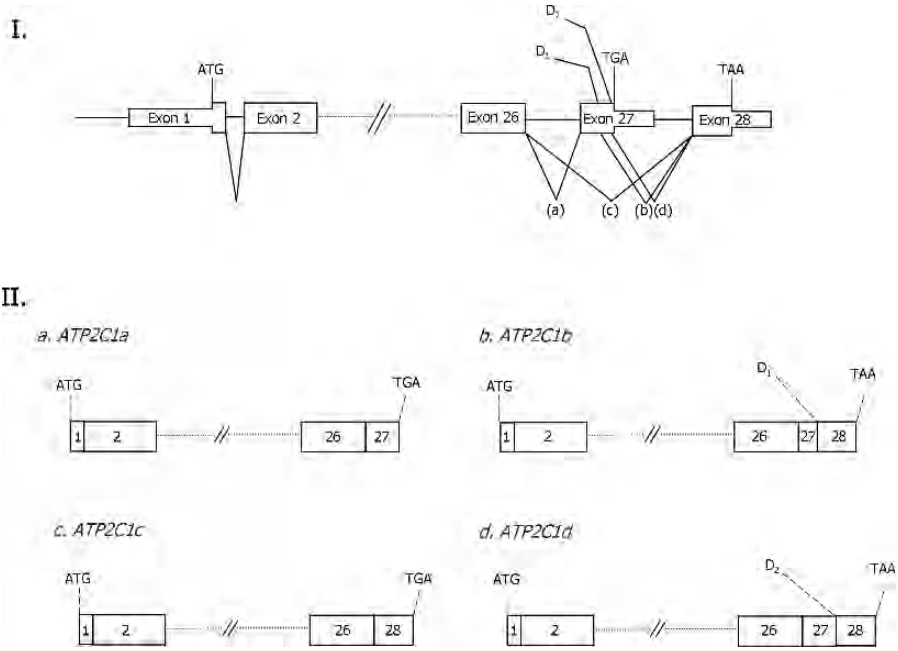

Figure 2. Gene structure of ATP2C1 and alternative splicing. I. Shows the structure of the ATP2C1 gene. Exons are represented by boxes, with wide boxes depicting the open reading frame. The thin horizontal line represents the position of introns. Diagonal lines illustrate the splicing patterns generating isoforms ATP2C1a-d. II. Structure of the different ATP2C1 splice variants: a. Isoform a results from the splicing of exon 26 to exon 27; b. Isoform b is the product of cryptic splicing at D_1; c. Exon 26 splices directly to exon 28 to produce isoform c; d. Activation of splicing at cryptic site D_2 produces isoform d

contains exon 28 and the partial portions of exon 27 up to the D_1 splice-donor site. Transcript *ATP2C1d* contains exon 28 and the partial portions of exon 27 up to the D_2 splice-donor site.

The resulting SPCA1 isoforms contain 919 (SPCA1a), 939 (SPCA1b), 888 (SPCA1c) and 949 amino acids (SPCA1d). Human SPCA1d exhibits 49% and 59% overall amino-acid sequence identity with, respectively, SPCA1 from *S. cerevisiae* and *C.elegans*.

A second human SPCA gene (*ATP2C2*) codes for SPCA2 (Xiang *et al*., 2005; Vanoevelen *et al*., 2005). *ATP2C2* is found at cytogenetic position q22.1 on chromosome 16. Corresponding SPCA2 genes are also present in birds, amphibians and mammals, but not in fish. *ATP2C2* consists of 27 exons. The intron/exon organizations of *ATP2C2* and *ATP2C1* are similar with conserved junctions at the interface between the constitutively spliced exons. They do not resemble those of SERCAs or PMCAs. The *ATP2C2* primary transcript is not known to be alternatively processed and, therefore, only one SPCA2 isoform of 946 amino acids is generated. Human SPCA1 and SPCA2 exhibit 64% amino-acid sequence identity.

Since the transcriptional activation and regulation of the *ATP2C2* promoter is so far unknown, the restricted tissue and cellular expression pattern of *ATP2C2* cannot be explained at the moment.

2.2. SPCA Proteins

Like for all P-type ATPases, the SPCA proteins are composed of an actuator (A), a nucleotide-binding (N) and a phosphorylation (P) domain together making up the large cytosolic head of the molecule as well as 10 hydrophobic helices (M1-M10) forming the membrane-spanning domain.

The amino acid sequence of some subdomains is highly conserved between SPCAs and SERCAs. The phosphorylation domain contains the typical SDKTGTLT sequence for P-type ATPases. The phosphorylated residues are Asp^{371}(*S. cerevisiae* PMR1), Asp^{336} (*C. elegans* PMR1), Asp^{333} (*D. melanogaster* SPCA1), Asp^{350} (human SPCA1) and Asp^{379} (human SPCA2). Mutation of $Asp^{371}$to Glu or Asn in yeast led to SPCA mutants without Ca^{2+}-transport activity, despite normal expression and proper targeting to the Golgi apparatus (Sorin *et al*., 1997). The D^{601}PPR loop connecting the N and P domains in SERCA1a is conserved as D^{546}PPR and D^{576}PPR in SPCA1 and SPCA2 respectively. The T^{189}GET and T^{220}GEA loops in domain A in, respectively, human SPCA1 and SPCA2, which correspond to the T^{181}GES loop in SERCA1a, are also highly conserved. The latter 4 amino-acid-long loops must move upwards to contact the catalytic site during dephosphorylation of the phosphoenzyme intermediate E_2-P.

Some structural differences between SPCAs and SERCAs should be highlighted. The SPCA1 peptide chain is shorter and the corresponding protein more compact than SERCA1a. In particular the luminal segments linking the membrane-spanning helices M1 and M2, M3 and M4, and M7 and M8 are shorter in SPCA1. Other loops in the N domain of SERCA1a are not conserved in SPCA1. The N-terminus, which is part of domain A, is longer in SPCA1 than in SERCA1. It contains a Ca^{2+}-binding EF hand-like motif, which is different from the actual ion-binding and transporting site located in the M domain. This motif modulates ion transport in *S. cerevisiae* (Wei *et al*., 1999). Our own unpublished Ca^{2+}-overlay experiments indicate that the EF hand-like motif in human SPCA1 also binds Ca^{2+}.

SERCA1 contains two Ca^{2+}-transport sites (site I and site II) (Clarke *et al*., 1989; Andersen, 1995; Toyoshima *et al*., 2000). Only site II is conserved in SPCA, like in PMCA (Glu^{329}, Asn^{774} and Asp^{778} in yeast SPCA, Glu^{308}, Asn^{738} and Asp^{742} in human SPCA1, and Glu^{337}, Asn^{768} and Asp^{772} in human SPCA2). Analysis of mutant SPCA pumps confirmed the importance of site II for the transport of Ca^{2+} and Mn^{2+} in yeast (Wei *et al*., 2000). The Hill coefficients (close to 1 and clearly lower than those in SERCA) deduced from the Ca^{2+} titration of phosphorylation also support the presence of a single ion-binding site per SPCA molecule (Dode *et al*., 2005). All these findings suggest that SPCAs transport only one Ca^{2+} ion per each hydrolysed ATP.

In contrast to SERCA1, SPCAs in yeast (Mandal *et al*., 2000a), worm (Van Baelen *et al*., 2001) and man (Fairclough *et al*., 2003; Xiang *et al*., 2005; Vanoevelen

et al., 2005) can also transport Mn^{2+} with high affinity. This function in the yeast SPCA depends on critical packing interactions at the cytoplasmic interface between the side chains of Gln^{783} in M6 and Val^{335} in M4 (Mandal *et al*., 2000a,b). These residues are conserved in the human SPCA1 as Gln^{747} and Val^{314}, and in human SPCA2 as Gln^{777} and Val^{343}. The Gly^{309}-residue in M4 of human SPCA1 (corresponding to Gly^{330} in yeast SPCA and Gly^{338} in human SPCA2) may also be important for Mn^{2+} binding at the cytoplasmic side (Fairclough *et al*., 2003).

Some differences between human SPCA1 and SPCA2 should be stressed. As already mentioned both proteins show 64% overall amino-acid sequence identity. The N-terminus of human SPCA2 is longer (32 amino acids) than in human SPCA1 (Xiang *et al*., 2005) and, as part of the A domain, may be involved in intramolecular conformational rearrangements altering ion affinity as proposed for the defective copper-ATPases typical of Wilson and Menkes diseases (Huster and Lutsenko, 2003). The EF hand-like motif, which in SPCA1 binds Ca^{2+}, is less conserved in human SPCA2 (C^{70}VDLHTGLSEFS, where the conserved residues are underlined) and apparently lost its Ca^{2+}-binding capacity. The C-terminus of SPCA2 is more divergent relative to SPCA1a. It contains a dileucine (L^{925}L) motif, which may be a retrieval signal from the plasma membrane (Xiang *et al*., 2005). Moreover, its extreme C-terminal P^{943}EDV stretch might function as a PDZ-binding motif (Heilker *et al*., 1996; Wang *et al*., 2004). These two C-terminal motifs could be involved in localization and shuttling of SPCA2 between the stacks of the *trans*-Golgi network, vesicles derived from this network, and even the plasma membrane.

2.3. Tissue and Cellular Expression

SPCA1 is a housekeeping enzyme and is therefore expressed in all cells (Vanoevelen *et al*., 2005). Results on the relative expression level in various tissues are still conflicting. Expression levels, both at the mRNA and at the protein level, in rat brain and testis were much higher than in other tissues like lung and liver (Wootton *et al*., 2004). Such relatively high expression in brain and testis was not observed at the mRNA level in humans (Vanoevelen *et al*., 2005). The cell-type dependent expression of SPCA1 in various animal species thus deserves further study.

SPCA1 is highly expressed in human epidermal keratinocytes (Hu *et al*., 2000). The epidermis consists of undifferentiated keratinocytes in the basal layer that develop into the differentiated cells of the more superficial spinous, granular and cornified layers (Eckert *et al*., 1997). Yoshida *et al*. (2006) reported a specific localization of SPCA1 in the basal layer, with low expression levels in differentiated keratinocytes in suprabasal layers. They correlated the preferential localization of SPCA1 in the undifferentiated cells of the basal layer with the induction of differentiation markers upon suppression of SPCA1 expression. This effect could be mimicked by addition of the ionophore Br-A23187, which has a higher affinity for Mn^{2+} than for Ca^{2+}, but not with the classical Ca^{2+} ionophore A23187. As a possible link between Mn^{2+} and differentiation, the authors suggest the requirement of integrin-dependent binding processes for Mn^{2+}

(Banères *et al*., 2000), considering the orchestrating role integrin-mediated signaling plays in the regulation of keratinocyte differentiation (Levy *et al*., 2000). It should however be remarked that these results on the preferential localization of SPCA1 in the undifferentiated cells of the basal layer contrast with the immunohistochemical data of Porgpermdee *et al*. (2005) and with our own unpublished observations, showing a clear labeling of both basal and suprabasal cells.

SPCA2 expression is more restricted than that of SPCA1. In mammals the mRNA for SPCA2 is abundant in the various segments of the gastrointestinal tract (Vanoevelen *et al*., 2005; Dmitriev *et al*., 2005). Its mRNA is also found to be enriched in trachea, salivary gland, thyroid, keratinocytes, prostate, mammary gland, brain and testis (Vanoevelen *et al*., 2005; Xiang *et al*., 2005). SPCA2 levels in the mammary gland increase during lactation (Dmitriev *et al*., 2005). Together with the observation that SPCA2 is mainly expressed in the mucus-secreting goblet cells in human colon (Vanoevelen *et al*., 2005), these data suggest a role of SPCA2 in the regulated secretory pathway. There is now an urgent need to complement the SPCA2 mRNA expression pattern with an analysis at the protein level.

2.4. Subcellular Localization

Antebi and Fink (1992) localized the yeast SPCA in the Golgi compartment. The SPCA from *C. elegans* heterologously expressed in COS-1 cells (Van Baelen *et al*., 2001; Missiaen *et al*., 2002) and the human SPCA1 overexpressed in CHO cells (Ton *et al*., 2002) also appeared in the Golgi area. These findings on overexpressed pumps were later confirmed for the endogenous SPCA1 in cultured cells and tissues. SPCA1 in rat liver cosedimented with the Golgi-localized Ca^{2+}-binding protein CALNUC (Reinhardt *et al*., 2004). Immunofluorescence microscopy of the endogenously expressed SPCA1 in HeLa cells (Van Baelen *et al*., 2003) and in PC12 cells (Reinhardt *et al*., 2004) also revealed a Golgi-like distribution. Intruigingly, the different splice variants of the *Drosophila* orthologue (SPoCk) all reside in a different subcellular compartment. The SPoCk-A variant is targeted to the Golgi apparatus while the SPoCk-B isoform is located in the membrane of the endoplasmic reticulum. Finally, the peroxisomes are equipped with the third splice variant, SPoCk-C (Southall *et al*., 2006). This contrasts with the situation in human where, with the notable exception of SPCA1c which is an inactive isoform retained in the endoplasmic reticulum for degradation, SPCA1a, -b, -c all are sent to the Golgi apparatus (Dode *et al*., 2005).

Ca^{2+} uptake in the Golgi apparatus is partially inhibited by low concentrations of thapsigargin, suggesting that also SERCAs contribute to the Ca^{2+} uptake in this compartment (Taylor *et al*., 1997; Pinton *et al*., 1998; Lin *et al*., 1999; Van Baelen *et al*., 2003; Vanoevelen *et al*., 2004). The relative contribution of SERCA and SPCA pumps to the uptake of Ca^{2+} into the Golgi is cell-type dependent. Among various cultured cells, the highest SPCA-dependent Ca^{2+} uptake (about 67%) was observed in human keratinocytes (Callewaert *et al*., 2003).

Because SERCAs are typically found in the endoplasmic reticulum and SPCAs in the downstream compartments of the secretory pathway, it is tempting to speculate that the SERCAs are preferentially localized in the upstream *cis*- and *medial*-Golgi. Behne *et al*. (2003) indeed observed an enrichment of SERCA and SPCA1 in, respectively, *cis*- and *trans*-Golgi membranes isolated from human keratinocytes. The differential localization of the Ca^{2+} pumps to either the proximal or distal regions of the secretory pathway may be determined by the cholesterol gradient. The cholesterol concentration is low in the endoplasmic reticulum and increases in the distal direction of the secretory pathway. Membranes of the *trans*-Golgi compartments are therefore thicker and more rigid. Both parameters are important determinants of Ca^{2+}-ATPase function and possibly of their localization as well (Li *et al*., 2004). A localization in the *trans*-Golgi implicates that SPCA1 may also appear in the more distal parts of the secretory pathway. Subcellular fractionation of ß-cell-derived lines indeed revealed SPCA1 immunoreactivity in fractions enriched in dense-core secretory vesicles (Mitchell *et al*., 2004).

SPCA1 in human spermatozoa is localized in an area behind the nucleus which extends into the midpiece (Harper *et al*., 2005). SPCA1 may represent the only intracellular Ca^{2+} pump in these cells because no evidence for the presence of SERCAs could be obtained.

There are only few reports on the subcellular localization of SPCA2 and no clear picture emerges from them. SPCA2 in human goblet cells colocalizes with Golgi markers in a compact structure near the apical pole of the nucleus (Vanoevelen *et al*., 2005). These observations contrast with those on cultured mouse hippocampal neurons, which show a punctate distribution of SPCA2 in the cell body and in the dendrites, with a partial colocalization with the *trans*-Golgi marker TGN38 (Xiang *et al*., 2005). SPCA2 in hippocampal neurons may therefore be localized in post-Golgi segments of the secretory pathway.

2.5. Functional Properties

SPCAs reversibly cycle between an E_1- and E_2-conformation, in line with the current E_1/E_2-catalytic model of P-type ATPases (De Meis and Vianna, 1979). The E_1-state binds cytoplasmic Ca^{2+} or Mn^{2+} with high affinity, resulting in several conformational changes enabling ATP to phosphorylate a conserved aspartyl residue. Concomitant with the release of occluded ions, the high-energy phosphoenzyme $E_1{\sim}P(Ca^{2+})$ or $E_1{\sim}P(Mn^{2+})$ also undergoes a conformational change to a low-energy E_2-P phosphoenzyme. The binding sites for Ca^{2+} or Mn^{2+} are now exposed to the lumen and are characterized by a low affinity. Hydrolysis of E_2-P and regeneration of the Ca^{2+}- or Mn^{2+}-free E_2-enzyme complete the reaction cycle.

The three functionally active splice variants of the human SPCA1 (SPCA1a, SPCA1b and SPCA1d) have a much higher apparent Ca^{2+} affinity ($K_{0.5}$ = 9–10 nM) than the yeast SPCA ($K_{0.5}$ = 70 nM) and SERCA1a ($K_{0.5}$ = 284 nM) (Dode *et al*., 2005). The high apparent affinity for Ca^{2+} of SPCA1 is due to a slower $E_1{\sim}P(Ca^{2+})$ to E_2-P transition, which also in SPCAs constitutes the most critical rate-limiting

step of the reaction cycle. The apparent Ca^{2+} affinity of SPCA2 ($K_{0.5}$ = 25 nM) is about 2.5-fold lower than that of SPCA1d (Dode *et al*., 2006) and might be explained by an enhanced rate of $E_1{\sim}P(Ca^{2+})$ to E_2-P transition and by a decreased rate of the Ca^{2+}-binding transition.

The maximal turnover rates of the ATPase activity for SPCA1a (20 s^{-1}), SPCA1b (23 s^{-1}) and SPCA1d (27 s^{-1}) differ slightly (Dode *et al*., 2005). This represents the first evidence for functional differences between the human SPCA1 isoforms. The physiological significance of this finding, and, implicitly, of the SPCA1 isoform diversity remains however to be further elucidated. The maximal turnover rate of the ATPase activity for SPCA2 (41 s^{-1}) is markedly higher than those of the SPCA1 isoforms (Dode *et al*., 2006).

SPCA1c, one of the four splice isoforms of human SPCA1, is not functionally active (Dode *et al*., 2005). Heterologous expression of human SPCA1c in HEK-293 cells resulted in a protein that was expressed at very low levels. Since this isoform has a truncated transmembrane segment M10, it could lead to an improperly folded protein that might be sensitive to enhanced cellular degradation. SPCA1c cannot be phosphorylated, and, therefore, cannot couple the energy from the energy-donating ATP molecule to the vectorial ion transport.

The SPCAs are less sensitive to inhibition by thapsigargin than the SERCAs. *E.g*. the K_d for thapsigargin inhibition of SPCA1d is 28 μM *versus* 0.031 nM for SERCA1a (Dode *et al*., 2006). This is most likely explained by the fact that Phe^{256}, which in SERCA1a is essential for binding thapsigargin, is not conserved in the SPCAs. SPCA2, with its faster rate of E_2-P dephosphorylation and its decreased rate of E_2 to E_1 transition relative to SPCA1d, has a higher thapsigargin sensitivity (K_d = 2 μM), because the E_2-form that binds thapsigargin will accumulate to a higher extent in SPCA2 than in SPCA1d.

SPCA pumps have some unique catalytic adaptations, optimizing them to function in the Golgi and more down-stream compartments. (*i*) The maximal turnover rates of the ATPase activity for the various SPCA1 isoforms (20–27 s^{-1}) and for SPCA2 (41 s^{-1}) are lower than that of SERCA1a (130 s^{-1}). The low turnover rate of the SPCAs could represent necessary adaptations to function in the Golgi compartments, where a higher rate of ion transport is not required because the depletion of Ca^{2+} occurs only during the slower process of downstream trafficking of Ca^{2+}-rich vesicles. On the other hand, the higher turnover rate may be required for SPCA2 in secretory cells, where the trafficking of Ca^{2+}-rich vesicles must be faster than in non-secretory cells. (*ii*) The relatively high apparent Ca^{2+} affinity of the SPCAs, with a $K_{0.5}$ below the resting $[Ca^{2+}]_c$, ensures a rapid refilling of the Golgi compartment with cytosolic Ca^{2+}, even in the absence of transient rises in $[Ca^{2+}]_c$. (*iii*) SPCAs, in contrast to SERCA1a, do not counter transport luminal protons because their Ca^{2+}-binding pocket has less potentially charged residues that can bind protons (Glu^{308} and Asp^{819} in SPCA1, and Glu^{337} and Asp^{850} in SPCA2) than in SERCA1a (Glu^{58}, Glu^{309}, Glu^{771}, and Glu^{908}). Protons, which are needed for several vital functions in the Golgi apparatus, are therefore kept inside the lumen of this organelle during accumulation of Ca^{2+} and Mn^{2+}. (*iv*) Mn^{2+} is also transported with high affinity

by SPCAs from yeast (Mandal *et al*., 2000a), worm (Van Baelen *et al*., 2001) and man (Fairclough *et al*., 2003; Xiang *et al*., 2005; Vanoevelen *et al*., 2005). Mn^{2+} is indispensable for normal Golgi function (see next section).

2.6. Role of Ca^{2+} and Mn^{2+} in the Golgi Apparatus

Many enzymatic reactions in the lumen of the Golgi apparatus require Ca^{2+} and/or Mn^{2+}. Glycosyltransferases, which add sugars to oligosaccharides, glycoproteins and glycolipids, often show a clear preference of Mn^{2+} over Ca^{2+} used as a cofactor (Kaufman *et al*., 1994; Dürr *et al*., 1998). This may explain the defects in glycan processing of secreted thyroglobulin when SPCA1 is downregulated with small interfering RNA (Ramos-Castaneda *et al*., 2005). Sulfotransferases transfer a sulfuryl group from 3'-phosphoadenosine 5'-phosphosulfate to a tyrosyl residue in a protein or to an alcohol or amino group of complex glycoconjugates (Negishi *et al*., 2001). Their basal activity is enhanced by Mn^{2+} (Rens-Domiano and Roth, 1989; Lo-Guidice *et al*., 1995; Spiro *et al*., 1996; Mishiro *et al*., 2004; Seko *et al*., 2005).

Ca^{2+} is needed for the proteolytic processing by pro-hormone convertases in the Golgi apparatus. Furin and pro-hormone convertase type 1 (PC1) require millimolar Ca^{2+} for half-maximal activation, whereas pro-hormone convertase type 2 (PC2) requires only ~5 to 100 μM Ca^{2+} (Davidson *et al*., 1988; Molloy *et al*., 1992; Zhou and Lindberg, 1993; Austin and Shields, 1996). In pancreatic β-cells, the high-affinity PC2 enzyme is expressed in the Golgi compartment, whereas the low-affinity PC1 is found in the insulin-containing secretory granules (Davidson *et al*., 1988). Also proteases that cleave precursors of plasma proteins in liver (Oda, 1992) and a furin-like convertase responsible for processing the beta-site APP cleaving enzyme (BACE) depend on Ca^{2+} (Bennett *et al*., 2000). Casein kinase in the Golgi apparatus also requires a sufficiently elevated $[Ca^{2+}]_l$ (Creemers *et al*., 1998).

Secretory vesicles already have a high $[Ca^{2+}]_l$ when they bud off from the *trans*-Golgi network. These vesicles may subsequently lose and take up Ca^{2+} via specific Ca^{2+}-release channels and Ca^{2+}-uptake mechanisms. Ca^{2+} uptake relies on Ca^{2+} pumps including SPCAs (Mitchell *et al*., 2001, 2004) and on Ca^{2+}/H^+ exchangers (Gonçalves *et al*., 1999; Moreno *et al*., 2005) and H^+/Na^+ in combination with Na^+/Ca^{2+} exchangers, using the energy of the proton gradient (Mahapatra *et al*., 2004). Exocytosis of these vesicles could represent a detoxification pathway for removing excess Ca^{2+} and Mn^{2+} from the cell. Secretion of Ca^{2+} could be especially important in yeast, which lacks the well-defined plasma-membrane Ca^{2+}-extrusion pumps.

2.7. Role of the Golgi Complex in Setting up Cytosolic Ca^{2+} Signals

There are so far no specific inhibitors of SPCAs. The role of this pump in setting up cytosolic Ca^{2+} signals can be assessed by downregulating SPCA1 with small interfering RNA. SPCA1 was not absolutely needed to set up agonist-elicited cytosolic

Ca^{2+} oscillations in HeLa cells, but contributed to the fine tuning of the shape of the Ca^{2+}spikes (Van Baelen *et al*., 2003). Pancreatic β-cells with depleted SPCA1 had also modified Ca^{2+} oscillations in response to glucose (Mitchell *et al*., 2004). These results indicate rather subtle effects of the SPCA1-containing Ca^{2+} store on cytosolic Ca^{2+} oscillations. Human spermatozoa may represent a notable exception. In these cells bis-phenol, a blocker of microsomal Ca^{2+}-ATPase (Brown *et al*., 1994), inhibited progesterone-induced Ca^{2+} oscillations (Harper *et al*., 2005). This important role of SPCA1 in spermatozoa could be related to the absence of SERCAs in these cells.

3. HAILEY-HAILEY DISEASE

Familial benign chronic pemphigus or HHD (OMIM 169600) is a skin disorder with an incidence of 1:50,000. The disease was first described by the Hailey dermatologist brothers (Hailey and Hailey, 1939). The symptoms typically arise after puberty or after the third or fourth decade. It is a blistering skin disease with vesicles and itchy erosions. Pain and an unpleasant smell become dominant symptoms as fissures and maceration develop. The distribution of cutaneous lesions is usually symmetrical. They mainly occur in the bodily folds, particularly the groin and axillar regions. Mucosal surfaces are rarely involved. The disease has a fluctuating course with remissions and exacerbations triggered by friction, sweating, heat, stress, infection, ultraviolet radiation or tissue damage like in allergic contact dermatitis. Many patients have fingernails with asymptomatic longitudinal white lines. The skin lesions may develop into squamous cell carcinomas (Chun *et al*., 1988; Cockayne *et al*., 2000; Holst *et al*., 2000). Sporadic diseases associated with HHD are affective disorder (Korner *et al*., 1993; Wilk *et al*., 1994; Yokota and Sawamura, 2006) as well as liver injury (Amagai *et al*., 2001). HHD patients also display a higher frequency of contact allergies (Reitamo *et al*., 1989).

Histologically, the disease is characterized by a loss of adhesion between suprabasal keratinocytes (acantholysis) and abnormal keratinisation (dyskeratosis) of the epidermis. In HHD, acantholysis is the most prominent histological feature while in the clinically related Darier disease (OMIM 124200), dyskeratosis is much more apparent. Ultrastructural analysis of acantholytic cells reveals perinuclear aggregates of keratin filaments that have retracted from desmosomes (Harada *et al*., 1994; Hashimoto *et al*., 1995; Metze *et al*., 1996).

3.1. Genetics

Both sexes are equally affected by HHD, which is inherited in an autosomal dominant manner with a family history in 70% of the patients. HHD is caused by germ-line mutations inactivating one allele of *ATP2C1*, the gene encoding SPCA1 (Hu *et al*., 2000; Sudbrak *et al*., 2000). Close to 100 mutations are known (Van Baelen *et al*., 2004). They are scattered throughout the *ATP2C1* gene, indicating no 'hotspot' or clustering of mutations in the gene. There are no genotype-phenotype

correlations between the nature of the *ATP2C1* mutation and the clinical features (Ikeda *et al*., 2001; Chao *et al*., 2002). The variation of disease characteristics observed in patients belonging to the same family or in two unrelated individuals sharing the same mutation suggests that the phenotype is strongly influenced by genetic modifiers and/or environmental factors (Dobson-Stone *et al*., 2002). HHD shows many similarities with Darier disease, in which the *ATP2A2* gene encoding SERCA2 is mutated (Sakuntabhai *et al*., 1999).

Poblete-Gutiérrez *et al.* (2004) described HHD patients with more severely affected skin lesions appearing already at the age of 3 months. They occur in a mosaic form along the Blaschko lines. These unilateral patterned skin lesions correspond to clones of keratinocytes descending during embryogenesis from a common precursor that as a result of a somatic mutation had lost the only remaining wild-type *ATP2C1* allele. The exacerbated areas are therefore superimposed on the ordinary symmetrical germ-line transmitted HHD phenotype.

3.2. Effect of HHD Mutations on the SPCA1 Protein

Fairclough *et al*. (2003, 2004) introduced several of the HHD mutations into the human SPCA1d isoform and functionally investigated the consequences of the expressed mutant pumps in COS-1 cells. More than half of the investigated mutants resulted in low levels of protein expression despite normal mRNA levels and a correct targeting of the pump to the Golgi compartments (*e.g*. $Leu^{341} \rightarrow Pro$, $Cys^{344} \rightarrow Tyr$, $Cys^{411} \rightarrow Arg$, $Ala^{528} \rightarrow Pro$, $Thr^{570} \rightarrow Ile$ and $Gly^{789} \rightarrow Arg$). This finding is consistent with the markedly reduced SPCA1 expression observed in keratinocytes of HHD patients (Behne *et al*., 2003). Porgpermdee *et al*. (2005) reported a 50% reduction of SPCA1 in uninvolved HHD epidermis and a 70–80% reduction in involved HHD epidermis *versus* normal epidermis. Other mutants resulted in a lack of ion transport. This was due to defects in Ca^{2+} and Mn^{2+} binding ($Asp^{742} \rightarrow Tyr$ and $Gly^{309} \rightarrow Cys$) or to the inability of the phosphoenzyme intermediate to undergo the major $E_1 \sim P(Ca^{2+})$ to E_2-P conformational transition ($Ile^{580} \rightarrow Val$). The $Pro^{201} \rightarrow Leu$ mutant displayed wild-type properties with respect to its intracellular targeting and catalytic abilities. Expression of the latter mutant in yeast restored the cellular phenotype of a yeast strain lacking *PMR1* (Ton and Rao, 2004). It is therefore unclear how this mutant leads to the HHD defect. This mutation may represent a benign polymorphism. The actual defect might be associated with the non-coding parts of *ATP2C1*.

3.3. Physiopathology of HHD

The precise link between altered expression or functionality of SPCA1 and the development of skin lesions has not yet been established. First, we do not know whether the symptoms are due to a defect in Ca^{2+} or Mn^{2+} transport by SPCA1. However, the similarity between HHD and Darier disease points to a major role of Ca^{2+} ions because SERCA2 is hardly capable of transporting Mn^{2+}relative to

SPCA1 (Gomes da Costa and Madeira, 1986). Secondly, we do not know whether the main defect in ion homeostasis is located in the lumen of the secretory pathway or in the cytosol. The lower $[Ca^{2+}]_l$ in the Golgi apparatus and possibly in post-Golgi secretory compartments may affect post-translational processing and sorting of key molecules involved in cell-cell adhesion. The defect may also be due to abnormal Ca^{2+} signaling in the cytosol, but here there is no consensus on how HHD mutations affect the $[Ca^{2+}]_c$. Hu *et al*. (2000) observed an elevated basal $[Ca^{2+}]_c$ in diseased keratinocytes, while basal levels remained unchanged in the experiments of Leinonen *et al*. (2005).

The rise in $[Ca^{2+}]_c$ induced by thapsigargin was smaller in HHD keratinocytes (Leinonen *et al*., 2005) as compared to normal keratinocytes. The size of the SERCA-dependent Ca^{2+} store was therefore decreased. It is an intriguing question whether SERCA levels would be decreased in HHD keratinocytes. If this were the case, the similarities between Darier disease and HHD would be easily explained. *Vice versa*, the expression of SPCA1 might be changed in Darier keratinocytes. SPCA1 might be upregulated and partially compensate for the lower SERCA activity (Foggia and Hovnanian, 2004). In contrast, Porgpermdee *et al*. (2005) observed a moderate decrease of SPCA1 levels in the affected skin of Darier patients, but not in the unaffected skin. On the other hand, our own observations indicate that overexpression of SPCA2 in COS-1 cells does not influence the expression levels of the other endogenous intracellular Ca^{2+} pumps (SPCA1 and SERCA2b) (Vanoevelen *et al*., 2005).

Aronchik *et al*. (2003) reported defective actin re-organization in HHD keratinocytes and a marked decrease in cellular ATP. These findings implicate inadequate ATP stores as an additional factor in the pathophysiology of HHD.

Although HHD patients with affective disorder have been described (Korner *et al*., 1993; Wilk *et al*., 1994; Yokota and Sawamura, 2006), the symptoms in most patients seem to be restricted to the skin. This is intuitively not expected for a mutated protein that is ubiquitously expressed. One explanation may be that the relative contribution of SPCA and SERCA Ca^{2+} pumps to the Ca^{2+} uptake in the Golgi apparatus is cell-type dependent. Reliance on SPCA1 was much higher (roughly two-thirds of the Ca^{2+} uptake into the Golgi apparatus is resistant to thapsigargin) in human keratinocytes than in many other cells (Callewaert *et al*., 2003). Additional factors might also be involved, like, amongst others, the disturbed glycosylation state of components involved in keratinocyte-specific cell-to-cell contacts, or modifications of specific keratins. Alternatively, compensatory mechanisms that protect non-cutaneous tissues may be lacking in keratinocytes.

The mere loss of one functional *ATP2C1* allele on its own is not sufficient to cause the HHD symptoms. Indeed, the skin of HHD patients is typically symptom-free during the first decades of life and even thereafter the symptoms are only seen in certain areas of the skin in response to sweating, local warming or friction. The symptoms in HHD patients can be elicited in a matter of minutes by locally rubbing the skin. This effect was also confirmed *in vitro*. Functional knockdown of *ATP2C1* in human keratinocytes renders them defective in post-translational processing and

makes these cells hypersensitive to ER stress (Ramos- Castaneda *et al*., 2005). The complete absence of functional SPCA1 in the patients reported by Poblete-Gutiérrez *et al.* (2004) still allows keratinocytes to proliferate and to survive, at least for some time. Clearly other external factors contribute to the development of the phenotype.

3.4. The Epidermal Ca^{2+} Gradient

A unique Ca^{2+} gradient is present within the normal epidermis, with the lowest $[Ca^{2+}]$ in the basal layer and the highest $[Ca^{2+}]$ in the granular layer (Mauro *et al*., 1998; Elias *et al*., 2002). Although proton-induced X-ray emission analysis does not discriminate between intra- and extracellular Ca^{2+}, Ca^{2+}-precipitation studies suggest that the increased $[Ca^{2+}]$ in the outer epidermis is mainly extracellular (Menon *et al*., 1985). This gradient of extracellular Ca^{2+} has profound effects on the behaviour of keratinocytes. Primary keratinocytes cultured in the presence of a low extracellular $[Ca^{2+}]$ proliferate and fail to develop intercellular contacts. Cell proliferation under normal conditions is therefore confined to the undifferentiated keratinocytes in the basal layer of the skin where the local extracellular $[Ca^{2+}]$ is kept low. Increases in extracellular $[Ca^{2+}]$ above 0.1 mM activate the Ca^{2+}-sensing receptor (CaR) in keratinocytes, stop proliferation and trigger differentiation. This is what happens in the more apical layers of the skin where keratinocytes slowly differentiate, move upwards and eventually enter terminal differentiation to form the cornified upper layer of the skin.

This unique Ca^{2+} gradient is no longer present in the skin of HHD patients, even not in the non-affected areas of the skin (Hu *et al*., 2000; Behne *et al*., 2003). The basal and suprabasal layers of the epidermis in HHD contain similar amounts of Ca^{2+} as in normal skin. Also the total $[Ca^{2+}]$ in the granular layer of the epidermis is significantly decreased in HHD skin. Kellermayer (2005) proposed that the absence of this unique epidermal Ca^{2+} gradient is a key pathogenic feature of HHD. In normal skin, noxious stimuli that disrupt the epidermal permeability barrier dissipate the epidermal Ca^{2+} gradient. Extracellular $[Ca^{2+}]$ in the lower layers of the epidermis rises resulting in a direct, post-translational effect that stabilizes desmosomal integrity. The elevated extracellular $[Ca^{2+}]$ also triggers CaR-mediated processes leading to cell-to-cell adhesion and differentiation. In HHD epidermis, noxious stimuli no longer increase the extracellular $[Ca^{2+}]$ in the suprabasal layer of the epidermis. The absence of a $[Ca^{2+}]$ rise results in the failure to reinforce the desmosomal connections and no activation of the CaR. Finally, Golgi functions are decreased as a result of the decreased Golgi $[Ca^{2+}]_l$ and $[Mn^{2+}]_l$ caused by the defective SPCA1. All these effects lead to a loss of cell-to-cell adhesion resulting in acantholysis.

The absence of an epidermal Ca^{2+} gradient in the epidermis in HHD may have direct effects on the expression of SPCA1. Extracellular Ca^{2+} may up regulate the expression of SPCA1 in keratinocytes (Kawada *et al*., 2005; Mayuzumi *et al*., 2005). According to one view, a rise in extracellular $[Ca^{2+}]$ would, at least in normal keratinocytes, result in a concomitant increase in $[Ca^{2+}]_c$ followed by the

translocation of Sp1 from the cytosol to the nucleus where it activates enhancing elements in the *ATP2C1* promoter and thereby markedly increases SPCA1 mRNA levels. In HHD keratinocytes, the rise of $[Ca^{2+}]_c$ is suppressed, the translocation of Sp1 to the nucleus is blunted and the transcriptional upregulation of SPCA1 mRNA impaired (Kawada *et al*., 2005). In other reports, however, either no change (Yoshida *et al*., 2006) or a decrease in SPCA1 expression (Ramos-Castaneda *et al*., 2005) was seen when the extracellular $[Ca^{2+}]$ increased.

4. CONCLUSIONS

We have reviewed the properties of the SPCA pumps. SPCAs are now recognized as important players in the regulation of cellular ion homeostasis, along with the SERCAs and PMCAs. We begin to understand their role in the Ca^{2+} and Mn^{2+} homeostasis in the Golgi apparatus and downstream compartments of the secretory pathway. This role seems to be crucial, since mutations in the SPCA1 gene (*ATP2C1*) cause HHD. There is now an urgent need to establish how mutations in SPCA1 lead to the clinical phenotype. A major problem to be resolved is why HHD only becomes manifest relatively late in life and why only the skin is affected by *ATP2C1* haploinsufficiency.

Due to its unique catalytic properties and restricted expression pattern, SPCA2 must have a special role to play in cellular ion homeostasis, a role which should be further investigated.

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CHAPTER 15

CALCIUM SIGNALLING AND CANCER CELL GROWTH

T. CAPIOD[1], Y. SHUBA[2], R. SKRYMA[1] AND N. PREVARSKAYA[1]

[1]*INSERM U800, Laboratoire de Physiologie Cellulaire, Université des Sciences et Technologies Lille 1, 59655 Villeneuve d'Ascq Cedex, France*

[2]*Bogomoletz Institute of Physiology, NASU, Bogomoletz Str, 4, 01024 Kiev-24, Ukraine*

Abstract: Cancer is caused by defects in the mechanisms underlying cell proliferation and cell death. Calcium ions are central to both phenomena, serving as major signalling agents with spatial localization, magnitude and temporal characteristics of calcium signals ultimately determining cell's fate. There are four primary compartments: extracellular space, cytoplasm, endoplasmic reticulum and mitochondria that participate in the cellular Ca^{2+} circulation. They are separated by own membranes incorporating divers Ca^{2+}-handling proteins whose concerted action provides for Ca^{2+} signals with the spatial and temporal characteristics necessary to account for specific cellular response. The transformation of a normal cell into a cancer cell is associated with a major re-arrangement of Ca^{2+} pumps, Na/Ca exchangers and Ca^{2+} channels, which leads to the enhanced proliferation and impaired ability to die. In the present chapter we examine what changes in Ca^{2+} signalling and the mechanisms that support it underlie the passage from normal to pathological cell growth and death control. Understanding this changes and identifying molecular players involved provides new prospects for cancers treatment

Keywords: Apoptosis, proliferation, calcium channels

1. INTRODUCTION

Cancer is caused by defects in the mechanisms underlying cell proliferation and cell death. The development of tumours results from excessive cell proliferation combined with inhibition of cell apoptosis that eventually leads to imbalances in tissue homeostasis and uncontrolled growth. The molecular machineries of proliferation and apoptosis are different, with proliferation relying on cyclin-dependent protein kinases (CDKs) – regulators of cell division cycle (Nigg, 1995) – and apoptosis primarily dependent on caspases – cysteine proteases executing a cell death programme (Nicholson, 1999). Nevertheless, calcium ions are central to both phenomena, serving as major signalling agents. From these observations, it is clear

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that changes in cytosolic free calcium ($[Ca^{2+}]_i$) alone are insufficient for governing such diverse processes deciding cell fate. Therefore, the amplitude, spatial localization and temporal characteristics of calcium signals are of major importance in determining death, survival and proliferation (Berridge, 1995, Berridge, et al., 1998, Kahl, et al., 2003, Lipskaia, et al., 2004, Munaron, et al., 2004, Orrenius, et al., 2003). Enzymatic reactions and gene activation obviously occur in the cytoplasm and nucleoplasm, and there is recent unequivocal evidence that calcium levels within the endoplasmic reticulum (ER) and mitochondria are at least as important as $[Ca^{2+}]_i$ changes. The formation of local signalling complexes due to restricted calcium diffusion (Allbritton, et al., 1992) may well allow even greater specialization of the cellular responses controlled by this divalent cation.

Cell transition from the normal to the malignant state is a multistage process characterized by a major reorganization of the molecular machinery of active and passive Ca^{2+} transport across cellular and subcellular membranes, and also Ca^{2+} buffering, accumulation and extrusion by various intracellular organelles (Berridge, et al., 2003). In this chapter we outline the major mechanisms of Ca^{2+} signalling and examine their involvement in the control of cell death and proliferation. However, the relationships to cancer development are not always straightforward and direct comparisons of normal and tumour cells are rare; indeed, cell lines have been widely used to understand some of the processes leading to abnormal cell proliferation.

2. EXTRACELLULAR CALCIUM AND CANCER

Extracellular Ca^{2+} is essential for a number of vital processes as diverse as bone formation and muscle contraction. The G protein-coupled Ca^{2+}-sensing receptor (CaSR) can sense extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) over the range of 0.05–5 mM, which makes it a key mediator of cellular responses to physiologically relevant changes in extracellular Ca^{2+} (Msaouel, et al., 2004). It is an essential component of the homeostatic system regulating parathyroid hormone secretion, Ca^{2+} excretion by kidney, and bone remodeling. For many cell types, including intestinal epithelial cells, breast epithelial cells, keratinocytes, and ovarian surface epithelial cells, changes in $[Ca^{2+}]_o$ in the 0.05–5 mM range can switch cellular behaviour from proliferation to terminal differentiation or quiescence. As cancer is the consequence of a disordered balance between proliferation, differentiation, and apoptosis, disruption of the function of the CaSR is a likely contributor (Rodland, 2004). Loss of the growth suppressing effects of elevated extracellular Ca^{2+} have been demonstrated in parathyroid hyperplasias and in colon carcinoma, and have been correlated with changes in the levels of CaSR. Diminished CaSR expression results in abnormal differentiation and progression of colorectal carcinoma. For instance, the human colon adenocarcinoma-derived cell line, Caco-2, responds to low ambient $[Ca^{2+}]_o$ by activation of the protein kinase C-signalling pathway ultimately leading to upregulation of c-myc mRNA production and release from the G1/S phase control of the cell cycle (Kallay, et al., 2000). This proliferative response can be abolished by activation of CaSR either through raising $[Ca^{2+}]_o$ or by using

the CaSR agonist Gd^{3+} as a substitute for Ca^{2+}. The inhibitory effect of $[Ca^{2+}]_o$ on cell replication has an IC_{50}=0.045 mM, indicating the existence of a highly sensitive CaSR operating at low ambient $[Ca^{2+}]_o$, and specific immunostaining demonstrates CaR-positive cells in the crypt epithelium of normal human colonic mucosa and in glandular (i.e. differentiated) structures of carcinomatous lesions (Kallay, et al., 2000). CaSR has been also implicated in increased expression and secretion of parathyroid hormone-related peptide (PTHrP), a primary causal factor in hypercalcaemia-induced malignancy and a contributor to metastatic processes involving bone. In parathyroid tissue, CaSR expression has been linked to proliferation of both parathyroid adenomas and carcinomas (Manning, et al., 2006). Activation of CaSR in H-500 rat Leydig cancer cells under conditions of model hypercalcaemia stimulates PTHrP release and proliferation (Tfelt-Hansen, et al., 2005). This effect was attributed to CaSR-mediated transactivation (by phosphorylation) of epidermal growth factor (EGF)-receptor tyrosine kinase. It was concluded that in Leydig cancer cells, calcium activates the EGF receptor, possibly through the CaSR, to regulate downstream signalling events and major biological functions.

CaSR may also influence the proliferative and apoptotic status of the cells indirectly via modulation of cell volume homeostasis. Indeed, stimulation of CaSR in human epithelial cells induces upregulation of volume-regulated anion channels (VRAC) via a G protein-mediated increase in intracellular cAMP (Shimizu, et al., 2000). Proliferation and apoptosis are associated with essential volume perturbations [e.g., (Lang, et al., 2000)] and VRAC, a key component of homeostatic volume regulation, has been directly implicated in proliferation (Chen, et al., 2002, Doroshenko, et al., 2001, Shen, et al., 2000, Wang, et al., 2002) and apoptosis (Lemonnier, et al., 2004, Okada, et al., 2001, Okada, et al., 2006, Shen, et al., 2002). Consequently, extracellular Ca^{2+} may affect carcinogenesis via the CaSR-VRAC-cell volume links. The Ca^{2+}-permeable store-operated channel (SOC) is directly and functionally coupled to VRAC in an androgen-dependent LNCaP human prostate cancer epithelial cell line (Lemonnier, et al., 2002), evidence for another, CaSR-unrelated, potential mechanism for extracellular Ca^{2+} involvement in proliferative and apoptotic events.

There is convincing laboratory and clinical evidence that calcium and vitamin D reduce the risk of colorectal cancer (Chia, et al., 2004): colon cancer incidence is inversely proportional to the intake of calcium. The same is true for mammary cancers (Lipkin, et al., 1999). The molecular events and cellular actions of these micronutrients that contribute to their tumour-modulating effects include a complex series of signalling events that affect the structural and functional organization of colon cells (Lamprecht, et al., 2003). The value of calcium as dietary supplement for the prevention of colon tumorigenesis can in part be explained by action via CaSR. However, the intimate link between calcium, vitamin D and cancers was clearly revealed following the cloning of a channel-like transporter mediating intestinal calcium absorption (Peng, et al., 1999): it was initially called the Ca^{2+} transporter type 1 (CaT1) and then epithelial calcium channel 2 (ECaC2), but once its relation to the transient receptor potential (TRP) channel family was established it was

renamed TRPV6. TRPV6 is a highly selective calcium entry channel suggested to be responsible for apical calcium entry into the vitamin D-regulated transcellular pathway of Ca^{2+} absorption. TRPV6 is found in the apical membrane of intestinal absorptive cells, suggesting that it is an apical entry channel in the gastrointestinal tract (Zhuang, et al., 2002). In addition, TRPV6 protein is abundant in a number of exocrine organs including pancreas, prostate, and mammary gland implicating it as a component of transcellular calcium transport mechanisms in many tissues. Moreover, TRPV6 is more abundant in various prostate, breast, thyroid, colon, and ovarian carcinomas than in normal tissues (Zhuang, et al., 2002). There is strong evidence that TRPV6 is involved in store-operated calcium entry (SOCE) by serving as a major constituent of CRAC-(calcium release-activated channel)-type store-operated channel (Yue, et al., 2001), although any such involvement is not likely in all cell types. Work with LNCaP human prostate cancer cells implicates TRPV6 in prostate-specific SOC (Vanden Abeele, et al., 2004, Vanden Abeele, et al., 2003). Interestingly, TRPV6 expression correlates with prostate cancer grade (Fixemer, et al., 2003, Peng, et al., 2001, Wissenbach, et al., 2001). Its expression is also strongly vitamin D dependent (Bouillon, et al., 2003), suggesting that it may be important in the pro-apoptotic action of vitamin D-receptor ligands on prostate, and other, cancer cells (Guzey, et al., 2002). By acting via a vitamin D receptor, these ligands suppress the expression of Bcl-2-related anti-apoptotic proteins and stimulate cytochrome c (Cyt-c) release in LNCaP and ALVA-31 prostatic cell lines (Guzey, et al., 2002). It is plausible that TRPV6 is involved in providing the Ca^{2+} entry required for the mitochondrial permeability transition leading to Cyt-c release. The elevation of the cytosolic calcium concentration associated with vitamin D-induced apoptosis of mammary MCF-7 cells results not from Ca^{2+}entry, but from Ca^{2+} release from the ER (Mathiasen, et al., 2002). Interestingly, increased cytosolic Ca^{2+} in this case stimulated the caspase-independent apoptotic pathway, which relied solely on the activation of Ca^{2+}-dependent cysteine proteases of the calpain family (Mathiasen, et al., 2002). In normal mammary cells, vitamin D-receptor ligands trigger a transient Ca^{2+} response via activation of voltage-gated calcium channels, which are absent from breast cancer cells. The normal cells, but not breast cancer cells, also expressed the Ca^{2+}-binding/buffering protein calbindin-D and were capable of buffering $[Ca^{2+}]_i$ increases induced by a mobilizer of the ER Ca^{2+} stores, thapsigargin (TG), or a Ca^{2+} ionophore, ionomycin (Sergeev, 2005).

TRPV6 overexpression also increases the rate of Ca^{2+}-dependent cell proliferation which is a prerequisite for its potential role in tumour progression (Schwarz, et al., 2006).

3. CYTOSOLIC CALCIUM AND CANCER

The cytosol is the cell compartment in which most mechanisms of Ca^{2+} signalling converge to produce the specific spatial-temporal pattern of the signal appropriate for targeting its effectors. The resting concentration of calcium in the cytosol is low,

around 10^{-7} M, but there is fine regulation of the amplitude, localization, propagation and timing of changes. Plasma membrane (PM) Ca^{2+}-ATPases (PMCA), Na^{+}-Ca^{2+} exchangers (NCX), voltage-gated (VGCC), receptor-gated (RGCC) and store-operated (SOC) Ca^{2+} channels are the major components of calcium shuttling between the cytosol and extracellular space.

Increases in $[Ca^{2+}]_i$ are associated with progression through the cell cycle: the exit from quiescence in early G1 phase, the G1/S transition, and other checkpoints during S and M phases (Berridge, 1995, Munaron, et al., 2004). Calcium exerts its regulatory role by acting as a ubiquitous allosteric activator or inhibitor of several intracellular enzymes in the cytosol, of which probably the most relevant calcium decoder for cell proliferation is calmodulin, regulating various processes and in particular the family of Ca^{2+}-calmodulin dependent kinases type II (CaMKII) and several membrane channels. There is a substantial amount of data indicating that CaMKII is directly involved at several transition points during cell cycle progression (Kahl, et al., 2003). Calcium-dependent enzymes also regulate the activation of several nuclear transcription factors and factors involved in the DNA division machinery, including CDKs and cyclins (Santella, 1998).

Sustained cytosolic Ca^{2+} overload usually results in a different route leading to cell death. It mainly relies on the activation of the calcium/calmodulin (CaM)-dependent phosphatase, calcineurin. Calcineurin-catalyzed dephosphorylation promotes apoptosis by regulating the activity of a number of downstream targets, including the pro-apoptotic Bcl-2 family member, Bad (Wang, et al., 1999), and transcription factors of the NFAT (nuclear factor of activated T cells) family (Rao, et al., 1997). There are also other Ca^{2+}-dependent enzymes contributing to the apoptotic events, and they include several DNA-degrading endonucleases (Robertson, et al., 2000) and Ca^{2+}-activated cystein proteases of the calpain family essential for the enzymatic activation of the crucial pro-apoptotic effectors (Altznauer, et al., 2004).

Depending on cell type, Ca^{2+} influx pathways mostly involve combinations of various subtypes of VGCCs, RGCCs and SOCs. Data concerning the expression and function of PM Ca^{2+}-permeable channels during malignant transformation are diverse such that consideration of each specific case is required. For instance, differentiation of androgen-dependent LNCaP prostate cancer cells into the malignant, terminally differentiated, apoptotic resistant neuroendocrine (NE) phenotype, characteristic of incurable androgen-independent prostate cancer, is associated with strong overexpression of voltage-gated T-type Ca^{2+} channels and in particular their α1H (Cav3.2) isoform (Mariot, et al., 2002). By providing constant Ca^{2+} influx around the resting potential, these channels seem to be involved in the formation of neuronal-like morphological features (i.e., neurite outgrowth). However, whether or not these channels contribute to the enhanced anti-apoptotic potential of NE cells is not clear. NE cells, and another androgen-independent, apoptosis-resistant prostate cancer cell phenotype overexpressing antiapoptotic Bcl-2 protein, are characterized by reduced SOCE most likely due to a diminishing density of SOCs (Vanden Abeele, et al., 2002, Vanoverberghe, et al., 2004). In general, reduced SOCE seems

to be a common feature of enhanced resistance to apoptosis, or at least that due to Bcl-2 overexpression (Pinton, et al., 2000), although there are also contradictory reports (Williams, et al., 2000).

Recent preliminary results suggest that novel T-type calcium channel blockers suppress the growth of some human cancer cells by blocking Ca^{2+} influx through this type of VGCC involved in cell cycle progression and proliferation via an unknown mechanism (Lee, et al., 2006). Findings concerning the value of pharmacological modulation of T type Ca^{2+} channels in cytostatic cancer chemotherapy are controversial (Gray, et al., 2006). The elevation of mRNA for another VGCC – the cardiac isoform of the voltage-gated L-type calcium channel (α1C) — has been detected in colon cancer (Wang, et al., 2000). The increase in α1C protein was higher in nonconfluent and dividing than other colonic cells, which may account for the increase in cancer.

Controlling intracellular Ca^{2+} homeostasis is a potential strategy to prevent or enhance programmed cell death. This could be accomplished, for instance, by overexpressing the Na^+-Ca^{2+} exchanger, an ubiquitous mechanism for Ca^{2+} extrusion from excitable and non-excitable cells (Blaustein, et al., 1999). The Na^+-Ca^{2+} exchanger couples the electrogenic transport of three Na^+ ions down their electrochemical gradient for Ca^{2+} ion against the electrochemical gradient. In some cell types including cardiac myocytes (Reuter, et al., 2005), Na^+-Ca^{2+} exchange is the major mechanism for Ca^{2+} extrusion participating in the control of $[Ca^{2+}]_i$.

Overexpression of the Na^+-Ca^{2+} exchanger in insulin-secreting tumoral BRIN-BD11 cells results in the depletion of ER Ca^{2+} stores, a phenomenon that leads to ER stress, activation of caspase-12, and an increase in Ca^{2+}-dependent and -independent apoptosis. ER depletion is probably the major reason for apoptosis, as it was enhanced despite the reduction in $[Ca^{2+}]_i$ (Diaz-Horta, et al., 2002, Diaz-Horta, et al., 2003). Reduction in ER Ca^{2+} stores due to an increase in Ca^{2+} extrusion, also explains why overexpression of NCX1.7 Na^+-Ca^{2+} exchanger isoform or PMCA2 results in a decrease in cell proliferation (Diaz-Horta, et al., 2002, Diaz-Horta, et al., 2003). Thus, because excessive cell proliferation and decreased ability to undergo apoptosis are the two characteristics of cancer, overexpression of Na^+-Ca^{2+} exchanger in cancer cells by gene therapy is a potential approach to cancer therapy.

Cardiac glycosides, which are mostly known for their positive inotropic effects on heart function by indirectly enhancing the reverse mode of the Na^+-Ca^{2+} exchanger, also exert a potent pro-apoptotic effect on prostate cancer cells, and this involves a perturbation of Ca^{2+} homeostasis (Huang, et al., 2004, McConkey, et al., 2000). Using the androgen-independent PC-3 cell line as a model system, it was demonstrated that cardiac glycosides induce Ca^{2+} influx (Huang, et al., 2004, McConkey, et al., 2000) probably due to reversed Na^+-Ca^{2+} exchange activity. This influx was accompanied by the loss of the mithochondrial membrane potential (Huang, et al., 2004) and release of Cyt-c followed by caspase-3 and -8 processing (McConkey, et al., 2000).

Another plasmalemmal pathway for decreasing the cytosolic Ca^{2+} concentration is provided by the PMCA pump (Carafoli, 1994). There is increasing awareness

that PMCA alterations are associated with tumorigenesis, including that of the mammary gland (Reinhardt, et al., 2000, Reinhardt, et al., 1999). Four PMCA isoforms (PMCA1–4) encoded by genes mapping on different chromosomes have been identified (Carafoli, 1994). PMCA1 and PMCA4 are ubiquitously expressed, whereas PMCA2 and PMCA3 are generally found in excitable tissues (Strehler, et al., 2001). PMCA expression is increased in cancer cells and PMCA2 levels are 100-fold higher in tumorigenic MCF-7 and MDA-MB-231 human breast cancer cell lines than in non-tumorigenic MCF-10A human breast epithelial cells (Lee, et al., 2005a). Although not to the same degree, mRNAs coding for PMCA1 and PMCA4 are also more abundant in tumorigenic cells (Lee, et al., 2002, Lee, et al., 2005a). Conversely, antisense-mediated depletion of PMCA inhibits proliferation of MCF-7 cells (Lee, et al., 2005b). However, as PMCA overexpression reduces the Ca^{2+} content of ER and mitochondria, and also increases the rate of Ca^{2+} extrusion from the cytosol (Brini, et al., 2000), it is not clear whether PMCA is directly or indirectly involved in regulation of tumorigenic breast cancer cell proliferation (Lee, et al., 2005a).

PMCA expression and activity are also enhanced in hepatocarcinomas (Delgado-Coello, et al., 2003, Mas-Oliva, et al., 1991). PMCA activity is more efficient in AS-30D ascites hepatocarcinoma than in the membrane fraction isolated from normal mouse liver (Mas-Oliva, et al., 1991) and this may be due to changes in the synthesis of mRNA for several PMCA isoforms (Delgado-Coello, et al., 2003).

4. ER CALCIUM AND CANCER

The ER is central to a variety of normal cellular processes. It is the principal site of protein synthesis, folding and proper targeting (Rao, et al., 2001). The ER is also the site of biosynthesis of steroids, cholesterol and other critical lipids (Rao, et al., 2001). The ER can accumulate and release calcium and is a major Ca^{2+} storage organelle involved in calcium signalling and maintenance of intracellular calcium homeostasis (Rao, et al., 2001). In eukaryotic cells, surface and secreted proteins are translocated in an unfolded state through the specialized protein channel in the ER where they encounter an oxidising and calcium-rich environment, in which they become modified (Cooper, et al., 1997, Ma, et al., 2004). The maturation of nascent proteins requires high calcium levels, and the ER resident chaperone proteins are required for proper protein folding (Ma, et al., 2004). Properly folded proteins leave the ER and progress down the normal secretory pathway, whereas unfolded or misfolded proteins are exported from the ER and degraded by the cytoplasmic proteosomes (Ma, et al., 2004).

The ER is highly sensitive to even minor perturbations in its environment, and is particularly sensitive to alterations in calcium content. ER stress, resulting from the disruption of its Ca^{2+} homeostasis or accumulation of excess proteins within the ER, causes the activation of caspase-12 (Van de Craen, et al., 1997) that triggers apoptosis (Nakagawa, et al., 2000). A variety of physiological conditions including glucose deprivation, oxidative stress, ischaemia and infection can affect

ER function, as can external agents such as calcium ionophores and chemical toxicants (Rao, et al., 2001) which can cause ER stress. ER responds to stress by triggering a specific signalling pathway termed the unfolded protein response (UPR). The UPR serves to protect the cell from normal variations that occur in the cellular environment (Ma, et al., 2004, Rao, et al., 2001). Recent evidence suggests that the UPR can also be activated in tumours (Ma, et al., 2004).

The ER is endowed with multiple mechanisms facilitating effective uptake, storage and release of Ca^{2+}. The primary molecular determinants involved in Ca^{2+} handling by the ER include: inositol trisphosphate (IP_3R) and ryanodine (RyR) receptors permitting active Ca^{2+} release, the ER leak channels underlying passive loss of Ca^{2+}, the Sarco-Endoplasmic Reticulum Ca^{2+}-Mg^{2+}-ATPases (SERCA) providing for Ca^{2+} reuptake back into the ER, and Ca^{2+}-binding proteins (chaperones) participating in intraluminal Ca^{2+} storage. ER leak channels, SERCA pumps and intraluminal Ca^{2+}-binding chaperones function in concert to maintain the optimal basal Ca^{2+} concentration within the ER required for protein synthesis and processing.

Sustained SERCA inhibition by thapsigargin (TG) or similar agents induces the ER stress response and simultaneous activation of apoptotic pathways within the ER and the mitochondria. However, as SERCA is a ubiquitous protein, thapsigargin is significantly toxic to both normal and cancer cells. The cytotoxicity of thapsigargin can be circumvented by coupling it to a carrier peptide to produce an inactive prodrug that is activated only in targeted malignant cells. In prostate cancer, prostate cancer-specific serine protease and prostate-specific antigen (PSA) have been used: *in vivo* efficacy studies demonstrated that daily or continuous treatment with such a prodrug resulted in complete block of PSA-producing LNCaP tumour growth during the period of exposure (Denmeade, et al., 2003). These prodrugs are currently undergoing preclinical evaluation as potential targeted therapy for prostate cancer, and they use SERCA pumps as a novel therapeutic target in cancer treatment (Denmeade, et al., 2005).

Thapsigargin, by blocking SERCA activity, induces ER stress. This effect is also produced by some other agents including diindolylmethane (DIM) derived from indole-3-carbinoal found in cruciferius vegetables (Savino, et al., 2006). DIM, like thapsigargin, rapidly mobilizes ER Ca^{2+} in cancer cell lines, but induces apoptosis in a cell-specific manner. In the androgen-independent DU-145 human prostate cancer cell line, DIM-induced apoptosis required both a decrease in the ER Ca^{2+} content and an increase in cytosolic calcium, whereas in the C33A cervical cancer cell line ER depletion was sufficient by itself (Savino, et al., 2006). Therefore, it was concluded that alteration of Ca^{2+} homeostasis within the ER can induce apoptosis via different mechanisms depending on the cell lines used.

Infection by human hepatitis B or C virus is a major risk factor for the development of liver cirrhosis and hepatocellular carcinoma (HCC) (Brechot, et al., 2000, Colombo, et al., 2003). Although they act via different mechanisms, both viruses directly influence the control of Ca^{2+} homeostasis in human hepatocytes. One of the proteins encoded by the HBV genome, the hepatitis virus X protein

(HBx), is central to HBV replication and may contribute to liver oncogenesis. HBx is a multifunctional protein (Murakami, 2001), and HBx expression deregulates cell growth (Lee, et al., 2001). HBV DNA has been found integrated into the host cell genome in more than 90% of HBV-related HCCs (Paterlini, et al., 1993). Paterlini-Brechot's group demonstrated that HBV DNA integration into the gene coding for SERCA1 in a liver tumour causes the expression of mutated protein, which results in a decreased ER Ca^{2+} content and an associated increase in apoptosis (Chami, et al., 2000). SERCA1 splice variants similar to the mutated proteins are also expressed in normal liver, but overexpression of these truncated pumps increases Ca^{2+} leakage from the ER, decreased its Ca^{2+} content and induced apoptosis (Chami, et al., 2001). Moreover, HBx itself directly modifies calcium homeostasis in several cell lines including the human hepatoma cell line Hep G2 (Chami, et al., 2003). Overexpression of HBx augmented cytosolic Ca^{2+} signals in response to inositol trisphosphate-(IP_3)-producing agonists most probably due to enhancement of caspase-dependent cleavage of plasma membrane Ca^{2+} ATP-ase without any modification of either basal ER Ca^{2+} filling or the kinetics of Ca^{2+} release (Chami, et al., 2003). HBx also caused major morphological changes to the mitochondria (fragmentation and swelling) and reduced mitochondrial Ca^{2+} uptake. It was concluded that HBx perturbs intracellular Ca^{2+} homeostasis mainly by acting on extrusion mechanisms, and that this effect plays an important role in the control of HBx-related apoptosis (Chami, et al., 2003).

The HCV core has recently been implicated in liver oncogenesis (Pawlotsky, 2004). It has been reported to interfere with Ca^{2+} signalling (Giannini, et al., 2003) and may directly induce apoptosis in transfected cells (Realdon, et al., 2004). The HCV core interferes with Ca^{2+} accumulation in the ER without affecting ER Ca^{2+} leakage or ER depletion (Benali-Furet, et al., 2005). Although calreticulin (CRT) overexpression was observed in HCV core-transfected cells, this probably leads to inhibition of SERCA2b function (John, et al., 1998) rather than to an increase in the ER Ca^{2+} content (Benali-Furet, et al., 2005). These observations are consistent with the HCV core directly triggering apoptosis in a proportion of transfected cells by inducing ER stress and ER Ca^{2+} depletion (Benali-Furet, et al., 2005) without any parallel decrease in store-operated calcium influx (Pinton, et al., 2000).

ER Ca^{2+} filling is largely determined by the expression of SERCA pumps. Several SERCA gene families have been identified; SERCA3 represents the largest family with numerous and species-specific isoforms (Bobe, et al., 2005), but their roles in cell physiology are still poorly defined. However, SERCA3 is not expressed in colon carcinomas but is abundantly expressed in normal colonic and gastric epithelial cells (Gelebart, et al., 2002). Consistent with this, intracellular calcium homeostasis becomes progressively anomalous during colon carcinogenesis, as reflected by deficient SERCA3 expression (Brouland, et al., 2005). A similar phenomenon is characteristic of prostate cancer. The only known tissue-specific SERCA-pump isoform detected in androgen-dependent human prostate cancer LNCaP cell line was the widely distributed phospholamban-dependent SERCA2b (Legrand, et al., 2001, Vanden Abeele, et al., 2002). Transformation of LNCaP cells

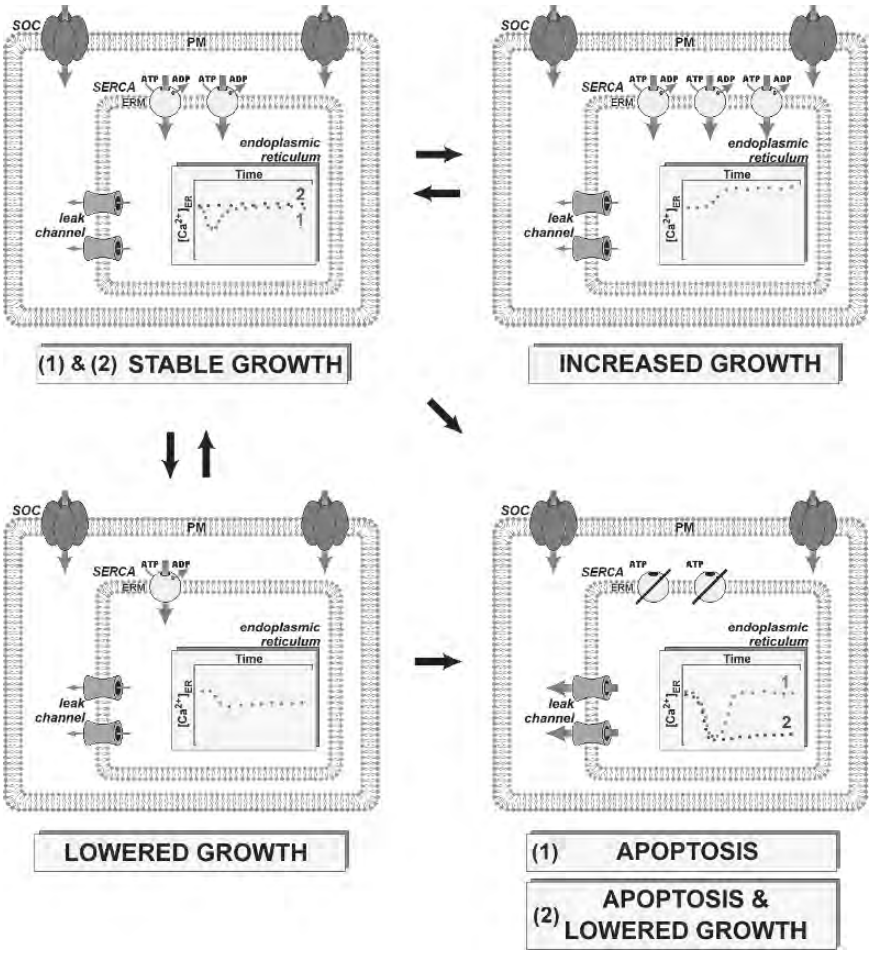

Figure 1. Endoplasmic reticulum Ca^{2+} pool as a key determinant of cell destiny. Each panel shows cellular plasma membrane (PM) and endoplasmic reticulum membrane (ERM) incorporating key Ca^{2+}-handling molecules: store operated channels (SOC), SERCA pump and ER leak channels. The inset graphs show ER calcium concentration ($[Ca^{2+}]_{ER}$) characteristic of each depicted state. **Stable growth** (upper left panel) is characterized by balanced expression of the SERCA pump and ER leak channel that provide optimal basal calcium filling of the ER (blue symbols marked by "2" in the inset graph). Transient decreases in the $[Ca^{2+}]_{ER}$ caused by physiological stimuli (red symbols marked by "1" in the inset graph) do not impair stable growth. **Increased growth** (upper right panel) is characterized by sustained elevation in $[Ca^{2+}]_{ER}$ (red symbols in the inset graph) mainly due to SERCA pump overexpression; the result is stimulated cell proliferation. **Lowered growth** (lower left panel) is characterized by a small decrease in the basal $[Ca^{2+}]_{ER}$ (red symbols in the inset graph) due to SERCA pump underexpression, which slows cell proliferation, but does not induce apoptosis yet. **Apoptosis** (lower right panel) can be induced by massive reduction in $[Ca^{2+}]_{ER}$ (red symbols marked by "1" in the inset graph); big long-lasting reduction in $[Ca^{2+}]_{ER}$ (blue symbols marked by "2" in the inset graph) due to substantial decrease in SERCA pump expression (marked by slash) and increased leak (marked as thiker arrows through the channels) shifts the whole balance towards growth arrest and enhanced programmed death. Black arrows indicate possible transitions between the states (See Colour Plate 23)

to androgen-independent phenotypes, characterized by increased malignancy and apoptotic resistance, results in the decreased expression of the SRCA2b Ca^{2+} pump. This decrease is one of the reasons for chronic underfilling of the ER Ca^{2+} pool and enhanced resistance of androgen-independent cells to the ER stress-induced apoptosis (Vanden Abeele, et al., 2002, Vanoverberghe, et al., 2004).

SERCA pumps also play a crucial role in cell proliferation (Lipskaia, et al., 2004). Proliferation of vascular smooth muscle cells (VSMC) is associated with a marked decrease in SERCA2a expression and overexpression of SERCA2a inhibited VSMC proliferation in a balloon injury model of the rat carotid artery (Lipskaia, et al., 2005). SERCA2a overexpression also resulted in an increase in the ER Ca^{2+} content, but reduced P2Y purinoceptor-stimulated rise in cytosolic calcium, largely due to the extremely fast rate of Ca^{2+} reuptake (Lipskaia, et al., 2005). The role of ER Ca^{2+} filling in the induction of apoptosis is generally well defined but data concerning its involvement in cellular proliferation is ambiguous. Using LNCaP cells, a model of human prostate cancer, we established that modulation of cell growth by such factors as epidermal growth factor (EGF), serum and androgens correlates with ER Ca^{2+} content: the increased growth rate induced by EGF was associated with a higher Ca^{2+} content and growth inhibition by androgens or serum deprivation was associated with a lower Ca^{2+} content (Legrand, et al., 2001). An increase or decrease of the ER load was accompanied by the increase or decrease of SERCA2b expression, respectively. Moreover, IGF was unable to enhance proliferation of TG-treated cells. Qualitatively and quantitatively similar results have been reported for other growth modulators: insulin growth factor (IGF), which stimulates proliferation, and tumour necrosis factor-α (TNF-α), which reduces proliferation and induces apoptosis (Humez, et al., 2004). As ER depletion is the key determinant of LNCaP cells apoptosis (Skryma, et al., 2000), these various findings allowed us to propose a general scheme for the involvement of ER Ca^{2+} load in the growth and death regulation of at least prostate cancer cells: stable growth is characterized by optimal filling due to balanced expression of the SERCA2b Ca^{2+} pump and ER leak channels, increased proliferation is characterized by overfilling due to increased expression of SERCA2b, inhibited proliferation is characterized by underfilling due to decreased expression of SERCA2b, and apoptosis is characterized by depletion due to decreased expression of SERCA2b and increased leak (Figure 1).

5. MITOCHONDRIAL CALCIUM AND CANCER

Mitochondria are essential for a number of cellular processes including those important for cancer progression such as growth, division, energy metabolism, and apoptosis. As organelles possessing the necessary apparatus for initiating suicidal process, they are a central integration point for the signals regulating a cell's destiny. Cellular Ca^{2+} overload, which may be triggered by various initial stimuli, promotes mitochondrial Ca^{2+} uptake. Excessive Ca^{2+} accumulation within the mitochondrial matrix is one of the primary reasons for mitochondrial permeability transition,

which is at least partly mediated by the opening of a permeability transition pore (PTP) – a multi-protein complex located at the contact sites between the inner and the outer mitochondrial membranes. PTP opening permits the release of mitochondrial apoptogenic factors, particularly cytochrome c (Cyt-c) and an apoptosis-inducing factor (AIF), into the cytoplasm where they activate a death-executing caspase cascade. Mitochondrial permeability in general, and the PTP complex in particular, are regulated by members of Bcl-2 family of proteins, of which those preventing apoptogenic factor release, Bcl-2 per se, Bcl-x_L, and Mcl-1, protect against apoptosis, whereas those promoting it, Bax and Bak, act as apoptosis enhancers (Antonsson, et al., 2000).

Effective Ca^{2+} transport mechanisms permit mitochondria to take up and release Ca^{2+} across their inner membrane (Rizzuto, et al., 2000) thereby controlling local and overall cytosolic Ca^{2+} concentration. The PTP together with H^+-Ca^{2+} and Na^+-Ca^{2+} exchange mechanisms form Ca^{2+} efflux pathways from mitochondria, whereas mitochondrial Ca^{2+} uptake mainly occurs via a channel-like uniporter (Kirichok, et al., 2004). Mitochondria are phically close to specialized cellular Ca^{2+} compartments (Rizzuto, et al., 1998) and Ca^{2+} channels (Varadi, et al., 2004) and are determinant in the regulation of vital Ca^{2+}-dependent processes, including activation of transcription factors and gene expression (Chen, et al., 2001, Dolmetsch, et al., 1997). In T lymphocyte leukaemia cells, gating of Ca^{2+} release-activated Ca^{2+} channels (CRAC), as well as the kinetics and magnitude of Ca^{2+} current (I_{CRAC}) through these type of store-operated Ca^{2+} channels, are determined by the mitochondrial status, underscoring the significance of mitochondria in defining cell cycle progression (Gilabert, et al., 2000, Hoth, et al., 2000, Hoth, et al., 1997).

A potential-dependent Ca^{2+} uptake into energized mitochondria reduces cytosolic Ca^{2+} in the proximity of CRAC channels, promoting channel opening (Gilabert, et al., 2000, Hoth, et al., 2000). Conversely, in cells with de-energized mitochondria and reduced mitochondrial Ca^{2+} uptake, the elevated $[Ca^{2+}]_i$ maintains store-operated channels closed, consistent with the Ca^{2+}-dependent inactivation of I_{CRAC} (Hoth, et al., 2000, Parekh, 1998). As mitochondria have the potential to regulate intracellular Ca^{2+} signalling and determine the transcriptional potential of a cell (Hoth, et al., 2000), targeting mitochondria has recently been exploited to control I_{CRAC}-mediated Ca^{2+} influx and associated cell proliferation (Enfissi, et al., 2004, Holmuhamedov, et al., 2002, Mignen, et al., 2005, Nunez, et al., 2006).

Drugs such as carboxyamidotriazole (CAI) or salicylate, which inhibit mitochondrial calcium import (Mignen, et al., 2005, Nunez, et al., 2006), or diazoxide, which displays a predilection for the inner mitochondrial membrane (Holmuhamedov, et al., 2002), can arrest cell cycle progression and block cell proliferation. The mitochondrial membrane potential must surge before proliferating cells can engage in S phase (Sweet, et al., 1999). Therefore, clamping the mitochondrial membrane potential at a depolarized level with diazoxyde provides a mechanistic basis for the antiproliferative action and cell arrest in the G0/G1 phase, thereby avoiding S phase.

6. CELL CYCLE BLOCK AND CALCIUM SIGNALLING

The role of growth factor-driven signalling in the pathogenesis of human cancers is well established and cancer is often associated with overexpression of growth factor receptors (Aaronson, 1991). Serum withdrawal may therefore block the cell cycle and mimic the difference between normal and cancerous cells. Serum deprivation for 48–72 hours blocks cell proliferation and has several consequences on the levels of expression of proteins involved in calcium signalling, including IP_3R, plasma membrane calcium channels, Ca^{2+} pumps, and Na^+-Ca^{2+} exchangers (Gill, et al., 1996, Golovina, et al., 2001, Smith, et al., 1994, Vallot, et al., 2000, Waldron, et al., 1994, Yu, et al., 2004). In Jurkat cells for instance, withdrawal of serum for 72 h has no significant effect on IP_3R or cyclin 1, but causes a 5-fold increase in the expression of calcium homoeostasis endoplasmic reticulum protein (CHERP), which could be completely reversed by restoring serum to the culture medium (O'Rourke, et al., 2003). CHERP is an integral ER membrane protein involved in the regulation of IP_3-mediated Ca^{2+} release (Laplante, et al., 2000). The initial arrest of cell proliferation due to the absence of serum allows comparison of Ca^{2+} mobilization in CHERP-depleted cells with that in control cells, to determine whether the lack of proliferation is a consequence of decreased Ca^{2+} mobilization. Serum deprivation for 72 h did not inhibit the rise in $[Ca^{2+}]_i$ in response to phytohaemagglutinin (PHA) or thrombin. The increase in the steady-state $[Ca^{2+}]_i$ due to the SERCA inhibitor cyclopiazonic acid was larger in the absence of serum than in its presence. The principal finding of these experiments is that an antisense-mediated depletion of CHERP impaired the ability of Jurkat cells to increase $[Ca^{2+}]_i$ in response to activation by PHA or thrombin. There was a direct correlation between the extent of CHERP depletion and the amplitude of Ca^{2+} transients. Reduction of CHERP expression by half reduced the $[Ca^{2+}]_i$ rise in response to PHA, but had no effect on Ca^{2+} influx, whereas a larger depletion of CHERP (>70%) further diminished the PHA-induced rise of $[Ca^{2+}]_i$, decreased Ca^{2+} influx and reduced TG-sensitive ER Ca^{2+} store filling. These various observations indicate that severe depletion of CHERP affected not only the size of Ca^{2+} mobilization, but also the magnitude of the ER Ca^{2+} load and the mechanism for activation of store-operated Ca^{2+} entry (SOCE). Cell proliferation was also affected by CHERP. The antisense-mediated knockdown of CHERP arrested proliferation of Jurkat cells and HEL cells (Laplante, et al., 2000). It was accompanied by a substantial decline in cyclin D1 concentration. Similarly, the ER Ca^{2+} content declines and proliferation is attenuated in other cell types under serum deprivation (Golovina, et al., 2001, Sweeney, et al., 2002), and these experimental conditions result in a notable drop in SERCA expression levels (see above).

Serum withdrawal affects the expression of plasma membrane calcium channels in diverse ways. Voltage-dependent calcium channels have their expression increased in the absence of serum in the culture medium (Ihara, et al., 2002, Kushmerick, et al., 2001, Patel, et al., 2005), whereas non voltage-dependent channels are down regulated (Golovina, 1999, Golovina, et al., 2001, Sweeney, et al., 2002, Yu, et al., 2004, Yu, et al., 2003).

The major changes in cell physiology during normal cell transformation to cancer cells suggest that the cells adapt their calcium signals to their new needs. However, calcium signals are highly organized and often restricted to specific sites to allow the diversity of normal and pathological cellular responses.

7. SPECIFICITY OF CA^{2+} SIGNALS

Various cellular Ca^{2+}-dependent processes rely on the specific spatial and temporal patterns of Ca^{2+} signalling (Thomas, et al., 1996). However, the type and manner of their organization during carcinogenesis is not completely understood. As Ca^{2+} signalling is an essential part of growth regulation, it is important to understand how various growth factors, neurotransmitters and hormones that control physiological and pathological cell proliferation, trigger specific Ca^{2+} signals. For instance, stimulation of two receptors on prostate cancer epithelial cells, α1-adrenoceptor (α1-AR) and metabotropic purinergic receptor (P2Y-R), produce divergent effects on cell proliferation: α1-AR stimulation enhances proliferation (Thebault, et al., 2006), and stimulation of P2Y-R results in growth arrest (Vanoverberghe, et al., 2003). These divergent effects on proliferation are surprising, given that both receptors act via a common phospholipase C (PLC)-catalyzed inositol phospholipid breakdown signalling pathway that results in the derivation of two second messengers important for Ca^{2+} signalling (Marshall, et al., 1999, von Kugelgen, et al., 2000), IP_3 and diacylglycerol (DAG). Our recent work with primary human prostate cancer epithelial cells brought some insight into these puzzling observations (Thebault, et al., 2006). Ca^{2+} signalling controlled by the two receptors relies on different Ca^{2+}-entry pathways ultimately targeting different intracellular effectors. It appeared that stimulation of α1-AR activates plasma membrane non-specific cationic channels via direct DAG gating (Thebault, et al., 2003) without affecting ER Ca^{2+} stores. In contrast, P2Y-R stimulation brings about IP_3R -mediated ER store depletion and activation of SOCs (Vanoverberghe, et al., 2003). Consistent with these peculiarities the α1-AR agonist, phenylephrin, stimulated oscillatory-type intracellular Ca^{2+} signalling involving DAG-gated cationic channels, whereas the P2Y-R agonist, ATP, induced a transient $[Ca^{2+}]_i$ rise followed by a smaller sustained elevation due to store depletion and SOC activation. The two Ca^{2+} entry pathways also appeared to have different molecular origins with the first mostly relying on TRPC6, a DAG-gated representative of the transient receptor potential (TRP) channel family, and the second on TRPC1 and TRPC4, both store-dependent members of the same family.

The expression of genes involved in cell proliferation and cell death is regulated by nuclear transcriptional factors. NFAT (Nuclear Factor of Activated T cells) proteins are a family of Ca^{2+}-dependent transcription factors (Crabtree, 2001), whose nuclear translocation and transcriptional activity is regulated by Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin (Crabtree, 2001). Thus, NFAT proteins can potentially be activated by diverse stimuli that lead to increased intracellular calcium levels. The NF-kB (nuclear factor kappa B) family

is another ubiquitously expressed family of transcription factors dependent on Ca^{2+} homeostasis, especially on the filling status of Ca^{2+} ER stores (Li, et al., 2002) (Clapham, 2003). α1-AR stimulation enhances prostate cancer epithelial cell proliferation by inducing store-independent, TRPC6-mediated Ca^{2+} entry resulting in NFAT activation (Thebault, et al., 2006). TRPC6 antisense knockout has effects similar to those of pharmacological α1-AR inhibition, i.e. suppression of agonist-induced Ca^{2+} entry, cessation of oscillatory-type Ca^{2+} signalling and consequent termination of cell proliferation. Furthermore, chronic treatment with α1-agonists enhanced TRPC6 protein expression, and altered the expression of two cell-cycle regulators, CDK4 and cyclin-dependent kinase inhibitor p27; this provides direct evidence for the α1-AR–TRPC6–NFAT–cell proliferation link. In contrast, Ca^{2+} entry associated with P2Y-R stimulation by extracellular ATP and related growth arrest did not involve either activation of TRPC6 channels or NFAT translocation. Our findings demonstrate that the α1-AR-dependent Ca^{2+} signalling which promotes proliferation of prostate cancer epithelial cells specifically requires the activation of TRPC6 channels coupled to NFAT, and thereby suggest TRPC6 as a new potential therapeutic target for controlling prostate cancer cell proliferation.

Clinical studies also implicate α1-AR antagonists as pro-apoptotic agents capable of inducing apoptosis of human prostate cancer epithelial and smooth muscle cells without affecting their cellular proliferation (Kyprianou, et al., 2000b). However, existing evidence indicate that these effects may be unrelated to α1-AR (Benning, et al., 2002, Kyprianou, et al., 2000a) and associated Ca^{2+} signalling.

Interestingly, recent evidence suggests that the expression of TRPC1, TRPC3 and TRPV6 proteins in androgen-dependent prostatic LNCaP cells is under the control of ER Ca^{2+} filling: after a prolonged (24–48 h) depletion of the stores with thapsigargin, which generally exerts pro-apoptotic action, expression of these factors was enhanced. The upregulation of TRPC1 and TRPC3 involved the activation of the Ca^{2+}/calmodulin/calcineurin/NFAT pathway (Pigozzi, et al., 2006). Functionally, cells overexpressing TRPC1, TRPC3 and TRPV6 channels after a prolonged depletion of the stores showed an increased $[Ca^{2+}]_i$ response to α-adrenergic stimulation, although the SOCE was unchanged. The selected overexpression of TRPV6 (without overexpression of TRPC1 or TRPC3) did not produce this increased response to agonists, suggesting that TRPC1 and/or TRPC3 proteins are responsible for the response to α-adrenergic stimulation but that TRPC1, TPRC3 and TRPV6 proteins, expressed alone or concomitantly, are not sufficient for SOC formation. Increased expression of store-dependent TRP members under ER store depletion is difficult to reconcile with the findings that androgen-independent, apoptosis-resistant prostate cancer cell phenotypes, for which chronic underfilling of the ER Ca^{2+} pool represents a new equilibrium helping them to withstand ER stress-mediated apoptosis, are characterized by reduced SOCE (Prevarskaya, et al., 2004, Vanden Abeele, et al., 2002, Vanoverberghe, et al., 2004). It is, therefore, likely that native SOC in prostate cancer epithelial cells is a much more complex entity whose functional expression does not correlate directly with any of the TRP members implicated.

Recent evidence points to the important functions of NFAT in other, non-prostatic human carcinomas as well. Specifically, NFAT is active in human breast and colon carcinoma cells. In human breast epithelial cells, NFAT1 is connected with breast cancer metastasis, and can be activated by a non-transforming secreted Wingless (Wnt) signalling protein, Wnt-5a, through a non-canonical Ca^{2+} signalling pathway (Dejmek, et al., 2006). NFATc1 is commonly overexpressed in pancreatic carcinomas and enhances the malignant potential of tumour cells through transcriptional activation of the c-myc proto-oncogene, ultimately resulting in increased cell proliferation and enhanced anchorage-independent growth (Buchholz, et al., 2006). Conversely, c-myc transcription and anchorage-dependent and -independent cell growth is significantly attenuated by inhibition of Ca^{2+}/calcineurin signalling or siRNA-mediated knock down of NFATc1 expression. These various results demonstrate that ectopic activation of NFATc1 and the Ca^{2+}/calcineurin signalling pathway is an important mechanism of oncogenic c-myc activation in pancreatic cancer (Buchholz, et al., 2006).

8. CONCLUSION

The transformation of a normal cell into a cancer cell is associated with a major re-arrangement of Ca^{2+} pumps, Na/Ca exchangers, and plasma and reticular calcium channels. They form signalling complexes that can deliver Ca^{2+} signals with the spatial and temporal characteristics necessary to account for the new cellular processes. Differential transcription and expression mechanisms underlie the passage from normal to pathological cell growth and proliferation. Expression of a new member of the Ca^{2+}-signalling kit specifically in cancer cells is therefore a potential therapeutic target as long as its block results in a clear inhibition of cell proliferation. However, most of these targets are expressed in a large number of cell types and organs where they may have a different function. Therefore, inhibitors or blockers of these channels, pumps or exchangers must be specifically addressed to cancer sites. Nevertheless, various approaches to the development of novel cancer therapies can be envisaged: prodrugs activated by specific proteases, for example serine protease prostate-specific antigen (PSA); and transfection reagents based on SiRNA designed to target tissue-specific membrane receptors which thereby regulate the expression of channels, pumps or exchangers.

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CHAPTER 16

CALCIUM MISREGULATION AND THE PATHOGENESIS OF MUSCULAR DYSTROPHY

F.W. HOPF[1], P.R. TURNER[2] AND R.A. STEINHARDT[3,*]

[1]*Ernest Gallo Clinic and Research Center, University of California, San Francisco, 5858 Horton St., Suite 200, Emeryville, CA 94608, woody@egcrc.net*
[2]*Panomics, 6519 Dumbarton Circle, Fremont, CA, 94555, paulturner727@btopenworld.com*
[3]*Department of Molecular and Cell biology, University of California, Berkeley, Berkeley, CA 94720-3200*

Abstract: Although the exact nature of the relationship between calcium and the pathogenesis of Duchenne muscular dystrophy (DMD) is not fully understood, this is an important issue which has been addressed in several recent reviews (Alderton and Steinhardt, 2000a, Gailly, 2002, Allen et al., 2005). A key question when trying to understand the cellular basis of DMD is how the absence or low level of expression of dystrophin, a cytoskeletal protein, results in the slow but progressive necrosis of muscle fibres. Although loss of cytoskeletal and sarcolemmal integrity which results from the absence of dystrophin clearly plays a key role in the pathogenesis associated with DMD, a number of lines of evidence also establish a role for misregulation of calcium ions in the DMD pathology, particularly in the cytoplasmic space just under the sarcolemma. A number of calcium-permeable channels have been identified which can exhibit greater activity in dystrophic muscle cells, and existing evidence suggests that these may represent different variants of the same channel type (perhaps the transient receptor potential channel, TRPC). In addition, a prominent role for calcium-activated proteases in the DMD pathology has been established, as well as modulation of other intracellular regulatory proteins and signaling pathways. Whether dystrophin and its associated proteins have a direct role in the regulation of calcium ions, calcium channels or intracellular calcium stores, or indirectly alters calcium regulation through enhancement of membrane tearing, remains unclear. Here we focus on areas of consensus or divergence amongst the existing literature, and propose areas where future research would be especially valuable

Keywords: dystrophin-glycoprotein complex, sarcolemma, membrane tears, calcium channels, calcium-activated proteases

*R.A.Steinhardt, ricksteinhardt@berkeley.edu

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1. STRUCTURE AND FUNCTION

1.1. The Dystrophic Genome

There is an excellent understanding of the different muscular dystrophies at the gene level. The genetic changes responsible for DMD are found on the X chromosome (Koenig et al., 1987, Monaco et al., 1986), specifically at Xp21 (Boyd and Buckle, 1986). This location was confirmed using DNA markers (Davies et al., 1983). The disease was shown to be allelic with the milder Beckers Muscular Dystrophy (BMD) (Kingston et al., 1983). The DMD gene is the largest and one of the most complex yet described, spanning ~2.5 Mb of genomic sequence and composed of 79 exons (Coffey et al., 1992, Monaco et al., 1992, Roberts et al., 1983). The full-length 14-kb mRNA transcript is predominantly expressed in skeletal and cardiac muscle, with smaller amounts found in the CNS (Monaco et al., 1986, Koenig et al., 1987, Monaco et al., 1987). The protein product encoded by this transcript was named dystrophin as the lack of it causes dystrophy (Hoffman et al., 1987, Koenig et al., 1987). Expression of the full 14-kb dystrophin transcript is controlled by three independent promoters. The brain, muscle, and Purkinje promoters each consist of unique first exons spliced to a common set of 78 exons (Nudel et al., 1989, Chelly et al., 1990, Klamuth et al., 1990, Boyce et al., 1991, Mackover et al., 1991, Gorecki et al., 1992). The brain promoter regulates expression in cortical neurons and the hippocampus (Barnea et al., 1990, Chelly et al., 1990, Gorecki et al., 1992), the Purkinje promoter drives expression in the cerebellar Purkinje cells and also in skeletal muscle (Gorecki et al., 1992, Holder et al., 1996), and the muscle promoter causes high levels of expression in skeletal muscle and cardiomyocytes, and low levels in some glial cells in the CNS (Barnea et al., 1990, Chelly et al., 1990). Although the majority of studies have examined calcium regulation in dystrophic skeletal muscle, loss of dystrophin also alters the function of cardiac (Yasuda et al., 2005) and smooth muscle (e.g., Serio and Mule, 2001). In fact, loss of dystrophin may have a more detrimental effect on cardiac than skeletal muscle; cardiac failure is the second leading cause of death in DMD patients (Muntoni, 2003).

1.2. Dystrophin Isoforms and Splice Variants

Alternative splicing at the 3'-end of the dystrophin gene can give rise to a number of isoforms (Feener et al., 1989, Bies et al., 1992). In addition, the DMD gene has four internal promoters, whose activity can give rise to truncated COOH-terminal isoforms. Each promoter utilizes a unique first exon that generates proteins of 260 kDa (Hoffman et al., 1987), 140 kDa (Lidov et al., 1995), 116 kDa (Byers et al., 1993), and 71 kDa (Blake et al., 1992, Hugnot et al., 1992, Lederfein et al., 1992) respectively. The 71 kDa isoform is detected in most nonmuscle tissues including brain, kidney, liver, and lung (Blake et al., 1992, Schofield et al., 1994, Howard et al., 1998, 1999, Sarig et al., 1999) while the remaining short isoforms are primarily expressed in the central and peripheral nervous system (Byers et al., 1993, Schofield et al., 1994, Lidov et al., 1995). These truncated proteins contain binding

sites for a number of dystrophin-associated proteins (DAPs), and they are thought to be involved in the stabilization and function of non-muscle dystrophin-like protein complexes (DPCs). Alternative splicing at the 3'-end is also found in the shorter isoforms. This differential splicing may regulate the binding of dystrophin to DAPs at the membrane (Crawford et al., 2000).

1.3. Protein Structure

Dystrophin is 427-kDa protein with homology to the β-spectrin/α-actinin protein family (Koenig et al., 1988). Dystrophin contains at least four separate regions: the actin-binding domain at the N-terminus, a central rod domain containing spectrin like repeats, a cysteine-rich domain, and the COOH-terminal domain. The NH2 terminus and a region in the rod domain bind directly to, but do not cross-link, cytoskeletal actin (Winder et al., 1995, Rybakova et al., 1996). The flexible rod domain is composed of 24 spectrin-like repeats. These α-helical repeats are interrupted by four proline-rich hinge regions (Koenig and Kunkel, 1990), and at the end of the 24th repeat, the fourth hinge region is followed by a WW (tryptophan) binding domain. This is found in several signaling and regulatory molecules (Bork and Sudol, 1994, Macias et al., 1996). Although a specific ligand for the WW domain of dystrophin has not been determined, this region mediates the interaction between β-dystroglycan and dystrophin. The entire WW domain of dystrophin does not appear to be required for this interaction with dystroglycan. The 71 kDa dystrophin isoform which contains only part of the WW domain, can bind to β-dystroglycan (Rosa et al., 1996). Interestingly, transgenic mice overexpressing the 71 kDa isoform in dystrophin-deficient muscle restore α-dystroglycan and the DPCs at the membrane but do not prevent muscle degeneration (Cox et al., 1994, Greenberg et al., 1994). The cysteine rich domain contains two EF-hand motifs that could bind intracellular Ca^{2+} (Koenig et al., 1988). The ZZ domain is also part of this cysteine-rich domain and contains conserved cysteine residues that can form coordination sites for divalent metal cations such as Zn^{2+} (Ponting et al., 1996). This ZZ domain zinc finger of dystrophin binds to calmodulin in a Ca^{2+}-dependent manner (Anderson et al., 1996). Thus the ZZ domain may represent a functional calmodulin-binding site and may have implications for calmodulin binding to other dystrophin-related proteins. The COOH terminus of dystrophin contains two polypeptide stretches that are predicted to form α-helical coiled coils similar to those in the rod domain (Blake et al., 1995). Each coiled coil has a conserved repeating heptad domain similar to those found in leucine zippers (Lupas, 1996, Burkhard et al., 2001). This domain has been named the CC (coiled coil) domain. The CC region of dystrophin forms the binding site for dystrobrevin and may modulate the interaction between syntrophin and other DAPs (Blake et al., 1995, Saoulet-Puccio et al., 1997).

Dystrophin is linked to the sarcolemma of normal muscle by a protein complex composed of at least 10 different proteins (McNeil and Steinhardt, 2003). In contrast to spectrin that appears operate as a heterodimer, the dystrophin complex is monomeric (Rybakova and Ervasti, 1997). This complex spans the membrane and

links the actin-based cytoskeleton to the muscle basal lamina (Figure 1). Thus the DPC can be thought of as a scaffold connecting the inside of a muscle fiber to the outside. The DPC can be divided into several separate subcomplexes based on their location within the cell and their physical association with each other. Using detergent extraction and two-dimensional gel electrophoresis, Yoshida et al. (1994) showed that the DPC could be dissociated into three distinct complexes. These complexes are 1) the dystroglycan complex, 2) the sarcoglycan/sarcospan complex and 3) the cytoplasmic, dystrophin-containing complex.

Dystroglycan was the first component of the DPC to be cloned (Ibraghimov-Beskrovnaya et al., 1992). The single dystroglycan gene produces a precursor protein that is processed by an unidentified protease to produce α- and β-dystroglycan. The dystroglycan gene is composed of only two exons, and there is no evidence of alternative splicing, although several glycoforms are produced (Ibraghimov-Beskrovnaya et al., 1993). The relative molecular weights of α-dystroglycan differ in different tissues as a result of the aforementioned differential glycosylation. In muscle, β-dystroglycan has a molecular mass of 156 kDa, whereas β-dystroglycan is 43 kDa. In brain, β-dystroglycan has a molecular mass of 120 kDa and was independently identified as a protein called cranin (Smalheiser and Schwartz, 1987, Smalheiser and Kim, 1995). Dystroglycan has a single transmembrane domain and is inserted into the muscle plasma membrane with the COOH terminus on the cytoplasmic side. In contrast, β-dystroglycan is located

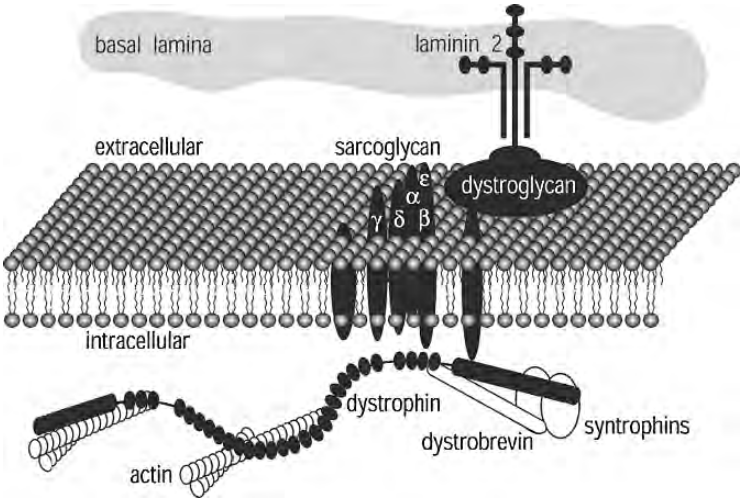

Figure 1. The muscle dystrophin-glycoprotein complex. The dystrophin-glycoprotein complex normally spans the plasma membrane of the skeletal muscle cell and may stabilize the sarcolemma and cytoskeleton to allow force transduction between the intracellular cytoskeleton (F-actin filaments) and the extracellular matrix. The molecules indicated are core components of the dystrophin-glycoprotein complex. Laminin 2 is the predominant laminin isoform in skeletal muscle basement membranes. Modified from McNeil and Steinhardt (2003)

in the extracellular matrix where it is thought to be directly associated with α-dystroglycan through multiple covalent interactions. The extreme C-terminus of α-dystroglycan contains several proline residues that are required for dystroglycan binding to dystrophin (Suzuki et al., 1992, 1994, Jung et al., 1995, Rentschler et al., 1999). The last 15 amino acids of β-dystroglycan appear to bind directly to the cysteine-rich region of dystrophin. This region of β-dystroglycan is proline rich and contains a site for tyrosine phosphorylation (James et al., 2000). Recently, the crystal structure of β-dystroglycan bound to dystrophin has been determined (Huang et al., 2000). The structure of this region of dystrophin shows that dystroglycan forms contacts with both the WW domain and EF-hands of dystrophin. The C-terminus of β-dystroglycan also binds to the adaptor protein Grb2 (Yang et al., 1995). This interaction is mediated by the SH3 domain of Grb2 that binds to proline-rich sequences in the cytoplasmic tail of β-dystroglycan. Thus β-dystroglycan may participate in the transduction of extracellular-mediated signals to the muscle cytoskeleton (Yang et al., 1995).

The dystrophin complex also interacts with neuronal-type nitric oxide synthase (nNOS), whose biological product, NO, regulates contraction in skeletal muscle (Stamler and Meissner, 2001). Another protein associated with the DGC, although not essential for the biogenesis of the complex itself, is caveolin-3 (Cav-3), a member of the caveolin protein family. Caveolins are the main structural components of caveolae, which are cholesterol- and sphingolipid-rich vescicular invaginations of the plasma membrane (Williams and Lisanti, 2004). The complete loss of dystrophin perturbs the structural composition of the DGC, such that all members of the DGC complex are greatly reduced in skeletal muscle fibers from DMD patients and from *mdx* mice (see above). The only exception is Cav-3, which was shown to be up-regulated by 2-fold in dystrophin-deficient skeletal muscle (Repetto et al., 1999). Caveolin regulation of sarcolemmal integrity and muscle regeneration in skeletal, cardiac, and smooth muscle are likely impaired in dystrophin-deficient muscle (Rando, 2001, Volonte et al., 2003, Halayko and Stelmack, 2005).

Finally, although much work has focused upon dystrophin and the associated DAG as a structural scaffold protecting the sarcolemma and intracellular contractile machinery, it is also clear that the DGC plays an important role in regulation of intracellular signaling, in particular through a number of kinase cascades implicated in cell survival (reviewed in Rando, 2001).

1.4. Mutations in DMD

Mutations that cause DMD generally result in the absence, or greatly reduced levels, of dystrophin protein, while BMD patients generally make some partially functional protein. There is some correlation between mutations in the DMD gene and the resulting phenotype. The study of such mutations has revealed the importance of a number of the structural domains of dystrophin and facilitated the design of dystrophin "mini-genes" for gene therapy approaches (Amalfitano et al., 1997). Approximately 65% of DMD and BMD patients have gross deletions of the DMD

gene (Monaco et al., 1985, Koenig et al., 1989). Monaco et al. (1988) argue that if a deletion leads to the expression of an internally truncated transcript without shifting the normal open reading frame, then a smaller, but functional version of dystrophin can be produced. This scenario would be consistent with a BMD phenotype. If, on the other hand, the deletion creates a translational frameshift, then premature termination of translation will result in the synthesis of a truncated protein. This latter scenario is often associated with extremely low levels of dystrophin expression and a DMD phenotype. There are exceptions (Malhotra et al., 1988, Baumbach et al., 1989, Winnard et al., 1993): complete dystrophin deficiency may be associated with a relatively benign phenotype (Hattori et al., 1999). The vast majority of large deletions detected in BMD and DMD cluster around two mutation "hot spots" (Koenig et al., 1989). Deletion cluster region I spans exons 45–53 (Beggs et al., 1990) and removes part of the rod domain, while deletion cluster region II spans exons 2–20 and removes some or all of the actin-binding sites together with part of the rod domain (Liechti-Gallati et al., 1989). One-third of DMD cases are caused by very small deletions and point mutations, most of which introduce premature stop codons (Hoffman et al., 1987, Lenk et al., 1993). These small deletions and point mutations appear to be evenly distributed throughout the gene (Beggs and Kunkel, 1990, Chamberlain, 1992, Roberts et al., 1994, Prior et al., 1995). Although it might be predicted that such mutations would give rise to normal amounts of truncated protein, usually very little or no protein is detected (see below for results with proteosome inhibition) (Vainzof et al., 1993).

A small number of useful mutations have been identified that generate mutated or truncated proteins. At the N-terminus of dystrophin, the importance of the actin-binding domain was demonstrated by the identification of a missense mutation (Arg for Leu-54) that resulted in a DMD phenotype associated with reduced amounts of protein (Prior et al., 1995). The rod domain of dystrophin has been found to accommodate large in-frame deletions without serious clinical consequences. The most notable example was the discovery of a patient with an in-frame deletion of 46% of the dystrophin coding sequence which resulted in only a mild case of BMD (England et al., 1990). This observation suggests that the rod domain acts merely as a spacer between the actin binding domain and the cysteine-rich and COOH-terminal domains of dystrophin. This deletion has been the basis of a dystrophin mini-gene used for transfer to muscle fibers *in vivo* (Acsadi et al., 1991, Dunckley et al., 1991, Ragot et al., 1993), which is able to restore the normal muscle phenotype in transgenic *mdx* mice (Phelps et al., 1995, Wells et al., 1995). The substitution of a conserved cysteine residue with a tyrosine at position 3340 results in reduced but detectable levels of dystrophin. This mutation alters one of the coordinating residues in the ZZ domain that is thought to interfere with the binding of the dystrophin-associated protein β-dystroglycan (Lenk et al., 1996). Another reported substitution, this time a histidine residue for an aspartate residue at position 3335, is also thought to affect the β-dystroglycan binding site, and although normal localization and amounts of dystrophin were detected, a severe phenotype resulted (Goldberg et al., 1998). The cysteine-rich domain is never deleted in BMD patients,

suggesting that this domain is critical for dystrophin function (Hoffman et al., 1991, Bies et al., 1992, Helliwell et al., 1992, Rafael et al., 1996), and presumably reflects the interactions with other dystrophin-associated proteins. Finally, cases of X-linked cardiomyopathy are caused by mutations in the DMD gene that abolish the cardiac gene expression of dystrophin, while retaining expression in skeletal muscle. This condition involves ventricular wall dysfunction, dilated cardiomyopathy, and cardiac failure in the absence of skeletal myopathy (Ferlini et al., 1994). Mutations in the muscle-specific M-promoter selectively abolish expression in the heart.

1.5. The mdx Mouse

The discovery of dystrophin has allowed the identification and targeted disruption of orthologous genes in other species. Thus far, dystrophin deficient cats, mice and dogs, have all been identified, and the gene has also been targeted in *C. elegans* (Bessou et al., 1998). All of these animal models have potential for the study of the pathophysiology of DMD. The dystrophin-deficient mouse (*mdx*) was initially identified because of raised serum creatine kinase levels, an enzyme released following damage to muscle fibres (see below), and these mice were also found to have significant muscle pathology (Bulfield et al., 1984). The *mdx* mouse lacks full-length dystrophin (Hoffman et al., 1987), a point mutation in exon 23 having formed a premature stop codon (Sicinski et al., 1989), but animals retain expression of some COOH-terminal dystrophin isoforms. Subsequent studies with ethyl-nitroso-urea induced and insertional mutagenesis have produced similar genetic lesions, with resultant mice phenotypically similar to the *mdx* mouse (Chapman et al., 1989, Cox et al., 1993, Im et al., 1996, Wertz and Fuchtbauer, 1998). The full-length dystrophin is therefore the functionally significant isoform.

However, the validity of this model for the study of DMD has been questioned (Dangain and Vrbova, 1984), because obvious animal weakness is not evident unless subjected to exercise, and, unlike the case in humans, the life span of *mdx* mice is not greatly reduced (Pastoret and Sebille, 1995, Lunch et al., 2001). Nonetheless, *mdx* skeletal muscles are dysfunctional (Rafael et al., 2000), with force production and power output both significantly reduced (Carlson and Makiejus, 1990, Lynch et al., 2001). Significant muscle fiber necrosis occurs, particularly at 3–4 wks of age (Tanabe et al., 1986). This is balanced in part by a vigorous regenerative response, with fibers now expressing the fetal myosin heavy chain isoform and with centrally located nuclei, which also occurs when normal muscle regenerates after a nonspecific insult (Hall-Craggs and Seyan, 1975, Coulton et al., 1988, Dimario et al., 1991). After this initial crisis period, many fibers remain centrally nucleated, but expression of the fetal myosin heavy chain declines. Degeneration and regeneration of muscle fibers continues apace (Jin et al., 2000) particularly in the diaphragm (Stedman et al., 1991, Pagel and Partridge, 1999), with the proliferating satellite muscle precursor cells expressing markers of activation (Jin et al., 2000). Fibrosis does eventually become apparent in the limb muscles of older *mdx* mice (Pastoret and Sebille, 1993). Thus, *mdx* mice show many features of DMD, but often at

later times relative to humans. Why this should be is not clear but may relate to differences in the murine biology of muscle regeneration (Gorospe et al., 1997). Despite these differences, the *mdx* mouse has been a key resource in the exploration of dystrophic pathophysiology, and *mdx* mice have already been used to screen for potential therapeutic treatments (Allamand and Campbell, 2000, Granchelli et al., 2000).

2. INCREASED FRAGILITY OF THE DYSTROPHIC SARCOLEMMA IS CRITICAL FOR ENHANCED MUSCLE DAMAGE

It is widely accepted that the sarcolemma of dystrophic muscle fibers is more susceptible to membrane tearing than tissue from control muscle (for review, see Allen et al., 2005). In normal muscle, dystrophin and the complex of DAGs is thought to anchor the intracellular cytoskeleton of the muscle fiber to the extracellular matrix. In the absence of this mechanical support, the sarcolemma is subject to excess mechanical strain during contractile activity, leading to tearing of the membrane. A number of lines of evidence demonstrate the increased fragility of the sarcolemma in dystrophic muscle, especially studies of damage to control and dystrophic muscle during strong contractile activity (discussed below). Membrane damage is most pronounced after eccentric contractions, where the intracellular contractile machinery shortens while the overall muscle length increases, which places particular strain upon the sarcolemma (Allen et al. 2005). Membrane fragility is not an intrinsic property of the sarcolemma, since the amount of pressure required to rupture the membrane is not different between dystrophic and normal muscle (Franco-Obregon and Lansman, 1994). Thus, any increased tearing or disruption of the sarcolemmal integrity likely results from altered sarcolemmal-cytoskeletal interactions, perhaps with weakening at particular regions of the sarcolemma where spectrin is less dense (Franco-Obregon and Lansman, 1994).

Early studies noted increased levels of creatine kinase in the blood of dystrophic patients and mice (for review, see Gillis, 1999). Since creatine kinase normally resides within the muscle cell, it could only leave the myofiber through tears in the membrane. In control muscle, creatine kinase is primarily observed outside of muscle fibers after intense or eccentric exercise. In contrast, dystrophic humans and mice have elevated creatine kinase levels under basal conditions, and levels are especially exaggerated after prolonged muscle contraction. Similar results are observed using loss of basic fibroblastic growth factor from dystrophic muscle as an indicator of membrane permeability (Clarke et al., 1993). In addition, endogenous or exogenous membrane-impermeant molecules, such as albumin, Flourescein-Lycine polymer, procion orange or Evans blue (Clarke et al., 1993, Petrof et al., 1993, Matsuda et al., 1995, Doran et al., 2006), do not normally pass through the fatty sarcolemmal membrane. Thus, any conditions under which these molecules are able to enter a muscle cell indicate that the integrity of the sarcolemma is compromised. Entry of these dyes into dystrophic muscle is far greater than control in unexercised

animals (Clarke et al., 1993, Matsuda et al., 1995), and exercise dramatically enhances dye entrance in dystrophic muscle, with a much more moderate effect in control muscle (Petrof et al., 1993). Increased susceptibility of dystrophic muscle to osmotic shock (Menke and Jockusch, 1991, Imbert et al., 1996, Vandebrouck et al., 2001) also suggests that enhanced mechanical disruption of the dystrophic sarcolemma is a primary contributor to the dystrophic pathology. However, it is likely that any membrane tears that do occur in control or dystrophic muscle heal rapidly (Carpenter and Karpati, 1979, Clarke et al., 1993, Bansal et al., 2003), otherwise membrane-impermeant dyes would not be retained within the cell free within the cytoplasm. In support of the primary importance of wounding and membrane resealing, loss of dysferlin decreases membrane resealing and results in a DMD-like pathology (Bansal et al., 2003, Doherty and McNally, 2003). Thus, increased dye entry and creatine kinase loss suggest decreased integrity of the sarcolemma in the absence of the dystrophin protein.

In addition to dye flux studies, a number of groups have measured the loss of force generation after eccentric contractions (contraction with stretching) as a measure of muscle damage, and have generally observed that the degree of loss of force generation is greater in dystrophic muscle (for review, see Allen et al., 2005, but see Sacco et al., 1992). Markers of degeneration and apoptosis are also more prevalent in dystrophic muscle after exercise (Weller et al., 1990, Matsuda et al., 1995, De Luca et al., 2003), supporting the idea that muscle damage after exercise is greater in dystrophic tissue. The loss of force and necrosis which typify the dystrophic pathology could result from increased sarcolemmal permeability or damage to the myofibrils of the intracellular contractile machinery in the absence of dystrophin. However, after permeabilization, which essentially removes the sarcolemma, muscle damage after contraction and stretching is identical and pronounced in both dystrophic and control muscle (Lynch et al., 2000). Thus, muscle damage after exercise likely occurs secondarily to sarcolemmal disruption, which is more severe in dystrophic muscle. In agreement, dye entry correlates with loss of force generation (Petrof et al., 1993) and with the presence of markers for apoptosis (Matsuda et al., 1995). Further, restoring dystrophin expression in dystrophic muscle reduces both dye influx and the loss of force during eccentric contraction (Deconinck et al., 1996). It has also been widely noted that dystrophic muscles are larger and heavier, with larger fibers, perhaps as a consequence of regeneration (e.g., see Tutdibi et al., 1999). Despite this greater size, dystrophic muscle develops less force relative to the cross-sectional area. This may be due to accumulation of damage from which there is no recovery (Woods et al., 2005).

Cardiac muscle may be more sensitive than skeletal muscle to the loss of dystrophin, and heart failure is the second leading cause of death among DMD patients (see Yasuda et al., 2005). Interestingly, the decreased compliance and increased stretch-induced calcium overload in dystrophic cardiac muscle are reduced by poloxamer 188, a membrane sealant (Yasuda et al., 2005), suggesting that enhancing sarcolemmal integrity could significantly reduce cardiac damage and represent a novel therapeutic intervention for DMD.

We should note that the exact mechanism underlying the loss of force after eccentric contractions remains unclear (see Allen et al., 2005), with a possible role for impaired excitation-contraction coupling (e.g., depolarization and partly inactivation of voltage-dependent calcium channels (Imbert et al., 2001, Friedrich et al., 2004), to calcium-dependent processes, and/or to damage to contractile machinery. In fact, increased membrane fragility might be secondary to increased intracellular free calcium levels (Iwata et al., 2003, Allen et al., 2005), although calcium-induced enhancement of membrane permeability does not involve proteases in normal muscle (Jackson et al., 1984) and overexpression of a calpain inhibitor reduces necrosis without altering sarcolemmal permeability (Spencer and Mellgren, 2002).

3. IS INTRACELLULAR CALCIUM ALTERED IN DYSTROPHIC MUSCLE CELLS?

As described below, there has been some controversy as to whether calcium levels are elevated within muscle cells lacking dystrophin. However, it is clear from several lines of evidence that free calcium levels are elevated in dystrophic muscle cells in a region lying just under the sarcolemma, and are also elevated in the bulk cytoplasm in some cases. This elevated calcium likely results from increased influx through calcium-permeable channels (described in section 4), and in turn contributes to calcium-dependent proteolysis (described in section 5) which facilitates muscle cell necrosis. Importantly, calcium influx through increased levels of muscle tearing in dystrophic muscle (described in section 2) can activate certain calcium channels, providing a cellular mechanism linking the increased fragility in the absence of dystrophin which is clearly important for the pathogenesis of muscular dystrophy to the misregulation of calcium which also facilitates muscle cell death.

3.1. Sub-Sarcolemmal Calcium Levels are Elevated in Dystrophic Muscle

Early studies showed that the total muscle calcium content is enhanced in dystrophic myofibers (for review, see Gillis, 1999), suggesting that dysregulation of calcium could contribute to the dystrophic pathology. There were, however, a number of questions, in particular the route of entry of the calcium, and whether calcium loading occurred early or late in the progression of myofiber necrosis. Since then, considerable work from a number of groups has examined whether dysfunction in calcium regulation contributes to the dystrophic pathology. Although there has been some controversy concerning the role of calcium, significant evidence supports the hypothesis that there is increased calcium influx into dystrophic muscle, which results in enhanced proteolysis and myofiber apoptosis and/or necrosis.

Work from our group was the first to demonstrate that resting intracellular free calcium ($[Ca^{2+}]_i$) levels are elevated, both in isolated, non-enzymatically-treated myofibers (Turner et al., 1988) and in cultured myotubes (Fong et al., 1990). Since

that time, many other studies have examined $[Ca^{2+}]_i$ levels, with many finding elevated calcium levels (Bakker et al., 1993, Imbert et al., 1995, Tutdibi et al., 1999, Robert et al., 2001, Fraysse et al., 2004) but several others not (Gailly, 1993, Head, 1993, Pressmar et al., 1994, Collet et al., 1999, DeBacker et al., 2002). A number of issues could contribute to such discrepancies. Some are critical technical concerns regarding fluorescent calcium indicators, including the fact that loading esterified forms of the dyes at higher concentrations or temperatures will enhance dye compartmentalization and sequestration into calcium stores, resulting in erroneous measurements of $[Ca^{2+}]_i$ because compartmentalized dye contributes to fluorescence but does not measure cytoplasmic $[Ca^{2+}]_i$. Use of the compartmentalization-resistant fura-PE3 negates many of these problems (see Hopf et al., 1996b), as does microinjection of the free form of the dye (Turner et al., 1988).

Variables related to the activity of the muscle are also important. For example, human dystrophic DMD myotubes only develop differences in $[Ca^{2+}]_i$ relative to control when cultured in the presence of spinal cord tissue, which induces muscle contractions (Imbert et al., 1995). This requirement is not necessary for myotubes cultured from the *mdx* mouse, which spontaneously contract (Hopf et al., 1996b). In both cases, chronic but not acute inhibition of contraction with tetrodotoxin returns resting $[Ca^{2+}]_i$ levels in dystrophic muscle to normal levels. In addition, osmotic shock causes elevations in $[Ca^{2+}]_i$ primarily in dystrophic muscle (Imbert et al., 1996).

A critical variable that could be negated by bulk cytoplasmic measurements of $[Ca^{2+}]_i$ is the possibility that $[Ca^{2+}]_i$ levels just under the sarcolemma are significantly higher in dystrophic muscle. This has been confirmed using the calcium indicator aequorin targeted to the sarcolemma (Robert et al., 2001, Basset et al., 2004) using single channel activity of the large calcium-activated potassium channel, BK (Mallouk et al., 2000), and using fura-2 measures of $[Ca^{2+}]_i$ (Turner et al., 1991). In fact, Mallouk and colleagues (2000) found significantly higher sub-sarcolemmal $[Ca^{2+}]_i$ with no apparent difference in bulk cytoplasmic $[Ca^{2+}]_i$ levels. In addition, Robert et al. (2001) found that enhanced mitochondrial accumulation of calcium precedes the development of enhanced cytosolic $[Ca^{2+}]_i$ levels in dystrophic muscle. Vandebrouck et al. (2006) also found an interaction of increased sarcolemmal cation flux after store depletion and mitochondrial regulation of $[Ca^{2+}]_i$ levels. Thus, elevated cytosolic $[Ca^{2+}]_i$ may only become apparent once the tremendous calcium buffering capacity of the muscle cell becomes saturated.

Increased $[Ca^{2+}]_i$ could be due to altered intracellular calcium uptake or buffering, and/or increased calcium flux across the sarcolemma in dystrophic muscle. In support of the latter, measurements of sarcolemmal cation influx have generally observed greater flux into dystrophic muscle. These studies have primarily examined influx of manganese, which quenches the fluorescence of calcium indicators at a rate proportional to the entry of manganese, and have been carried out both in cultured myotubes (Hopf et al., 1996b) and myofibers (Tutdibi et al., 1999, Fraysse et al., 2004). The increased rate of quenching in dystrophic muscle is prevented by nickel, a non-specific blocker of calcium channels (Fraysse et al., 2004), confirming

that manganese influx occurs through channels and not tears in the membrane. The nature of these channels is discussed in detail below.

The controversy regarding possible differences in calcium regulation in dystrophic and normal muscle has continued into the present decade. Several recent studies did not observe differences in manganese flux in collagenase-isolated fibers (De Backer et al., 2002) or acutely dissected fibers (Carlson et al., 2003), and also did not find differences in $[Ca^{2+}]_i$. However, Frayyse et al. (2004) examined acutely dissected muscle fibers, and found both elevated resting $[Ca^{2+}]_i$ and manganese flux rates, as well as differential modulation of manganese influx by exercise. Thus, there are clearly a number of lines of evidence, including targeted calcium sensors and measurements of calcium-activated potassium channels, that indicate that subsarcolemmal calcium is elevated, but whether this can be partly or fully explained by increased flux through calcium-permeable channels (section 4) or alterations in other calcium regulatory pathways will require more experimentation, with particular care taken in regard to technical issues such as the method of calcium measurement and the exact state of the muscle tissue under investigation.

3.2. Are Calcium Regulatory Pathways Altered in Dystrophic Muscle?

Detecting differences in $[Ca^{2+}]_i$ in dystrophic and normal muscle might be complicated by the huge capacity for calcium uptake and buffering in skeletal muscle, which could efficiently compensate for any increased calcium influx. Skeletal muscle resting $[Ca^{2+}]_i$ levels are under complex and potent regulation (for review, see Gillis, 1999, Gailly, 2002). In addition to sarcolemmal calcium pumps (Ca^{2+}-ATPases) and mitochondrial calcium uptake, skeletal muscle has large amounts of sarcoplasmic reticulum (SR) which is rich in Ca^{2+} pumps. Strong and rapid regulation of $[Ca^{2+}]_i$ is critical for repeated, efficient muscle contraction. Thus, misregulation of calcium regulatory pathways could contribute to elevated $[Ca^{2+}]_i$ levels and the downstream negative consequences for muscle viability.

The existing evidence regarding the possibility of differences in various calcium pumps or store calcium release is diverse, and thus it is more difficult to definitively conclude whether intracellular components of the excitation-contraction and calcium release machinery are altered in dystrophic muscle. For example, a number of groups have shown no differences in calcium kinetics after stimulation with a single action potential (Head, 1993, Tutdibi et al., 1999), which involves a number of steps in the excitation-contraction process including T-tubule depolarization, voltage-dependent calcium channels (VDCC) and subsequent ryanodine receptor activation, and calcium release from the SR. Other studies of store calcium release also have not observed differences in the caffeine-releasible pool (Imbert et al., 2005, Collet et al., 1999, Fraysse et al., 2004). In agreement, ryanodine receptor and SR Ca^{2+}-ATPase levels are not altered in dystrophic skeletal muscle (Culligan et al., 2002, but see Divet et al., 2005), although they are reduced in dystrophic cardiac muscle (Rohman et al., 2003). In contrast, Woods et al. (2005) have found a reduced AP amplitude, and through a series of careful studies have shown normal

electrical propagation but reduced SR calcium release in dystrophic muscle (see also Nicolas-Metral et al., 2001). Tutdibi et al. (1999) also reported slower decay in $[Ca^{2+}]_i$ after stimulation in some but not all, suggesting decreased uptake. Finally, Divet et al. (2002, 2005) found reduced SR calcium uptake in fast-twitch but not slow-twitch muscle that correlated with increased expression of a slow Ca-ATPase isoform, in agreement with Kargacin and Kargacin (1996) who showed decreased maximal velocity of calcium uptake in dystrophic muscle. Possible differential regulation of intracellular calcium stores is discussed in further detail below.

Calcium buffering and other calcium regulatory proteins, including calsequestrin or a calsequestrin-like protein, sarcalumenin, and regucalcin, are reduced in dystrophic muscle (Culligan et al., 2002, Doran et al, 2004, Dowling et al., 2004, Doran et al., 2006). Thus, enhanced calcium levels in dystrophic muscle might be secondary to decreased calcium buffering or altered regulation of Ca^{2+}-ATPases. However, a number of other proteins are also altered in dystrophic muscle (Ge et al., 2004, Doran et al., 2006), making it unclear whether changes in calcium-regulatory proteins are a primary cause or a secondary consequence of the myofiber degeneration.

4. ARE CALCIUM-PERMEABLE CHANNELS MORE ACTIVE IN DYSTROPHIC MUSCLE?

Increased sub-sarcolemmal calcium in dystrophic muscle cells could result from increased influx through a calcium-permeable ion channel in the sarcolemma. A significant amount of work has gone into trying to identify the calcium-permeable channels that might underlie the enhanced cation influx and increased subsarcolemmal $[Ca^{2+}]_i$ levels described above. These studies have been remarkable for the diversity of different channels which have been observed. We will describe here the evidence for several major types of channels, mechanosensitive (MS) channels, store-activated channels (SOC), calcium leak channels, and growth factor-regulated channels (GRC), all of which have been reported to be differentially active in dystrophic muscle. A major challenge is understanding the relationship among these different types of channels, and the potential contribution of each to the dystrophic pathology. However, we believe there is enough evidence to suggest potential relationships between the channels, as well as different conditions under which the contribution or activity of particular channels might be enhanced in dystrophin-deficient muscle tissue (Figure 2).

4.1. The Mechanosensitive (MS) Channel

Stretch-activated MS channels are observed in a number of types of cells, including skeletal muscle. Original studies from Lansman and colleagues showed that a lower-activity, stretch-activated channel is present in both control and *mdx* muscle, with greater open probability in *mdx* myotubes and collagenase-isolated fibers (Franco

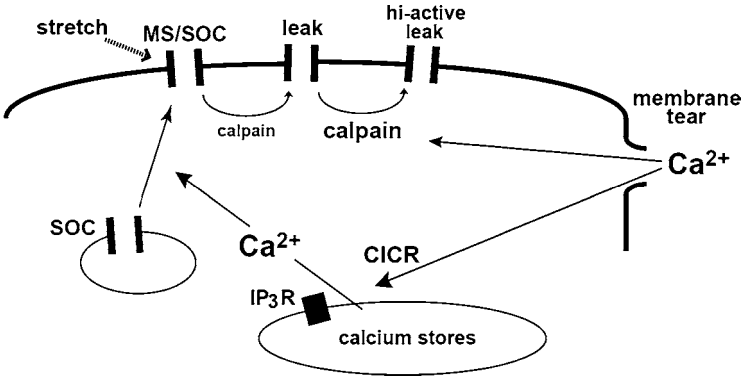

Figure 2. A proposed model demonstrating several different prominent calcium-related pathways whose activity may be altered in dystrophic muscle. Increased activity of mechanosensitive channels (MS) and store-activated channels (SOC), which are likely derived from the same gene product (TRPC), and the calcium leak channel, which could represent a proteolyzed TRPC SOC channel. Decreased mechanical coupling between L-type VGCC and ryanodine receptors may increase basal calcium release from calcium stores (not shown). Further, increased IP_3 and IP_3 receptor levels may also enhance basal and stimulated calcium-induced calcium release (CICR) from calcium stores. Calcium store depletion can increase translocation of SOCs from intracellular vesicles to the sarcolemma. Finally, the relationship between increased membrane fragility and tearing is less certain, but calcium influx through sarcolemmal tears could lead to calcium-dependent proteolysis and increased activity of calcium leak channels, as well as proteolysis of other targets, and increased release of calcium from intracellular stores through CICR. This model is not meant to be comprehensive, and other calcium-related molecules are discussed in the text

and Lansman, 1990, Franco-Obregon and Lansman, 1994). In addition, a high-activity, stretch-inactivated channel is present only in *mdx* muscle cells (Franco and Lansman, 1990). More recent studies from this group (Franco-Obregon and Lansman, 2002) show that stretch-activated and stretch-inactivated channels likely represent the same channel, since strong suction caused some channels in *mdx* muscle to irreversibly switch into a high open probability mode that is inactivated by stretch, and stretch-activated and -inactivated channels exhibit very similar voltage-dependence of gating. These authors thus conclude that decreased stability of the sarcolemma in dystrophic muscle facilitates the transition of channels to a high-activity state after strong mechanical strain and loss of cytoskeletal regulation.

We should note that there is some diversity among studies examining MS channels in dystrophic muscle. MS channel abundance (the number of membrane patches containing channels) is greater in cultured myotubes but not in acutely isolated myofibers (Franco-Obregon and Lansman, 1994). Also, stretch-inactivated channels are observed in myotubes but not myofibers. In addition, Vandebrouck et al. (2002b) reported a channel with some similar biophysical properties as an MS channel. However, channels from dystrophic muscle show an increased abundance but no differences in open probability, somewhat divergent from the observations of Lansman and colleagues (Franco and Lansman, 1990,

Franco-Obregon and Lansman, 1994); several other properties are also different, including increased activity with depolarization in Lansman (and other studies of canonical MS channels) but decreased activity with depolarization in Vandebrouck. Finally, McBride and Hamill (1992) observed stretch-activated channels with no clear evidence for stretch-inactivated channels in dystrophic muscle, although the number of channels studied might not have been sufficient to observe such channels.

Others have observed indirect evidence for MS channels. For example, Tutdibi et al (1999) showed that manganese flux is not blocked by a putative SOC inhibitor, and is not enhanced by nifedipine, an activator of calcium leak channels (Coulombe et al., 1989), but is inhibited by Gd^{3+}, a MS channel blocker. Thus, they attribute the greater cation flux in dystrophic muscle to MS channels. Increased activity of MS channels is also suggested by studies showing elevated resting sodium levels in dystrophic muscle (Dunn et al., 1993, Yeung et al., 2003), although this was not observed by Turner et al. (1991) in non-enzymatically isolated muscle fibers. MS channels are cationic, passing sodium as well as calcium, and MS channel blockers such as Gd^{3+} and streptomycin reduce resting sodium and calcium levels in *mdx* muscle with no effect in controls (Yeung et al., 2003, 2005, but see Carlson et al., 2005). Yeung et al. (2003) have proposed that contraction in the absence of mechanical stability in dystrophic muscle enhances the transition of MS channels into a high-activity state (as described in Franco-Obregon and Lansman, 2002), resulting in greater tonic MS activity and thus sodium and calcium influx.

A number of lines of evidence suggest that MS channels play a critical role in exercise-induced alterations in muscle function, both in control and *mdx* muscle. Eccentric muscle contraction with stretching leads to persisting depolarization and enhancement in sodium and $[Ca^{2+}]_i$ levels in control muscle (McBride et al., 2000, Yeung et al., 2003, 2005), which is greater in dystrophic muscle and is normalized by blocking MS channels. In addition, MS blockers prevent the loss of force normally produced by eccentric contractions, suggesting that ion influx through MS channels is causal to the reduced force generation in normal muscle and is more pronounced in dystrophic muscle. Also interesting is the observation that, in the absence of dystrophin, muscle stretching induces aberrant activation of MAP kinase pathways through SR calcium store release and a Gd^{3+}-sensitive conductance, which could contribute to necrosis (Kumar et al., 2004).

Several studies have tried to address the therapeutic value of reducing MS channel activity on the dystrophic pathology. Two week treatment of *mdx* mice with the MS blocker streptomycin protects against muscle damage (Yeung et al., 2005). On the other hand, Squire et al. (2002) showed that partial expression of utrophin in the *mdx* mouse normalized MS properties and assembly of the DAG, but led to only partial recovery of force loss after eccentric contractions and partial reduction in the number of central nuclei, an indicator of necrosis and regeneration. These MS channels are of the type reported in Vandebrouck et al. (2002b). Thus, abnormal calcium influx through MS channels may partly contribute to the dystrophic pathology,

but inhibiting MS channels only may not be sufficient to reverse the dystrophic muscle wasting, and thus other calcium sources are clearly also important. Further, De Backer et al. (2002) found elevated MS channel activity but no differences in manganese flux or resting $[Ca^{2+}]_i$, suggesting that a pathway other than the MS channel might carry a considerable portion of the Mn^{2+} flux observed in dystrophic fibers. In addition, to test the contribution of MS channels to resting $[Ca^{2+}]_i$, Turner et al. (1991) stretched adult myofibers and examined changes in $[Ca^{2+}]_i$. Although a small increase in $[Ca^{2+}]_i$ is observed in control fibers, and a small decrease in $[Ca^{2+}]_i$ in *mdx* fibers, these authors concluded that the changes in $[Ca^{2+}]_i$ are too small to fully account for the differences in resting $[Ca^{2+}]_i$ between *mdx* and control muscle.

4.2. The Store-Operated Channel (SOC)

SOCs, also known as ICRAC (calcium-release activated channels), have been observed in a wide range of cell types (Parekh and Penner, 1997). The defining property is that depletion of intracellular calcium stores results in increased calcium influx at the plasma membrane. The actual SOC that carries this calcium influx may vary between cells, and cloning studies have identified "transient receptor potential channel" (TRPC) (Parekh and Penner, 1997, Vandebrouck et al., 2002b) and CRACM1 (Peinelt et al., 2006) as candidate genes. Also, the exact mechanism by which SOCs are activated by store depletion has only been partly elucidated, with a role suggested for a calcium sensor on the endoplasmic reticulum (see Peinelt et al., 2006) and for IP_3 (Kiselyov et al., 1998).

Work from Vandebrouck and colleagues (2002b) have significantly advanced our understanding of the role of SOCs in muscular dystrophy, and helped clarify the relationship between the different channels observed in dystrophic muscle. They describe a channel with many similar biophysical properties as the MS channel, whose abundance is greater in dystrophic muscle. The activity of this channel is significantly enhanced by depletion of calcium stores with thapsigargin or caffeine. In addition, antisense knockdown of the genes for several TRPC isoforms (TRPC1 or 4) significantly reduces the occurance of both the putative MS channel and the ability of store depletion to enhance channel activity. Recently, a stretch-activated MS channel with ~40 pS conductance in normal Ringer's was shown to be encoded by the TRPC1 gene (Maroto et al., 2005). Taken together, these results suggest that MS and SOC channels represent the same channel, whose activity is greater in dystrophic muscle. However, this channel is not inhibited by 2-aminoethoxydiphenyl borate (2-APB), which inhibits SOCs in other cell types (see Wang et al., 2005), suggesting that muscle SOCs may differ from those in other cell types.

The calcium leak channel (described below) is also a SOC, since the single channel activity of the leak channel and manganese influx through the leak channel are greatly increased in cultured myotubes by depletion of the intracellular calcium stores (Hopf et al., 1996a).

4.3. Altered Regulation of Calcium Stores?

Since SOCs might be important for the enhanced calcium influx in dystrophic muscle, lack of dystrophin and disruption of the integrity of the cytoskeleton might impair the tight regulation of calcium release from the SR. Normally, SR calcium release is tightly regulated by L-type VDCCs in the T-tubules: SR calcium is only released when depolarization enters the T-tubules, activating VDCCs and in turn ryanodine receptors on the SR, resulting in calcium release from the SR. Disruption of this regulation might allow a persisting leak from dystrophic calcium stores, which could either directly elevate $[Ca^{2+}]_i$ levels, or could activate SOCs and increase $[Ca^{2+}]_i$.

An interesting study in this regard comes from Wang et al. (2005), who examined calcium sparks, considered elementary events in intracellular calcium release. These small, highly localized events are necessary for rhythmic activity of cardiac muscle, but few calcium sparks are normally observed in skeletal muscle, perhaps to maintain calcium stores quiescent and ready for release during contractile events. Wang et al. used osmotic changes to cause contraction or expansion of the sarcolemma. In control muscle, calcium spark activity is induced by osmotic changes, and disappears within 5–10 minutes of returning to normal osmotic solution. However, in *mdx* muscle, calcium spark activation is essentially irreversible. Also, store release after osmotic challenge is reduced in *mdx* muscle, suggesting persistent store depletion. Osmotic shock also increases $[Ca^{2+}]_i$ in both normal and *mdx* muscle, with a greater increase in *mdx* tissue. Finally, mild exercise produces calcium sparks in *mdx* tissue in the absence of osmotic shock, and heavy exercise leads to calcium sparks in normal and *mdx* tissue, but still significantly more events in *mdx* muscle. Taken together, these results strongly suggest that enhanced SOC activity in dystrophic muscle might be secondary to impaired regulation of calcium stores, a condition which is also observed in normal muscle after heavy exercise.

Altered inositol trisphosphate (IP_3) regulation might also contribute to differential calcium store activity in dystrophic muscle. Kiselyov et al. (1998) used inside-out excised patches, where a piece of the cell plasma membrane is removed from the cell, to show that activation of the IP_3 receptor by IP_3 can activate a SOC. Thus, altered regulation of IP_3 signaling in dystrophic muscle might enhance calcium influx. Calcium store release in control skeletal muscle occurs predominantly through the ryanodine receptor on the SR. However, both IP_3 receptor abundance and IP_3 levels are increased in *mdx* muscle (Liberona et al., 1998), and carbachol-induced increases in sub-sarcolemmal calcium are significantly greater in dystrophic muscle and only involve IP_3 in dystrophic muscle (Basset et al., 2004). Thus, regulation of IP_3 signaling in dystrophic muscle may be impaired, and enhanced IP_3 levels might increase SOC activity directly (as described by Kiselyov et al., 1998) and indirectly by releasing calcium from SR calcium stores. Also, bcl-2, an apoptosis inhibitor, reduces $[Ca^{2+}]_i$ and mitochondrial calcium levels in dystrophic muscle by inhibiting IP_3 receptors (Basset et a., 2006). However, we should note that several studies have not found differences in the magnitude of

intracellular calcium stores, for example, caffeine-induced store release in normal versus dystrophic muscle (Imbert et al., 2005, Collet et al., 1999, Fraysse et al., 2004). In contrast, using a calcium sensor aequorin targeted to the SR, Robert et al. (2001) found higher steady-state SR calcium levels in dystrophic muscle. Also, these studies do not strictly rule out the possibility of a small increased tonic calcium release from SR in dystrophic muscle, which has been observed by Divet and Huchet-Cadiou (2002). In fact, even partial store depletion is sufficient to activate SOCs in skeletal muscle (Kurebayashi and Ogawa, 2001). In addition, store depletion leads to greater sarcolemmal cation flux in DMD relative to normal myotubes, suggesting that a small basal efflux from the calcium stores might have a disproportionate effect in dystrophic muscle (Vandebrouck et al., 2006).

4.4. The Growth Factor-Regulated Channel (GRC)

A recent paper from Iwata et al. (2003) described a channel of the TRP family which is activated in response to growth factors or stretch, and whose activity is elevated in dystrophic skeletal and cardiac muscle. Under normal conditions, the GRC is located on vesicles in the cytoplasm, but translocates to the sarcolemma in response to growth factor signaling or stretch. This translocation is calcium-dependent, and, interestingly, requires a Gd^{3+}-sensitive conductance, which could represent a basal level of GRC activity or some other perhaps MS-type channel. In addition, the translocation is inhibited by ruthenium red, a blocker of ryanodine receptor-dependent release of SR calcium, suggesting that store calcium release is also necessary for sarcolemmal GRC insertion. Whether the GRC is an SOC was not reported.

Most interestingly, creatine kinase loss (an indicator on membrane tearing) is greater in dystrophic tissue, as widely observed, and is significantly reduced by antisense for the GRC, or by channel inhibition with Gd^{3+}. These results support the interesting possibility that enhanced susceptibility to membrane tearing might be under direct and acute control of calcium influx through GRC, in agreement with previous studies suggesting that increasing $[Ca^{2+}]_i$ enhances membrane permeability (see Allen et al., 2005). This idea significantly refines our understanding of the etiology of dystrophy: other hypotheses (including our own) suggest that increased calcium channel activity is secondary to membrane tearing (McCarter and Steinhardt, 2000), but the results of Iwata suggest that calcium dysregulation could be the primary dysfunction. It is also likely that there is a positive feedback cycle through which membrane tearing enhances channel activity, and channel activity enhances sarcolemmal tearing.

4.5. The Calcium Leak Channel

Studies from our lab have identified a calcium-permeable, voltage-independent channel, whose probability of opening is significantly greater in cultured myotubes derived from *mdx* mouse or human DMD tissue (Fong et al., 1990) and in isolated,

non-enzyme-treated myofibers (Hopf et al., 1996b). These channels have several properties similar to previously described calcium leak channels (Coulombe et al., 1989). In particular, leak channels are activated by the dihydropyridine agonist nifedipine, which also enhances resting $[Ca^{2+}]_i$ levels in control and dystrophic cultured myotubes and non-enzymatically isolated adult fibers (Fong et al., 1990, Turner et al., 1991), and manganese influx in myotubes (Hopf et al., 1996b) and myofibers (Fraysse et al., 2004), confirming the presence of such channels in adult muscle.

Since calcium-dependent proteolysis is also increased in dystrophic muscle (discussed in detail below), the relationship between proteolysis and leak channel activity has been extensively investigated, and the calcium leak channel has been shown to play an important role both in regulation of proteolysis and regulation by proteolysis. Inhibition of leak channel activity reduces levels of calcium-dependent proteolysis to normal (Alderton and Steinhardt, 2000b). In addition, inhibitors of calcium-dependent proteolysis reduce calcium leak channel activity and resting $[Ca^{2+}]_i$ in *mdx* myotubes to normal levels (Turner et al., 1993). These results suggest a positive feedback cycle, whereby calcium influx activates leak channels through proteolysis, further enhancing calcium influx and proteolysis, in a vicious cycle that perhaps culminates in muscle cell death. Since the dystrophic sarcolemma is more susceptible to membrane tearing and could act as a calcium source for proteases, membrane tears were generated by forming a seal with a patch electrophysiology pipette and then pulling the electrode away. Leak channels near this wound are significantly more active than more distant channels, and this increase in activity is prevented by an inhibitor of calcium-activated proteases (McCarter and Steinhardt, 2000). Thus, calcium influx through membrane tears can activate proteases and the leak channel, which would further increase the calcium load in dystrophic muscle. In agreement, Fraysse et al. (2004) found that exercise significantly increases the ability of the leak channel activator nifedipine to enhance manganese influx in *mdx* but not control muscle.

In myotubes, about 50% of patches contained leak channels. In contrast, in adult non-enzyme-treated myofibers, only about 5% of patches contained leak channels. Thus, in adult muscle, calcium leak channels might be clustered or restricted to specific regions of the fiber. Interestingly, leak channels from DMD myotubes are significantly more active than those from *mdx* mice, and there is the increased muscle wasting in DMD relative to *mdx* mice. Decreased mechanical stress on muscles due to the lighter weight of the mouse might lead to less membrane tearing, resulting in less activation of leak channels, and thus less development of proteolysis and necrosis.

4.6. Voltage-Dependent Calcium Channels

The L-type VDCC plays a critical role in excitation-contraction coupling, as voltage activation of VDCC results in activation of ryanodine receptors and release of calcium from the SR. Studies of VDCC in dystrophic muscle have produced

somewhat mixed results. The total number of dihydropyridine receptor binding sites is unaltered (Pereon et al., 1997). In contrast, several groups have reported reduced total L-type VDCC currents (Imbert et al., 2001, Friedrich et al., 2004, but see Desnuelle et al., 1986, Trautman et al., 1986), which might contribute to the reduced overall force generation in dystrophic muscle (see above). However, there are reports of unaltered voltage-dependence of inactivation (Friedrich et al., 2004, in *mdx* myotubes) or reduced voltage-dependence of inactivation (Imbert et al., 2001, in DMD myotubes). The latter condition could contribute to enhanced calcium influx in dystrophic muscle under conditions of depolarization. In cardiac muscle, the role of the L-type VDCC is clearer. Several groups have observed a decrease in L channel inactivation in dystrophic muscle, with no change in peak current (Sadeghi et al., 2002, Woolf et al., 2006), as well as several other kinetic changes. In agreement, there is a prolonged QT interval in the electrocardiogram from *mdx* mice (Sadeghi et al., 2002).

4.7. Do the Different Channels Contribute under Different Conditions?

How can these observations of apparently different types of channels contributing to the dystrophic pathology be reconciled? One possibility is that the different channel types are all present in muscle cells, and that they contribute to the dystrophic pathology under different conditions. In this regard, Fraysse et al. (2004) performed a particularly interesting study of the effects of exercise on dystrophic muscle. This study is valuable in part because it examined calcium regulation in muscle fibers which are dissected and pinned down but not collagenase-isolated; this perhaps represents the closest model to the *in vivo* situation, without possible disruption by enzymes or the artificial development which might occur during culturing of myotubes.

Fraysse et al. found that manganese influx is greater in dystrophic tissue at rest, and is increased in dystrophic and normal muscle by exercise. However, using Gd^{3+} as an indicator of MS channel activity, they found that the Gd^{3+}-sensitive portion of the manganese flux is ~20% in basal control, and ~40% in exercised control and in *mdx* with or without exercise. Thus, flux through MS channels is greater in *mdx* at rest, but not enhanced in *mdx* by exercise. On the other hand, the ability of nifedipine to enhance manganese flux (an indicator of calcium leak channel activity) is low and not different between normal and dystrophic muscle at rest, but is significantly enhanced by exercise only in dystrophic muscle. Taken together, these results suggest that the greater $[Ca^{2+}]_i$ in resting muscle is due to MS channel activity, but that any changes in muscle function in dystrophic animals after chronic exercise (such as decreased force generation) is more likely related to increased leak channel activity. In this regard, utrophin expression in *mdx* mice normalized the activity of MS channels, but did not reverse the progression of muscle degeneration, raising the question of the "sole involvement of this channel type in the progression of the pathology" (Squire et al., 2002)

4.8. Are the Different Channels in Fact Variants of the Same Channel?

Another possible explanation for the apparently different channel types is that they in fact are derived from the same initial gene product, but that proteolysis or other forms of regulation alter the properties of the channel. In fact, Allen et al. (2005) have suggested that all the channel types represent a TRP-type SOC, although the muscle SOC channel is likely to be different from the canonical SOC, since it is not inhibited by APB (Wang et al., 2005) or carboxyamidotriazole (Tutdibi et al., 1999), and some SOCs show a single-channel conductance an order of magnitude smaller than reported for muscle leak and MS channels (Parekh and Penner, 1997). Nonetheless, the work of Vandebrouck et al. (2002b) has shown that at least some SOC variants are MS channels. Loss of cytoskeletal regulation may facilitate transition of some of these MS/SOCs into persistently active, stretch-inactivated channels. In addition, we propose that activation of calcium-dependent proteolysis above some threshold level, especially after eccentric contractions and membrane tearing, might convert these SOC/MS channels to the calcium leak channels (McCarter and Steinhardt, 2000).

In support of a relationship between MS/SOC and calcium leak channels, calcium leak channels exhibit evidence of being both MS channels (Fong et al., 1990) and SOCs (Hopf et al., 1996a). Also, Vandebrouck et al. (2001, 2002a) described a leak/MS channel that had greater abundance and open probability in DMD myotubes. Mechanical stretch inactivates a portion of these channels, so that the abundance of patches of channels is now the same in dystrophic and normal tissue, although the open probability of the remaining channels is still significantly elevated in dystrophic muscle. Since all channels had the same single channel properties, this may indicate that the MS channels represented a subset of the calcium leak channels.

We should also note that Gu et al. (2001) showed mechanosensitivity in a Shaker-type channel, and suggest that mechanosensitivity might be a general property of "multiconformational" channels. Thus, the presence of mechanosensitivity might not be evidence of channel relatedness.

Arguing against the hypothesis that MS and leak channels derive from a SOC is the observation that the different channels have different biophysical properties (see Allen et al., 2005). For example, the calcium leak channel is relatively impermeable to sodium, while the other channel types are more permeable to sodium. This permeability difference may merely reflect that Franco and Lansman (1990) tested for sodium permeability in the absence of calcium, a condition under which channels can lose specificity. Further, few leak channels observed by our lab exhibited the degree of activity reported for MS channels (Franco and Lansman, 1990, Franco-Obregon and Lansman, 2002, Vandebrouck et al., 2002b). However, the fact that the TRPC1 gene contributes both to MS channels and SOC (Vandebrouck et al., 2002b, Maroto et al., 2005), and that these channels have somewhat different properties (Allen et al., 2005), suggest that a particular channel could in fact undergo an as-of-yet undefined transition into a different channel species.

4.9. Channels: Conclusions

Although there is diversity in the types of channels whose activity is reported to be different in dystrophic vs. normal muscle tissue, it is clear that some of these channels may in fact represent variants of the same channel type, where, e.g., channel properties might be modified slightly by proteolysis. In addition, inhibition of these channels has been shown to reduce proteolysis and muscle degeneration. Thus, it is likely that altered regulation of calcium-permeable channels plays a primary role in the elevations in $[Ca^{2+}]_i$ and calcium-dependent proteolysis and cellular degeneration observed in dystrophic muscle.

5. PROTEASE ACTIVATION

To date the available data supports altered cell membrane integrity as a key component of the pathological progression of DMD and *mdx* dystrophies. Reports from our lab and others of increased $[Ca^{2+}]_i$ levels led to the hypothesis that muscle cell death results from activation of proteases and in particular calpains (Turner et al., 1988, Spencer et al., 1995, Mariol and Segalat, 2001, Alderton and Steinhardt, 2000b). Calpains are cytosolic, nonlysosomal, cysteine proteases with high homology to the papain protease family (Sorimachi and Suzuki, 2001). Skeletal muscle expresses three isoforms of calpain called calpain 1, calpain 2 and calpain 3 (Sorimachi et al., 1989). Calpains 6, 7 and 10 (Sorimachi and Suzuki, 2001) have not been identified in muscle. Abnormal levels of several proteases are a feature of a wide variety of muscle diseases (Pearson and Kar, 1979, Sorimachi et al., 1989, Voisin et al., 1996, Kumamoto et al., 1997, Helliwell et al., 1998), so changes in protease expression or activity in DMD or *mdx* muscle may be a nonspecific feature, causally far removed from the primary pathological process (Kar and Pearson, 1978, Zeman et al., 1985, Furono and Goldberg, 1986, Kumamoto et al., 2000). In terms of gain of function experiments, net protein degradation rates in isolated normal muscle as assessed by tyrosine release can be raised or lowered by manipulations that raise or lower $[Ca^{2+}]_i$ (Turner et al., 1988). Turner et al. (1988), having found a raised $[Ca^{2+}]_i$ in *mdx* myofibers, therefore studied tyrosine release rates in isolated *mdx* muscle. The net rate was elevated 80% above normal, an effect that could be blocked by leupeptin (a cysteine and serine protease inhibitor protease inhibitor). Loss of function studies were also striking: reducing $[Ca^{2+}]_i$ levels in *mdx* muscle to close to those in normal muscle abolished the increase in net proteolysis. Alderton and Steinhardt (2000b) went on to show that leupeptin not only blocked the extra proteolysis of *mdx* myotubes but also normalized their $[Ca^{2+}]_i$ and the open probability of their calcium leak channels. The increase in $[Ca^{2+}]_i$ seen in *mdx* myotubes after hypo-osmolar shock is also abolished by a protease inhibitor (Leijendekker et al., 1996). Furthermore, *in vivo* studies using leupeptin to treat *mdx* mice by intramuscular injection showed a reduction in muscle pathology as assessed by a decrease in centrally nucleated (regenerated) fibers (Bell and Cohen, 1968). A variety of proteolysis inhibitors showed that most of the extra proteolysis was not due to lysosomal or proteosomal pathways (Turner et al., 1988).

There is a body of evidence which specifically implicates calpains in the pathology of muscular dystrophy (Spencer and Tidball, 1992, Combaret et al., 1996). Candidates for this proteolytic activity include calpains I and II (Spencer et al., 1995). Studies *in vitro* using *mdx* myotubes have demonstrated that elevations in extracellular calcium result in increased proteolysis of a calpain substrate (Alderton and Steinhardt, 2000b). Alderton and Steinhardt (2000b) used hydrolysis of a fluorogenic calpain substrate to confirm that calpain-mediated proteolysis was elevated in *mdx* myotubes relative to controls, and showed that this can be prevented by lowering external calcium concentrations or by using an antagonist of calcium leak channel activity. In addition, whole-muscle extracts showed an increase in expression of m-calpain in *mdx* mice (Spencer et al., 1995) and DMD (Reddy et al., 1986). However, calpain assays of whole-muscle extracts are probably not indicative of *in vivo* activity, because of the presence of calpastatin, the specific inhibitor of calpains I and II. In an attempt to overcome this, examination of the calpain propeptide was used to assess activation of calpains *in vivo* (Spencer and Tidball, 1992), and propeptide cleavage results in activation of the protease. The ratio of cleaved propeptide to intact calpain I was higher in 4-week *mdx* mice than in controls, while m-calpain propeptide cleavage remained unchanged, suggesting that m-calpain was specifically activated during the dystrophic process (Spencer and Tidball, 1992). It is also of interest to note that calpains have an altered distribution in the cell during muscle necrosis and regeneration of *mdx* myofibers (Spencer and Tidball, 1996), which may be a result of the increased calcium flux near the sarcolemma.

Several interesting studies have addressed the effects of calpain inhibition *in vivo* on muscle wasting. Calpain inhibitors reduced signs of necrosis (Badalamente and Stracher, 2000) and reduced the loss of muscle strength in exercised *mdx* mice (Granchelli, 2000). Also, transgenic *mdx* mice have been produced to overexpress the calpain inhibitor calpastatin in muscle (Spencer and Mellgren, 2002). Interestingly, calpastatin expression ameliorated dystrophic symptoms, although it did not repair the primary membrane defect. Taken together, the existing data strongly imply that decreased sarcolemma integrity is a primary aspect of dystrophic muscle, and that intracellular calpain activity is necessary for increased sarcolemmal fragility to translate into muscle damage, and that inhibition of calpain is a potentially promising treatment for DMD. These are not the results that would have been expected if the abnormalities of calcium influx were a direct result of dystrophin deficiency. Transient membrane ruptures may allow an influx of calcium which then causes local activation of proteases, which modify calcium leak channels in turn to cause further calcium influx. McCarter and Steinhardt (2000) simulated the initial steps in this process by using a patch clamp electrode to rupture the membrane of a normal myotube. The patch clamp was then resealed either close to (5 μm) or far from (50 μm) the initial rupture, and the calcium leak channel activity was measured. Channels close to the lesion had a fourfold increased open probability, an effect that was abolished by incubation with leupeptin.

Calpains may be only part of the story. Similar to other tissues, skeletal muscle has at least three different pathways for protein degradation: 1) proteolysis by lysosomal proteases, such as the cathepsins, 2) proteolysis by non-lysosomal Ca^{2+}-dependent proteases, such as calpain, and 3) proteolysis by non-lysosomal ATP-ubiquitin-dependent proteases, such as the multi-catalytic protease complex (or proteasome). Inhibition of the proteasome (Bonuccelli et al., 2003) has produced some striking results. *mdx* mice were treated *in vivo* with the inhibitor MG-132 by both local injections and systemic treatment using an osmotic pump that delivered different concentrations of the proteasomal inhibitor, over an 8-day period. By immunofluorescence and Western blot analysis, MG-132 effectively rescued the expression levels and plasma membrane localization of dystrophin, α-dystroglycan, β-dystroglycan, and α-sarcoglycan in skeletal muscle fibers from *mdx* mice. Furthermore, treatment reduced muscle membrane damage, as revealed by vital staining (with Evans blue dye) and ameliorated the histopathological signs of muscular dystrophy, as judged by hematoxylin and eosin staining (Bonuccelli et al., 2003). As yet, no reports of effects on channel activity or calcium handling have been made. In addition, several groups have noted the presence of apoptotic fibers even when necrosis is not yet evident (see Robert et al., 2001), and Robert et al. (2001) have suggested that the elevated steady state calcium within the mitochondria or SR may contribute to this apoptosis. Further, the anti-apoptotic molecule bcl-2 decreases the calcium overload in the cytoplasm and mitochondria of dystrophic muscle (Basset et al., 2006). Thus, enhanced calcium-dependent proteolysis may be only one of several mechanisms which contribute to cell death during dystrophic pathology.

6. FUTURE QUESTIONS

A preponderance of evidence suggests that there is elevated $[Ca^{2+}]_i$ levels in the subsarcolemmal space of muscle cells lacking dystrophin, and that increased activity of calcium-permeable channels in the sarcolemma as well as altered regulation of $[Ca^{2+}]_i$ levels by intracellular proteins could contribute to this elevated $[Ca^{2+}]_i$. However, there are many important and unresolved questions regarding the role of calcium in the dystrophic pathology. For example, a number of groups discuss the possible importance of calcium-dependent proteolysis, but, in fact, only a few studies have directly examined the consequences of proteolysis, including ours examining calcium leak channel regulation by proteases (Turner et al., 1993, Alderton and Steinhardt, 2000b, McCarter and Steinhardt, 2000) and others examining decreases in muscle damage after inhibition of calcium-dependent proteases (Badalamente and Stracher, 2000, Granchelli et al., 2000, Spencer and Mellgren, 2002). It would be interesting to determine whether the activity of MS, SOC, or GRC channels is also modulated by proteases, and in particular whether the transition of MS channels to the high-activity form (Franco-Obregon and Lansman, 2002) might depend on proteolysis. It would also be interesting to determine whether

the dependence of muscle tearing on GRC activity (Iwata et al.,2003) requires proteolysis. Although Jackson et al. (1984) found that calcium-induced enhancement of membrane permeability did not require calcium-dependent proteases, an additional protease-dependent process might be present in dystrophic muscle. On the other hand, overexpression of the calpain inhibitor calpastatin decreased necrosis without altering membrane tearing (Spencer and Mellgren, 2002). Such studies could provide significant insight into the interrelationship of the intracellular dysregulations that contribute to the dystrophic pathology, and also might clarify the relationship between the observed channel types.

There are several important technical questions to be addressed. Very useful would be a deterministic study to examine the effect of collagenase treatment on channel properties and calcium regulation in isolated adult fibers. In particular, dystrophin links the intracellular cytoskeleton to the extracellular matrix through the DAG, and provides mechanical support for the sarcolemma through this linkage. Thus, removal of the extracellular matrix with enzymes might be predicted to alter the consequence of loss of dystrophin. To address this, fibers could be mechanically isolated and pinned out, then treated with collagenase.

Finally, the relation between loss of the dystrophin-DAG complex and the misregulation of $[Ca^{2+}]_i$ levels, calcium-permeable channel activity, and calcium store integrity, remain elusive. Whether there are direct associations between DAG proteins and the various calcium-related proteins remains largely undetermined. Although calcium influx through membrane tears, and the subsequent proteolysis, may explain some of the cellular changes associated with muscular dystrophy, direct experimental demonstration of the link between increased fragility and altered regulation of intracellular or surface proteins is needed. Some changes could occur through altered gene transcription (perhaps calcium-dependent) or could represent post-translational modifications. In addition, the membrane sealant poloxamer 188, which reduces degeneration in dystrophic cardiac tissue (Yasuda et al., 2005), could provide very important insights into the role of membrane tearing versus simply the lack of the DAG in the cellular alterations which result in necrosis in dystrophic tissue.

7. OVERALL CONCLUSIONS

It is clear that there is a great deal more understood about the primary gene and protein changes in DMD patients than there is about the cellular mechanisms which drive the necrosis/apoptosis and muscle wasting with such tragic results for DMD patients. Although increased muscle tearing is clearly of primary importance in the dystrophic etiology, increased subsarcolemmal calcium levels and enhanced calcium-dependent proteolysis also seem to be critical contributors to muscle degeneration. A number of calcium regulatory molecules, including calcium-permeable channels, IP_3 receptors and calcium stores, and calcium buffering proteins, are likely altered in dystrophic muscle. Further, since muscle cells have such tremendous calcium buffering capacity, changes in all these pathways may be required for

elevations in calcium to drive proteolysis and cellular degeneration. Although much remains to be learned about the different contributors to the pathogenesis of muscular dystrophy, there is enough information to suggest several exciting targets for therapeutic intervention to prevent the muscle wasting of DMD.

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CHAPTER 17

CALCIUM AND CELL DEATH

A. VERKHRATSKY

Faculty of Life Sciences, The University of Manchester, 1.124 Stopford Building, Oxford Road,Manchester M13 9PT, UK
Email: alex.verkhratsky@manchester.ac.uk

Abstract: Calcium signalling system controls majority of cellular reactions. Calcium signals occurring within tightly regulated temporal and spatial domains, govern a host of Ca^{2+}-dependent enzymes, which in turn determine specified cellular responses. Generation of Ca^{2+} signals is achieved through coordinated activity of several families of Ca^{2+} channels and transporters differentially distributed between intracellular compartments. Cell damage induced by environmental insults or by overstimulation of physiological pathways results in pathological Ca^{2+} signals, which trigger necrotic or apoptotic cellular death

Keywords: Calcium; Ca^{2+} signalling; apoptosis; necrosis; cell death

Claude Bernard was the first to realise that life is a relentless struggle for constancy of "milieu interior", the concept, which we now know as homeostasis. When homeostasis fails, the organism dies. This, however, does not always stand true for the elementary units of the organism that is for single cells. For many cells the death is pre-planned, which occurs for instance upon **programmed cell death**, necessary for orderly succession of cells in development or in **physiological cells death**, which controls cell turnover in the adulthood. The injury-induced or pathological cell death also may take two distinct routes; the cells may exit through the process of **apoptosis** ("falling away"; (Kerr et al., 1972)) that is also often responsible for programmed and physiological cell death, or by cell destruction achieved by **necrosis**, which destroys the cells and triggers release of harmful cellular content; this invariably affects surrounding tissues and ends up with more or less long-lasting and unpleasant consequences. The process of apoptosis is genetically controlled and relies on numerous intracellular signalling systems. The apoptotic cell death is triggered by activation of distinct cascades and undergoes several clearly defined stages, which eventually turn the dying cell into encapsulated apoptotic bodies,

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easily removed by tissue macrophages without leaving a trace. Conversely, necrosis invariably leads to the loss of membrane integrity and proceeds to cell lysis and release of cell components, which in turn trigger inflammatory tissue reactions. Despite these differences both apoptosis and necrosis are regulated by a single signalling system, which uses Ca^{2+} ions as a universal messenger; even more strikingly the same signalling system controls a vast majority of cellular activities during everyday life.

The multiple roles of calcium, which controls birth, life and death were identified in the late 19 century, when Sydney Ringer discovered that Ca^{2+} ions control heart contractions, regulate fertilisation and development of tadpole and determine survival of fishes (Ringer 1883a, b, 1886, 1890; Ringer and Sainsbury 1894). In late 1960-es/ early 1970-es it has been recognised that cell death is associated with an increase in cell calcium content (see (Farber 1981) for review), and in 1974 the key role of excessive plasmalemmal Ca^{2+} entry and subsequent Ca^{2+} overload in triggering ischemic death of cardiomyocytes was hypothesised (Fleckenstein et al., 1974). Several years later massive translocation of Ca^{2+} from the extracellular space into neural cells was identified in ischemic cerebellum (Nicholson et al., 1977). In the following 30 years the role of Ca^{2+} overload and cell Ca^{2+} dyshomeostasis in initiation and regulation of various death pathways was firmly established (see e.g. (Nicotera et al., 1992; Orrenius and Nicotera, 1994; Choi, 1995; Kristian and Siesjo, 1998; Ermak and Davies 2002; Eisner et al., 2006)). In this chapter I shall present the overall view on Ca^{2+} homeostasis and dyshomeostasis, discuss the role of various Ca^{2+} regulating pathways on pathological developments and briefly overview the Ca^{2+}-dependent systems involved in controlling apoptosis and necrosis.

1. CALCIUM HOMEOSTASIS IN VARIOUS INTRACELLULAR COMPARTMENTS

Calcium ions act as both extra- and intra-cellular messenger. The fluctuations of extracellular Ca^{2+} are picked up by a broad family of Ca^{2+} sensors, whose role still remains largely unknown (Riccardi 2004). At the same time Ca^{2+} is the most versatile intracellular messenger discovered so far, since it is involved in the regulation of almost all known cellular functions and reactions (Petersen et al., 2005). There are exceptions, but they are few, the most notable probably being the propagation of nerve action potentials, which depends on Na^+ and K^+ channels that are not acutely Ca^{2+}-regulated. The most important properties of Ca^{2+} signalling are the promiscuity with respect to its effector systems and its autoregulation. Indeed, Ca^{2+} regulates a truly remarkable variety of intracellular processes, within extremely different temporal domains, from microseconds (e.g. exocytosis) to months or even years (e.g. memory processes). At the same time, the actual molecular systems responsible for producing Ca^{2+} signalling events are limited to only a few protein families (Ca^{2+} channels and transporters), and these systems appear to be very well conserved and ubiquitously expressed within the cellular

kingdom. Most importantly, all these systems are regulated by Ca^{2+} itself, thus making a very robust, albeit versatile and adaptable piece of molecular machinery.

Fundamentally, calcium homeostasis is a result of coordinated transportation of Ca^{2+} ions through several sets of membranes, which delineate distinct cellular compartments; these compartments maintain very different Ca^{2+} concentrations, and have a specific role in both physiological and pathological Ca^{2+} signalling (Petersen et al., 1994; Berridge et al., 2003; Carafoli 2004; Verkhratsky 2005).

These compartments are represented by the cytosol, by endoplasmic reticulum, which is often connected with nuclear envelope and complex Golgi, by mitochondria and by the nucleus (Figure 1). Although each of these compartments has its own Ca^{2+} homeostatic pattern, they all rely on Ca^{2+} movements across relevant membranes, which are governed by relatively restricted number of Ca^{2+} channels and Ca^{2+} transporters. Calcium-binding proteins, whose Ca^{2+} affinities vary between several nM and 0.5–1.0 mM, represent second important element, which controls Ca^{2+} traffic within the said compartments. The Ca^{2+} binding proteins also act as Ca^{2+} sensors, which control cellular biochemistry and execute cellular reactions. Calcium fluxes between different cellular compartments occur either by diffusion down the concentration gradient, or by active energy dependent transport against the latter.

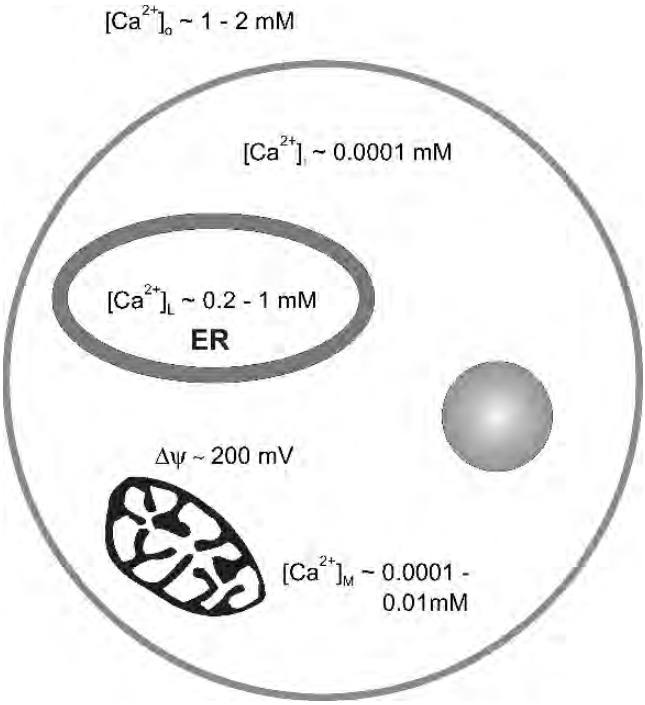

Figure 1. Compartmentalisation of cellular Ca^{2+} homeostasis

The Ca^{2+} concentration gradients, build across different membranes are, as a rule, quite steep, which ensures the high signal to noise of Ca^{2+} signalling system; simultaneously these steep gradients present a danger of rapid Ca^{2+} overload upon disruptions of Ca^{2+} homeostasis. The extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) varies within the range of 1.2–1.5 mM. The cytosolic Ca^{2+} concentration (or $[Ca^{2+}]_i$) is 4 orders in magnitude lower, being set around 30–100 nM. Free Ca^{2+} concentration in the lumen of endoplasmic reticulum ($[Ca^{2+}]_L$) can reach 0.5–1.0 mM, being thus comparable with $[Ca^{2+}]_o$. Mitochondria have another degree of complexity, added by an electrical gradient between cytosol and mitochondrial matrix, which can reach up to 200 mV, and thus favour Ca^{2+} influx down the electrogenic gradient.

Passive "downhill" calcium transport (down to concentration- or electrical gradients) between the compartments occurs via several sets of Ca^{2+} permeable channels (Figure 2). Plasmalemmal Ca^{2+} influx is a function of voltage- ligand- and store-operated Ca^{2+} channels, which have a variable Ca^{2+} selectivity (the highest for voltage- and store-operated, the intermediate for ligand-operated) and through non-selective cation channels, also permeable to Ca^{2+}. The endomembrane (which forms the ER) contains several sets of intracellular Ca^{2+} channels, the ryanodine receptors (RyRs), the $InsP_3$ receptors ($InsP_3Rs$) and possibly the NAADP receptors (Bezprozvanny 2005; Galione and Ruas 2005; Hamilton 2005). Activity of these channels is controlled by intracellular messengers (RyRs by cyclic ADP ribose; $InsP_3Rs$ by $InsP_3$) and by Ca^{2+} ions, which directly activate RyRs and modulate the openings of $InsP_3Rs$. Finally, mitochondria contain the Ca^{2+} uniporter (which is in fact a highly selective Ca^{2+} channel – (Kirichok et al., 2004)), and a

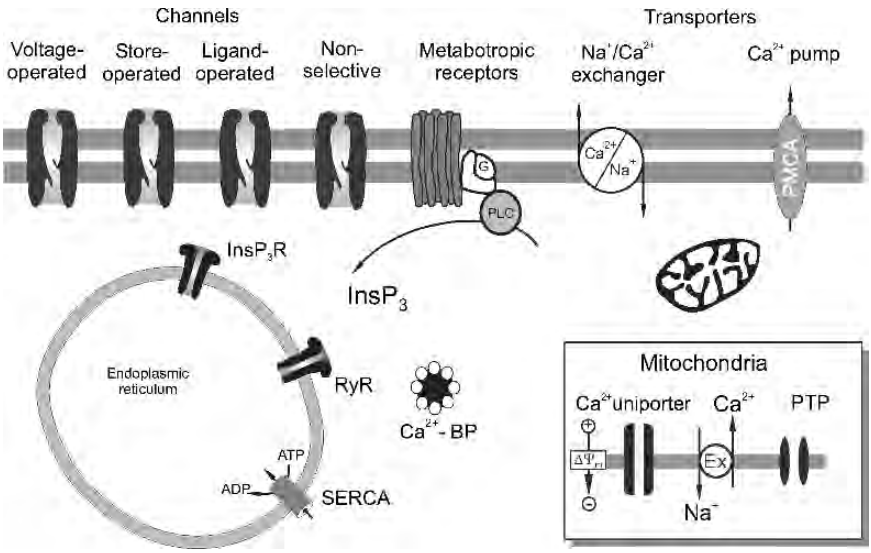

Figure 2. Molecular cascades responsible for Ca^{2+} homeostasis and Ca^{2+} signalling

complex permeability transition pore, which can allow Ca^{2+} flux. The uphill Ca^{2+} transport against concentration gradient is the function of Ca^{2+} pumps, represented by plasmalemmal variety PMCA (plasmalemmal Ca^{2+} ATPases – see (Wuytack et al., 2002; Guerini et al., 2005)), by endomembrane variety SERCA (sarco-endoplasmic reticulum Ca^{2+} ATPases – (Wuytack et al., 2002; Vangheluwe et al., 2005)) and by several other pumps (e.g. PMR1 pump) residing for instance in the membrane of Golgi complex (Sorin et al., 1997). The second energy-dependent transmembrane Ca^{2+} transporter is a sodium-calcium exchanger, the NCX, present in plasma membrane and in mitochondria; the NCX exchanges Ca^{2+} for Na^{+} and can operate in forward and reverse mode depending on the transmembrane Na/Ca^{2+} gradients and membrane polarisation (Guerini et al., 2005).

All molecular systems involved in Ca^{2+} homeostatic processes are regulated by Ca^{2+} itself, which operates numerous feedback loops. That is, increase in cytosolic Ca^{2+} concentration induces a Ca^{2+}-dependent inactivation of voltage-operated plasmalemmal Ca^{2+} channels. Fluctuations in $[Ca^{2+}]_L$ differentially regulate Ca^{2+} transport across the endomembrane: elevation of $[Ca^{2+}]_L$ increases the susceptibility of RyRs and $InsP_3Rs$ to activation, and inhibits the SERCAs; when $[Ca^{2+}]_L$ falls, the SERCA pumping is much activated, and the Ca^{2+} release channels are inhibited (Burdakov et al., 2005; Burdakov and Verkhratsky 2006).

Physiological cell stimulation triggers openings of different sets of channels, which results in a rapid Ca^{2+} fluxes aimed at the cytosol; Ca^{2+} can originate either from extracellular space and enter the cell through plasmalemmal channels, or from the ER via intracellular Ca^{2+} channels. This Ca^{2+} influx triggers the cytosolic Ca^{2+} signals, which are transient in nature and often are spatially localised. The preferred routes vary between different cell types: in excitable cells such as neurones and muscle cells the leading role in Ca^{2+} signal generation belongs to plasmalemma; in non-excitable cells the main source of Ca^{2+} ions is intracellular. The role of plasmalemmal Ca^{2+} channels is particularly important for neurones, where depolarisation brought by action potential activates several sets of voltage-operated calcium channels, which rapidly and effectively increase the $[Ca^{2+}]_i$; alternatively the plasmalemmal Ca^{2+} influx can be also achieved through ligand-operated channels, gated by various neurotransmitters. In this way plasmalemmal Ca^{2+} channels swiftly transform membrane events into cytosolic Ca^{2+} signals. In cardiac muscle cells plasmalemmal Ca^{2+} influx acts mostly as a trigger event, which activates massive Ca^{2+} release from the sarcoplasmic reticulum that actually delivers bulk of Ca^{2+} necessary for triggering contraction. In non-excitable cells the ER takes the leading role: activation of numerous plasmalemmal receptors initiates synthesis of $InsP_3$, which opens $InsP_3Rs$ and induces intracellular Ca^{2+} release; the Ca^{2+} release depletes the ER lumen, and releases a yet unidentified signal (but see (Liou et al., 2005; Soboloff et al., 2006)), which opens store-operated Ca^{2+} channels residing in the plasma membrane (Parekh and Putney 2005). Thus, by combining different pathways and connecting various Ca^{2+} handling molecules the versatility and adaptability of Ca^{2+} signalling machinery is achieved.

Upon entering cellular compartments Ca^{2+} ions are subject to buffering by Ca^{2+}-binding proteins, which to a very large extent determine the shape of resulting Ca^{2+} signals. Cytosolic Ca^{2+} buffers are characterised by high (in a submicromolar range) affinity to Ca^{2+} ions (Lewit-Bentley and Rety 2000; Ikura et al., 2002). These high affinity cytosolic buffers tend to hamper diffusion and localise cytosolic Ca^{2+} events thus assisting in forming a focal micro- or even nano-domains of high (1–100 μM) $[Ca^{2+}]_i$, which in turn control highly focal and rapid cellular reactions, such as e.g. neurotransmitter release (Barclay et al., 2005; Rizzuto and Pozzan 2006).

To the contrary, the Ca^{2+} binding proteins residing in the ER lumen (e.g. calsequestrin, calreticulin or calumenin), have a very low affinity, their K_D being around 0.5–1 mM (Michalak et al., 2002). This low affinity is, however, combined with very high capacity to bind Ca^{2+} (each molecule of calsequestrin or calreticulin may bind up to 20–50 Ca^{2+} ions). This arrangement allows unhampered diffusion, and thus permits much freedom for intra-ER Ca^{2+} movement, as compared to the cytosol (Mogami et al., 1999). As the ER lumen is internally continuous (Park et al., 2000; Solovyova and Verkhratsky 2003), the $[Ca^{2+}]_L$ can rapidly equilibrate, providing thus a system for long-range and relatively rapid Ca^{2+} movements within the cell (Mogami et al., 1997; Petersen et al., 2001; Verkhratsky 2005).

Finally, the termination of cellular Ca^{2+} signals achieved through energy dependent Ca^{2+} transporters, which restore the physiological distribution of Ca^{2+} within various compartments, either by exporting Ca^{2+} into the extracellular space (PMCA and NCX – (Guerini et al., 2005)), or accumulating Ca^{2+} into the lumen of the ER (SERCA's – (Wuytack et al., 2002)). Importantly, part of Ca^{2+} ions entering the cytosol during Ca^{2+} signal generation are accumulated by mitochondria; Ca^{2+} elevation within the mitochondrial matrix activates enzymes of citric acid cycle, hence stimulating production of ATP (Rizzuto et al., 2004); this is a fundamental signalling cascade which links cellular activity and ATP production. The mechanisms of mitochondrial Ca^{2+} homeostasis and role of mitochondrial Ca^{2+} signalling in controlling the cell death are, however, covered by another chapter of this volume, and therefore will not be discussed in this essay.

A broad family of Ca^{2+} sensors, which are in effect Ca^{2+}-regulated enzymes, accomplishes the decoding of calcium signals and execution of Ca^{2+}-regulated processes. When binding/unbinding Ca^{2+} these enzymes change their activity, and thus control cellular responses. These enzymes are distributed between different compartments, and quite obviously, display different sensitivity to Ca^{2+}. For example, intra-ER chaperons, assisting protein folding require 100 μM of free Ca^{2+} in the ER lumen to perform; many cytosolic Ca^{2+} sensors are governed by low-μM changes in $[Ca^{2+}]_i$, yet some of them (e.g. proteins controlling exocytosis) are activated by $[Ca^{2+}]$ levels approaching 10–100 μM, which may be reached in focal $[Ca^{2+}]_i$ microdomains.

All systems described above, operate physiologically and provide for normal cell function; yet the very same systems are also involved in generation of pathological signals activated upon cell injury.

2. CALCIUM DYSHOMEOSTASIS UPON CELL INJURY

Cell injury results from two conceptually different sources. First, cells can be injured by disruption of endogenous structures or processes, which can lead either to pathological over(under)stimulation (i.e. hyper-release of glutamate in the brain) or to deprivations to extracellular environment (i.e. anoxia, ischemia, glucose deprivation etc.). Second, cells can be injured by exogenous toxins, substances or physical factors representing the environmental attack. Irrespective to the injury signal, the cytotoxicity is invariably accompanied by a sustained and long-lasting elevation in cellular Ca^{2+} content. Increases in intracellular Ca^{2+} are observed upon toxicity of various organic and non-organic substances, from cyanides to mercury; it is detected during cell death initiated by toxic components of viruses wall, or even by mechanical trauma. Similarly, intracellular Ca^{2+} increase accompany ischemic cell death, glutamate toxicity, hormonal killing of immunocompetent cells and complement- or T-cells dependent cytolysis (see (Orrenius et al., 1991; Nicotera et al., 1992; Orrenius and Nicotera 1994) for review and relevant references).

All in all, cell injury almost invariably results in generation of pathological Ca^{2+} signals, which usually appear in a form of prolonged elevations of cytosolic Ca^{2+} concentration. These pathological Ca^{2+} signals originate from increased plasmalemmal Ca^{2+}entry, increased Ca^{2+} release, compromised Ca^{2+} extrusion or

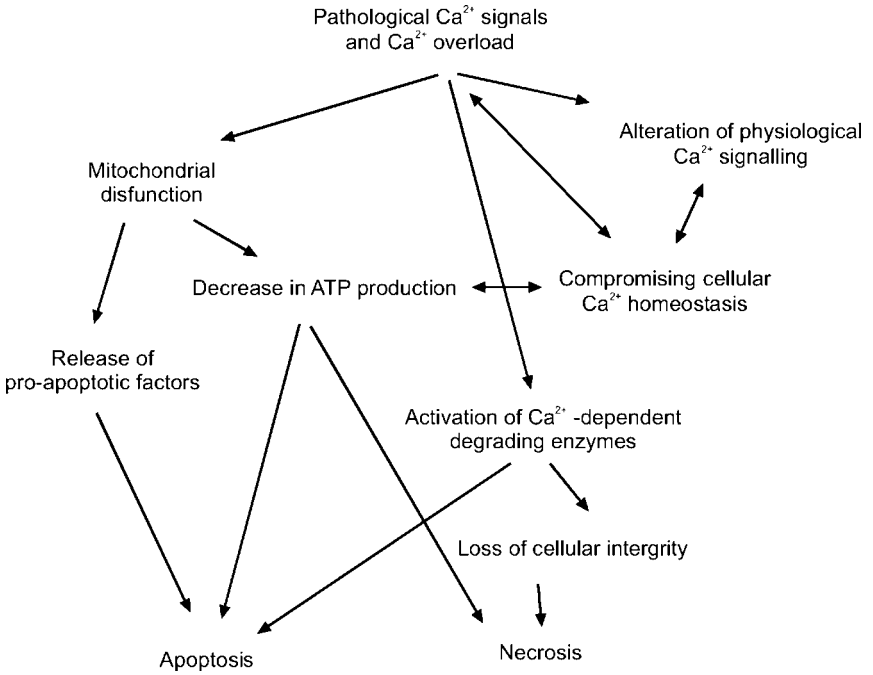

Figure 3. Pathological Ca^{2+} signalling regulate cell death via apoptosis or necrosis

combinations of the above. Whatever the nature of triggering event, cell Ca^{2+} overload compromises Ca^{2+} homeostatic machinery, which eventually sets up a vicious circle of positive feedbacks further exacerbating Ca^{2+} overload and leading to irreparable damage. The pathological Ca^{2+} signals also set into motion the cell death process, which may proceed either via benign apoptotic route or end up in necrosis. Conceptually, the choice between the two depends on the intensity of injury and the status of the cell - e.g. the resting concentration of ATP and the mitochondrial status very much affect the mode of cellular death (Orrenius et al., 2003).

To conclude, pathological Ca^{2+} signals result from cell overstimulation and/or failure of Ca^{2+} homeostasis. These pathological Ca^{2+} signals activate several intracellular enzymatic cascades, which in turn trigger or execute death subroutines (Figure 3). Depending on the type of cell, severity of pathological signalling or cell status, the same cascades may initiate either apoptotic or necrotic cell death.

3. GENERATION OF PATHOLOGICAL CA^{2+} SIGNALS: PLASMALEMMAL CA^{2+} ENTRY

Massive Ca^{2+} entry trough the plasmalemma may result in very rapid and severe cellular Ca^{2+} overload. This Ca^{2+} entry may occur either from overstimulation of existing ion channels or through the formation of pathological ion channels; alternatively chronic diseases may affect expression of Ca^{2+} channels and thus modulate Ca^{2+} entry.

Increased Ca^{2+} influx through **voltage-operated calcium channels** (VOCCs) is implicated in many forms of both apoptotic and necrotic cell death. For example, the physiological apoptosis of spermatocytes is controlled to a large extend by Ca^{2+} entry through VOCCs (Barone et al., 2004). From another hand, an excessive or prolonged activation of VOCCs may trigger Ca^{2+} overload and necrotic cell death. This happens, for instance in chromaffin cells, where Ca^{2+} entry through L-type channels induces rapid cell death (Cano-Abad et al., 2001) or in organotypic hippocampal neurones, where T channels significantly contribute to ischemia-induced pathological Ca^{2+} entry (Nikonenko et al., 2005). The chronic changes in expression of VOCCs occur upon physiological ageing (Landfield 1996; Toescu et al., 2004) and in certain forms of experimental Alzheimer disease-like pathologies (Toescu et al., 2004); elevated Ca^{2+} entry may underlie long-term damage of cells in these conditions.

Sometimes pathological Ca^{2+} overload may result from Ca^{2+} influx through other types of channels, as for example was shown for DEGF/ENaC sodium channel, which could generate a Ca^{2+} influx, sufficient to trigger intracellular Ca^{2+} release and thus induce the necrotic cell death (Bianchi et al., 2004).

Ligand-operated Ca^{2+} channels (LOCCs), known also as ionotropic receptors, are important progressors of pathological Ca^{2+} entry, especially in the brain tissue. The best example of toxic plasmalemmal Ca^{2+} entry is represented by glutamate-induced excitotoxic death of neural cells (or glutamate excitotoxicity) in damaged

brain, initially descried in late 1960-s (Olney 1969; Olney and Ho 1970). Every type of brain insult results in substantial release of glutamate; the latter in turn activates numerous glutamate receptors, which provide a pathway for excessive Ca^{2+} entry. The Ca^{2+} entry mainly occurs through highly Ca^{2+}-permeable NMDA subtype of glutamate receptors; these receptors conduct not only Ca^{2+} ions, but have very slow desensitisation, which results in a long-lasting periods of Ca^{2+} influx (Siesjo 1989; Choi 1995; Leist and Nicotera 1998). Glutamate excitotoxicity represents the main mechanism of neural cell death upon ischemia and stroke. Other types of LOCCs may also trigger pathological Ca^{2+} overload: Ca^{2+} entry resulting in a cell death may occur for example via AMPA/KA glutamate receptors (Kwak and Weiss 2006) or though various subtypes of P2X purinoreceptors (Franke et al., 2006), with particular role for $P2X_7$ receptor (Sperlagh et al., 2006).

Transient receptor potential or TRP channels belong to a diverse super-family of cationic non-voltage gated channels, present in almost all types of eukaryotic cells (Pedersen et al., 2005). Many of TRP channels have substantial Ca^{2+} permeability and can mediate relevant Ca^{2+} fluxes. Mechanisms of TRP channels activation are sundry; many of them are sensitive to environmental factors such as e.g. temperature; alternatively, diacylglycerols or relative substances, which occur following activation of G-protein coupled metabotropic receptors, activate many of TRP channels. Pathological activation of TRP channels may create cell Ca^{2+} overloads, which can cause cellular death; the later was documented for photoreceptors (Yoon et al., 2000). Massive Ca^{2+} influx through TRPM7 channels was also proposed as a key mechanism of anoxia-induced death of cortical neurones (Aarts et al., 2003).

4. GENERATION OF PATHOLOGICAL CA^{2+} SIGNALS: INTRACELLULAR CA^{2+} STORES

The intracellular Ca^{2+} stores, mostly associated with the ER also play important role in generation of pathological Ca^{2+} signals and in controlling cell death. The role of ER Ca^{2+} homeostasis in cellular pathology is complex: firstly ER generates Ca^{2+} release, which can contribute to cytosolic Ca^{2+} overload and is sensed by mitochondria; secondly, disruptions of ER Ca^{2+} homeostasis can also generate relevant death signals. These two processes often go in parallel, as aberrant activity of Ca^{2+} release channels can simultaneously trigger $[Ca^{2+}]_i$ increases and deplete the $[Ca^{2+}]_L$. The intra-ER Ca^{2+} homeostasis is critical for many ER functions, most notably for posttranslational processing of proteins; failures in ER Ca^{2+} handling affect chaperones and alter protein folding. This results in generation of ER stress response, which generally appears in two forms, the unfolding protein response (UPR) and ER overload response (EOR), see (Pahl and Baeuerle 1997; Patil and Walter 2001; Glazner and Fernyhough 2002). The ER stress response, in turn generates several signals aimed towards the nucleus, which are meant to assist the protein folding recovery. Under severe stress, however, these signals became detrimental and may initiate apoptotic or necrotic cell death. Particularly important is an activation of nuclear factor NK-κB, which regulates production of cytokines

and interferons; the latter being important factors in triggering cell death (Ferri and Kroemer 2001). Prolonged ER stress can also stimulate pro-caspase-12, localised in the endomembrane; when activated the caspase 12 stimulates other pro-apoptotic caspases (Nakagawa et al., 2000).

The cell death signals can be associated with malfunctioning of both RyRs and $InsP_3Rs$. For example, an aberrant activity of RyRs was shown to trigger apoptotic and necrotic cell death in CHO cell lines and in prostate cancer cell line LNCaP (Mariot et al., 2000; Pan et al., 2000). Incidentally, cytosolic Ca^{2+} buffering prevented necrosis, but did not affect apoptosis, indicating a specific role of ER Ca^{2+} depletion in the initiation of the latter (Pan et al., 2000).

Particular importance in regulation of apoptotic death pathway belongs to $InsP_3Rs$, activation of which stimulates various pro-apoptotic factors. The $InsP_3R$ mediated Ca^{2+} release increases mitochondrial Ca^{2+}, with subsequent release/activation of numerous pro-apoptotic molecules such as cytochrom C, apoptosis-inducing factor (AIF), and second mitochondrial activator of caspases (SMAC/Daiblo) (Hanson et al., 2004). In addition $InsP_3$-induced Ca^{2+} release may activate calcineurin with subsequent phosphorylaytion of pro-apoptotic protein Bad (Jayaraman and Marks 1997). Genetic removal of $InsP_3Rs$ reduces the susceptibility of cells to apoptosis (Sugawara et al., 1997).

Activation of $InsP_3Rs$ relevant to apoptosis can occur through physiological pathway, by stimulation of relevant metabotropic receptors. This mechanism, for example, ensures the extinction of immature B cells, which is essential for immunological self-tolerance. In this case the $InsP_3$ induced Ca^{2+} release is activated by stimulation of B-cell receptors (Hanson et al., 2004). Alternatively, the $InsP_3$ receptors can be activated indirectly, for instance through proteolysis of $InsP_3R$ type 1 by caspase 3; the truncated $InsP_3R$ remains in the endomembrane and provides for a continuous Ca^{2+} leak from the ER (Nakayama et al., 2004).

The role of ER Ca^{2+} leak in generation of cell death signals is not entirely understood, mostly because the nature of the ER leak pathways remain enigmatic (Camello et al., 2002), yet it may play a key role in various types of cellular pathology, such as e.g. in Alzheimer disease-related neurodegeneration. In the latter case the aberrant Ca^{2+} leak may be controlled by mutant presenilins (Tu et al., 2006). Another important and relevant for pathology ER Ca^{2+} leak pathway may be associated with recently discovered channels of pannexin family, which can form Ca^{2+} permeable channels in the endomembrane (Vanden Abeele et al., 2006). All in all the pathological role of ER Ca^{2+} leakage needs further attention.

5. GENERATION OF PATHOLOGICAL CA^{2+} SIGNALS: CA^{2+} EXTRUSION SYSTEMS

Excess of Ca^{2+} is removed from the cytosol by plasmalemmal Ca^{2+} pumps and sodium-calcium exchangers. The abnormal function of both systems can result in alteration of basal $[Ca^{2+}]_i$ and prolongation of evoked Ca^{2+} signals. Inhibition of PMCA results in pathological Ca^{2+} homeostasis in several cellular preparations

(Waring 2005). The activity of NCX can be even more important for maintenance of physiological Ca^{2+} signalling, as cleavage and incapacitation of NCX in brain ischemia was instrumental for triggering the excitotoxic cell death (Bano et al., 2005). The intracellular SERCA pumps not only remove excess of Ca^{2+} from the cytosol, but also control the ER Ca^{2+} homeostasis; inhibition of SERCA pumping has a prominent detrimental effect on many cells, and often triggers rapid cell death (see e.g. (Paschen 2003; Paschen and Mengesdorf 2005)). Mutation of SERCA2 pumps, which underlies the autosomal-dominant Darier disease, is associated with severe neurodegeneration, manifested by mental retardation, schizophrenia, epilepsy and progressive encephalopathy (Missiaen et al., 2000).

6. CA^{2+} SENSORS IN CELL DEATH REACTIONS

Fluctuations in $[Ca^{2+}]_i$ affect multiple enzymes, some of which are directly associated with initiation of cell death. These enzymes control the breakdown of various cellular constituents, and their pathological activation can have fatal consequences.

Ca^{2+}-activated proteases. Ca^{2+}-activated proteases or calpains are ubiquitously distributed in the cytosol (Nicotera et al., 1992). Upon activation they may degrade various intracellular proteins, including components of cytoskeleton. Inhibition of calpains by specific inhibitors greatly reduces the proteolysis and toxic cell death (Nicotera et al., 1986; Lee et al., 1991).

Calcineurin. The calcineurin, or Ca^{2+}-calmodulin dependent protein phosphatase (Aramburu et al., 2004) mediates apoptosis through at least two routes. First, this action can be achieved through steroid receptor Nur77 and CD95 ligand; this pathway was found in lymphoid cells (Shi et al., 1989). Alternatively, calcineurin dephosphorylates a pro-apoptotic protein Bad (a member of Bcl-2 family), which in turn translocates into mitochondria and triggers release of cytochrome C and activation of caspases (Wang et al., 1999).

Endonucleases. Endonucleases provide for DNA cleavage into small (200 base pairs) fragments, which is an essential step in apoptotic cascade (Wyllie 1980; Wyllie 1998). Endonucleases are stimulated by Ca^{2+} and their activation was detected in several cell types undergoing apoptosis (McConkey et al., 1988; Aw et al., 1990). The intimate mechanisms of endonucleases action remain not fully described; at least in part they can be explained through the involvement of caspase-3 activated endonuclease (or caspase-activated DNAase - (Enari et al., 1998)). Nonetheless, the chromatin fragmentation was also observed in cell (and caspase)-free system, when isolated nuclei were treated with Ca^{2+} and ATP, suggesting the existence of caspase-independent DNA cleavage mechanism (Jones et al., 1989).

Phospholipases. Calcium controls several phospholipases, most notably the phospholipase C (PLC), which produces $InsP_3$ and diacylglycerol (Berridge 1981), and phospholipase A_2 (PLA_2), which regulates the liberation of arachidonic acid from phospholipids (Exton et al., 1991). Overstimulation of

phospholipases, therefore, may cause a further increase in Ca^{2+} release from the ER stores, extensive membrane breakdown and generation of toxic substrates (Nicotera et al., 1992). In particular, Ca^{2+}-dependent over-activation of PLA_2 was observed upon ischemia and following treatment with some toxins (Nicotera et al., 1992).

Nitric oxide synthase. The NO synthase (NOS) can generate both pro- and anti-apoptotic effects. The deleterious effects of excessive NO production are well documented in neuronal damage following ischemia and glutamate excitotoxicity (Dawson et al., 1991; Huang et al., 1994).

Transglutaminases. Ca^{2+}-dependent transglutaminase activation was detected in dying and apoptotic cells; its actual role can be related to cross-linking of plasmalemmal proteins with cytoskeleton (Orrenius et al., 2003).

7. CONCLUSIONS

Calcium signalling systems play central role in regulation of cell fate and in determining the choice of cell death. This role is inseparable from a general importance of Ca^{2+} signalling, which controls most of cellular reactions. By tight coordination of several molecular cascades, expressed in various cellular compartments, Ca^{2+} signals achieve a remarkable versatility in governance over multitude of biochemical reactions. The temporal and spatial appearance of Ca^{2+} signals determines the outcome and conductance of cellular processes. Similarly, pre-orchestrated Ca^{2+} signals control the programmed cell death, which is vitally important for development and survival of multicellular organisms. The overstimulation of Ca^{2+} signalling system either by endogenous factors or by environmental insults triggers cell death, which may occur in apoptotic or necrotic disguise.

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CHAPTER 18

CALCIUM AND CELL DEATH: THE MITOCHONDRIAL CONNECTION

P. BERNARDI AND A. RASOLA

Department of Biomedical Sciences, University of Padova, Viale Giuseppe Colombo 3, 35121 Padova, Italy

Abstract: Physiological stimuli causing an increase of cytosolic free Ca^{2+} $[Ca^{2+}]_c$ or the release of Ca^{2+} from the endoplasmic reticulum invariably induce mitochondrial Ca^{2+} uptake, with a rise of mitochondrial matrix free $[Ca^{2+}]$ ($[Ca^{2+}]_m$). The $[Ca^{2+}]_m$ rise occurs despite the low affinity of the mitochondrial Ca^{2+} uptake systems measured *in vitro* and the often limited amplitude of the cytoplasmic $[Ca^{2+}]_c$ increases. The $[Ca^{2+}]_m$ increase is typically in the 0.2–3 μM range, which allows the activation of Ca^{2+}-regulated enzymes of the Krebs cycle; and it rapidly returns to the resting level if the $[Ca^{2+}]_c$ rise recedes due to activation of mitochondrial efflux mechanisms and matrix Ca^{2+} buffering. Mitochondria thus accumulate Ca^{2+} and efficiently control the spatial and temporal shape of cellular Ca^{2+} signals, yet this situation exposes them to the hazards of Ca^{2+} overload. Indeed, mitochondrial Ca^{2+}, which is so important for metabolic regulation, can become a death factor by inducing opening of the permeability transition pore (PTP), a high conductance inner membrane channel. Persistent PTP opening is followed by depolarization with Ca^{2+} release, cessation of oxidative phosphorylation, matrix swelling with inner membrane remodeling and eventually outer membrane rupture with release of cytochrome *c* and other apoptogenic proteins. Understanding the mechanisms through which the Ca^{2+} signal can be shifted from a physiological signal into a pathological effector is an unresolved problem of modern pathophysiology that holds great promise for disease treatment

Keywords: Ca^{2+}; channel; mitochondria; permeability transition pore; apoptosis

1. INTRODUCTION

Mitochondria play a central role in a variety of biological processes, including ATP synthesis, steroid hormone synthesis, the urea cycle, lipid and amino acid metabolism, and cellular Ca^{2+} homeostasis. Ca^{2+} is an essential regulator of vital processes, such as secretion, motility, metabolic control, synaptic plasticity, proliferation, gene expression and apoptosis. Therefore, the location, amplitude,

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and kinetics of free Ca^{2+} transients within the cell are tightly controlled. Ca^{2+} enters cells using three channel families: (i) voltage-gated channels; (ii) ligand-operated channels; and (iii) capacitative channels, which are controlled by the filling state of the cellular Ca^{2+} stores; whereas it is extruded by either high-affinity plasma membrane Ca^{2+}-ATPase pumps or lower affinity Na^{+}-Ca^{2+} exchangers (Carafoli, 2004). Eukaryotic cells reduce resting cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_c$) to below the micromolar level also by storing it into intracellular organelles. These operations generate a 10,000-fold concentration gradient between the extracellular spaces and the cytosol, in which resting free $[Ca^{2+}]$ is 100–200 nM.

Cells use a variety of mechanisms to transiently increase Ca^{2+} in the cytosol in response to specific signals (Gunter et al., 2004). The cytosolic concentration of Ca^{2+} may be much higher in discrete microdomains, for instance in the cytosolic regions close to the Ca^{2+} channels and immediately beneath the plasma membrane, and near the sarco/endoplasmic reticulum sites where Ca^{2+} release occurs. Hot spots of high Ca^{2+} concentration in the vicinity of the organelles result in highly efficient mitochondrial Ca^{2+} accumulation (Nicholls and Budd, 2000; Vandecasteele et al., 2001). In intact cells, apposition of mitochondria to the ER is a common finding and involves a significant portion of the mitochondrial surface (Rizzuto et al., 1998). Given their variable distribution, mitochondria actively orchestrate the spatiotemporal profiles of intracellular Ca^{2+} by using a machinery of exquisite precision and complexity. Mitochondrial Ca^{2+} accumulation allows to cope with potentially lethal Ca^{2+} loads, as the mitochondrial matrix can accumulate large amounts of Ca^{2+} without significantly changing its free matrix concentration due to the coupled accumulation of inorganic phosphate (Pi). In addition, mitochondrial Ca^{2+} satisfies cell energy demands by stimulating ATP production (Hajnoczky et al., 1995). To add a further layer of complexity to Ca^{2+} signalling, all organelles so far studied including nucleus, Golgi, lysosomes, endoplasmic reticulum (ER) and mitochondria, can act as mobilizable Ca^{2+} stores, and many of these appear to interact with each other (Michelangeli et al., 2005).

Physiological stimuli causing an increase of $[Ca^{2+}]_c$ or the release of Ca^{2+} from the ER invariably induce mitochondrial Ca^{2+} uptake, with a rise of mitochondrial matrix free $[Ca^{2+}]$ ($[Ca^{2+}]_m$) despite the low affinity of the mitochondrial Ca^{2+} uptake systems measured *in vitro* and the often limited amplitude of the measured cytoplasmic $[Ca^{2+}]_c$ increases (Duchen, 1999). The $[Ca^{2+}]_m$ increase is typically in the 0.2–3 μM range, which allows the activation of Ca^{2+}-regulated enzymes of the Krebs cycle (Hajnoczky et al., 1995); and the $[Ca^{2+}]_m$ transient rapidly returns to the resting level if the $[Ca^{2+}]_c$ rise recedes. Indeed, the mitochondrial efflux mechanisms and the matrix Ca^{2+} buffering activity keep $[Ca^{2+}]_m$ relatively stable, allowing mitochondria to accumulate Ca^{2+} and to efficiently control the spatial and temporal shape of global cellular Ca^{2+} signals (Brini, 2003; Szabadkai et al., 2006). On the other hand this situation exposes mitochondria to the hazards of Ca^{2+} overload.

Mitochondrial Ca^{2+}, which is so important for metabolic regulation, can indeed become a death factor through induction of the permeability transition (PT). The PT is a permeability increase of the inner mitochondrial membrane (IMM) to ions and solutes mediated by the PT pore (PTP), a voltage-dependent, high-conductance channel that requires a permissive load of matrix Ca^{2+} for opening. Persistent PTP opening is followed by depolarization with Ca^{2+} release, cessation of oxidative phosphorylation, matrix swelling with IMM remodeling and eventually outer mitochondrial membrane (OMM) rupture with release of cytochrome *c* (cyt *c*) and other apoptogenic proteins (Bernardi, 1999; Crompton, 1999; Bernardi et al., 2001; Halestrap et al., 2002; Kim et al., 2003a; Bernardi et al., 2006). The early consequences of PTP-dependent mitochondrial dysfunction are easily appreciated in contractile tissues such as the heart where the combination of elevated cytosolic $[Ca^{2+}]$ and decreased ATP levels prevent muscle relaxation with hypercontracture, sarcolemmal damage and myocyte death (Di Lisa and Bernardi, 2006).

Understanding the mechanisms through which Ca^{2+} can be shifted from a physiological signal into a pathological effector is one of the most fascinating problems in modern pathophysiology. Here we will focus on the role of mitochondrial Ca^{2+} homeostasis in this process with some emphasis on the PTP. The reader is referred to previous reviews (Gunter et al., 1994; Pozzan et al., 1994; Rizzuto et al., 1998; Bernardi, 1999; Duchen, 1999; Crompton, 1999; Nicholls and Budd, 2000; Rizzuto et al., 2000; Bernardi et al., 2001; Vandecasteele et al., 2001; Halestrap et al., 2002; Orrenius et al., 2003; Brini, 2003; Kim et al., 2003a; Gunter et al., 2004; Carafoli, 2004; Michelangeli et al., 2005; Bernardi et al., 2006; Di Lisa and Bernardi, 2006; Szabadkai et al., 2006) and to other articles in this issue for an exhaustive coverage of the multiple facets of mitochondrial and cellular Ca^{2+} homeostasis.

2. MITOCHONDRIAL Ca^{2+} UPTAKE

In energized mitochondria, electrophoretic Ca^{2+} uptake across the IMM is mediated by the mitochondrial Ca^{2+} uniporter (MCU) and/or the rapid uptake mode (RaM) (Gunter et al., 2004). In addition, the OMM voltage-dependent anion channel (VDAC) may regulate Ca^{2+} access to the mitochondrial intermembrane space (Gincel et al., 2001) and thus contribute to the control of mitochondrial Ca^{2+} fluxes.

(i) The MCU transports Ca^{2+} and Sr^{2+} but not Mg^{2+}. It is inhibited by ruthenium red (RR) and it is activated by external Ca^{2+} itself, and it undergoes deactivation upon removal of extramitochondrial Ca^{2+} (Kröner, 1986). *In vitro* at least, activation/deactivation of the uniporter are slow processes, requiring the order of 1 min to be achieved, and the rate of Ca^{2+} uptake via the uniporter is zero at $[Ca^{2+}]_c$ less than about 200 to 300 nM. Thus, at low $[Ca^{2+}]_c$ the activity of the uniporter could be extremely low (Gunter and Pfeiffer, 1990), due also to inhibition by adenine nucleotides (ATP> ADP>AMP) (Litsky and Pfeiffer, 1997). The activity of the uniporter could be affected by (de)phosphorylation, as suggested by the stimulation observed after treatment with the p38 mitogen-activated protein kinase inhibitor SB202190 (Montero et al., 2002). Single

channel recording from mitoplasts (organelles where the outer membrane has been largely removed) identified a highly selective Ca^{2+} channel exhibiting a complex gating mechanism with multiple sub-conductance states, which has ion-conductive properties identical to those of the MCU studied in intact mitochondria (Kirichok et al., 2004).

(ii) RaM is a transport mechanism that might contribute to mitochondrial Ca^{2+} uptake from $[Ca^{2+}]_c$ transients *in vivo*. It was observed that Ca^{2+} uptake is more efficient when energized mitochondria are exposed to trains of Ca^{2+} pulses of about 400 nM rather than to an identical steady Ca^{2+} concentration ($[Ca^{2+}]_o$) for the same overall time (Sparagna et al., 1995). *In vitro* at least, mitochondrial Ca^{2+} uptake via the RaM is faster than via the MCU and RaM inhibition is also very rapid. It is blocked by $[Ca^{2+}]_o$ above 100–150 nM, while no uptake takes place via the RaM at a steady state $[Ca^{2+}]_o$ above 180–200 nM, suggesting that RaM is inactivated by the drop of $[Ca^{2+}]_o$ below 100–150 nM. Hence, as RaM-mediated uptake depends more on the frequency of the signal than on its amplitude, mitochondria may store Ca^{2+} via the RaM in a frequency-modulated fashion (Sparagna et al., 1995). Uptake via the RaM is inhibited by uncouplers, suggesting that it is driven by the Ca^{2+} electrochemical gradient as is the case for the MCU (Gunter et al., 2000). It was suggested that the very rapid Ca^{2+} uptake via the RaM may function in a non equilibrium manner to produce high, transient $[Ca^{2+}]_m$ even when total Ca^{2+} is low, allowing the storage of enough matrix Ca^{2+} to activate metabolism (Gunter et al., 2000; Buntinas et al., 2001). Therefore, rapid mitochondrial sequestration of Ca^{2+} from physiological transients might stimulate ADP phosphorylation to allow matching of energy production with energy use. Notably, increased $[Ca^{2+}]_m$ enhances both the rate of F_OF_1 ATP synthase activity and the rate of ATP hydrolysis (Territo et al., 2000).

As Ca^{2+} sequestered into the matrix by both the MCU and the RaM is capable of modulating the shape and amplitude of cytosolic Ca^{2+} transients (Gunter and Gunter, 2001) and to rapidly increase rate of NADH production (Hajnoczky et al., 1995), oxygen use (Territo et al., 2000; Territo et al., 2001) and ATP levels (Jouaville et al., 1999), the question arises of why different modes for Ca^{2+} uptake have evolved. Different hypotheses have been proposed to explain the connection between mitochondrial Ca^{2+} uptake and regulation of ATP production. The uniporter hypothesis postulates that the MCU sequesters enough Ca^{2+} because mitochondria are located in hotspots close to the release sites for Ca^{2+} of ER or plasma membrane (Rizzuto et al., 2000). Only when $[Ca^{2+}]$ increases in a small region near the mitochondria, the MCU is markedly activated. Conversely, when Ca^{2+} transients are averaged across the cell around the micromolar level, the uniporter alone is usually insufficient to sequester enough Ca^{2+} for the activation mitochondrial metabolism (Gunter et al., 1998). The RaM hypothesis holds that the RaM and the MCU both sequester the Ca^{2+} necessary to stimulate ATP production, but the latter would prevail when cytosolic Ca^{2+} pulses are longer-lasting (Sparagna et al., 1995). In a third model it was proposed that influx via the RaM creates a

short period of high $[Ca^{2+}]_m$ before equilibration in the matrix, and that this high $[Ca^{2+}]_m$ transients would suffice to stimulate mitochondrial metabolism (Buntinas et al., 2001); under slower accumulation conditions, the total amount of Ca^{2+} required would be much higher. Remarkably, in the hotspot theory mitochondria close to the Ca^{2+} release sites could receive dangerous amounts of Ca^{2+}that may lead to induction of the PT. It is possible that inactivation of both MCU and RaM protects mitochondria against unnecessary Ca^{2+} uptake in these cases (Gunter and Pfeiffer, 1990; Sparagna et al., 1995; Buntinas et al., 2001). However, the MCU could have a primary role in PT induction because the amount of RaM-mediated uptake per Ca^{2+} pulse is limited. Moreover, the MCU only sequesters Ca^{2+} when $[Ca^{2+}]_o$ rises above about 200–300 nM. Altogether, both the MCU and the RaM are probably involved in the response to physiological Ca^{2+} transients or pulses, with the uniporter playing a greater role as the duration of the transients increases (Gunter and Gunter, 2001).

Given that cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_c$) in most cell types is about 100 nM (Pozzan et al., 1994), and that the IMM potential ($\Delta\Psi_m$) is of the order of 180 mV (negative inside), equilibrium $[Ca^{2+}]_m$ should be at least 0.1M (Azzone et al., 1977) whereas most measurement indicate a basal $[Ca^{2+}]_m$ level in the sub- to micromolar range (Wan et al., 1989; McCormack et al., 1989). Displacement from equilibrium is due to the fact that Ca^{2+} distribution is modulated by kinetic rather than thermodynamic parameters (Azzone et al., 1977), and that Ca^{2+} uptake is matched by Ca^{2+} efflux via distinct pathways.

3. MITOCHONDRIAL Ca^{2+} EFFLUX

Respiring, coupled mitochondria (which maintain a high transmembrane potential) release Ca^{2+} through two pathways that can be studied in isolated mitochondria after the addition of RR to block Ca^{2+} reuptake.

(i) Na^+-independent Ca^{2+} efflux. Respiring mitochondria in vitro maintain a steady-state extramitochondrial free $[Ca^{2+}]$ at a constant value of 0.25–1.0 μM. If RR is added to block the MCU and RaM, a process of Ca^{2+} efflux ensues suggesting that a RR-insensitive Ca^{2+} efflux (coupled to MCU-mediated reuptake) was also occurring prior to the addition of RR (Vasington et al., 1972). The interpretation of many studies of RR-insensitive Ca^{2+} efflux from mitochondria is complicated by the often unrecognized contribution of the PT. Indeed, a small fraction of mitochondria may undergo reversible opening of the PTP. In the steady state, Ca^{2+} released from this permeabilized fraction would be taken up by polarized mitochondria. Addition of RR would prevent the reuptake process resulting in what appears to be net Ca^{2+} efflux from the whole mitochondrial population, while release is occurring from the subset of permeabilized mitochondria (Riley and Pfeiffer, 1985). The properties of the Na^+-independent pathway for Ca^{2+} efflux in rat liver mitochondria have therefore been reassessed under conditions where occurrence of the PT could be excluded. These important experiments established that this pathway saturates

at Ca^{2+} loads of 25 nmol x mg protein^{-1}; that its V_{max} does not exceed a rate of 1.2 nmol Ca^{2+} x mg protein^{-1} x min^{-1} and is not affected by the concentration of Pi; and that this system releases Ca^{2+} against a gradient that is much higher than thermodynamically permissible to an electroneutral H^+-Ca^{2+} exchanger (Wingrove and Gunter, 1986a). Thus, either Ca^{2+} efflux occurs via a nH^+-Ca^{2+} exchanger with n>2, or it has an active component as also suggested by the finding that steady state Ca^{2+} efflux is inhibited rather than stimulated by small depolarizations (Bernardi and Azzone, 1983).

(ii) Na^+-dependent Ca^{2+} efflux. After the addition of RR, the rate of Ca^{2+} efflux can be substantially stimulated by the addition of Na^+, which is evidence that mitochondria possess a Na^+-Ca^{2+} exchanger mediating physiological Ca^{2+} cycling (Crompton et al., 1976; Crompton et al., 1978). Its V_{max} ranges between 2.6 nmol Ca^{2+} x mg protein^{-1} x min^{-1} in the liver and 18 nmol Ca^{2+} x mg protein^{-1} x min^{-1} in the heart. The dependence on Na^+ is sigmoidal, with typical K_m values of about 8–10 mM Na^+. Ca^{2+} efflux is inhibited by Sr^{2+}, Ba^{2+}, Mg^{2+} or Mn^{2+}, by RR above 5 nmol x mg protein^{-1}, by submicromolar concentrations of the membrane potential probe triphenylmethylphosphonium, and by a variety of compounds of pharmacological interest such as amiloride, trifluoperazine, diltiazem, verapamil, clonazepam, bepridil and CGP37157, while it is stimulated by short-chain alcohols (Wingrove and Gunter, 1986b; Cox et al., 1993). It is modulated by matrix pH (optimum at pH 7.6) and by *in vivo* treatment with glucagon and β-adrenergic agonists (Goldstone et al., 1983). Ca^{2+} efflux is inhibited by antimycin A and protonophores, indicating that the transmembrane potential stimulates the exchange (Crompton et al., 1976). This is consistent with studies indicating that the likely stoichiometry is $3Na^+$:Ca^{2+} (Wingrove and Gunter, 1986b; Jung et al., 1995).

4. CONTROL OF MATRIX Ca^{2+} THROUGH REGULATION OF Ca^{2+} FLUXES

As discussed above, the rise of $[Ca^{2+}]_m$ following stimulation is rapidly followed by a return to baseline levels. The $[Ca^{2+}]_m$ decrease could depend on a decreased activity of the Ca^{2+} uptake systems, on an increased activity of the Ca^{2+} efflux systems, or both (Nicholls and Crompton, 1980). The consequences are however quite different depending on whether the former or the latter mechanism prevails. If net efflux results from stimulation of the efflux pathways, the increase of $[Ca^{2+}]_o$ will also increase the rate of uptake through the MCU with increased rate of Ca^{2+} cycling and therefore energy drain. This can be experimentally observed by adding electroneutral H^+-Ca^{2+} ionophores to respiring mitochondria that have accumulated Ca^{2+}, a condition where all the respiratory capacity is diverted into Ca^{2+} cycling (Heaton and Nicholls, 1976; Bernardi and Pietrobon, 1982). Thus, as long as the membrane potential is high, net Ca^{2+} efflux through stimulation of the efflux pathways has a very high energetic cost. The low V_{max} and saturation of the efflux pathways by $[Ca^{2+}]_m$ are probably designed

to pose a limit to the energy that can be spent in regulation of $[Ca^{2+}]_m$ and $[Ca^{2+}]_o$ through "Ca^{2+} cycling". On the other hand this situation exposes mitochondria to the risks of Ca^{2+} overload, which could be prevented by Ca^{2+} release through transient openings (flickering) of the PTP (Bernardi and Petronilli, 1996) as we discuss in the following paragraph.

5. POTENTIAL ROLE OF THE PTP IN THE REGULATION OF MITOCHONDRIAL Ca^{2+} HOMEOSTASIS

If there is a $[Ca^{2+}]$ concentration gradient between the matrix and the external medium (or the cytosol), opening of the PTP will lead to Ca^{2+} release. The possibility that the PT might serve as a mitochondrial Ca^{2+} release channel has been discussed in the field (Bernardi and Petronilli, 1996) but it has also been criticized based on the lack of channel selectivity. Emergence of channels with different ion selectivity has been an evolutionary process, which is reflected by the high degree of homology among the main channel subfamilies. For example, both the voltage-gated and the cyclic nucleotide-gated K^+ channels have evolved from an ancestral channel, followed by further evolution into the more complex voltage-gated Ca^{2+} and Na^+ channels by small mutations coupled to gene duplication events. Yet, only the existence of Ca^{2+} and Na^+ gradients across the plasma membrane conferred an evolutionary advantage to the independent emergence of different ion selectivities. As already noted elsewhere, PTP opening could lead to selective Ca^{2+} release without major consequences on K^+ and Na^+ homeostasis because no K^+ and Na^+ concentration gradients are maintained across the IMM and therefore no evolutionary pressure may have existed for the development of cation selectivity (Bernardi, 1999). A similar function could be performed by the ryanodine receptor (RyR) isoform 1, which was found to localize to the IMM in excitable cells (Beutner et al., 2001).

Another important point is that Ca^{2+} efflux down its concentration gradient via a selective channel (like the MCU; this could occur if mitochondria depolarize) is opposed by the buildup of a Ca^{2+} diffusion potential (Åkerman, 1978). According to the Nernst equation, the magnitude of the Ca^{2+} diffusion potential is -30 mV per log of the Ca^{2+} concentration difference. The magnitude of the Ca^{2+} diffusion potential can be decreased (leading in turn to an increased rate of Ca^{2+} efflux) by charge-compensating currents, but given the very low IMM permeability to charged species the basal rate of Ca^{2+} efflux through the MCU would be extremely low. The PTP lack of selectivity and its large size would provide charge compensation within the channel itself, thus allowing maximal Ca^{2+} flux (*i.e.*, at zero potential). This would in turn allow fast Ca^{2+} release even for small $[Ca^{2+}]$ gradients; and regulation of the Ca^{2+} efflux rate could be achieved through modulation of the pore open time (Bernardi and Petronilli, 1996; Bernardi, 1999).

We would like to stress that transient PTP openings, which could correspond to lower conductance states of the pore (Ichas and Mazat, 1998), have been documented in isolated mitochondria (Hüser et al., 1998; Hüser and Blatter, 1999), at the single channel level as conductance "flickerings" (Kinnally et al., 1989; Petronilli et al., 1989;

Szabó and Zoratti, 1991) and in intact cells (Petronilli et al., 1999). Transient openings are not associated with irreversible mitochondrial alterations, and PTP flickering could be responsible for the spontaneous transient depolarizations ranging from <10 mV to >100 mV measured in individual mitochondria *in situ* (O'Reilly et al., 2003). This mode of PTP operation could also mediate the CsA-sensitive cristae remodeling that follows apoptotic stimulation and may precede overt swelling (Scorrano et al., 2002). In our view, a rigorous testing of the hypothesis that pore flickering takes part in regulation of mitochondrial Ca^{2+} release under physiological conditions awaits the identification of PTP blockers or of PTP inactivation by genetic means. Indeed, CsA does not block the PTP, but rather desensitizes it with an inhibitory effect that can be overcome by increasing the Ca^{2+} and Pi load (Bernardi et al., 1992).

6. PTP REGULATION BY Ca^{2+}

Several pore regulators may modify (directly or indirectly) the PTP sensitivity to Ca^{2+}. OMM VDAC is modulated by Ca^{2+}, and this could in turn affect the amount of Ca^{2+} delivered to the matrix via the MCU and RaM (Gincel et al., 2001) irrespective of whether VDAC takes directly part in PTP formation (Krauskopf et al., 2006). Cyclophilin D (CyP-D, the mitochondrial ligand for CsA) appears to be involved in the modulation of PTP affinity for Ca^{2+} since (i) Ca^{2+} displaces CsA from high-affinity binding sites in rat liver mitochondria (McGuinness et al., 1990); (ii) higher concentrations of CsA are required to inhibit spreading of the PTP to a population of mitochondria when the Ca^{2+} load is increased (Bernardi et al., 1992); and (iii) ablation of CyP-D doubles the threshold Ca^{2+} load required to open the PTP in the presence of Pi (Baines et al., 2005; Nakagawa et al., 2005; Basso et al., 2005; Schinzel et al., 2005). Finally, the PTP of adenosine nucleotide translocator (ANT)-null mitochondria requires threefold higher levels of Ca^{2+} for activation when compared to control mitochondria (Kokoszka et al., 2004).

Matrix Ca^{2+} acts as a permissive factor for PTP induction, apparently for all pore inducers. We have reported that phenylarsine oxide induces PTP opening in the absence of added Ca^{2+} (Lenartowicz et al., 1991; Bernardi, 1992), yet depletion of endogenous Ca^{2+} abolishes the inducing effects of phenylarsine oxide (P. Bernardi, unpublished observations). The PTP dependence on matrix Ca^{2+}represents somewhat of a paradox, however, because there is no obvious correlation between $[Ca^{2+}]_m$ and onset of the PT. Indeed, decreasing Pi from 5 mM to 2 mM increased the apparent threshold for PTP opening from 1.8 μM to 5.0 μM free $[Ca^{2+}]_m$ in rat brain mitrochondria (Chalmers and Nicholls, 2003). Based on these results, we suspect that the PTP Ca^{2+}-binding sites saturate at very low matrix free $[Ca^{2+}]$ and that it is unlikely that PTP opening may be caused by Ca^{2+} overload as such. We consider more likely that the uptake of substantial amounts of Ca^{2+} causes further modifications that independently sensitize the PTP to opening, like

the matching changes of matrix H^+ and Pi concentration (Bernardi, 1992; Chalmers and Nicholls, 2003), the generation of lipid mediators (Scorrano et al., 1999; Kristal and Brown, 1999; Garcia-Ruiz et al., 2002; Penzo et al., 2004; Gadd et al., 2006) and of reactive oxygen species (Kowaltowski et al., 1998; Colell et al., 2004), as well as additional factors whose effect may be mediated by (de)phosphorylation reactions (Juhaszova et al., 2004).

7. THE STATUS OF MATRIX Ca^{2+}

Already 40 years ago it was observed that mitochondria (particularly those isolated from muscle) display an enormous capacity to accumulate Ca^{2+} (up to 1 micromole x mg protein^{-1}, which corresponds to a total matrix concentration of about 1 M) (Rossi and Lehninger, 1964; Lehninger et al., 1967; Lehninger, 1974). This is made possible by the simultaneous accumulation of Pi, which precipitates with Ca^{2+} in the alkaline ambient of the matrix in the form of deposits in which, quite remarkably, calcium does not become crystalline but remains indefinitely amorphous thus facilitating its discharge (Carafoli et al., 2001; Carafoli, 2004). When $[Ca^{2+}]_c$ is maintained below a certain set point there is too little matrix Ca^{2+} accumulation to form the calcium-phospate complexes, and $[Ca^{2+}]_m$ varies with $[Ca^{2+}]_c$ allowing for the Ca^{2+}-dependent control of matrix metabolism. When $[Ca^{2+}]_c$ rises above the set-point, mitochondria accumulate Ca^{2+} and Pi, the calcium-phosphate complexes form in the matrix and $[Ca^{2+}]_m$ becomes invariant with matrix Ca^{2+} load (Nicholls and Chalmers, 2004). A major conundrum is that calcium-phosphate complexes are extremely difficult to redissolve *in vitro*, whereas the addition of a protonophore to Ca^{2+}-loaded mitochondria leads to an extremely rapid efflux of Ca^{2+} via the PTP (Igbavboa and Pfeiffer, 1988; Petronilli et al., 1993). Thus, the calcium–phosphate complex that forms within the matrix is rapidly dissociable. The structure of the matrix calcium–phosphate complex might be either hydroxyapatite [$Ca_5(PO4)_3OH$] or tricalcium phosphate [$Ca_3(PO4)_2$]. Probably matrix pH plays a key role in controlling the stability of these complexes. The Pi carrier transports H_2PO4^- in exchange for OH^-, which is thermodynamically equivalent to the electroneutral transport of H_3PO_4. Because dissociation of three protons is required to form PO_4^{3-}, at constant extramitochondrial Pi the concentration of this species is inversely proportional to the cube of the proton concentration in the matrix (Chalmers and Nicholls, 2003). The uniport activity increases as the 2.5 power of $[Ca^{2+}]_c$ (Zoccarato and Nicholls, 1982). Because the concentration of PO_4^{3-} increases with pH, an increase in matrix pH from 7 to 8 would increase the matrix PO_4^{3-} concentration by 1000-fold. In this model, protonophore addition leads to a drastic acidification of the matrix, increasing $[Ca^{2+}]_m$ 100-fold (Nicholls and Chalmers, 2004; Nicholls, 2005). Importantly, this mitochondrial Ca^{2+}buffer system only activates when $[Ca^{2+}]_c$ rises above 0.5 μM, whereas basal $[Ca^{2+}]_c$ is about 0.1 μM. This difference protects from an otherwise inexorable loading of the matrix with Ca^{2+}, which could rapidly lead to cell death.

8. THE PT AS AN EFFECTOR MECHANISM OF CELL DEATH

As previously mentioned, the mitochondrial PT can be defined as a sudden increase of IMM permeability to solutes with molecular masses up to 1500 Da, and is due to the opening of a voltage- and Ca^{2+}-dependent, cyclosporin A (CsA)-sensitive, high-conductance channel (Bernardi, 1999; Crompton, 1999; Bernardi et al., 2006). Following prolonged PTP opening, equilibration across the IMM of ions and of solutes with molecular masses below the pore size induces massive release of the Ca^{2+} stored in the matrix and extensive swelling of mitochondria, given the colloidosmotic pressure exerted by matrix proteins. As a consequence, the unrestricted cristae unfolding causes breaches in the OMM and release of intermembrane proteins including cyt *c* (Petronilli et al., 1994) and other proapoptotic factors like apoptosis inducing factor (Susin et al., 1996), Smac-Diablo (Du et al., 2000; Ekert et al., 2000), Omi/HtrA2 (Hegde et al., 2001) and endonuclease G (Li et al., 2001).

PTP opening has major consequences on energy conservation. The initial uncoupling would be followed by inhibition of respiration due to the release of pyridine nucleotides (Vinogradov et al., 1972; Di Lisa et al., 2001), and cessation of mitochondrial ATP synthesis would be matched by hydrolysis of glycolytic ATP by the F_OF_1 ATPase working "in reverse" because of the collapse of the proton gradient. The combination of the bioenergetic deficit, altered Ca^{2+} homeostasis and release of apoptogenic proteins easily explains why prolonged PTP opening has detrimental and potentially lethal consequences on cell viability (Bernardi et al., 2006).

For individual mitochondria the PT is an all-or-nothing phenomenon. *In situ*, however, a subpopulation of mitochondria may have a lower threshold for opening and therefore open the PTP first. In this scenario there may be enough release of cyt *c* and other proapoptotic factors from these permeabilized mitochondria to activate apoptosis; and enough ATP production by the subpopulation of mitochondria that has not undergone the PT to support the energy requirements of apoptosis signalling. At later stages, Ca^{2+} or other diffusible signals released by the permeabilized mitochondria might propagate a wave of PTP openings, eventually culminating in permeabilization of the whole mitochondrial population (Ichas et al., 1997; Pacher and Hajnoczky, 2001) and possibly to a switch of the mode of cell death to necrosis due to the concomitant worsening of ATP depletion (Ankarcrona et al., 1995).

9. ROLE OF BCL-2 FAMILY PROTEINS IN PTP REGULATION AND MITOCHONDRIAL Ca^{2+} LOADING

It is generally accepted that Bcl-2 family proteins localize to the OMM where they act as "gatekeepers" of the permeability to cyt *c*, with Bcl-2 inhibiting the formation of the permeation channels by Bax and/or Bak multimers (Antonsson et al., 2000; Scorrano and Korsmeyer, 2003). The anti-apoptotic effects of Bcl-2 (and Bcl-X_L) have been originally interpreted as the result of direct PTP modulation at the OMM. The first indication that the PT could be modulated by Bcl-2 family

proteins was obtained in 1996 by Kroemer and collaborators, who found that PTP opening by specific stimuli was inhibited by Bcl-2 with matching inhibition of the release of AIF (Susin et al., 1996). In addition, Bcl-2/X_L were proposed to interact with VDAC, within the framework of a model where the PTP forms at contact sites through the interaction of ANT with VDAC (Susin et al., 1996; Zamzami and Kroemer, 2001). Although genetic evidence questions the role of ANT (Susin et al., 1996) and VDAC1 (Krauskopf et al., 2006) in PTP formation, the idea that Bcl-2 directly modulates the pore has recently been reinforced by the finding that a set of chemically unrelated Bcl-2 ligands facilitates the PT in isolated mitochondria (Milanesi et al., 2006).

When evaluated in a cellular context, however, it is clear that Bcl-2 family members can also influence the PTP indirectly by modulating the mitochondrial Ca^{2+} load through an effect exerted on ER Ca^{2+} homeostasis. The ER is a multifunctional signaling organelle that controls a wide range of cellular processes and acts as the main cellular Ca^{2+} store. Ca^{2+} homeostasis in the ER is complex, and it is regulated by ryanodine and InsP3 receptors, SERCA pumps and luminal Ca^{2+}-binding proteins, including calreticulin, GRP78 and GRP94 (Berridge et al., 2003). Certain stimuli induce apoptosis by promoting matrix uploading following release of Ca^{2+} from ER stores. In this context interorganelle Ca^{2+} movements appear to be regulated by Bcl-2 family proteins, and the PTP could integrate different Ca^{2+} signals switching their output towards death or survival (Ferrari et al., 2002; Pinton et al., 2002; Scorrano et al., 2003; Scorrano and Korsmeyer, 2003; Demaurex and Distelhorst, 2003; Annis et al., 2004). Waves of mitochondrial depolarization and Ca^{2+} release might also propagate through the cell, resulting in cyt *c* release and apoptosis (Pacher and Hajnoczky, 2001). Travelling mitochondrial waves are prevented by anti-apoptotic Bcl-2 family members, perhaps by reducing the transfer of Ca^{2+} from the ER to mitochondria, and are likely to involve PT activation since they can be blocked by CsA (Pacher and Hajnoczky, 2001). Wave propagation is promoted by pro-apoptotic members of the Bcl-2 family, which sensitize the PT to Ca^{2+} and therefore promote mitochondrial Ca^{2+} release (Pacher and Hajnoczky, 2001; Csordas et al., 2002; Forte and Bernardi, 2005).

Indeed, Bcl-2 family members are localized both in mitochondria and in the ER, and contribute to the modulation of intracellular Ca^{2+} homeostasis with important implications for the death/survival equilibrium. Several reports indicate that Bcl-2 inhibits Ca^{2+} release from the ER and preserves ER luminal Ca^{2+}, whereas other studies suggest that Bcl-2 increases the leakage of Ca^{2+} across the ER membrane, thereby reducing luminal Ca^{2+}. Consistent with the first model, it was found that disruption of intracellular Ca^{2+} homeostasis by the SERCA inhibitor thapsigargin induces apoptosis, which is inhibited by Bcl-2 overexpression (Lam et al., 1994; Pan et al., 2000). Following the second model, Bcl-2 overexpression would decrease the ER [Ca^{2+}] pool (Foyouzi-Youssefi et al., 2000; Pinton et al., 2000; Vanden Abeele et al., 2002), thus protecting cells from apoptosis by limiting the amount of Ca^{2+} released into the cytoplasm and its subsequent uptake into the mitochondria

(Ferrari et al., 2002). Overexpression of Bcl-X_L has also been reported (i) to decrease apoptosis through decreased expression of IP3 receptors and the matching decrease of IP3-dependent Ca^{2+}signalling (Li et al., 2002), and (ii) to favor the opening of VDAC (Vander Heiden et al., 2001) which may in turn affect mitochondrial Ca^{2+} uptake (Gincel et al., 2001; Rapizzi et al., 2002). These conflicting findings may be due to the study of overexpressed rather than endogenous Bcl-2, which may in turn lead to substantial differences in its expression levels and subcellular localization (Distelhorst and Shore, 2004).

Whatever the model, there is general agreement that Bcl-2 family proteins are involved in ER-to-mitochondria communication to modulate both cell survival and death. The BH3-only proteins Bax, Bak, Bik and tBid can induce apoptosis from the ER (Germain et al., 2002; Mathai et al., 2002; Nutt et al., 2002a; Scorrano et al., 2003; Zong et al., 2003). Bax- or Bak-overexpression prompts an early increase in mitochondrial Ca^{2+}, whose inhibition abrogates Bax/Bak-induced apoptosis (Nutt et al., 2002a; Nutt et al., 2002b). The picture is made more complex by the observations that Bik integrates in the ER membrane, facing the cytosolic side, and stimulates ER Ca^{2+} depletion in a Bax-Bak dependent manner (Mathai et al., 2005; Walter and Hajnoczky, 2005); and that tBid also propagates the IP3-induced Ca^{2+}signal to mitochondria (Csordas et al., 2002).

In summary, the regulation of steady state ER [Ca^{2+}] by pro- and anti-apoptotic Bcl-2 family proteins is an important mechanism underlying the sensitivity of cells to apoptosis, possibly through the matching changes of mitochondrial [Ca^{2+}] that affect PTP opening. The resulting modulation of Ca^{2+} release from the ER and Ca^{2+} uptake into mitochondria in turn is pivotal in regulating the cell death/survival balance (Scorrano and Korsmeyer, 2003; Szabadkai and Rizzuto, 2004).

10. THE PTP IN PATHOLOGY

Mitochondria are involved in many human pathologies. These do not include only primary mitochondrial diseases (Di Mauro, 2004), but also conditions where mitochondria play a key role in disease onset, often through derangement of Ca^{2+} homeostasis and abnormal PT induction (Bernardi et al., 2006). PTP involvement in cell death was initially established in a series of seminal studies published in the early 1990s in hepatocytes subjected to oxidative stress (Broekemeier et al., 1992; Imberti et al., 1993), anoxia (Pastorino et al., 1993) or treatment with ATP (Zoeteweij et al., 1993), and in cardiomyocytes (Duchen et al., 1993) and isolated hearts (Griffiths and Halestrap, 1995) exposed to ischemia-reperfusion. The link with apoptosis was made in 1996, with the demonstration that the PT was involved in the release of an apoptogenic protease from the intermembrane space (Susin et al., 1996). After these pioneering papers, and largely based on the protective effects of CsA, PTP-dependent mitochondrial dysfunction and Ca^{2+} deregulation have been subsequently implied in so many cellular models of programmed and accidental cell death that we must refer the reader to recent reviews for a coverage of the original literature (Halestrap et al., 1998; Lemasters et al., 1998; Di Lisa

and Bernardi, 1998; Pfeiffer et al., 2001; Jordan et al., 2003; Weiss et al., 2003; Rama Rao et al., 2003; Tempestini et al., 2003; Kim et al., 2003b; Green and Kroemer, 2004; Kristal et al., 2004; Halestrap, 2004; Crompton, 2004; Di Lisa and Bernardi, 2005; Stavrovskaya and Kristal, 2005; Sullivan et al., 2005). The new frontier, however, is establishing the role of the PTP in models of disease *in vivo*, and to devise effective therapies based on its inhibition, issues that have seen a considerable progress over the last few years. We summarize below recent progress on the role of the PTP in diseases, and we refer the reader to a recent review for a more thorough coverage of this topic (Bernardi et al., 2006).

Myocardial ischemia-reperfusion. Paradoxically, in the heart mitochondrial function is not only essential for contraction but also for the sudden onset of cell death in post-ischemic reperfusion (Jennings and Ganote, 1976; Di Lisa et al., 1998; Halestrap et al., 1998). In this condition partial recovery of mitochondrial function may generate enough ATP for contraction but not for relaxation, resulting in hypercontracture and sarcolemmal rupture [4–6]. We assessed the role of PTP opening in heart ischemia-reperfusion injury through the redistribution of mitochondrial pyridine nucleotides, which do not readily permeate the inner membrane unless a PT occurs (Vinogradov et al., 1972). In isolated hearts subjected to ischemia-reperfusion pyridine nucleotides are released from the mitochondrial matrix into the intermembrane and the cytosolic spaces, where they become the substrate of a wide array of NAD^+ utilizing enzymes (Di Lisa et al., 2001). Formation of cyclic nucleotides from NAD^+ (such as cADP ribose) may profoundly affect cellular Ca^{2+} homeostasis and further increase the probability of PTP opening through the release of Ca^{2+} from the sarcoplasmic reticulum. Clear support for a role of the PTP in myocardial pathophysiology is provided by the reduced susceptibility to ischemic injury observed in mice lacking CyP-D (Baines et al., 2005; Nakagawa et al., 2005).

Liver diseases. The protective effects of CsA have been tested in several *in vivo* models of liver disease (Yoshiba et al., 1995; Wasaki et al., 1997; Pastorino et al., 1999; Kawakami et al., 2000) (Feldmann et al., 2000; Haouzi et al., 2002; Masubuchi et al., 2002; Crouser et al., 2002a; Crouser et al., 2002b; Hirakawa et al., 2003; Morin et al., 2004; Crouser et al., 2004; Soriano et al., 2004; Teckman et al., 2004; Masubuchi et al., 2005). Protection by CsA could be traced to PTP inhibition in a model of fulminant hepatitis caused by *Streptococcus* lipopolysaccharide plus D-Galactosamine, a treatment that sensitizes the liver to the proapoptotic effects of TNFα (Soriano et al., 2004). The PTP is also involved in a rodent model of hepatocarcinogenesis. Feeding rats with the arylamine 2-acetylaminofluorene (AAF) causes onset of liver tumors within 30–50 weeks (Neumann et al., 1994), which is preceded by a sequence of alterations that closely resembles the clinical course of chronic hepatitis gradually evolving into cyrrhosis (Neumann et al., 1994). Very early into AAF feeding an adaptative mitochondrial response takes place with upregulation of mitochondrial Bcl-2 and desensitization of the PTP and of the liver mitochondrial apoptotic pathway to TNFα *in vivo*. This adaptive response is a tumor-promoting event that may contribute to the selection of resistant hepatocytes in the population of chemically-transformed cells (Klöhn et al., 2003). Also other

conditions that predispose to liver cancer (alcoholic liver disease, chronic hepatitis C and cholestasis) may involve a mitochondrial response with involvement of the PTP. The hepatitis C virus core protein localizes to mitochondria, where it inhibits electron flow at Complex I causing increased ROS production and possibly increased PTP opening (Korenaga et al., 2005); and a similar sequence of events may take place in liver chronic alcohol exposure (Hoek et al., 2002) and after bile duct ligation (Lieser et al., 1998).

Neurological diseases. Many neurological disorders are caused or worsened by mitochondrial dysfunction. Based on the protective effects of CsA, the PTP has been implied in brain damage due to both hyperglycemia (Li et al., 1997; Folbergrova et al., 1997) and hypoglycemia (Friberg et al., 1998; Ferrand-Drake et al., 1999), to ischemia (Uchino et al., 1998; Yoshimoto and Siesjo, 1999; Matsumoto et al., 1999; Nakai et al., 2004), trauma (Okonkwo et al., 1999; Okonkwo and Povlishock, 1999; Scheff and Sullivan, 1999; Sullivan et al., 2000; Alessandri et al., 2002) and injection of 3-nitropropionic acid (Leventhal et al., 2000); in cell death following facial motoneuron axotomy in neonatal rodents (Vanderluit et al., 2003) and in photoreceptor apoptosis (Fox et al., 2003). CsA prolongs the survival of mouse models of amyotrophic lateral sclerosis (Keep et al., 2001; Karlsson et al., 2004), an effect that is also exerted by minocycline through inhibition of the PTP (Zhu et al., 2002). Protective effects that could be traced to inhibition of the PTP have been reported for promethazine in a mouse model of stroke (Stavrovskaya et al., 2004), and for topiramate in pilocarpine-induced epilepsy (Kudin et al., 2004). Independent evidence that the PTP may be involved in brain ischemic damage is the striking decrease of emispheric damage after middle cerebral artery occlusion in mice with genetic inactivation of the gene encoding for CyP-D (Schinzel et al., 2005). PTP-dependent mitochondrial defects appear to be involved in the pathogenesis of the Huntington's disease (HD) (Panov et al., 2002; Panov et al., 2005). Mutant huntingtin, containing an expanded polyglutamine tract, reduced mitochondrial membrane potential in normal mitochondria, thus impairing the capacity of mitochondria to take up Ca^{2+}. This finding partially reproduced the defects seen in HD patients and in HD mutant transgenic mice, whose mitochondria are much more prone to depolarize than control mitochondria (Piccioni et al., 2002).

Muscle diseases. The hypothesis that Ca^{2+}-dependent mitochondrial dysfunction could play a role in muscular dystrophies has been put forward 30 years ago (Wrogemann and Pena, 1976). A recent development has been the demonstration that the PT plays a key role in the pathogenesis of muscular dystrophy due to collagen VI deficiency (Irwin et al., 2003). Inherited mutations of collagen VI genes cause two muscle diseases in humans, Bethlem myopathy (Jöbsis et al., 1996) and Ullrich congenital muscular dystrophy (Camacho Vanegas et al., 2001). Collagen VI-deficient ($Col6a1^{-/-}$) mice display a muscle phenotype resembling Bethlem myopathy, with loss of strength associated with ultrastructural alterations of sarcoplasmic reticulum and mitochondria, and spontaneous apoptosis (Bonaldo et al., 1998). These defects are due to inappropriate PTP opening, and could be normalized by treatment with CsA, which rescued the muscle ultrastructural defects

and dramatically decreased the number of apoptotic nuclei *in vivo* in the first successful pharmacological treatment of an animal model of a genetic muscle disease (Bonaldo et al., 1998). We have found that the same defect is present in myoblast cultures from patients with Ullrich congenital muscular dystrophy (Angelin et al., 2007). This observation suggests that mitochondrial dysfunction triggered by PTP opening could be a component of the human disease as well, and opens new perspectives for treatment of the human collagen VI diseases.

11. PATHOLOGICAL CHANGES OF $[Ca^{2+}]_m$ UNRELATED TO THE PTP

The mitochondrion's high capacity to store and release Ca^{2+} is likely to play a critical role in a variety of disease processes also in a manner not directly linked to induction of PTP openings. Activity of the Na^+-dependent Ca^{2+} efflux pathway was shown to be increased, leading to lower than normal levels of $[Ca^{2+}]_m$, in mitochondrial complex I deficiency (Visch et al., 2004) and hereditary cardiomyopathy (Kuo et al., 2002). Mitochondrial Ca^{2+} response to agonists is drastically reduced in models of mitochondrial encephalomyopathy with ragged-red fibers (MERRF), causing a smaller increase in ATP levels upon agonist stimulation which could be restored by applying an inhibitor of the mitochondrial Ca^{2+} efflux pathways (Brini et al., 1999). The role of $[Ca^{2+}]_m$ in modulating ATP levels is fundamental in the insulin-secreting pancreatic β-cells (Rutter and Rizzuto, 2000), since ATP levels control the gating of ATP-sensitive K^+ channels and thus the initiation of the secretion process. A reduction in mitochondrial Ca^{2+} accumulation prompts a diminished insulin secretion (Maechler et al., 1998). Intriguingly, diabetes mellitus is very common in patients affected by mitochondrial encephalomyopathies, and a maternally inherited form of diabetes is associated to mtDNA mutations (Ballinger et al., 1992).

Depending on the ATP levels, excitotoxicity can induce necrosis or apoptosis in neuronal cells in response to overexposure to excitatory amino acids such as glutamate (Ankarcrona et al., 1995). In particular, prolonged activation of the N-methyl-D-aspartate (NMDA) receptor leads to $[Ca^{2+}]_c$ overload and cell death, which have been ascribed to $[Ca^{2+}]_m$ overload (Duchen, 2000). Moreover, during the progression to excitotoxic cell death, a late secondary increase in $[Ca^{2+}]_c$ occurs, termed "delayed Ca^{2+} deregulation" (DCD). Although the mechanisms underlying DCD are still under debate, it is agreed that the speed of DCD is strongly dependent on the magnitude of $[Ca^{2+}]_m$ accumulation (Brookes et al., 2004). It should be mentioned that earlier work suggested that the PTP was involved also in excitotoxicity (Schinder et al., 1996).

12. SUMMARY AND CONCLUSIONS

Mitochondria play a key role in cellular Ca^{2+} homeostasis, and Ca^{2+} plays a key role in regulation of mitochondrial metabolism, allowing a perfect match between varying metabolic demand and mitochondrial performance. This essential

integration is achieved through an extraordinarily complex network that involves Ca^{2+} fluxes across the mitochondrial membranes. This arrangement exposes the cell to severe risks, however. Primary derangements of cytosolic Ca^{2+} regulation may cause secondary mitochondrial dysfunction, with worsening of Ca^{2+} deregulation; and primary mitochondrial dysfunction will inevitably cause cellular Ca^{2+} deregulation, in a vicious circle that may precipitate cell death through the PTP. Understanding the mechanistic basis for the switch of mitochondrial Ca^{2+} homeostasis from physiology to pathology is a challenge that holds great promise for disease treatment.

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CHAPTER 19

ROLE OF CALCIUM IN THE PATHOGENESIS OF ALZHEIMER'S DISEASE AND TRANSGENIC MODELS

K.N. GREEN, I.F. SMITH AND F.M. LAFERLA*

Department of Neurobiology and Behavior, University of California, Irvine CA 92697-4545

Abstract: Alzheimer's disease (AD) is a progressive neurodegenerative disorder of the elderly that is characterized by memory loss. Neuropathologically, the AD brain is marked by an increased Aβ burden, hyperphosphorylated tau aggregates, synaptic loss, and inflammatory responses. Disturbances in calcium homeostasis are also one of the earliest molecular changes that occur in AD patients, alongside alterations in calcium-dependent enzymes in the post-mortem brain. The sum of these studies suggests that calcium dyshomeostasis is an integral part of the pathology, either influencing Aβ production, mediating its effects or both. Increasing evidence from *in vitro* studies demonstrates that the Aβ peptide could modulate a number of ion channels increasing calcium influx, including voltage-gated calcium and potassium channels, the NMDA receptor, the nicotinic receptor, as well as forming its own calcium-conducting pores. *In vivo* evidence has shown that Aβ impairs both LTP and cognition, whereas all of these ion channels cluster at the synapse and underlie synaptic transmission and hence cognition. Here we consider the evidence that Aβ causes cognitive deficits through altering calcium homeostasis at the synapse, thus impairing synaptic transmission and LTP. Furthermore, this disruption appears to occur without overt or extensive neuronal loss, as it is observed in transgenic mouse models of AD, but may contribute to the synaptic loss, which is an early event that correlates best with cognitive decline

Keywords: Alzheimer disease, calcium, long term potentiation, cognition, transgenic, amyloid beta

1. INTRODUCTION

Alzheimer's disease (AD) is neuropathologically characterized by a buildup of extracellular amyloid-β peptide (Aβ) plaques and of intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau, in addition to marked

*Frank M. LaFerla, Department of Neurobiology and Behavior, University of California, Irvine, 1109 Gillespie Neuroscience Bldg., Irvine, CA 92697-4545, E-mail: laferla@uci.edu

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neuronal and synaptic loss and other changes such as brain inflammation. Accompanying these neuropathological lesions are progressive memory loss and reduced cognitive function which the "amyloid cascade hypothesis" states occurs as a consequence of the progressive buildup of Aβ and the subsequent downstream changes such as tangle accumulation, eventually leading to neuronal toxicity. In support of this hypothesis, humans with familial mutations in proteins and enzymes involved in Aβ metabolism, such as the amyloid precursor protein (APP) and presenilin which releases Aβ via intramembraneous proteolysis, robustly develop all hallmarks of AD with nearly complete penetrance. Further support comes from transgenic mouse models of AD – animals that overexpress APP develop extensive plaque pathology, and show cognitive decline (Holcomb et al., 1998) illustrating that Aβ production is sufficient to cause memory impairment. Other key features of human AD are also mimicked in these Aβ overexpressing mouse models, such as inflammatory responses and oxidative stress (Benzing et al., 1999; Lim et al., 2001). This shows that overproduction of Aβ is enough to mimic many facets of the human disorder, placing the hydrophobic peptide firmly at the root of the disease.

Abundant production of the Aβ peptide alone in mice is not sufficient to fully mimic all aspects of AD, most notably tau pathology and neuronal loss which is likely due to differences between mice and humans and the duration of the disease in each species. Recently, we derived the 3xTg-AD mouse model that overexpresses mutant tau (P301L) as well as mutant APP (SWE) and presenilin 1 (PS1M146V) to better mimic the key neuropathological features of AD (Oddo et al., 2003a; Oddo et al., 2003b). The 3xTg-AD mice develop tau pathology, which first manifests with the accumulation of tau in the neuronal somatodendritic compartment, followed by its hyperphosphorlyation as a function of age, eventually leading to the formation of Gallyas-positive NFTs. Importantly, consistent with the predictions of the Aβ cascade hypothesis, removal of the Aβ pathology leads to clearance of the tau aggregates as well (Oddo et al., 2004), suggesting that pathological tau accumulates as a consequence of Aβ. Despite robust formation of both Aβ and tau lesions neuronal loss is not apparent, certainly not to the striking levels observed in the late stage human AD brain. Although this may be due to the longevity of the human disorder, which may last 20 years, compared to the 2 years that a mouse may live, it does present some interesting observations about the role that Aβ may play in the disease. Firstly, and most importantly, Aβ overproduction is sufficient to cause memory impairments and cognitive dysfunction in the *absence* of neuronal loss (Billings et al., 2005). Secondly, despite the lack of neuronal loss, Aβ causes many facets of the human disease. And thirdly, due to the longevity of AD, in which neuronal loss occurs, cell death is a slow gradual process which may or may not be a consequence of Aβ peptide.

In this review, we consider the evidence that disturbances in calcium triggered by Aβ may contribute to the synaptic loss in human AD. Furthermore, we discuss the likelihood that calcium dyshomeostasis in AD, and AD mouse models, may contribute to memory impairments. We have not discussed the role of the presenilins in calcium homeostasis as they have recently been reviewed (LaFerla, 2002; Smith

et al., 2005), and mounting evidence supports presenilins as physiological global calcium regulators which explains why various mutations lead to disrupted calcium signaling (Tu et al., 2006), rather than presenilin mediated calcium dysregulation being a pathological event.

Calcium is a vital cellular signaling ion whose concentration and localization are tightly regulated by a variety of membrane voltage-gated and ligand-operated channels, pumps and sequestering proteins (Toescu et al., 2004). Cytoplasmic concentrations are kept low by actively pumping cytosolic calcium into the endoplasmic reticulum, mitochondria or outside of the cell. In addition cytosolic proteins bind calcium, sequestering it and effectively crippling its transduction. Localized increases in cytosolic calcium can occur through ion channel entry across the plasma membrane, or from release from ER calcium stores within the cells. These controlled increases can activate a variety of enzymes, proteins and signaling transduction pathways as well as alter membrane potential and the properties of other ion channels upon which neural action potentials depend. It is therefore easy to see how a disruption in normal neuronal calcium homeostasis could lead to alterations in memory and cognition, and/or cell death.

2. APP

Many studies have focused on the effects of the various APP fragments on cellular calcium homeostasis. Of the several proteolytic byproducts, Aβ is by far the most widely characterized. Surprisingly, relatively little has been shown on how alterations in calcium could affect APP processing; AD patients have altered cellular calcium levels and responses early on in the disease (Gibson et al., 1987; Peterson et al., 1985; Peterson and Goldman, 1986; Peterson et al., 1986) and it would be useful to understand if these changes could underlie Aβ pathology, or if they are a consequence of it. Of note, increased cellular calcium levels could lead to an increase in either α- or β-cleavage of APP (Sinha and Lieberburg, 1999). Elevated calcium levels triggered by the ionophore ionomycin increased processing of APP by the non-amyloidogenic pathway, resulting in higher sAPPα levels, an effect which could be blocked by tyrosine kinase inhibitors (Petryniak et al., 1996). Furthermore, stimulation of α-secretase cleavage with phorbol esters, which activate PKC, have been shown to be dependent on both calcium and calpain activation (Chen and Fernandez, 2004), a calcium-dependent protease, whereas phorbol esters have been shown to elevate intracellular calcium levels and activate calpain (Kishimoto et al., 1989; Pontremoli et al., 1987). This therefore links elevated calcium levels with an increase in α-secretase processing of APP. It has been shown that an increase in α-secretase cleavage is usually accompanied by a concomitant decrease in Aβ production which is why activation of this pathway is a potential therapeutic for AD and is currently being targeted through muscarinic receptor agonists (Caccamo et al., 2006; Fahrenholz and Postina, 2006).

BACE1 is the sole identified β-secretase and is an aspartyl protease produced with a 46 amino acid pro-domain. This pro-domain must be removed for efficient

APP cleavage to occur (Shi et al., 2001), and removal is carried out by furin, a member of the pro-protein convertase enzymes (Bennett et al., 2000). Furin is a calcium dependent protease and so increases in calcium lead to an increase in activity. Treatment of cells with a calcium ionophore (A23187), which depleted intracellular stores of calcium, leads to a reduction in both furin and BACE activity (Bennett et al., 2000) and thus demonstrates one pathway through how intracellular calcium can regulate Aβ production through BACE.

Using the same calcium ionophore, but this time with calcium in the external media to increase cytosolic calcium levels Querfurth and Selkoe (Querfurth and Selkoe, 1994) showed that Aβ levels were increased 3-fold in HEK293 cells overexpressing APP. As caffeine, which causes calcium release from ER stores via the ryanodine receptor, also increased Aβ production they concluded that either calcium influx or release from stores into the cytosol could increase Aβ (Querfurth et al., 1997). Therefore, it seems that despite studies showing that elevated cytosolic calcium levels could increase α-secretase processing of APP, the effects on the β-secretase pathway are dominant, resulting in a net increase in Aβ. Conversely, we have found that SERCA (Sarco Endoplasmic Reticulum ATPase) pump activity, an intergral ER calcium pump which actively keeps cytosolic calcium low and ER calcium high by pumping calcium into the ER, is linked to Aβ production. Thapsigargin, an irreversible inhibitor of SERCA reduces Aβ production while overexpression of SERCA increases it (Akbari et al; manuscript in prep).

Capacitative calcium entry (CCE), is a way of replenishing intracellular calcium stores through tightly controlled plasma membrane channels. Our group was the first to demonstrate that mutations in presenilin attenuate CCE (Leissring et al., 2000). A potential linkage of CCE as a modulator of the length of Aβ was first suggested by Yoo et al. (Yoo et al., 2000), who showed that by using CCE inhibitors, they could increase the Aβ42:40 ratio without affecting total levels, in both HEK293 and CHO cells overexpressing APP, whereas blockers of either L- or N-type voltage gated calcium channels (VGCCs) had no effect. They also showed that although CCE affects APP processing, most likely by affecting the γ-secretase by shifting the cleavage site, elevated APP or Aβ42 did not affect CCE. As Aβ42 is more hydrophobic than its shorter cousin Aβ40, it aggregates more readily and is thought to be the more pathogenic species. Therefore, shifting the ratio of Aβ42:40 may be beneficial to AD patients and these results suggested that modulators of CCE could be therapeutic. Pierrot et al. (Pierrot et al., 2004) further showed that increasing intracellular calcium in rat cortical neurons through depolarization selectively increased Aβ42, through a CCE-dependent mechanism, which could be blocked by nimodipine – a selective L-type VGCC blocker. They reported that the higher calcium levels inhibited the α-secretase processing of APP, leading to enhanced Aβ production.

Figure 1 illustrates how modulators of cellular calcium may affect Aβ production in N2A cells and suggests that it is calcium entry through VGCCs which plays a key role in Aβ production, as blockers of VGCCs reduced production and activators

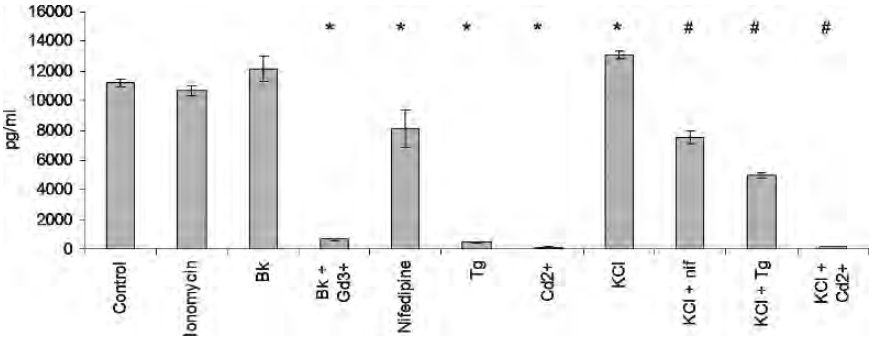

Figure 1. Aβ40 levels after 8 hours treatment with various calcium modulators in N2A cells stably overexpressing APP_{SWE} and PS1. We incubated N2A cells, a mouse neuronal cell line stably overexpressing APPSWE and PS1, with various calcium modulators for 8 hours and then the media collected and Aβ40 levels measured by sandwich ELISA. Ionomycin (10 μM), an ionophore had no effect on levels, while bradykinin (Bk; 100 nM) elevated levels but did not reach significance. The combination of bradykinin and 200 μM Gd^{3+}, a blocker of CCE and VGCCs, dramatically reduced Aβ levels. Likewise nifedipine (nif; 5 μM), reduced Aβ levels by about 30% indicating that calcium entry through the L-type VGCC contributes to Aβ production, while Cd^{2+} (200 μM), a non selective blocker of all VGCCs reduced Aβ levels to a similar level as Bk + Gd^{3+}, indicating that VGCC calcium entry is a key regulator of Aβ production. Thapsigargin (Tg; 1 μM) a potent inhibitor of SERCA effectively blocked all production of Aβ. Stimulation of calcium entry through VGCCs with KCl (50 mM), significantly increased Aβ production which was again reduced with either nifedipine, thapsigargin or Cd^{2+}. These results indicate that calcium influx into the cytosol through VGCCs can regulate Aβ production. ∗ indicates significance vs. controls ($p < 0.05$) and # indicates significance vs. KCl ($p < 0.05$)

enhance production. All of these studies suggest that elevations in calcium entry into cells, or increased release from intracellular stores can shift APP processing to the amyloidogenic pathway resulting in more Aβ production.

3. CALCIUM AND AD

Evidence for a disruption of calcium homeostasis in AD has been shown indirectly, through activation of calcium-dependent proteins obtained from post mortem brains and from studies on peripheral tissues from AD patients. Direct evidence of calcium disruption has not been shown due to the impossibility of using fresh human tissue for determining neuronal calcium dynamics, and problems associated with working with post-mortem tissue. However, using tissues from the periphery of AD patients, one of the first observations made was that calcium levels were altered compared to age-matched controls. As far back as 1986, Peterson et al. (Peterson et al., 1986) measured the free calcium concentration in fibroblasts using calcium-binding fluorescent dyes and found that AD patients had decreased levels compared to age-matched controls (70%), and even further decreased compared to young adults (81%). This study was followed up in 1988 by stimulating calcium release from stores, using bradykinin which stimulates release via the IP_3 receptor, in fibroblasts and recording the transients (Peterson et al., 1988). The rise and

amplitude of calcium release was slower and decreased in aged and AD patients compared to young adults, showing that calcium homeostasis was altered as a function of aging, but that it was the basal calcium levels in AD fibroblasts which were altered compared to controls. Huang et al (Huang et al., 1991) repeated these studies and found that bradykinin-induced IP_3 calcium transients were decreased with age but again found no difference between AD patients and age-matched controls. Despite these data, IP_3 receptor binding assays showed a decrease in AD brain tissue compared to age-matched controls, suggesting that there was decreased IP_3-mediated calcium release in the AD brain, which was not represented in the periphery (Young et al., 1988). In agreement, IP3 receptor binding assays of platelets from AD patients and controls showed no difference (Garlind et al., 1995). More recently, though, it has been shown that low doses of bradykinin almost exclusively elicit calcium transients in fibroblasts from AD patients while producing no response in age-matched controls (Hirashima et al., 1996). Discrepancies in these studies may have been due to different stages of the disease as well as not distinguishing between sporadic and familial AD, in which presenilin mutations are known to have diverse effects on calcium homeostasis in their own right. Addressing this, a study involving only sporadic AD patients and age-matched controls found that fibroblasts from sporadic AD patients had lower basal calcium levels than the controls and, interestingly, only fibroblasts from the controls elicited responses to Aβ (Palotas et al., 2001).

Platelets from mild AD patients display decreased basal calcium levels, in agreement with fibroblasts, compared to age matched controls in the absence of extracellular calcium (Ripova et al., 2004; Ripovi et al., 2000). Upon addition of 1 mM calcium into the extracellular media, intracellular calcium levels markedly increased in AD patients compared to a small increase in the controls. These results suggested that platelets from AD patients had compromised buffering capabilities that allowed intracellular calcium to rise to dangerous levels, but that this was an early event in the disease progression. Altered calcium homeostasis has also been reported in lymphocytes. Lymphocytes from sporadic AD patients exhibit elevated basal intracellular calcium levels versus age-matched controls and calcium transients induced by Aβ fragments were higher in the AD lymphocytes than controls (Ibarreta et al., 1997). Again, the authors concluded that the calcium buffering capacity of the lymphocytes from AD patients was impaired. In contrast, no difference in calcium response was seen in platelets from AD patients compared to age-matched controls when stimulated with Aβ fragments (Hedin et al., 2001). In conclusion, altered calcium homeostasis has been observed by many groups in peripheral tissues from AD patients compared to age-matched controls and appears to be an early event in the disease, even prompting some to suggest using altered calcium responses in platelets as a diagnosis tool for AD (Ripova et al., 2004).

Discrepancies in results between studies could be due to the disease stage at which the measurements were established as well any other underlying conditions or medications being taken by the participants in the studies, which is a common problem when utilizing human tissue. Unfortunately, no study has been published

on calcium dynamics in peripheral tissues of AD transgenic mouse models, in the absence of global presenilin mutations, which would help to determine if changes in peripheral tissue calcium homeostasis preceded Aβ pathology in humans or if increased Aβ leads to altered calcium homeostasis.

Data have also been published providing evidence for calcium dyshomeostasis in AD brain tissue, which offers invaluable insights since direct studies on human AD brain tissue calcium dynamics are impossible. These studies either reported altered activity or levels of proteins involved in calcium homeostasis, or activation of calcium-dependent proteases and other enzymes not found in control brains. For example, Colvin et al. (Colvin et al., 1991) demonstrated that there was increased Na^+/Ca^{2+} exchanger activity in AD patient brain tissue compared to age-matched controls, providing direct evidence of a facet of calcium homeostasis altered in AD. As already mention IP_3 receptor binding assays showed decreased receptor numbers in the AD brain (Young et al., 1988). L-type VGCCs were found to be increased in both the dentate gyrus and CA1 region of the hippocampus in AD brains compared to age-matched controls utilizing binding assays (Coon et al., 1999), which is consistent with *in vitro* data showing Aβ increases L-type VGCCs (Green and Peers, 2001; Ueda et al., 1997), whereas NMDA receptor binding and mRNA is decreased (Greenamyre et al., 1987; Ulas and Cotman, 1997). What these alterations in proteins involved in calcium homeostasis mean in the AD brain is unknown but suggests that calcium homeostasis is changed by the pathology. In support of calcium disturbances underlying neuronal death in AD, calcium-dependent calpain 1 is activated in the AD brain (Grynspan et al., 1997; Saito et al., 1993; Veeranna et al., 2004), as well as one of its substrates, calcineurin, being truncated (Liu et al., 2005). Calpain and calcineurin are amongst the most important calcium-dependent enzymes in the central nervous system. Calpain is a family of cysteine proteases which are activated by increased intracellular calcium concentration and have been implicated in the activation of cyclin-dependent protein kinase 5 (cdk5; (Lee and Tsai, 2001)). Cdk5 itself has been shown to cause hyperphosphorylation of tau leading to NFTs and neuronal death (Cruz et al., 2003), both of which are implicated in AD.

It seems that altered intracellular calcium homeostasis may be a crucial event in the initiation and progression of AD however the therapeutic value has thus far been mixed. For example, the use of calcium channel blockers, including dihydropyridines and non-dihydropyridines, has no effect on the risk of AD (Yasar et al., 2005), whereas the NMDA-antagonist memantine improves cognition in mild to moderate AD (Peskind et al., 2006).

4. Aβ, ION CHANNELS AND SYNAPTIC PLASTICITY

The *in vitro* effects of exogenous Aβ on a variety of ion channels have been widely documented and reviewed, including VGCCs (Price et al., 1998), in particular the L-type channel (Brown et al., 2005; Ekinci et al., 1999; Green and Peers, 2001; Green and Peers, 2002; Ueda et al., 1997), potassium channels

(Ramsden et al., 2001), NMDA receptors (Goto et al., 2006; Nomura et al., 2005; Snyder et al., 2005; Ye et al., 2004) and nicotinic receptors (Liu et al., 2001; Pettit et al., 2001; Takenouchi and Munekata, 1994; Wu et al., 2004), as well as forming its own novel calcium conducting ion channels (Arispe et al., 1993; Kawahara and Kuroda, 1997). Although direct evidence for the involvement of any of these channels is lacking in AD patients, evidence exists from transgenic mouse models as well as extensive *in vitro* studies from which we can postulate the underlying disturbances in the AD brain. Transgenic mouse models of AD, through overexpression of APP or Aβ, display cognitive impairments (Holcomb et al., 1998) in the absence of extensive neuronal loss and it is easy to see how disturbances in any of these ion channel activities could result in perturbed memory formation or consolidation. Of note, all of the above ion channels are involved in calcium regulation (potassium channels through determining the membrane potential upon which VGCC's depend) and are highly concentrated in both pre- and post-synaptic terminals, whereas synaptic loss in the AD brain correlates best with cognitive deficits (DeKosky et al., 1996; Terry et al., 1991) and is an early event. As synaptic activity underlies cognition and memory, it remains a highly relevant target for Aβ mediated disturbances in calcium to cause the clinical cognitive deficits seen in both AD and mouse models. For example APP overexpressing mice provide evidence that Aβ pathology can impair synaptic functioning as long term potentiation (LTP), a form of synaptic plasticity thought to underlie learning and memory (Bliss and Collingridge, 1993). Hippocampal synaptic transmission is impaired in mice containing Aβ plaques compared to age matched controls (Chapman et al., 1999). Utilizing the 3xTg-AD mouse model, our group demonstrated the same impaired hippocampal LTP at 6 months of age, compared to controls (Oddo et al., 2003b), whereas no differences were seen at 1 month of age. At 6 months of age, the 3xTg-AD have no Aβ plaque pathology but accumulate intraneuronal Aβ within the hippocampus (Billings et al., 2005). Moreover, they also display deficits in learning and memory at this age (Billings et al., 2005), specifically impaired retention of memories, rather than problems learning. The deficits in memory could be reversed by removing the intraneuronal Aβ via immunotherapy, demonstrating that intraneuronal accumulation of Aβ causes cognitive decline (Billings et al., 2005). Supporting these data in which non-plaque Aβ causes LTP and retention deficits, other groups have shown that direct injections of Aβ into the hippocampus impairs memory (Nabeshima and Nitta, 1994), whereas others have shown that exogenous application of Aβ to hippocampal slices impairs LTP (Chen et al., 2000). These observations have been taken further by showing that inhibition of LTP depends upon the aggregation state of Aβ and that it is the oligomeric form that inhibits, not the monomeric or fibrillar species (Walsh et al., 2005). Notably, we recently reported that the 3xTg-AD mice at 6 months of age, when appreciable memory and LTP deficits occur, coincides with increased Aβ oligomer levels (Oddo et al., 2006) providing strong *in vivo* evidence for naturally produced oligomers disrupting both LTP and cognition. As LTP is strongly dependent upon NMDA receptors (Morris et al., 1986), which conduct calcium into the post-synaptic terminal, as well as

neurotransmitter release, such as glutamate, which is dependent upon pre-synaptic calcium entry, Aβ oligomers disrupting any pre- or post-synaptic ion channel could impair LTP. Two recent articles have highlighted a role for Aβ in regulation of two crucial ion channels involved in LTP – the L-type VGCC and the NMDA receptor. One of the earliest documented effects of Aβ on any ion channel was that it could enhance calcium current through the L-type VGCC (Ueda et al., 1997), which was found to involve production of reactive oxygen species (Green and Peers, 2002), a feature central to AD pathology, but the significance or consequences of this were not reported. Scragg and colleagues (Scragg et al., 2005) took these observations further and found that endogenous produced, or exogenously applied, Aβ acted post translationally to promote channel insertion into the plasma membrane, through increased channel trafficking or insertion/retention into the membrane, resulting in an increase in channel activity. Although this observation alone, suggesting that Aβ has a physiological role in L-type VGCC regulation, which could go wrong in AD leading to deregulated calcium entry in the synapse inhibiting LTP and memory, is astounding, a further study has found that Aβ also regulates trafficking of the NMDA receptor (Snyder et al., 2005), another integral component of LTP and memory. Here the authors found that naturally secreted Aβ promoted endocytosis of NMDA receptors in cortical neurons, whereas mouse models of AD with increased Aβ production displayed reduced quantities of cell surface NMDA receptors. Interestingly endocytosis was found to be dependent upon another calcium conduction ion channel with which Aβ has been reported to interact - the α7 nicotinic receptor (Takenouchi and Munekata, 1994; Liu et al., 2001; Pettit et al., 2001). Although the effects of Aβ binding to the NMDA receptor remain controversial, it was found that application of Aβ led to the activation of PP2B which is a calcium sensitive enzyme known to regulate NMDA receptor transmission (Shi et al., 2000). In conclusion, Aβ may have a role in the regulation of a number of ion channels involved in synaptic transmission, which would explain why cognitive decline is seen in AD where Aβ levels are increased, and mouse models of the disease correlating with reduced LTP, highlighting the synapse as the major site of calcium dysregulation in the disease. This may also help to explain why synapse loss is an early event in AD and correlates best with cognitive decline, whereas plaque load does not (Braak and Braak, 1997).

In addition to Aβ altering the activity of a number of ion channels involved in synaptic transmission, it has also been shown to increase exocytosis of neurotransmitter containing vesicles, an event largely dependent upon calcium entry. Aβ was first shown to enhance exocytosis of MTT formazan (Liu and Schubert, 1997) by altering intracellular vesicle trafficking (Liu et al., 1998). Following this, it was shown that endogenously produced or exogenously added Aβ could markedly increase secretion of catcholamine-containing vesicles from PC12 cells (Taylor et al., 1999), an effect that was dependent upon calcium entry. However, this calcium entry was found to be very small – less than 1 pA/pF but coupled to a doubling in secretory vesicles (Green and Peers, 2001), suggesting that this calcium entry was located in very close proximity to the vesicles themselves, and

the docking machinery. Furthermore, it appeared that the calcium was entering the cell through a novel route not attributed to any VGCC, and was postulated to be through the novel Aβ channel as it could be blocked by Congo red, which disrupts Aβ aggregation, or antibodies against Aβ (Taylor et al., 1999). This demonstrated that Aβ could endogenously form channels which, upon membrane depolarization, would conduct tiny amounts of calcium into the cell but result in enormous secretion of neurotransmitter containing vesicles. Although Aβ increasing neurotransmitter secretion has not yet been reported in animal models of AD, it has been shown that incubating nerve terminals in physiological concentrations of Aβ42 increased both glutamate and noradrenaline exocytosis from rat cortical neurons (Bobich et al., 2004), of which oligomers probably mediated the effect. Of note, Demuro and colleagues (Demuro et al., 2005) have recently shown that application of Aβ oligomers rapidly increases intracellular calcium levels via entry across the plasma membrane not attributable to any VGCC. Supporting a role for Aβ in secretion of neurotransmitter containing vesicles has come from a recent *in vivo* study by Cirrito and colleagues (Cirrito et al., 2005). Using *in vivo* microdialysis alongside field potential recordings, the authors demonstrated that Aβ was secreted into the interstitial fluid dynamically with synaptic activity. Secretion of Aβ was dependent upon exocytosis suggesting that Aβ is present, or secreted, alongside neurotransmitter containing vesicles and due to the close proximity that Aβ must have with the vesicles and their docking machinery could support a role for modulating secretion as shown *in vitro*. Disruption of neurotransmitter release from the pre-synaptic membrane by Aβ is another way in which both memory and LTP could be impaired in AD and mouse models, alongside changes in intracellular calcium levels caused by Aβ deregulation of ion channels either pre- or post-synaptically.

5. CONCLUSIONS

Calcium may play a vital role in any number of aspects of AD – from modulating APP processing towards the amyloidogenic processing pathway, to underlying memory, and neuronal loss. As calcium dysregulation seems to be an early event in the disease, as shown from peripheral cells from AD patients, it seems likely that if this does not underlie the increased Aβ pathology, it certainly could contribute to it through a positive feedback loop. As the evidence for calcium involvement comes from peripheral cells, it also suggests that the effects of AD are not restricted to the CNS and more subtle global changes occur.

Many *in vitro* studies have highlighted the ability of the Aβ peptide to modulate ion channel function and activity, resulting in increased calcium influx into the cytosol. These observations make it highly likely that any changes in calcium seen in AD are as a result of the Aβ peptide, which may be because there are increased levels in the AD brain, or because the aggregation state is altered to a more predominantly oligomeric species which seems to mediate many of the effects on synaptic plasticity (Walsh et al., 2005). Regardless, *in vivo* evidence has shown that Aβ impairs LTP and induces learning and memory deficits highlighting

the synapse as a target. Synaptic transmission is highly dependent upon tightly regulated calcium at both the pre- and post-synaptic membranes and it is at these membranes all the ion channels Aβ has been reported to disrupt are clustered. Therefore, if any of the properties of Aβ to alter ion channel function reported in *in vitro* studies occurs *in vivo*, it will interfere with synaptic transmission, explaining the LTP and memory deficits observed in transgenic mice. Furthermore, evidence is accumulating that Aβ has a physiological role in ion channel regulation (Snyder et al., 2005), which would explain why calcium homeostasis is disrupted in AD once Aβ levels become deregulated.

Understanding the precise actions of Aβ on the ion channels that control calcium homeostasis will be crucial in the future and we will need to turn to sophisticated animal models to fully explore the effects on neuronal calcium, synaptic transmission, LTP and cognition. Indeed, the NMDA receptor antagonist memantine is currently being used as a therapeutic for AD, with patients displaying improved cognition (Peskind et al., 2006). This therapy is thought to target the actions of Aβ on ion channels underlying cognition rather than targeting the production of Aβ in the first place. Better understanding will lead to the realization of new drug targets to combat the pathological effects of Aβ, in the event that therapies designed to prevent or reduce the production of Aβ fail, or that preventing Aβ production does not prevent memory loss in AD, highlighting perhaps another APP fragment as the culprit.

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CHAPTER 20

CALCIUM AND CARDIOMYOPATHIES

E.G. KRANIAS[1,*] AND D.M. BERS[2]

[1] *Department of Pharmacology and Cell Biophysics, College of Medicine, University of Cincinnati, Cincinnati, OH 45267*

[2] *Department of Physiology, Loyola University Chicago, Stritch School of Medicine, Maywood, IL 60153*

Abstract: Regulation of Calcium (Ca) cycling by the sarcoplasmic reticulum (SR) underlies the control of cardiac contraction during excitation-contraction (E-C) coupling. Moreover, alterations in E-C coupling occurring in cardiac hypertrophy and heart failure are characterized by abnormal Ca-cycling through the SR network. A large body of evidence points to the central role of: a) SERCA and its regulator phospholamban (PLN) in the modulation of cardiac relaxation; b) calsequestrin in the regulation of SR Ca-load; and c) the ryanodine receptor (RyR) Ca-channel in the control of SR Ca-release. The levels or activity of these key Ca-handling proteins are altered in cardiomyopathies, and these changes have been linked to the deteriorated cardiac function and remodeling. Furthermore, genetic variants in these SR Ca-cycling proteins have been identified, which may predispose to heart failure or fatal arrhythmias. This chapter concentrates on the pivotal role of SR Ca-cycling proteins in health and disease with specific emphasis on their recently reported genetic modifiers

Keywords: calcium, sarcoplasmic reticulum, cardiomyopathy, mutations

1. INTRODUCTION

Excitation-contraction coupling in cardiac myocytes is initiated by the cardiac action potential (AP), where depolarization-activates an inward Ca current (I_{Ca}), that is called the Ca-trigger, as it promotes the sarcoplasmic reticulum (SR) Ca release. The combination of I_{Ca} and SR Ca release raises intracellular free [Ca] ($[Ca]_i$), allowing Ca to bind to the myofilament protein troponin C, which activates contraction. For

*Evangelia G. Kranias,University of Cincinnati, College Medicine, Department of Pharmacology & Cell Biophysics, 231 Albert Sabin Way, Cincinnati, OH 45267-0575, E-mail: Litsa.kranias@uc.edu

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relaxation to occur $[Ca]_i$ declines, causing Ca dissociation from troponin C. This $[Ca]_i$ decline is due to transport from the cytosol by four pathways: 1) SR Ca-ATPase; 2) sarcolemmal Na/Ca exchange (NCX); 3) sarcolemmal Ca-ATPase; and 4) mitochondrial Ca uniport. The most prominent of these is the SR Ca-ATPase. For the myocyte to be in a steady state with respect to Ca balance, the amount of Ca extruded from the cell during relaxation must be the same as the amount of Ca entry at each beat. Likewise, the amount of Ca released from the SR must equal that re-accumulated by the action of the SR Ca-ATPase.

During heart failure (HF), functional expression of different proteins involved in E-C coupling is altered, and these changes contribute to altered Ca transients, contractility and arrhythmias in HF. Furthermore, genetic mutations in the key Ca-cycling proteins have been recently identified, which contribute to heart failure and fatal arrhythmias. This chapter will concentrate in recent studies on genetic modifiers of cardiac function at the level of the sarcoplasmic reticulum.

2. SR CALCIUM-CYCLING

During cardiac relaxation, Ca is transported into the SR lumen by the SR Ca-ATPase, which is under reversible regulation by phospholamban (PLN). Dephosphorylated PLN binds to SERCA2a and inhibits the enzyme's apparent Ca-affinity. However, phosphorylation of PLN relieves the Ca-ATPase inhibition and enhances Ca-sequestration, associated with increased relaxation rates and contractility *(Simmerman et al., 1998; MacLennan et al., 2003)*. *In vivo*, PLN is phosphorylated by both cAMP-dependent and Ca-CaM-dependent protein kinases (PKA and CaMK) during β-adrenergic stimulation *(Kranias, et al., 1982; Wegener, et al., 1989; Talosi, et al., 1993; Lindemann, et al., 1983; Garvey, et al., 1988; Mundina-Weilenmann, et al., 1996)*. PLN is the major phosphoprotein mediating the positive inotropic and lusitropic effects of β-adrenergic receptor (β-AR) agonists *(Wegener, et al., 1989; Talosi, et al., 1993; Lindemann, et al., 1983; Garvey, et al., 1988; Mundina-Weilenmann, et al., 1996)*. Reversal of PLN phosphorylation occurs by the SR-associated type 1 phosphatase, which is regulated by an endogenous inhibitor-1 protein *(Kranias, et al., 1988)*.

Initiation of contractions occurs when a Ca-trigger through the outer cell membrane induces SR Ca-release through the RyRs (SR Ca release channels), which are coupled to other proteins at the luminal SR surface (triadin, junctin and calsequestrin) *(Zhang, et al., 1997)*. This quaternary Ca-signaling complex participates in both intra-SR Ca buffering and modulation of the Ca release process. Termination of SR Ca release most likely includes RyR inactivation (or adaptation) and a partial decline in $[Ca]_{SR}$. When the SR Ca load is elevated, it enhances the fraction of SR Ca that is released due to stimulation of RyR open probability *(Bassani, et al., 1995; Shannon, et al., 2000)* . In addition to effects of $[Ca]_{SR}$ on fractional release in response to Ca current during E-C coupling, elevation of $[Ca]_{SR}$ also increases the probability of spontaneous SR Ca release events that can propagate through the

myocyte as Ca waves and activate aftercontractions, transient inward current and delayed afterdepolarizations that are arrhythmogenic *(Bers, DM, 2001)*.

2.1. Regulation of SR Calcium-Cycling by Phospholamban

The functional significance of PLN in cardiac muscle has been elucidated through the generation of mouse models with altered PLN expression levels. Heterozygous (40% of PLN) and homozygous (no PLN) for PLN deficiency mice *(Luo, et al., 1994; Luo, et al., 1996)* indicated that the decreases in PLN levels were associated with a linear increase in the affinity of SERCA2a for Ca, *(Luo, et al., 1996)* and with a linear increase in contractile parameters of isolated cardiomyocytes, perfused hearts and intact mice *(Luo, et al., 1996; Wolska, et al., 1996; Li, et al., 1998; Lorenz, et al., 1997)*. The hyperdynamic cardiac function of PLN null hearts could be minimally stimulated by β-AR agonists. Furthermore, there were no effects of aging on the hyperdynamic cardiac function and there was no compromise of exercise performance *(Desai, et al., 1999)*. On the other hand, cardiac overexpression (two-fold) of PLN was associated with significant inhibition of cardiac function *(Kadambi et a., 1996; Dash et al., 2001)*. The inhibitory effects of PLN overexpression could be reversed by β-AR agonist stimulation, which resulted in phosphorylation of the increased PLN levels. These findings in genetically altered models indicate that PLN is a major regulator of basal cardiac Ca^{2+} cycling and contractile parameters (Figure 1). PLN is also a key determinant of β-AR agonist responses. Furthermore, only a fraction of the SERCA2 molecules in cardiac SR are functionally regulated by PLN *in vivo*.

2.2. Regulation of Cardiac Function by β-Adrenergic Receptor Signalling

β-adrenergic receptor stimulation of the heart increases cardiac contractility through enhanced Ca-cycling. The major substrates for the cAMP-PKA axis include PLN, L-type Ca channels, RyR, troponin I and myosin binding protein C (Figure 2). The relaxant effect of PKA is mediated mainly by phosphorylation of PLN and troponin I. PLN phosphorylation speeds up SR Ca reuptake, while phosphorylation of troponin I speeds up dissociation of Ca from the myofilaments.

Current evidence indicates that PLN phosphorylation appears to be dominant over troponin I phosphorylation *(Li, et al., 2000)*. The faster SR Ca uptake by phosphorylated PLN also contributes to increased SR Ca load, which is available for subsequent release, resulting in an inotropic effect. The increased I_{Ca} by PKA activation also contributes to the inotropic effects of the β-AR agonists. The myofilament effects of PKA appear to be almost entirely attributable to troponin I phosphorylation (vs. myosin binding protein C) because substitution of troponin I with a non-phosphorylatable troponin I abolishes myofilament effects of PKA *(Kentish, et al., 2001; Pi, et al., 2002)*.

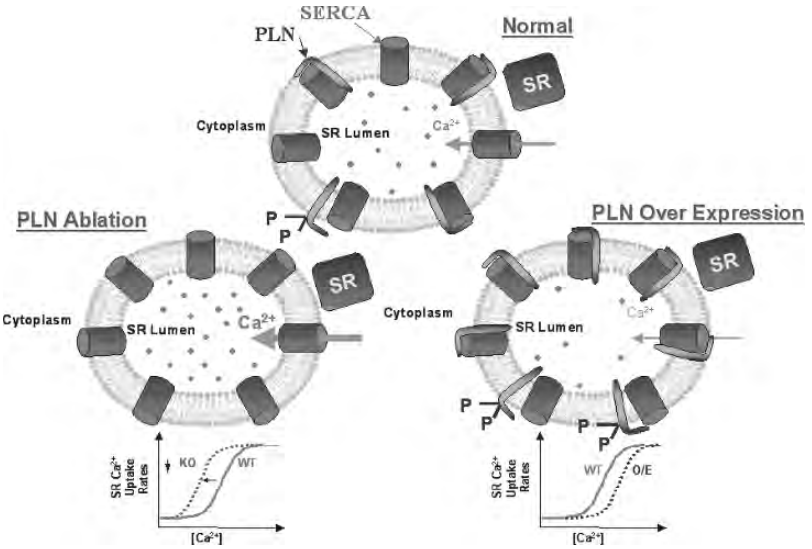

Figure 1. Schematic representation of PLN regulation of the SR Ca-ATPase Ca-affinity, which reflects altered SR Ca-load (green dots in SR) (See Colour Plate 24)

RyR phosphorylation by PKA also alters its open probability. In recordings of single RyR channels in lipid bilayers, PKA treatment enhanced the immediate RyR opening in response to a very rapid $[Ca]_i$ rise (meant to simulate I_{Ca} activation), but it decreased the steady state open probability at a given $[Ca]_I$ *(Valdivia, et al., 1995)*.

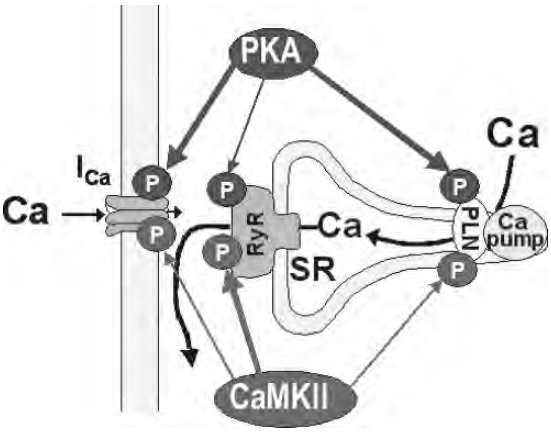

Figure 2. Both PKA and CaMKII have common molecular targets in E-C Coupling (I_{Ca}, RyR and PLN), The specific amino acids that are targets for phosphorylation (P) differ between PKA and CaMKII, as well as the intensity of functional regulation (indicated by arrow thickness). Both kinases may bind directly to the RyR and L-type Ca channel (via an anchoring protein for PKA) (See Colour Plate 25)

In contrast, Marx, *et al.*, *2000,* found that PKA enhanced steady state RyR open probability in bilayers, attributing this to RyR phosphorylation and consequent release of FKBP-12.6 from the RyR. PKA effects on diastolic RyR function remain equivocal because several groups have not found FKBP dissociation from RyR upon PKA-dependent phosphorylation, and Li, *et al.*, *2002*, found no effect of PKA-dependent RyR phosphorylation on Ca spark frequency in intact or permeabilized PLN-knockout myocytes (where SR Ca load was not increased) *(Li*, *et al.*, *2002; Stange*, *et al.*, *2003; Xiao*, *et al.*, *2004)*. During E-C coupling PKA effects on RyR are also somewhat mixed, and are generally complicated by simultaneous enhancement of I_{Ca} and SR Ca-ATPase and SR Ca content upon PKA activation. In a systematic E-C coupling voltage clamp study, where I_{Ca} and SR Ca content were controlled, PKA was found not to alter the amount of SR Ca released, but to increase the initial and maximal rate of Ca release and speed the shut-off of Ca release *(Ginsburg*, *et al.*, *2004)*.

2.3. Regulation of SR by Ca-Calmodulin Dependent Protein Kinase

Parallel to the long-studied regulation of I_{Ca}, SR Ca-ATPase/PLN and RyR by PKA-dependent phosphorylation (Figure 2), these three key targets are also phosphorylated by Ca-Calmodulin dependent protein kinase (CaMKII), and the phosphorylation occurs at different molecular sites *(Maier*, *et al.*, *2003)*. CaMKII is responsible for Ca-dependent facilitation of Ca current, which may contribute somewhat to the positive force-frequency relationship in heart *(Yuan*, *et al*, *1994; Xiao*, *et al.*, *1994; Anderson*, *et al.*, *1994)*. However, this is a quantitatively small stimulation of I_{Ca} compared to that produced by PKA activation. PLN phosphorylation (at Thr-17) by CaMKII also increases SR Ca-ATPase activity similar to PKA-dependent phosphorylation of Ser-16 on PLN. While sympathetic stimulation enhances phosphorylation at both of these sites, PKA-dependent phosphorylation seems to be functionally predominant *(Luo*, *et al.*, *1998)*.CaMKII also phosphorylates the RyR and appears to strongly activate SR Ca release, both during diastole and during E-C coupling *(Li*, *et al.*, *1997; Guo*, *et al.*, *2006)*. Both PKA and CaMKII are likely to be co-activated during normal sympathetic stimulation, creating synergy between these important regulatory signaling pathways.

3. HEART FAILURE (HF)

A major characteristic of human and experimental HF is depressed Ca-cycling in the cardiac myocyte. The differences in Ca-cycling and contractility between non-failing and failing myocytes are mainly observed at high heart rates and the force-frequency relationship is generally less positive in failing vs. non-failing hearts. This is mainly attributed to depressed SR Ca-transport and SR Ca content, as suggested by findings on alterations of the protein levels or activity of the key Ca-cycling proteins (e.g. I_{Ca}, SR Ca-ATPase, Na/Ca exchange, myofilament Ca sensitivity), *(Hasenfuss*, *et al.*, *1998; Richard*, *et al.*, *1998; Mukherjee*, *et al.*, *1998;*

Wickenden, et al., 1998; Nabauer, et al., 1998; Phillips, et al., 1998; de Tombe, et al., 1998; Houser, et al., 2000) and direct cellular measurements of both SR Ca-ATPase function and SR Ca content *(Pogwizd, et al., 2001; Hobai, et al., 2001; Piacentino, et al., 20003)*.

3.1. SERCA2A and PLN

Most reports indicate that the SR Ca-ATPase is functionally decreased in almost all HF models. However the PLN levels are not altered in HF, indicating decreased Ca-affinity of the SR Ca transport system *(Dash, et al., 2001)*. There are also data to suggest that the phosphorylation state of PLN may be reduced in HF *(Richard, et al., 1998; Huang, et al., 1999; Schwinger, et al., 1999)*. This would further reduce the [Ca]-sensitivity of SR Ca uptake and further slow Ca transport at physiological $[Ca]_i$. Reduced SR Ca-ATPase function fits well with the characteristic slowed relaxation and $[Ca]_i$ decline of HF. Moreover, when SERCA2 expression in myocytes or failing hearts is increased or PLN expression is decreased, by adenoviral gene transfer, relaxation and $[Ca]_i$ decline can be accelerated *(del Monte, et al., 1999; Miyamoto, et al., 2000)*. Thus, it seems clear that reduced SR Ca-transport function is important in the slowed relaxation and $[Ca]_i$ decline characteristic of HF, and correction of this depressed SR Ca-uptake may hold promise as a therapeutic approach in heart failure.

3.2. Ryanodine Receptor

Western blots and ryanodine binding generally indicate that the RyR protein levels are unchanged in heart failure *(Go, et al, 1995; Schillinger, et al., 1996; Sainte Beuve, et al., 1997)*. However, in the pacing-induced dog HF model and a rabbit pressure/volume overload HF model, there seems to be down-regulation of RyR *(Vatner et al., 1994; Yano, et al., 2000; Bossuyt, et al., 2005)*.

The regulation of RyR function may also be altered in HF since some studies have reported enhanced RyR phosphorylation by PKA and/or CaMKII. This increased RyR phosphorylation can enhance diastolic RyR open probability, and increased SR Ca leak has been measured in HF *(Marx, et al., 2000; Schwinger, et al., 1999; Bossuyt, et al., 2005)*. Whether this involves loss of FKBP binding to the RyR, *(Marx, et al., 2000; McCall, et al., 1996)* is controversial. Indeed, some investigators have indicated that PKA-dependent RyR phosphorylation has no effect on Ca sparks *(Li, et al., 2002)* and may not alter FKBP12.6 binding *(Stange, et al., 2003; Xiao, et al., 2004)*.

Buffering of Ca inside the SR is probably unaltered in HF, because calsequestrin (and calreticulin) does not seem to be altered in HF *(Maier, et al., 2003; Richard, et al., 1998)*. This means that if SR Ca content is lower in HF, free $[Ca]_{SR}$ may also be lower. Although there are few measures of SR Ca in HF under relatively physiological conditions, SR Ca content seems to be reduced in human, *(Piacentino, et al., 2003; Lindner, et al., 1998)* rabbit *(Pogwizd, et al., 1999 & 2001)* and dog,

(Hobai, et al., 2001) based on caffeine-induced Ca transients. Reduced SR Ca content is sufficient to largely explain the reduced twitch Ca-peak and contractile function in HF.

3.3. Human SERCA2 Mutations in Heart Failure

There is only one report on naturally occurring mutations in the human SERCA2 gene *(Schmidt, et al., 2003)*. This study concentrated on exons 8, 15, 16, 18, and 19, corresponding to the SERCA2-PLN interaction domains, as well as exons 10, 13, and 14, which covered the phosphorylation and the nucleotide binding/hinge domain of SERCA2, since mutations in these regions may predispose to the development of heart failure.

One hundred and sixty one patients with ischemic or idiopathic dilated cardiomyopathy (New York Heart Association functional class II-IV) were screened for SERCA mutations. Double strand sequencing revealed nucleotide changes in exons 8, 15 and 18. However, none of these naturally occurring genetic variants resulted in amino acid alterations. Furthermore, there were no mutations or single nucleotide changes observed in exons 10, 13, 14, 16, and 19. Thus, although the SERCA2 mRNA and protein levels are altered in human heart failure, the SERCA2 gene is highly conserved in patients with heart failure. There were only four nucleotide changes identified in the SERCA2 gene in three out of eight exons examined. All of these alterations were conservative *(Schmidt, et al., 2003)*. Thus, the SERCA2a gene is tightly regulated to maintain proper intracellular Ca^{2+} cycling. It is interesting to speculate that even minor alterations in the SERCA2a gene cannot be accommodated and result in premature death, which may not allow their discovery in adult heart failure.

3.4. Human PLN Mutations in Dilated Cardiomyopathy

Three PLN mutations in the coding region have been reported to date. Interestingly, all three appeared to be inherited in a familial manner. One of these is the mutation of R9C, which was associated with the inheritance of dilated cardiomyopathy in a large American family *(Schmitt, et al., 2003)*. Carriers of this mutation had a mean age of 25 years. The effects of R9C-PLN appeared to be linked to significant decreases in PLN phosphorylation. Transgenic mice overexpressing human PLN-R9C mutant exhibited dilated cardiomyopathy and early death. To elucidate the mechanisms underlying the detrimental effects of this mutant, the R9C-PLN was expressed in HEK cells. When the mutant-PLN was co-expressed with WT-PLN, it did not relieve the inhibition of SERCA2a by WT-PLN. The R9C mutant appeared to exhibit enhanced affinity for PKA, preventing the PKA-phosphorylation of WT-PLN. These findings suggest that the dominant effects of this mutation in affected individuals may be associated with chronic inhibition of SERCA2a. Thus, inhibition of PLN phosphorylation is sufficient to cause the onset of dilated cardiomyopathy in humans in their teenage years.

A second mutation, associated with a termination codon at amino acid 39 (L39stop) was discovered in two large families *(Franz, et al., 2001)*. Truncation of the 52 amino acid protein occurred in transmembrane domain II, which is highly conserved among species *(Mc Tiernan, et al., 1999)* and involved in PLN regulation of SERCA2a affinity for Ca^{2+} *(Brittsan, et al., 2000)*. In the first family, there were two homozygous individuals, which developed severe dilated cardiomyopathy and required cardiac transplantation at young age. Histopathological examination of both explanted hearts revealed fibrosis and myofibrillar disarrangement. The heterozygous individuals exhibited normal left ventricular function but some of them appeared with left ventricular hypertrophy. In the second family, there were two brothers identified, who were heterozygous for the L39stop-PLN mutation. Interestingly, both of them were diagnosed with cardiomyopathy. Their father had also died of dilated cardiomyopathy and their mother was homozygous for wild-type PLN. The rest of the heterozygous subjects in this family had normal left ventricular systolic function but some of them exhibited left ventricular hypertrophy, similar to the first family. These finding indicate incomplete penetrance of the cardiomyopathy phenotype.

The function of PLN-L39stop on SR Ca^{2+} transport was elucidated by expression studies in HEK cells. Co-expression of human wild-type PLN (PLN-WT) with SERCA2a resulted in decreased apparent affinity for Ca^{2+}, but co-expression of SERCA2a with PLN-L39stop had no effect. When the wild type and mutant PLN were co-expressed with SERCA2a, the decrease in the apparent Ca^{2+} affinity was similar to that observed in by WT-PLN, indicating that the PLN-L39stop mutant does not exert any effects on SERCA2a activity.

Furthermore, infection of adult rat myocytes with adenoviral vectors containing either wild-type or L39stop PLN cDNAs indicated that wild type PLN decreased the contractile parameters and calcium kinetics, compared to control cells infected with an adenovirus expression GFP. However, the PLN-L39stop did not alter myocyte mechanics or calcium cycling.

Western blots of microsomal fractions from transfected HEK-293 cells with the PLN-L39stop mutant, indicated that the PLN-L39stop protein could not be detected. In addition, confocal microscopy in HEK-293 cells transfected with PLN-L39stop revealed detectable immunoreactive protein signals in a small percent of cells and the PLN-mutant was mainly localized to the cell membrane, compared with PLN-WT, which localized to the endoplasmic reticulum. Consistent with these findings, human PLN-L39stop homozygous ventricles had no detectable PLN.

Thus, PLN is an important regulator of human SERCA2a due to the large cardiac reserve required for flight or fight situations, allowing 2–3 fold increases in heart rate. In contrast, the mouse exhibits heart rates ranging up to 800 bpm and it is operating close to its maximal rate, with low cardiac reserve. Intuitively, the rapid Ca-cycling in mouse hearts does not depend on PLN, while PLN is essential for normal function in the human heart.

More recently, another human PLN mutation, which deletes Arg 14 in the coding region was identified. This mutation is associated with inherited

human dilated cardiomyopathy and premature death. Some of the heterozygous individuals presented dilated cardiomyopathy with ventricular extra systolic beats and ventricular tachycardia. These symptoms progressed to congestive heart failure by middle age. However, other young heterozygous subjects were asymptomatic with normal echocardiography, indicating that the effects of this mutation may be age-dependent. In accordance, cardiac overexpression of PLN-R14Del in the mouse recapitulated human dialed cardiomyopathy with abnormal histopathology and premature death.

Expression of the heterozygous mutant-PLN in HEK-293 cells resulted in SERCA superinhibition. The dominant effect of the PLN-R14Del mutation could not be reversed, even after β-adrenergic stimulation. Thus, mutant-PLN remains a chronic inhibitor of SERCA and cardiac Ca-cycling. In accordance, cardiac overexpression of PLN-R14Del in the mouse recapitulated human dilated cardiomyopathy with abnormal histopathology and premature death. Increased PLN inhibition over a period of years may lead to cardiac remodeling, which may progress to failure in later years.

The superinhibitory effects of the PLN-R14Del mutant maybe due to its structure. One charged residue (Arg 14) is missing from the three charged Arg (at positions 9, 13, and 14) in wild-type PLN, which may influence the interaction of PLN with SERCA. Another effect of the Arg 14 deletion is partial disruption of the PLN pentameric structure. Increases in monomeric PLN are expected to gain inhibitory function on the apparent affinity of SERCA2a for Ca^{2+}, which may not be relieved even upon PKA-mediated phosphorylation. A mutation in the PLN promoter region, which increases PLN expression, has been also identified in human hypertrophic cardiomyopathy *(Minamisawa, et al., 2003)*. Consistent with findings in transgenic mice, an increase in the apparent stoichiometry of PLN/SERCA2 is expected to result in depressed Ca-cycling and contractility, which may lead to cardiac remodeling.

Collectively, the human PLN mutant studies indicate that chronic inhibition of either basal SERCA2a activity (PLN-R14Del mutant) or the β-adrenergic stimulation (PLN-R9C mutant) *(Schmitt, et al., 2003)* result in heart failure. On the other hand, absence of PLN inhibition by the PLN-L39stop mutant, associated with the lack of cardiac reserve, also results in heart failure (Figure 3). Thus, the identification of these human PLN mutations point to the paramount importance of PLN and its role in maintaining normal calcium homeostatic mechanisms in the human heart.

3.5. Human Calsequestrin Mutations

Calsequestrin (CSQ) is the most abundant Ca-binding protein in the SR lumen, acting as a low affinity but high capacity Ca buffer (Figure 4) *(Mitchell, et al., 1988)*. CSQ has also been suggested to have a regulatory effect on the activity of the RyR *(Schillinger, et al., 1996)*. Some insight into the physiological function of the protein came from mice overexpressing either canine or murine cardiac CSQ

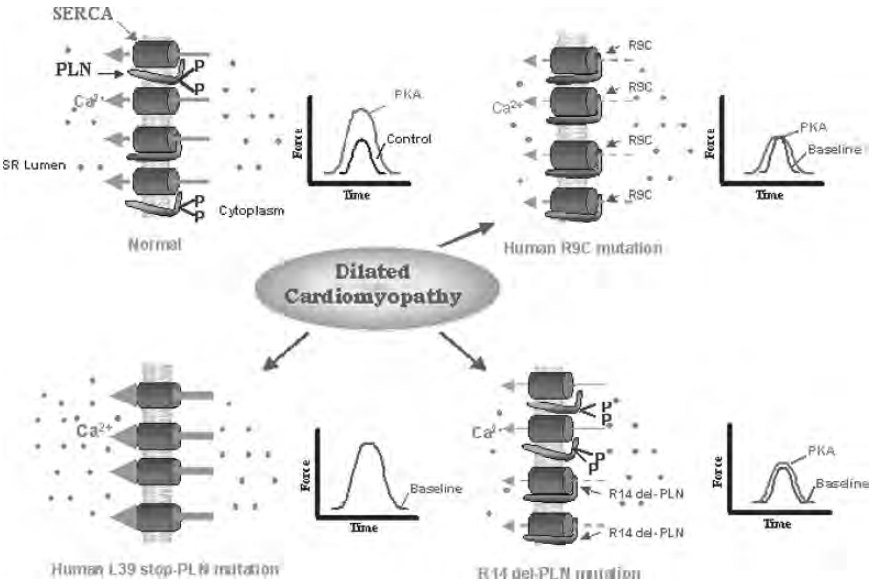

Figure 3. Schematic representation of the effects of Human PLN Mutations on the SR Ca-ATPase Activity and contractility under basal and isoproterenol (PKA)-stimulated conditions (See Colour Plate 26)

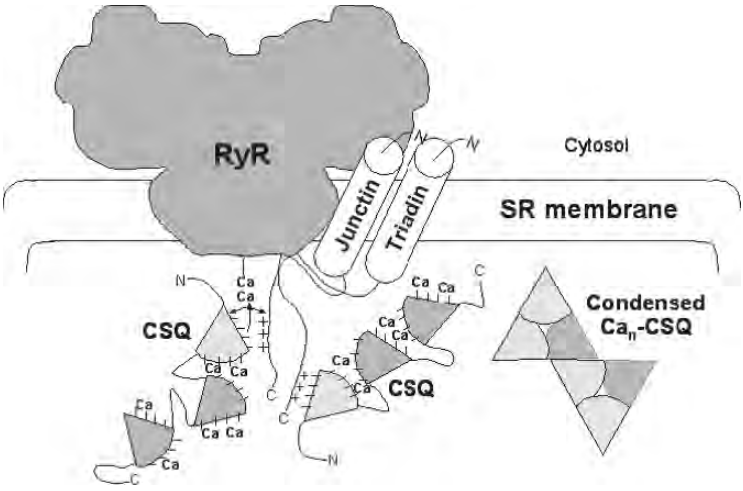

Figure 4. Calsequestrin (CSQ) is the main intra-SR Ca buffering protein, and its structure and interaction with other CSQ and partner proteins (triadin and junctin) is influenced by intra-SR [Ca]. In addition to its role as a low affinity Ca buffer, CSQ may also regulate RyR gating via its interaction with triadin and junctin (See Colour Plate 27)

in the heart. Overexpression of the heterologous protein resulted in hypertrophy which progressed to heart failure *(Solaro, et al., 1974)*. Overexpression of the homologous protein was also associated with cardiac hypertrophy and induction of a fetal gene expression program *(Sato, et al., 2001)*. These mice also exhibited depressed contractility and Ca transients even though the Ca storage capacity of the SR was enhanced, which may be due to increased SR Ca buffering.

There are currently two different CSQ genes, a skeletal isoform and a cardiac isoform *(Lehnart, et al., 2004)*. The cardiac isoform is highly conserved between species and is the only isoform expressed in the heart. The levels of CSQ are not altered in the developing heart and in many pathological disease states such as hypertrophic, ischemic or dilated cardiomyopathy *(Gyorke, et al., 2004; Wang, et al., 2001)*, suggesting that CSQ expression is under rigid genetic regulation. Interestingly, recent studies have indicated that human mutations in the CSQ gene may be responsible for catecholaminergic polymorphic ventricular tachycardia (CPVT). More specifically, a missense mutation was discovered that replaced aspartic acid (negatively charged residue) with histidine, (a positively charged residue), at position 307 *(Farrell, et al., 2003; Wehrens, et al., 2003)*. This residue is localized in a highly conserved Ca binding region and CPVT may therefore be triggered by disrupted Ca binding. However, it is possible that this mutation may also disrupt the interaction of CSQ with the RyR. Also, a nonsense mutation was described, which results in a truncated protein, associated with CSQ ablation *(Leenhardt, et al., 1995)*. In both situations, the patients present with recurrent syncope, seizures or sudden death following physical activity or emotional stress. The seizures associated with the disease are often misdiagnosed as epilepsy, especially since the patients may recover spontaneously without the need of any resuscitation. These patients appear to have structurally normal hearts and marked bradycardia under resting conditions. In general, these patients exhibit arrhythmogenic activity, when a threshold heart rate of 120 beats per minute (bpm) is exceeded. Although the mean age at which the first syncope occurs is around 7 years old, cases have been reported, where the patients were as young as 3 years old. Some insight into the cellular mechanism underlying this disease were obtained from experiments on reduction of CSQ levels, using antisense methodology or expression of the D307H mutant in myocytes. Both of these resulted in disturbances in rhythmic Ca transients with signs of delayed afterdepolarizations (DADs), when undergoing periodic electrical stimulation and exposure to isoproterenol *(Hoit, et al., 1995; Bassani, et al., 1995)*. Thus, it was suggested that the development of this arrhythmogenic disorder is due to impaired SR Ca storage as well as impaired release through the RyR, resulting in an increase in diastolic leak, which may cause delayed afterdepolarizations.

4. CONCLUSION

Many proteins contribute centrally to the delicate balance of Ca in cardiac myocytes that controls cardiac contractility and influence electrical activity. These include voltage-gated Ca channels, RyR, SERCA2, PLN, calsequestrin and regulatory

kinases (and phosphatases), Both genetic and acquired alterations in these Ca handling proteins in cardiac myocytes contribute to pathophysiological cardiovascular disease. Altered cellular Ca^{2+} handling can cause reduced systolic or diastolic cardiac function and also contribute to cardiac arrhythmias.

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CHAPTER 21

CALCIUM SIGNALLING AND CALCIUM TRANSPORT IN BONE DISEASE

H.C. BLAIR, P.H. SCHLESINGER, C.L.-H. HUANG, AND M. ZAIDI

Departments of Pathology and of Cell Biology and Physiology, University of Pittsburgh and Veterans' Affairs Health System, Pittsburgh, PA 15261, Department of Physiology and Cell Biology, Washington University School of Medicine, St. Louis, MO 63110, Physiological Laboratory, University of Cambridge, Cambridge, CB2 3EG, United Kingdom, and The Bone Program, Mount Sinai School of Medicine, New York, New York 10029

Abstract: Calcium transport and calcium signalling mechanisms in bone cells have, in many cases, been discovered by study of diseases with disordered bone metabolism. Calcium matrix deposition is driven primarily by phosphate production, and disorders in bone deposition include abnormalities in membrane phosphate transport such as in chondrocalcinosis, and defects in phosphate-producing enzymes such as in hypophosphatasia. Matrix removal is driven by acidification, which dissolves the mineral. Disorders in calcium removal from bone matrix by osteoclasts cause osteopetrosis. On the other hand, although bone is central to management of extracellular calcium, bone is not a major calcium sensing organ, although calcium sensing proteins are expressed in both osteoblasts and osteoclasts. Intracellular calcium signals are involved in secondary control including cellular motility and survival, but the relationship of these findings to specific diseases is not clear. Intracellular calcium signals may regulate the balance of cell survival versus proliferation or anabolic functional response as part of signalling cascades that integrate the response to primary signals via cell stretch, estrogen, tyrosine kinase, and tumor necrosis factor receptors

Keywords: Hypophosphatasia; chondrocalcinosis; osteopetrosis; osteoporosis

Although bone is not considered a major calcium sensing organ in humans, the cells of bone tissue control over 99% of the human body's calcium content. The principal calcium sensors that regulate bone calcium uptake and release are in the parathyroid glands. Bone function is also modified by vitamin D and by calcium transport in the kidney and intestine. These indirect mechanisms of controlling bone calcium metabolism are beyond the scope of our considerations here. In spite of processing

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such massive quantities of the Ca^{2+}, bone cells use calcium in their homeostatic control processes. The massive movement of calcium is carried out by specialized and regulated transporters. Defects in the transporters cause diseases with affect bone structure or function. Indeed, inborn errors have been very important in defining the calcium transport mechanisms in bone.

Additionally, calcium is used by bone forming and bone degrading cells as a secondary mediator of hormone and cytokine action. These actions include roles in intercellular communication within groups of osteoblasts, which are connected by gap junctions (Henriksen et al., 2006). These osteoblast groups function in a coordinated fashion in bone synthesis and maintenance, and are collectively known as the osteon. Osteoblasts in these groups are connected by gap junctions which are capable of propagating signals in the cell groups, including calcium waves (Xia and Ferrier, 1992). Calcium is also an important regulator of cellular attachment, motility, endosome function (Piper and Luzio, 2004) and survival (Davies and Madesh, 2004). These are all critical functions in bone bone degrading osteoclasts.

In this chapter, we will describe calcium transport and calcium regulation from the standpoint of bulk mineral transport, and then from the standpoint of cellular regulation. We will use inborn errors of metabolism where possible to illustrate the major calcium-related activities of the cells. After the framework of functional calcium transport is established, we will discuss calcium-dependent cellular regulatory functions.

1. BULK CALCIUM TRANSPORT BY THE OSTEOBLAST AND RELATED CELLS

The osteoblast is a mesenchymal stem cell derivative (Pittenger et al., 1999). Specialization of the osteoblast allows it to produce an extracellular matrix of type I collagen and accessory proteins including osteocalcin (Young 2003), which are present in minor quantities. The type I collagen is very dense and heavily crosslinked, creating an extremely high tensile strength. To provide strength to the skeleton, it comprises approximately 10% of the dry mass of bone. Osteocalcin is a calcium-binding low molecular weight protein, its calcium binding properties conferred by post-translational modification to produce γ–carboxyglutamate (Bugel, 2005; Weber, 2001). Osteocalcin is important in proper calcification of the matrix. Dozens of other accessory proteins found in bone matrix also play important, but secondary, roles in bone structure. The major component of bone matrix is bone mineral, which provides its resistance to compression, and in combination with type I collagen produces a skeleton of great strength and durability. However, the bone matrix is also a massive depot of minerals (Neuman and Neuman, 1958). This includes over 99% of the body's calcium, but the calcium-balancing anions, mainly PO_4^{3-}, are also important and, in the presence of a chronic acid load, amount to approximately 1.5 moles of available base equivalents for each calcium (Cho et al., 2003). When necessary the skeleton is sacrificed to maintain the pH of the extracellular fluid (Bushinsky, 2001; Carano et al., 1993).

How the osteoblast deposits mineral has long been an interesting mystery. Details of the process are still unclear, although some general principals can be derived from diseases of mineralization which point to the central mechanisms. The chemistry of bone mineral, hydroxyapatite, requires that any mechanism of bone formation include a supply of Ca^{2+} and $H_2PO_4^-$ and some way to dispose of 1.4 H+ per each Ca^{2+} deposited.

$$(1) \qquad 10Ca^{2+} + 6H_2PO_4^- + 2H_2O \leftrightarrow Ca_{10}(H_2PO_4)_6(OH)_2 + 14H^+$$

The need for local control of extracellular ion composition and pH buffering is reflected in the multicellular structure that regulates bone formation in the air-breathing vertebrates, which is called the "osteon". This is an extended group of gap-junction connected cells that are embedded in the bone matrix, together with a layer of osteoblasts "covering" the bone surface at the interface of the osteon with the bone marrow (Figure 1). This multicellular structure is of key importance. That the surface osteoblasts are connected to the earlier generations of osteoblasts (called osteocytes), which have become embedded in the matrix produced by the cells, allows the unit to be regulated by interaction with marrow cells and serum components. It has long been known that the access of water and ions to bone matrix is very restricted (Deakins and Burt, 1944; Neuman and Neuman, 1958). Thus the bone matrix is entirely surrounded by cells, which control the movement of constituent proteins of bone matrix and also of the ions which are deposited to produce bone mineral. Therefore it is mainly by active or facilitated transport that constituents in the extracellular fluid can reach the matrix. Note, for example, that tetracycline or calcein will accumulate very specifically in the mineralizing layer of bone at the surface of an active osteon, while bone surfaces deep within the osteon are unlabeled. When the osteon dies, as in osteonecrosis following high-dose glucocorticoid exposure, the cell-matrix surfaces can be labeled by tetracycline in an osteon specific geometry (Eberhardt et al., 2001). The control of this cellular array surrounding bone matrix requires both connexin43 and purinergic receptors (Civitelli et al., 1993; A. Hoebertz et al., 2003).

The mechanism of bulk calcium transport for bone formation is partly established from experimental work and is partly conjectural (Figure 1). The source of the calcium is obviously the extracellular fluid, and its transport through the active surface osteoblasts of a bone forming osteon is clearly regulated. However, the molecular mechanism is poorly understood. There is insufficient Ca^{2+}-ATPase activity for mineralization to be supported by direct transport (Gay and Lloyd, 1995), and this would be, energetically, very inefficient as well. It was long hypothesized that there was a calbindin-dependent calcium ferry (Balmain et al., 1989), but this is either not to be the case, or the calcium buffer system for facilitated transport involves a unique mechanism not yet described, in that calbindin-negative osteoblasts lose 25% of their calcium binding capacity but transport calcium at a normal rate (Turnbull et al., 2004). Osteoblasts express abundant sodium-calcium exchangers, however, in a pattern likely to correlate with bulk mineral transport (Stains et al., 2002), so it is probably premature to discard the calcium ferry

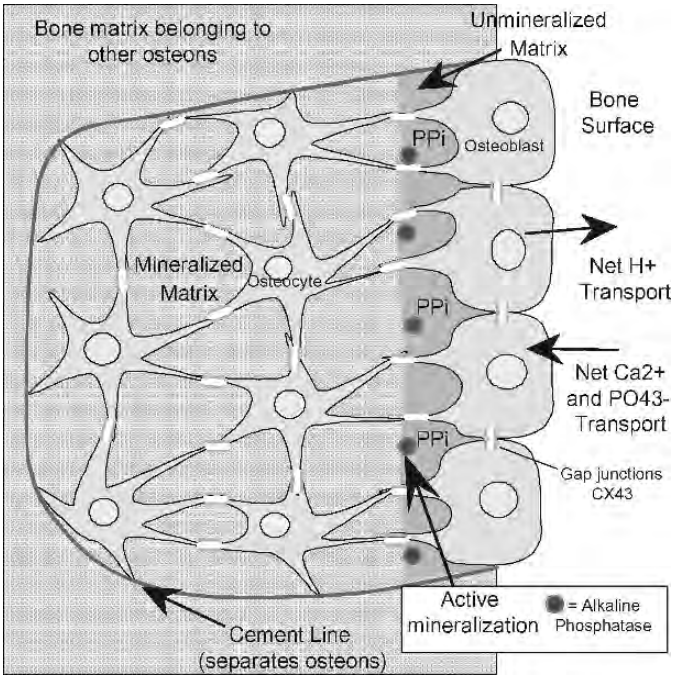

Figure 1. Bulk calcium transport by the osteoblast. While chondrocytes are individual cells embedded in an acellular matrix, and calcify the matrix focally by producing high local concentrations of phosphate, osteoblasts are arrayed in a three-dimensional organized matrix that allows calcium to be deposited in an efficient site-directed mechanism. The osteoblasts are connected into sheets of cells at the surface of bone by gap junctions containing connexin 43. The osteoblasts secrete an organic matrix comprised mainly of type I collagen, which is oriented in layers alternately along the axis of stretch of the bone and orthogonal to this axis. There are also minor proteins, including the calcium binding low molecular weight protein osteocalcin, which facilitate mineral deposition. Mineral deposition is driven by alkaline phosphatase activity which degrades pyrophosphate. Pyrophosphate can be transported either by membrane transporters including ANKH, or may be produced locally by nucleoside pyrophosphatase (PC-1) activity. The high phosphate produced is balanced by calcium transport and by alkalinization of the mineralization site, which are required for continuing mineral deposition, but the specific transporters involved in these activities are unclear (See Colour Plate 28)

hypothesis entirely, and it is more likely that a calbindin-independent mechanism of some sort exists.

The only essential components of the mineral deposition mechanism that are fairly certain at this time relate to phosphate. Even for phosphate, alternative mechanisms are proposed, which are not mutually exclusive but probably function in parallel, in the regulation of different aspects of skeletal calcium transport, and to some extent provide redundancy that allows many mineral transport disorders to be survivable. Alkaline phosphatase activity is essential to produce phosphate. Its major substrate is pyrophosphate. In the absence of the alkaline phosphatase, normally highly expressed as an ectoenzyme by osteoblasts, there is little matrix mineralization

and very high serum pyrophosphate accumulation occurs (Whyte et al., 1995). The pyrophosphate is, to a major extent, produced by a nucleoside pyrophosphatase, PC-1 (Lotz et al., 1995; Hessle et al., 2002). This gene is also expressed in other organs, where polymorphisms may be related to pathology, but mutations in the bone are not described. A multipass transmembrane protein related to progressive ankylosis (fusion of joints), ANK, is also major additional source of pyrophosphate (Ho, Johnson, and Kingsley, 2000). It is a pyrophosphate transporter, so presumably the source of the pyrophosphate is intracellular although the biochemical pathway is unknown. Mutations in its human homolog, ANKH, cause a group of diseases including craniometaphysial dysplasia and chondrocalcinosis (Reichenberger et al., 2001; Nurnberg et al., 2001; Williams et al., 2002; Pendleton et al., 2002).

2. A PROTON CONNECTION

The third component required for bone formation, removal of protons, is essential but it is frequently ignored in considerations of mineralization. High concentrations of phosphate and calcium at neutral pH will form an initial precipitate, but mineral formation is quickly limited as the pH falls below 5.6 (Neuman and Neuman, 1958). From the hydroxyapatite formation equation (Equation 1) it is clear that $\sim$1.5 moles of H^+ is produced by the combination of $H_2PO_4^-$, Ca^{2+} and H_2O. The exact stoichiometry depends on the pH at the deposition site, which determines the ratio of HPO_4^{2-} to $H_2PO_4^-$; this detail is excluded, for clarity, from Equation 1, where only the predominant phosphate ion, $H_2PO_4^-$, is shown; the phosphoric acid K_2 is 6.70. On the other hand, PO_4^{3-} and H_3PO_4 are of no importance biologically since the phosphoric acid K_1 is 1.96 and K_3 is 12.32.

The deposition of limited quantities of hydroxyapatite in extracellular matrix has been observed without bounding cells. Cartilage calcification is such a case where local pH control and Ca^{2+} are dependent upon diffusion and the rate of mineral deposition is driven by phosphate presentation. Chondrocytes produce alkaline phosphatase that generates the required phosphate, but cartilage is not delimited by any cellular structures and transfer of Ca^{2+} and H^+ is by diffusion from extracellular fluid.

Bone mineral, on the other hand, is deposited rapidly along a line of new bone within an osteon where the matrix is completely enclosed by cells. The calcium deposition is carefully orchestrated and precise, requiring regulated Ca^{2+} and H^+ transport (Figure 1). The existence of local alkalinization at the site of mineral deposition was long ago noted in using a pH indicator (Cretin, 1951). We noted in genescreen studies of human osteoblasts that many subunits of the vacuolar-type H^+-ATPase are highly expressed, but no clear vectorial localization of the pump (hypothetically in the opposite orientation as in the osteoclast, discussed below) has been observed. Other possibilities include an H^+-pyrophosphatase, which could make the requisite (outward) acid gradient as well as producing pyrophosphate for mineral formation, but such transporters have not been found in metazoans.

3. CALCIUM-DEPENDENT CELLULAR REGULATION IN THE OSTEOBLAST AND IN RELATED CELLS

As noted in the introduction, the bone is not a classical calcium sensing organ. However, osteoblasts express a G-protein coupled calcium receptor, GPRC6A (Pi et al., 2005), and purinergic receptors that mediate calcium uptake or regulation in the osteoblast (Hoebertz et al., 2003). The osteoblast may thus play a role in adaptation to abnormal extracellular calcium such as in diseases where the parathyroid calciostat is unable to maintain extracellular calcium activity within its normal narrow limits, and knockout of the calcium sensing receptor does impair calcium homeostasis independently of PTH (Kos et al., 2003). This mechanism will, however, require further study. Activating mutations of the calcium sensing receptor are associated with Bartter's syndrome (Watanabe et al., 2002), which is a renal calcium reabsorption defect, and no osteoblast-related phenotype is known. Purinergic receptors are known to be present on osteoblasts and ATP or UTP coordinate osteoblast activity in bone remodeling (Hoebertz et al., 2002). Similarly, in the mineralization of otoliths purines play an important role in mineral deposition by otoconial epithelial cells (Suzuki et al., 1997). From mice defective in vestibular function a new and unique family of membrane proteins has been identified, the otopetrins (Hurle et al., 2001). These membrane proteins are essential for otolith mineralization but are found in many tissues. We have studied the expression of otopetrin 1 in cultured cells and are studying it as a potential novel regulator of P2Y and P2X activity. Although the function of otopetrins in osteoblasts has not been studied, they are essential for mineralization in otolith formation (Hurle et al., 2003; Hughes et al., 2004).

As in most cells, osteoblasts express several calcium channels. The clearest role of calcium channels in functional osteoblast regulation is for L-type voltage-sensitive channels. These channels mediate changes in osteoblast intracellular calcium that vary with major osteoblast regulatory agents including vitamin D and PTH (Li et al., 1997; Gu et al., 2001; Ryder et al., 2001; Bergh et al., 2006). Pharmacological inhibitor studies of L-type calcium channels show mixed results in assays of osteoblast differentiation (Zahanich et al., 2005; Nishiya et al., 2002). However, the L-type calcium channels are probably essential intermediates in osteoblast intercellular calcium signals (Jorgensen et al., 2003). Additional calcium channels may be involved in metabolic coupling to osteoblastic intracellular calcium, including the ryanodine receptor, purinergic receptors, and IP3Rs (Sun et al., 2002; Jorgensen et al, 2003). There are also sodium/calcium channels such as ENaC that may respond to membrane swelling in the osteoblast (Kizer et al., 1997). These transporters may be important, and some associations with cytokine response are known (Kirkwood, et al., 1997; Bradford et al., 2000). However, physiological mechanisms are for the most part not clear.

Osteoblast intracellular calcium is regulated by membrane stretch or shear stress and by other mechanisms (Kamioka et al., 2006). Further, the absence of stretch causes atrophy. This effect is important, with acute and severe bone loss caused by disuse or unweighting (Bikle and Halloran, 1999). Stretch-induced calcium

transients, probably via L-type calcium channels, are important in the coupling of bone flexion to the activity and proliferation of osteoblasts and related cells (Hughes-Fulford, 2004). Skeletal flexion also promotes chondrocyte growth (Drescher et al., 2003). Osteoblastic cellular responses include cell proliferation (Riddle et al., 2006), as well as diverse, generally anabolic, effects on differentiation and activity.

It is likely that the calcium signal is secondary to potassium-dependent depolarization (Wiltink et al., 1994; Gofa and Davidson, 1996; Hattori et al., 2001; Jorgensen et al., 2003). Stretch-activated potassium channel activity has been demonstrated (Duncan and Misler 1989; Ypey et al., 1992; Davidson, 1993). This may be due to two-pore domain potassium channels (Chen et al., 2005; Hughes et al., 2006), although the mechanism is not firmly established. Other channels have been proposed, including activation by focal adhesion kinase or outwardly-rectifying BK potassium channels (Rezzonico et al., 2003). Stretch-activated channels are, in turn, co-regulated with cell attachment-related proteins. These may, in addition to the ion signals, modify the cell attachment and attachment-related kinases. This has been demonstrated in chondrocytes (Lee et al., 2000), and a similar mechanism in osteoblasts probably modifies the focal adhesion kinase (Boutahar et al., 2004).

While stretch activation of calcium signals involves interaction with attachment proteins, not all stretch-related signals require calcium. All mechanical stimuli activate ERK1/2, with downstream upregulation of pro-growth transcription factors, such as c-fos (Ruwhof and van der Laarse, 2000). The integrin β subunit is essential for ERK1/2 activation and downstream signals in response to mechanical stimulation (MacKenna et al., 1998). However, the cation channels may be dispensable for most of the gene expression effects (Sadoshima et al., 1992). On the other hand, in connected osteoblasts and osteocytes, calcium is a key intercellular signal.

Calcium fluxes in osteoblasts may have anabolic effects or may promote apoptosis, depending on the context of the signal. Survival mechanisms triggered along with cell stretch, including PI-3-kinase activity and phosphorylation of Akt (Danciu et al., 2003), are probably involved in the promotion of cell survival and anabolic effects in stretch related calcium signals. The IP3R receptor family may be important in changes of nuclear calcium transport with senescence (Huang et al., 2000). On the other hand, induction of calcium via NAD^+ and the RyR induces apoptosis (Romanello et al., 2001). This is not surprising in that this type of calcium signal is unrelated, as far as is known, to adhesion-related survival signals. Further, the RyR is a critical regulator of nuclear calcium in osteoblasts (Adebanjo et al., 1999). The anabolic/apoptotic decision may also be dependent on the type of calcium channel, and on the magnitude and location of the calcium current.

Abnormal calcium transport undoubtedly contribute to bone defects but specific examples are poorly described. For the major calcium transporters, significant defects are lethal in embryonic life, such as in the Cav1.2 L-type calcium channel

(Seisenberger et al., 2000). Interestingly, for the closely related Cav1.2 and Cav1.3 L-type calcium channels, which are likely to be the principal calcium channels in stretch-related calcium fluxes in osteoblasts, the Cav1.3 channel may partially compensate for loss of function of Cav1.2 (Xu et al., 2003). There are mutations of Cav1.2 with cardiac defects and a variety of other developmental abnormalities including syndactyly (fusion of fingers), but no clear bone phenotype at the level of bone matrix structure (Splawski et al., 2005). In other cases, knockouts are survivable, but, as in the IP3R1 knockout (Hirota et al., 1998), with complex defects including runting; specific skeletal changes have not been characterized.

4. BULK CALCIUM TRANSPORT BY THE OSTEOCLAST

The osteoclast is unique in mobilizing massive quantities of calcium from mineralized tissue. Dissolving hydroxyapatite requires the addition of protons, just as deposition of hydroxyapatite liberates acid (see Equation 1). To allow acidification, the osteoclast produces an isolated micro-compartment on the bone surface. This is achieved by close apposition to the matrix via adhesion of αv integrins to matrix RGD peptides, with β3 the major complementary subunit (Miyauchi et al., 1991). Inside the osteoclast the cytoskeleton (Akisaka et al., 2006), and transport activities (Vaananen et al., 2000; Schlesinger et al., 1994) are reorganized to support the resorption compartment.

The key metabolic activity within this sealed compartment is acid transport. It is driven by a V-type H^+-ATPase (Blair et al., 1989). This ATPase is comprised of two major subassemblies, membrane (Vo) and cytoplasmic (V_1). The Vo component consists of a hydrogen channel, 17 kDa, and large, 116 kDa, protein with several transmembrane domains. This protein is crucial for membrane insertion, and four homologous genes encode variants of it (Nishi & Forgac, 2002). The isoform TCIRG1 (ATP6i; A3) is amplified specifically in osteoclasts (Li et al., 1999; Mattsson et al., 2000). Defects in this protein are common causes of osteopetrosis in the human (Blair et al., 2004). Heterogeneity in TCIRG1 expression and function may also affect bone density (Carn et al., 2002). The V_1 assembly is essential to life. The proton pump mechanism is inferred by analogy to the homologous mitochondrial F–ATPase, which produces ATP from proton gradients rather than vice versa. It was described in detail in the 1980s and 90s (Boyer, 1997). The F_1 or V_1 assemblies are nano-motors that couple ATP hydrolysis to electrogenic H^+ translocation (Finbow and Harrison, 1997). The F and V-ATPases are electrogenic and transport H^+ without counter-ions. Thus, counter-transport of cations or co-transport of anions is required for H^+ transport in meaningful quantity, as for mineral removal. And the osteoclast must move an amazing amount of acid, since bone mineral requires addition of ~1.5 moles of H^+ per mole of calcium removed at pH 7.4, and the osteoclast can degrade approximately its own volume in bone mineral per day. Indeed, the quality of cultured osteoclasts in vitro is relatively easy

to judge from rapid acidification of the medium completely out of proportion from the quantity of cells (Carano et al., 1993).

Thus, calcium mobilization is dependent on and requires a cotransport of ions to balance the electrogenic proton pump. Studies of isolated osteoclast vesicles made it clear that this cotransport includes primarily chloride (Blair et al., 1991). A Cl^- channel was isolated from the avian osteoclast ruffled border (Schlesinger et al., 1997), which is a homologue of a human intracellular chloride channel 5 (CLIC5) belonging to a family of proteins which form chloride channels in their membrane conformation (Heiss & Poustka, 1997; Ashley, 2003). These proteins are structurally related to the omega family of glutathione S-transferases, and are required for development in *C. elegans* (Berry et al., 2003). However, transgenic mice deficient in an unrelated and also widely expressed chloride transporter, CLCN7 (Brandt and Jentsch, 1995), are osteopetrotic (Cleiren et al., 2001; Kornak et al., 2001). Polymorphisms in CLCN7 are associated with osteopetrosis in several families as reported in a number of studies (Blair et al., 2004). However, CLCN7 is a chloride-proton antiporter (Picollo & Pusch, 2005; Scheel, et al., 2005) rather than a chloride channel. This complicates the model for osteoclastic acid secretion a bit in that a chloride-proton antiporter will not transport chloride without a significant H^+ gradient (Diewald et al., 2002: Accardi et al., 2005). In an elegant experiment correcting CLCN7 expression in osteoclasts rescued bone metabolism and uncovered an underlying lysosomal defect (Kasper et al., 2005). Thus it is clear that multiple Cl^- transporters play important roles in osteoclast function (Jentsch et al., 2005). The CLIC family of intracellular proteins, which are chloride channels, have been identified with acidification in osteoclasts for some time (Blair & Schlesinger, 1990: Schlesinger et al., 1997). Recently, CLIC5, has been directly implicated in osteoclast bone resorption and H^+ transport (Edwards et al., 2006). In combination the CLCN7 exchanger and CLIC5 provide a H^+ leak and charge neutralization that are important in acidification [(Grabe & Oster, 2001).

This would extend our model of balanced HCl transport for mineral dissolution, but additional studies are required to understand the integration of this model (Blair et al., 2002), Figure 2. There is a pervasive cytoskeletal-src dependence of proper targeting for the ion transporters of osteoclasts (Zuo et al., 2006: Tehrani et al., 2006: Abu-Amer et al., 1997: Soriano et al., 1991). The actin-directed disposition of CLIC protein has also been observed in microvilli of placental cells (Berryman et al., 2004). In osteoclasts the coordinated disposition of V-ATPase and CLIC required for full expression of the bone resorption phenotype (Edwards et al., 2006). It is clear that much of the osteoclasts organization exists to support the massive acid secretion for bone calcium solubilization.

To complete bulk calcium transport, the high calcium solution at the osteoclast attachment (Silver et al., 1988) must be moved to and diluted in the extracellular space. Some calcium may be released when the osteoclast detaches, occurring under normal circumstances at intervals of roughly one day, but the volume of solublized bone producing calcium and phosphate is too great for this to be

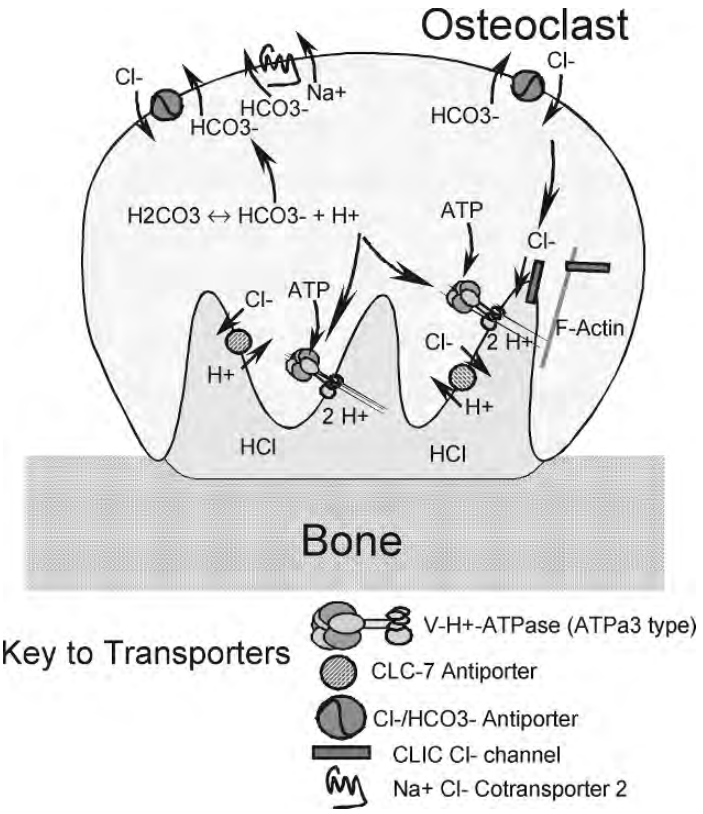

Figure 2. Bulk calcium transport by the osteoclast. Net acid transport is driven by the vacuolar-type H^+-ATPase with a specialized large membrane subunit. Transport is balanced by chloride transport, probably involving both a chloride channel (CLIC-5) and a chloride bicarbonate antiporter (CLCN7). Supporting transport processes include chloride-bicarbonate exchange. Insertion of transporters is specific for subcellular locations and involves interaction of transporters with specific cytoskeletal components, including actin (See Colour Plate 29)

the sole mechanism. Studies using confocal imaging and labeled matrix showed that the bulk transport of calcium and other degraded membrane components by vacuolar transcytosis through the osteoclast plays a role in this process (Nesbitt and Horton, 1997; Salo et al., 1997). However, there is also good evidence for calcium binding proteins and an epithelial calcium channel, TRPV5, that support a calcium-ferry mechanism (van der Eerden et al., 2005). The osteoclast has several other calcium transport proteins, including a Ca^{2+} ATPase (Bekker and Gay, 1990), which is expressed highly, as expected for a cell which may undertake significant cytoplasmic calcium transcytosis. Contrariwise, knockout of the TRPV5 calcium channel does not cause osteopetrosis, although rickets and hyperparathyroidism occur (Renkema et al., 2005). The massive calcium movement occurring

in bone resorption undoubtedly requires that the osteoclast have a mechanism for protecting cytoplasmic and organelle calcium content even if the bulk transport is vesicular. Therefore understanding the parallel contributions of transcytosis and cytoplasmic transport will require a quantitative analysis of their relative activities.

5. CALCIUM-DEPENDENT CELLULAR REGULATION IN THE OSTEOCLAST

5.1. Ca^{2+} Signalling in Osteoclasts

Osteoclasts show a cell membrane Ca^{2+} sensor function: this is not unexpected from the increases in ambient $[Ca^{2+}]$ to levels as high as 8–20 mM (Silver et al., 1988) following hydroxyapatite dissolution by osteoclastic activity. Thus, such extracellular $[Ca^{2+}]$ alterations alter bone resorptive activity in isolated rat osteoclasts (Malgaroli et al., 1989, Zaidi et al., 1989) following rapid and sustained changes in micro-spectrofluometrically determined cytosolic $[Ca^{2+}]$, cell retraction ('R' effect) and longer-term inhibition of enzyme release and bone resorption (Datta et al., 1989a correct to 1989, Zaidi et al., 1989, Moonga et al., 1990; Zaidi, 1990). These findings suggest an existence of long and short term feedback mechanisms on both enzyme release (Zaidi et al., 1989; Moonga et al., 1990) and osteoclastic bone resorptive activity (Datta et al., 1989) controlled by the increases in extracellular $[Ca^{2+}]$ that result from the latter process.

Cell physiological studies using fura-2 fluorescence to measure increases in free cytsolic $[Ca^{2+}]$ suggested that such signals likely arise both through release of intracellularly stored Ca^{2+} and extracellular Ca^{2+} entry. Thus, ionomycin applications elicited cytosolic $[Ca^{2+}]$ transients in osteoclasts exposed to Ca^{2+}-free external solutions that fully recovered to baseline, persisted following surface membrane potential manipulations but disappeared following repeated ionophore application as would have been expected by progressive depletion of Ca^{2+} stores. Restoration of extracellular $[Ca^{2+}]$ then elicited cytosolic $[Ca^{2+}]$ overshoots consistent with the capacitative Ca^{2+} entry reported in other cells. In contrast Ca^{2+} transients in osteoclasts studied in Ca^{2+}-containing bathing solutions decayed to sustained levels and persisted despite repeated ionophore application. (Shankar et al., 1994).

5.2. Evidence for a Surface Membrane Ca^{2+} Receptor (CaR)

Neither the increase in cytosolic $[Ca^{2+}]$ nor the associated cell retraction or inhibition of bone resorption appear to involve voltage-dependent L-type Ca^{2+} channels. These were largely insensitive to both dihydropyridine or phenylalkylamine Ca^{2+} channel specific reagents even under depolarizing conditions (Datta et al., 1990; see also: Zaidi et al., 1990). The available evidence favours specialist cellular mechanisms that sense ambient $[Ca^{2+}]$ that parallel situations described in

a number of other cell types, including CT-secreting thyroid parafollicular cells, parathyroid hormone (PTH)-secreting chief cells of the parathyroid gland (Brown et al., 1993), gastrointestinal enterocytes (Gama et al., 1997; Pazianas et al., 1995) renin-secreting renal juxtaglomerular and proximal tubular cells (Riccardi et al., 1995), neurones (Quinn et al., 1997), cytotrophoblasts (Lundgren et al., 1994), keratinocytes (Zaidi, 1990; Brown, 1991) and testicular Leydig cells (Adebanjo et al., 1998a).

Such a hypothesis would similarly suggest for the osteoclast an existence of *specific surface membrane* Ca^{2+} receptors (CaR) sensitive to higher, millimolar, $[Ca^{2+}]$ changes than some of the other examples suggested above. Nevertheless, one could then suggest an activation scheme in which Ca^{2+} acts both as extracellular regulator and intracellular messenger. This suggestion was compatible with the action of even some membrane-impermeant divalent or trivalent ions that similarly triggered cytosolic $[Ca^{2+}]$ changes: this would remain compatible with their interaction with a surface membrane CaR (Malgaroli et al., 1989; Zaidi et al., 1991; Zaidi et al., 1992a; Shankar et al., 1992a,b). Thus, applications of the divalent cation Ni^{2+} as surrogate extracellular trigger elicited rapid, concentration-dependent, cytosolic $[Ca^{2+}]$ elevations. These showed use-dependence inactivation, persisted despite extracellular $[Ca^{2+}]$ deprivation, and a dependence on agonist $[Ni^{2+}]$ suggesting a unity Hill coefficient. They were prevented by prior depletion of intracellular Ca^{2+} stores by ionomycin and modified by extracellular levels of the divalent cations, Ca^{2+} and Mg^{2+}. They were potentiated by acidification from pH 7.8 to 4 suggesting possible linkages between Ca^{2+} sensing and extracellular acidification. The latter effect persisted in Ca^{2+}-free, EGTA-containing solutions, implicating actions on the release of intracellularly stored Ca^{2+} as opposed to its entry from the extracellular space (Adebanjo et al., 1994). Finally, alterations of membrane voltage produced by altered extracellular $[K^+]$ in the presence of a valinomycin ionophore modified both the activation and inactivation kinetics of the $[Ca^{2+}]$ transients (Shankar et al., 1995a; Pazianas et al., 1993). Findings of this kind were clearly compatible with regulation of cytosolic $[Ca^{2+}]$ through a integral surface membrane receptor for the divalent cation regulators Ca^{2+} and Mg^{2+} that was also sensitive to the surrogate agonist Ni^{2+}, whose occupancy activated and subsequently inactivated release of intracellularly stored Ca^{2+}.

5.3. Functional Consequences of CaR Activation

Such a CaR activation were accompanied by a specific causally related set of functional and morphometric events culminated in a reduction of bone resorptive activity over hours (Bax et al., 1993; Bax et al., 1992; Zaidi et al., 1992b; Shankar et al., 1993) and reduced acid phosphatase release, whose extent depended on agonist concentration. These effects followed a pronounced cell retraction (R effect) but preserved granule movement, cell migration, and quantitative indicators of margin ruffling. Again, they could be reproduced following application of different alkaline

earth or other metal cations in a common potency sequence: $La^{3+} > Cd^{2+} > Ni^{2+} > Ca^{2+} > Ba^{2+} = Sr^{2+} > Mg^{2+}$ (Zaidi et al., 1991; Shankar et al., 1992a), again consistent with actions at a single surface membrane CaR (Zaidi et al., 1991).

The CaR activation mechanism also appeared to interact with other regulatory processes involving more systemic mechanisms that influence osteoclast activity. Physiological, femtomolar, CT concentrations reduced the sensitivity of the CaR system to both Ca^{2+} and Ni^{2+}. Amylin, calcitonin gene-related peptide, cholera toxin and dibutyryl-cAMP, all believed to act through the osteoclast cAMP signaling system exerted similar effects (Zaidi et al., 1996). Such cross coupling phenomena may also involve cytokine systems particularly those involving interleukin-6 (IL-6). Osteoclast surface membrane expresses IL-6 receptor, and IL-6 but not IL-11 reversed the inhibition of osteoclastic bone resorption induced by high extracellular Ca^{2+}. This effect was reversed by excess soluble IL-6 receptor. IL-6 also inhibited cytosolic $[Ca^{2+}]$ signals triggered by extracellular Ca^{2+} or Ni^{2+}. Conversely, elevated ambient $[Ca^{2+}]$ or their culture on a bony matrix increased osteoclastic IL-6 secretion as well as IL-6 receptor mRNA expression. Together such observations suggest that ambient $[Ca^{2+}]$ enhances secretion of IL-6 that in turn attenuates Ca^{2+} sensing and Ca^{2+} inhibition of bone resorption in an autocrine-paracrine loop that sustains osteoclastic activity despite local elevations in extracellular $[Ca^{2+}]$ generated by bone resorption. (Adebanjo et al 1998c).

Finally, both vitamin D-binding protein (DBP) and the macrophage-activating factor (DBP-MAF) left following removal of its sialic acid or galactose residue inhibit extracellular Ca^{2+} and cation sensing with the order of potency: native DBP = sialidase-treated DBP > beta-galactosidase-treated DBP (Adebanjo et al., 1998b).

5.4. Ca^{2+} Recovery in the Osteoclast

Mechanisms that then restore the basal cytosolic $[Ca^{2+}]$ levels remain unclear. Besides a Ca^{2+}-ATPase on the osteoclast dorsal surface relatively little is known of alternative or parallel methods for Ca^{2+} extrusion (Zaidi et al., 1993) although there is recent functional, evidence for a Na^+/Ca^{2+} exchanger that, in analogy to the regulation of cytoplasmic $[Ca^{2+}]$ in cardiac muscle could be linked to the proton extrusion that is a primary determinant of the rate and extent of bone resorption (Moonga et al., 2001).

In common with other cellular systems, refilling of Ca^{2+} stores following their release appears to depend upon a thapsigargin-sensitive Ca^{2+}-ATPase. Furthermore, such store depletion appears to induce a capacitative Ca^{2+} influx. Thus, the Ca^{2+}-ATPase inhibitor thapsigargin produced the expected elevation of cytosolic $[Ca^{2+}]$ in osteoclasts studied in Ca^{2+}-free extracellular solutions. Restoration of the extracellular $[Ca^{2+}]$ then produced a cytosolic $[Ca^{2+}]$ overshoot. Similar effects followed Ca^{2+} store depletion by ionomycin in cells bathed in EGTA-containing solutions when extracellular $[Ca^{2+}]$ was similarly restored. Both studies suggested capacitative Ca^{2+} influx processes from the extracellular space by a cytosolic route (Shankar et al., 1994).

5.5. Functional Evidence for Ryanodine Receptor (RyR) Participation in Osteoclast Ca^{2+} Sensing

Increasing evidence implicates the ryanodine-receptor (RyR) in the transduction processes described here. Initial indications for this came from experiments that investigated the effect of perchlorate ions, known to facilitates skeletal muscle excitation-contraction coupling processes through acting on the RyR-Ca^{2+} release channel, on osteoclast function. Its intravenous infusion reduced plasma [Ca] in rats. Perchlorate reduced in vitro cortical bone resorption, induced transient cytosolic free [Ca^{2+}] elevations, and marked and sustained cell retraction in isolated cultured osteoclasts whilst conserving cell motility parameters or of supernatant concentrations of tartrate-resistant (osteoclastic) acid phosphatase (Moonga et al., 1991). Conversely, the RyR-inhibitor dantrolene Na inhibits Ca^{2+}-induced cytosolic [Ca^{2+}] elevations (Miyauchi et al., 1990). Furthermore, ryanodine itself induced transient elevations of cytosolic [Ca^{2+}] in fura 2-loaded osteoclasts to extents dependent upon surface membrane potential. Conversely the RyR agonist caffeine triggered releases of intracellularly stored Ca^{2+} through a bell-shaped concentration-response curve that varied with extracellular [Ca^{2+}] whilst inhibiting Ni^{2+}-induced elevations in cytosolic [Ca^{2+}] [Shankar et al., 1995b].

These physiological findings prompted labeling and fluorescence studies that went on more directly to implicate a RyR, or at the very least a RyR-like molecule, that uniquely existed in the cell surface as opposed to the microsomal membrane of the osteoclast, in the process by which changes in extracellular [Ca^{2+}] become transduced into elevations of cytosolic [Ca^{2+}]. Certainly extracellular applications of the cell-impermeant RyR modulators ruthenium red and adenosine 3′,5′-cyclic diphosphate ribose (cADPr) both triggered elevations in cytosolic [Ca^{2+}] that were sensitive to manipulations of the surface membrane voltage. Both modulators additionally attenuated cytosolic [Ca^{2+}] responses to external Ni^{2+} applications (Adebanjo et al., 1996). This correlated well with binding and microscopy studies that demonstrated that [^{3}H]-ryanodine specifically bound to freshly isolated rat osteoclasts but was then displaced by ryanodine itself, the CaR agonist Ni^{2+} and the RyR antagonist ruthenium red. Labelled ryanodine inhibited but antisera raised to an epitope located within the channel-forming domain of the type II RyR potentiated Ni^{2+}-induced cytosolic Ca^{2+} elevations. Serial confocal sections and immunogold scanning electron microscopy localized a staining to the plasma membrane staining by antiserum directed to a putatively intracellular RyR epitope only staining fixed, permeabilized cells in a distinctive cytoplasmic pattern (Zaidi et al., 1995).

Additionally, possible roles for cADPr in regulation of a surface RyR were suggested by demonstrations of cytosolic mRNA for the multifunctional ADP-ribosyl cyclase, CD38 which catalyzes NAD^+ cyclization to cADPr, known in turn to gate Ca^{2+} release through microsomal membrane-resident ryanodine receptors (RyRs). Both confocal microscopy and Western blotting then localized the CD38 protein to the plasma membrane (Sun et al., 1999).

5.6. Is Ca^{2+} Sensing Coupled to Changes in Cytosolic [Ca^{2+}]?

A possible involvement of a unique cell surface-situated RyR by which extracellular [Ca^{2+}] is transduced into changes in intracellularly stored [Ca^{2+}] nevertheless leaves a number of mechanistic questions unresolved. It remains uncertain as to whether the RyR-2 also itself functions as the Ca^{2+} sensor, conceivably through its intraluminal low-affinity Ca^{2+} binding site (Anderson et al., 1989) or is coupled to a distinct intramembrane entity of the conventional 7-pass G-protein coupled types described elsewhere (Kameda et al., 1998). The mechanism by which such a surface event would induce a release of intracellularly stored Ca^{2+} remains unclear. Finally, the role of such a unique surface membrane site in the osteoclast for the RyR in longer term osteoclast regulation certainly merits further exploration (Adebanjo et al., 1999; Gerasimenko et al., 1995; Santella & Carafoli, 1997).

6. FINAL THOUGHTS

Calcium is a unique cation in living systems because of it dominant role in intracellular signaling. Therefore bone cells which must handle massive amounts of this mineral take special care in its regulation. Sustained elevation of intracellular calcium leads to cell death that is not effectively opposed by the usual regulators of apoptosis. We have undertaken to present what is known about how bone cells deal with calcium. However much remains to be learned and the acquisition of this knowledge will inform our treatment of many important medical conditions.

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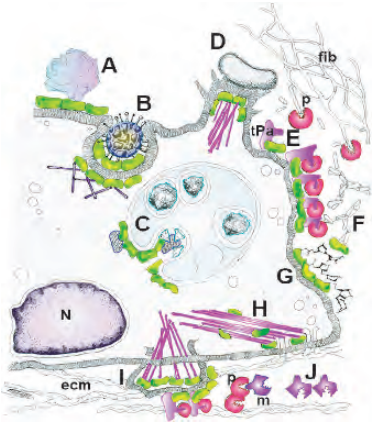

Plate 1. Schematic view of the putative roles of annexin 2 in disease pathology
A: Extracellular annexin 2 acts as a receptor for calcium oxalate crystals in collecting duct epithelial cells in Dent's disease. B: It acts as a receptor for respiratory syncytial virus and cytomegalovirus. C: It is necessary for maturation of the gag protein of HIV and virion maturation on the internal membranes of multivesicular bodies in macrophages. D: Annexin 2 is enriched in the PIP2 and actin-rich pedestals of non-invasive enteropathogenic E.coli. E: Annexin 2 acts as a 'fibrinolytic receptor' binding both tissue plasminogen activator (tPa) and Plasminogen (P) on the cell surface. Activated plamin (P) is then involved in degradation of fibrin (fib). F: Antibodies with specificity for annexin 2 (F) or the formation of Advanced Glycation Products (G) can interfere with the role of annexin 2 in fibrinolysis. Annexin 2 expression is often altered as normoplastic cells become cancer cells. This may reflect the role of annexin 2 in regulation of actin polymerisation. Dramatic changes in actin structure are associated with loss of focal adhesions (H) and the formation of invadopodia (I). Annexin 2 modulation of plamin activation (P) has knock-on effects on the activation of metalloproteinases (M) which degrade extracellular matrix (ECM) and encourage cellular migration. N is the nucleus. Annexin 2 is coloured green throughout (See Chapter 1 Figure 1, p. 3.)

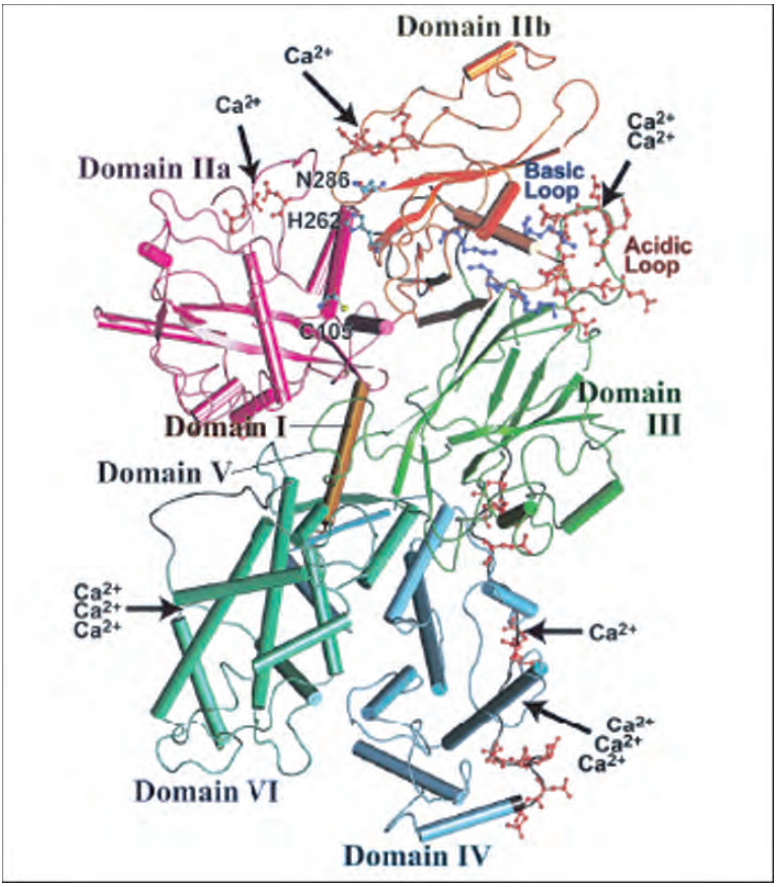

Plate 2. 3D structure of heterodimeric m-calpain, from Suzuki et al., 2004 (See Chapter 2 Figure 1, p. 32.)

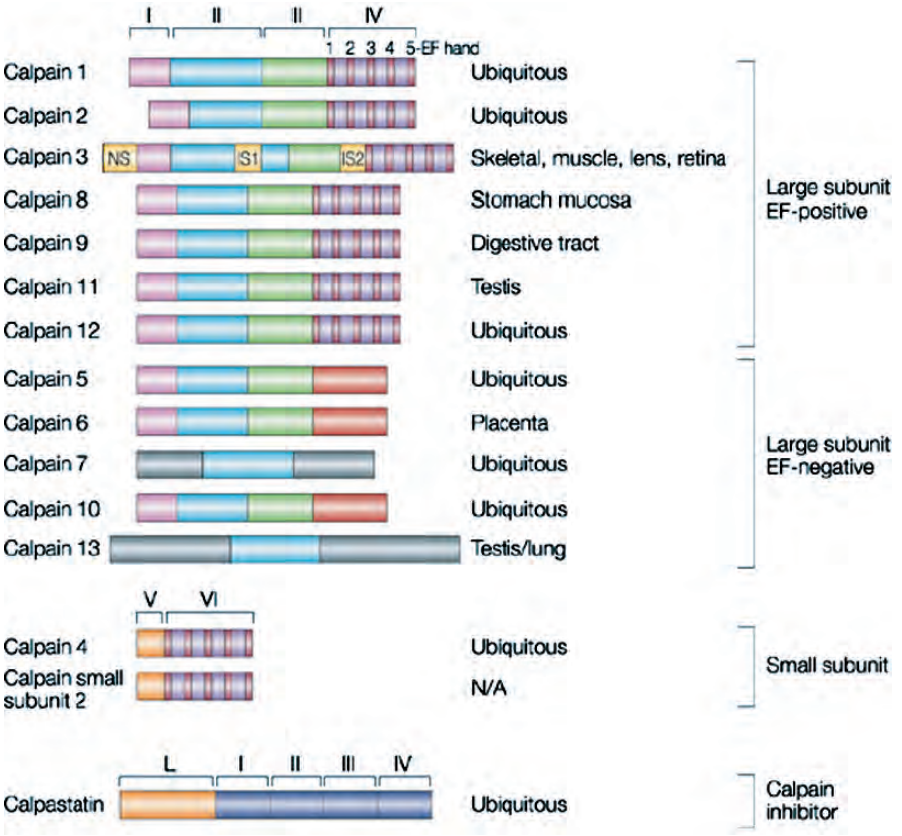

Plate 3. Domain organization of calpains from Branca, et al., 1999 (See Chapter 2 Figure 2, p. 34.)

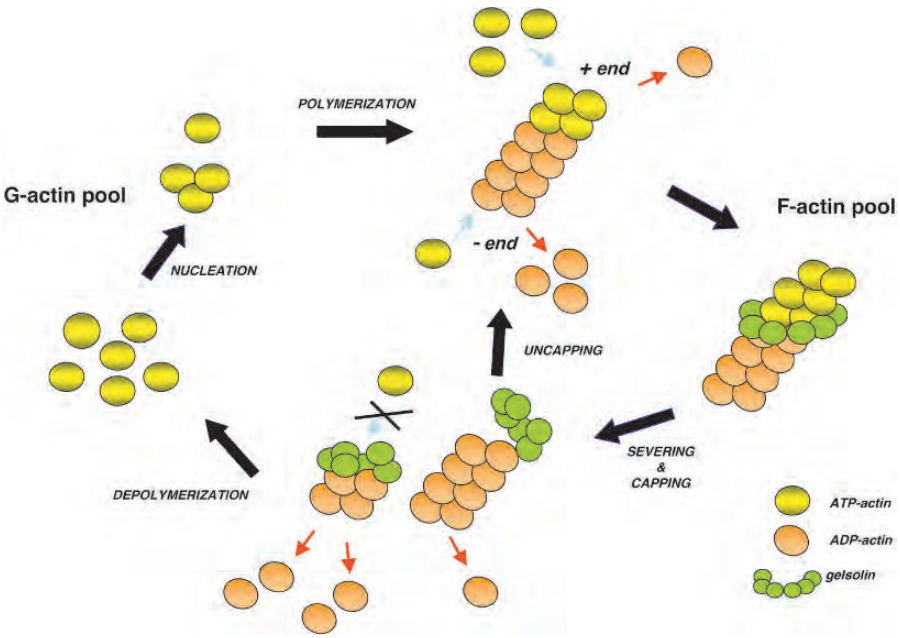

Plate 4. The life cycle of actin filaments.
Actin dynamics follows a complex kinetic that is regulated by actin binding proteins. ATP-bound globular actin (G-actin) first forms a trimer in a nucleation step after which rapid polymerization occurs into filamentous actin (F-actin). Due to the intrinsic ATP ase activity of actin the growing filament comprises an ATP-actin cap and an aged ADP-actin tail. Gelsolin preferentially binds to the ADP-actin containing filaments and severs them. Gelsolin then remains attached to the + end (barbed end) of filaments as a cap blocking further addition of actin monomers. Uncapping of gelsolin can liberate polymerization competent filament ends and thereby induce actin polymerization (See Chapter 3 Figure 1, p. 56.)

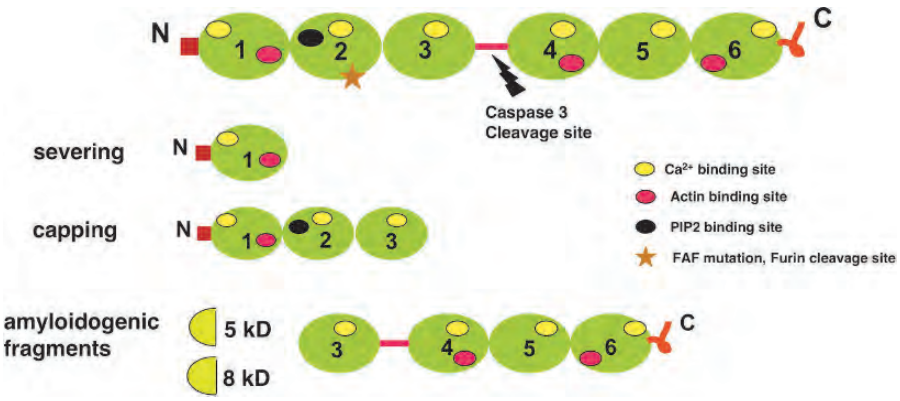

Plate 5. Gelsolin structure.
Gelsolin is composed of six domains, named S1-S6, that have arisen from of an ancestral gene encoding a protein of about 15 kDa. Gelsolin activity is regulated by calcium binding (yellow ovals) and by phosphatidylinositol 4,5-bisphosphate (PIP2) binding (black ovals). The isolated domain S1 contains severing activity, while domain S1-3 has capping activity. Biochemicals studies have identified three actin-binding sites (red ovals), a caspase-3 cleavage site (black arrow) and a furin cleavage site. In the Finnish type familial amyloidosis (FAF) a point mutations in gelsolin (orange star) activates the furin cleavage site eventually leading to aberrant intracellular cleavage and the production of amyloidogenic fragments (See Chapter 3 Figure 2, p. 57.)

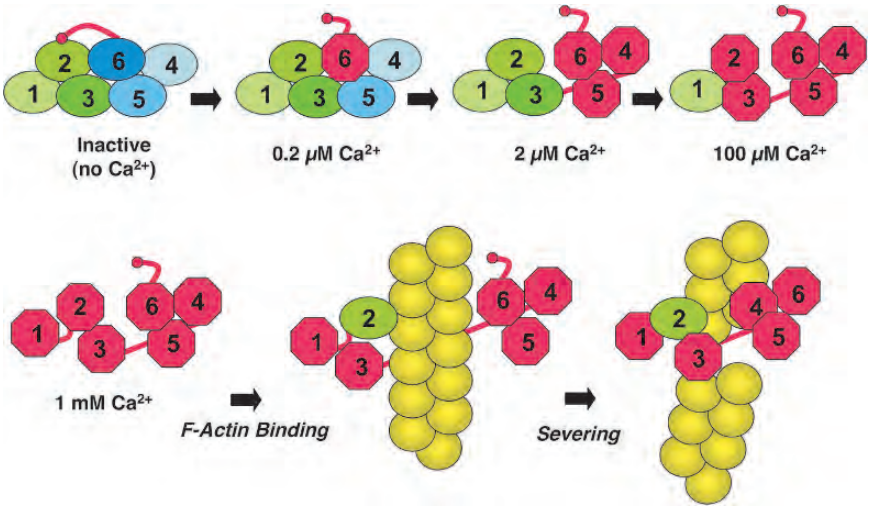

Plate 6. Gelsolin activation by Ca^{2+}.
Gelsolin is a Ca^{2+-}-activated actin filament severing protein. Depending on the Ca^{2+-}-concentration, gelsolin becomes gradually activated by controlled unfolding of the individual domains. Ca^{2+} opens up gelsolin by inducing a conformational change in the C-terminus (S4-6) to expose the actin binding sites on the N-terminus. This is called the "tail latch hypothesis".
In the presence of low calcium concentration, Ca^{2+} binds to the S6 domain, induces release of the C- tail domain and frees the actin binding sites present in domains S1-2. In order for severing to occur, higher concentrations of the ion are required. Additional calcium binding, most likely to domains S1, S2 and S4, fully activates the molecule, thus allowing severing to take place.
Activated gelsolin domains are shown as red hexagons and calcium ion concentrations required for each conformation change are indicated. F-actin is shown in yellow (See Chapter 3 Figure 3, p. 59.)

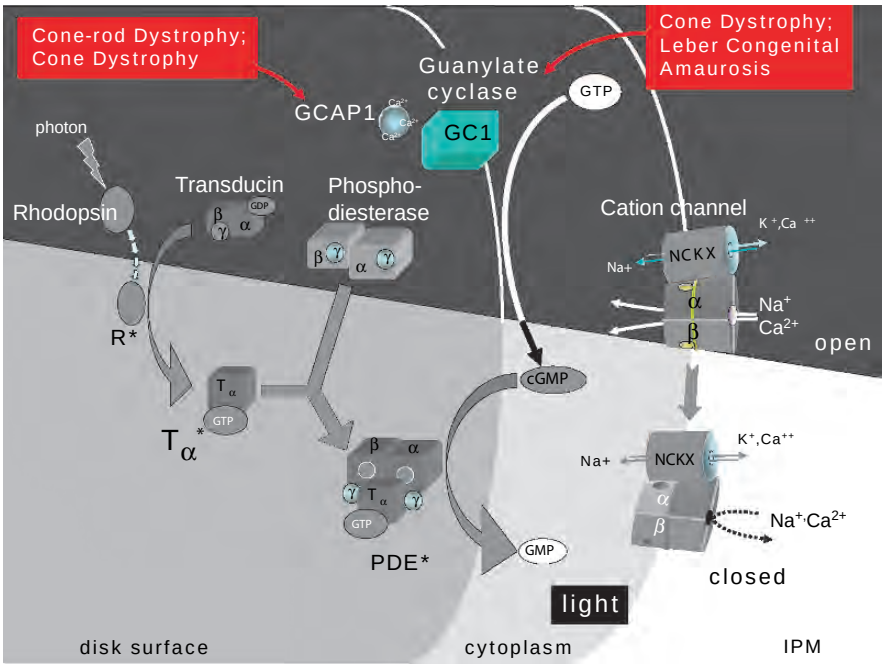

Plate 7. The rod phototransduction cascade and human diseases associated with mutations in GC1 and GCAP1. The main proteins of the cascade are shown in gray. GC1 and GCAP1 represent the main components of the Ca^{2+}-regulated GC in rods and cones responsibel for cGMP synthesis (See Chapter 4 Figure 2, p. 73.)

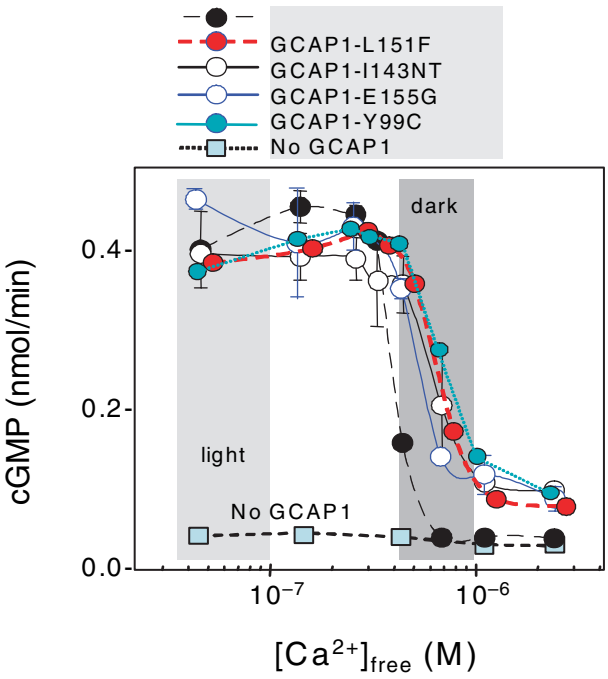

Plate 8. Stimulation of GC activity in ROS membranes by normal and mutant GCAP1s as a function of Ca^{2+}. The dark shaded area indicates low $[Ca^{2+}]_{free}$ expected in the dark-adapted photoreceptors, the lighter shaded area reflects high $[Ca^{2+}]_{free}$ expected in the light. Mutant GCAP1s are active at Ca^{2+} concentrations which inactivate normal GCAP1 (See Chapter 4 Figure 8, p. 83.)

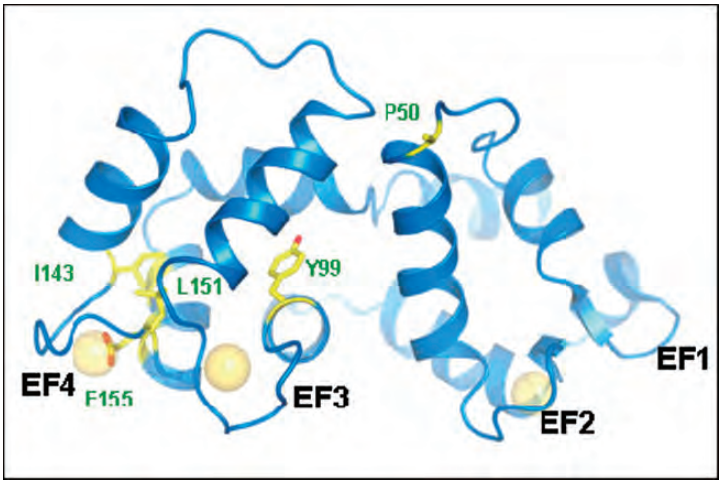

Plate 9. The structure of GCAP3 (Stephen et al., 2006). Residues that correspond to pathogenic mutation in GCAP1, the structure of which is unknown, is shown in yellow with the side chains. Ca^{2+} is shown as yellow spheres. Molecular graphics representations were created with PYMOL (Warren L. DeLano "The PyMOL Molecular Graphics System." DeLano Scientific LLC, San Carlos, CA, USA. http://www.pymol.org) (See Chapter 4 Figure 9, p. 84.)

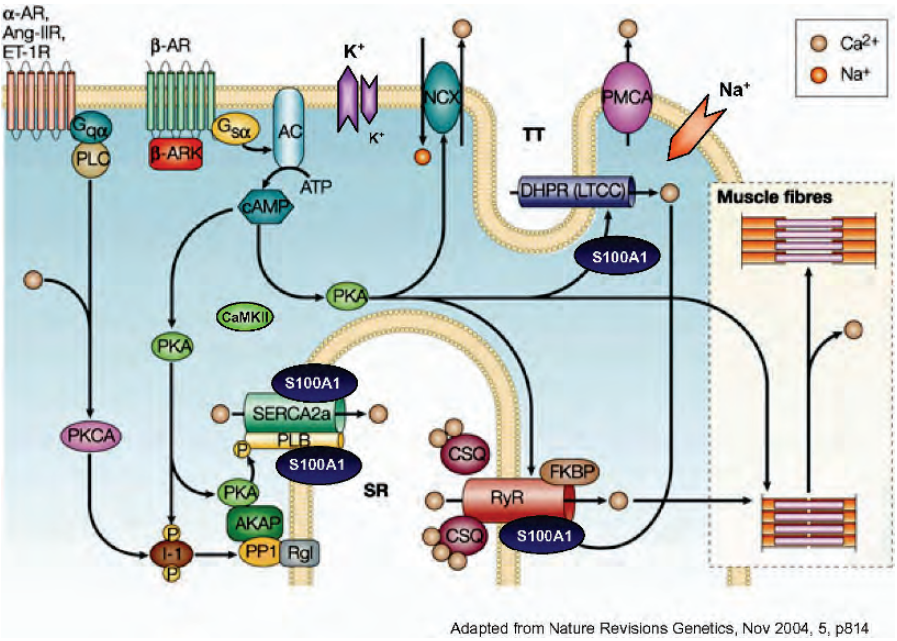

Plate 10. Calcium cycling in the heart: The role of S100A1
Legend: S100A1 occurs in the cytoplasm and was shown to physically interact and functionally modulate the sarcoplasmatic reticulum calcium ATPase (SERCA2a) and the ryanodine receptor (RyR). In addition, S100A1 can bind phospholamban (PLB). Our preliminary in vivo results indicate that loss of S100A1 results in reduced Ca^{2+}-induced Ca^{2+} release and prolonged Q-T intervals upon (-adrenergic stimulation (Ackermann et al. 2006, unpublished data). Abbreviations: adenylate cyclase (AC), A-kinase anchoring protein (AKAP), angiotensin II receptor (Ang-IIR), alpha and beta adrenergic receptor (α/β-AR), beta adrenergic receptor kinase (β-ARK), calcium/calmodulin-dependent protein kinase II (CaMKII), calsequestrin (CSQ), dihydropyridine receptor (DHPR) or L-type calcium channel (LTCC), endothelin-1 receptor (ET-1R), FK506-binding protein (FKBP), G-proteins (Gqα and Gsα), phosphatase inhibitor 1(I-1), sodium/calcium exchanger (NCX), phospholipase C (PLC), plasma membrane calcium ATPase (PMCA), protein kinase A (PKA), protein kinase C alpha (PKCA), protein phosphatase 1 (PP1), PP1 regulatory subunit 3A (Rgl), sarcoplasmatic reticulum (SR), transverse tubule (TT) (See Chapter 5 Figure 1, p. 106.)

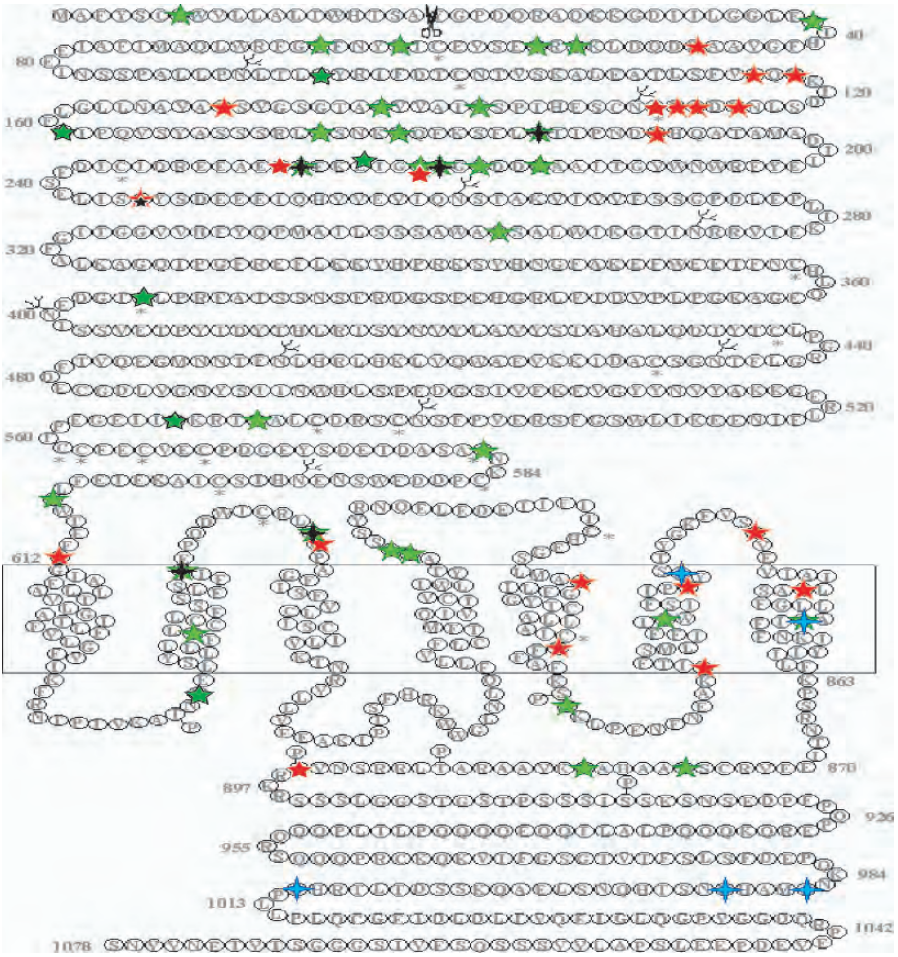

Plate 11. Topology of the CaR showing naturally occuring mutations
Scissors indicate end of signal peptide, circled P's at amino acids T646, S794, S915, S895, T888 are protein kinase C sites, blue symbols are polymorphisms, green are inactivating mutations, red are activating mutations, and black on top of green are two inactivating mutations (See Chapter 6 Figure 1, p. 142.)

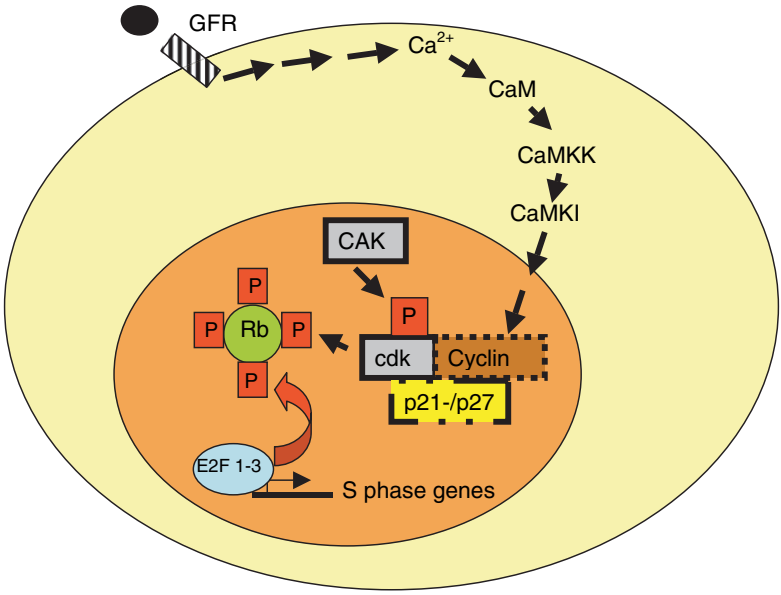

Plate 12. Schematic representation of the activation of the cdk4/cyclin D complex during G1. Evidence shows that CaMKI alpha, which can be activated by either CaMKK alpha or beta, activates the cdk4/cyclin D complex, leading to the phosphorylation of Rb and therefore de-repression of E2F transcription factor members. The mechanism by which CaMKI regulates cdk4/cyclin D remains to be determined. GFR, receptor for a growth factor; CAK, cdk activating kinase; p21/p27, members of the inhibitory Cip and Kip families, respectively; E2F 1-3, transcription factors 1-3 of the E2F family; Rb, Retinoblastoma protein; P, phosphorylation event (See Chapter 7 Figure 2, p. 178.)

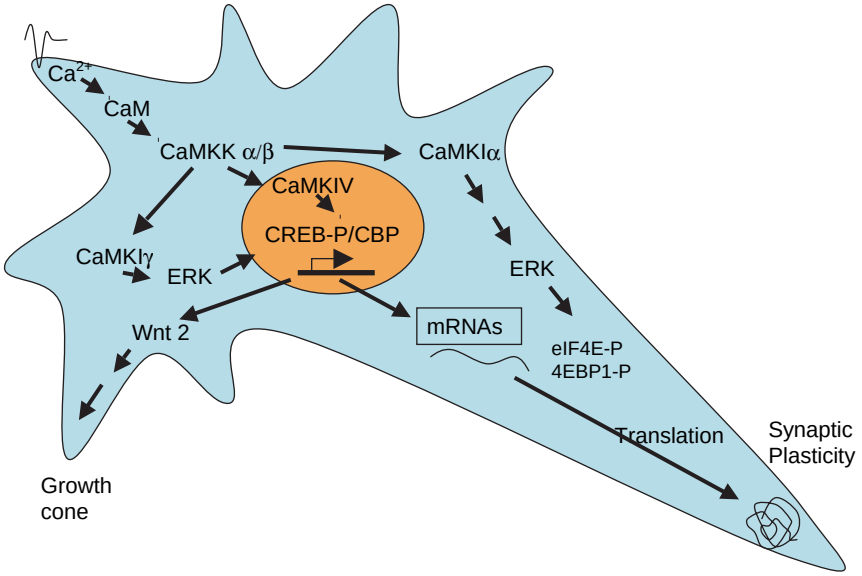

Plate 13. Neuron representing different CaMK cascade pathways that might be activated after electrical stimulation of the cell. Electrical stimulation (represented as a wave on the top left corner) induces a surge of Ca^{2+}, which binds to calmodulin and activates CaMKK a or β, which in turn activate CaMKIV (both activate CaMKIV, although in different neuron types), which proceeds to induce the expression of some genes, possibly by CREB phosphorylation. On the other hand, a CaMKK has been proposed to activate CaMK1 α, which would crosstalk with the MAPK/ERK pathway and enhance translation of the mRNAs whose synthesis is regulated by the CaMKIV pathway. The production of such proteins is necessary for synaptic plasticity. Also, there is evidence that the CaMKK/CaMKI γ pathway is involved in dendrite growth, in particular stimulating the growth cone of the dendrites through activation of Wnt 2 expression. This figure a summary of data collected from the different mouse models and neuron types studied, and details of some of the CaMKK/CaMKI pathways still require clarification (See Chapter 7 Figure 3, p. 188.)

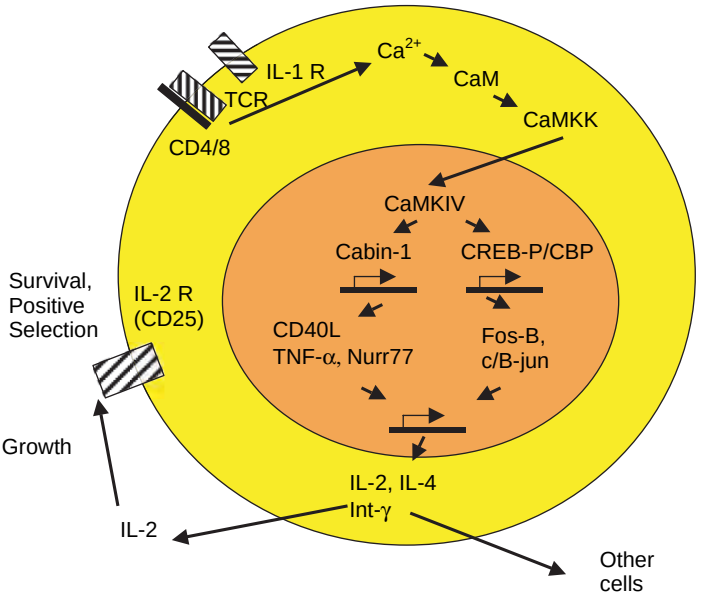

Plate 14. Schematic representation of the Ca^{2+}/CaM/CaMKK/CaMKIV/CREB/immediate early genes/IL pathway in T cells. Activation only takes place after the triple stimulation of the TCR, IL-1R and CD4/8 receptors. The end point effect of CaMKIV, which is the synthesis of interleukins and other growth factors, is common to T cells and thymocytes. Interleukines and other growth factors may have an autocrine or a paracrine effect. The different pathways included here have been addressed using Camk4-/- mice models. Since the role of the CaMK cascade in hematopoietic stem cells and neutrophils is a little different, they are not included here (See Chapter 7 Figure 4, p. 197.)

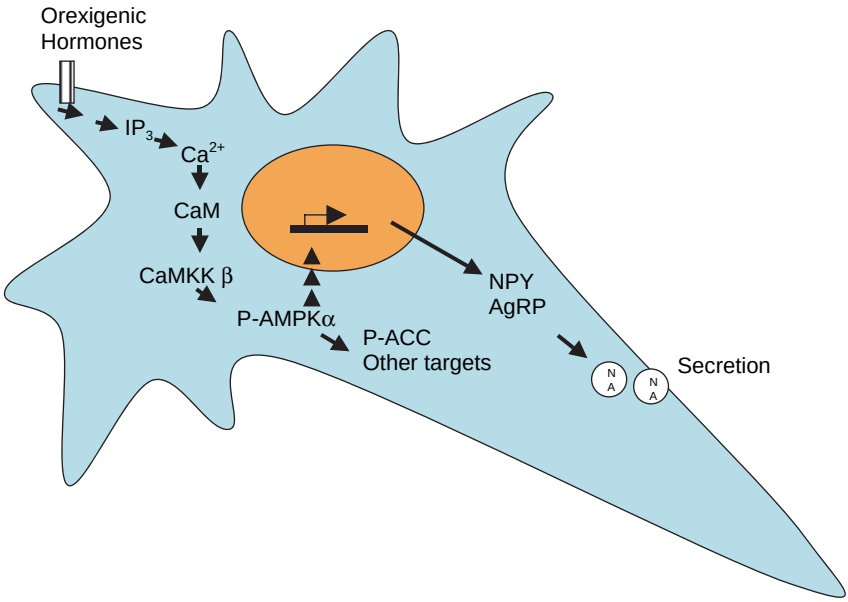

Plate 15. Arcuate nucleus neuron representing a CaMKKβ/AMPK cascade pathway that might be activated after hormonal stimulation. Stimulation by hormones such as ghrelin induce a surge in cytoplasmic Ca^{2+}, which binds to calmodulin and activates CaMKKβ, which in turn activates AMPK a by phosphorylation. Active AMPK can either promote transcription of some orexygenic factors such as neuropeptide Y (NPY) and agouti-related peptide (AgRP), or can phosphorylate metabolic enzymes such as acetyl-CoA carboxylase (ACC). Both NPY (N) and AgRP (A) are paracrine factors that will target other neurons of the central nervous system. This pathway is based on observations in Camkk2–/– mice as well as on several cell lines. Intermediate steps that are currently unknown are represented by several arrows in a row (See Chapter 7 Figure 5, p. 204.)

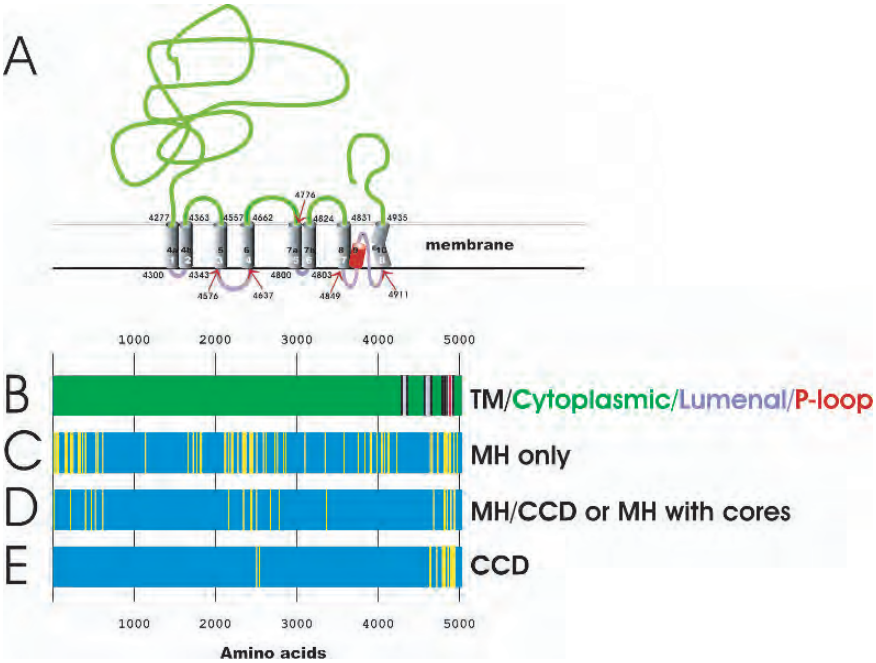

Plate 16. RyR1 Sequences and Disease Mutations. A) Arrangement of RyR1 in the SR membrane, indicating the transmembrane domains. B) Location of transmembrane, cytoplasmic, P-loop, and lumenal regions in the primary sequence of RyR1. C-E) Location of MH, MH + CCD, and CCD mutations in the primary amino acid sequence of RyR1 (See Chapter 10 Figure 1, p. 276.)

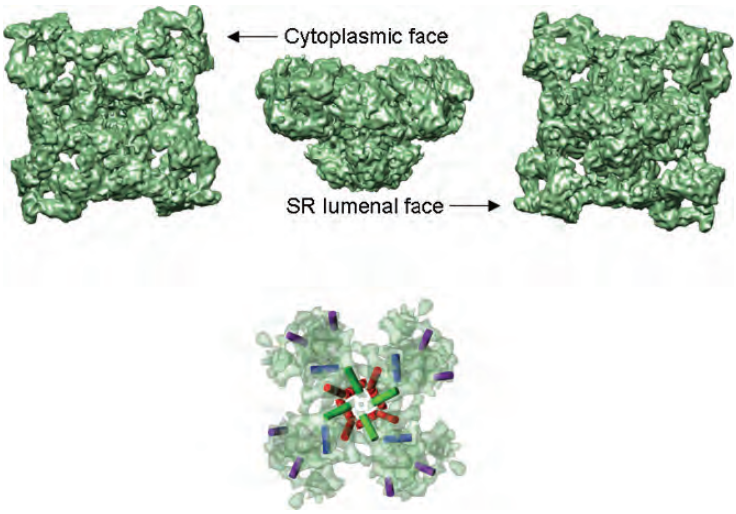

Plate 17. RYR1 structure at 9.5 Å. The color ribbons represent a helices, the inner helix is labeled red, the pore helix green, and other helices are colored blue or purple (See Chapter 10 Figure 2, p. 276.)

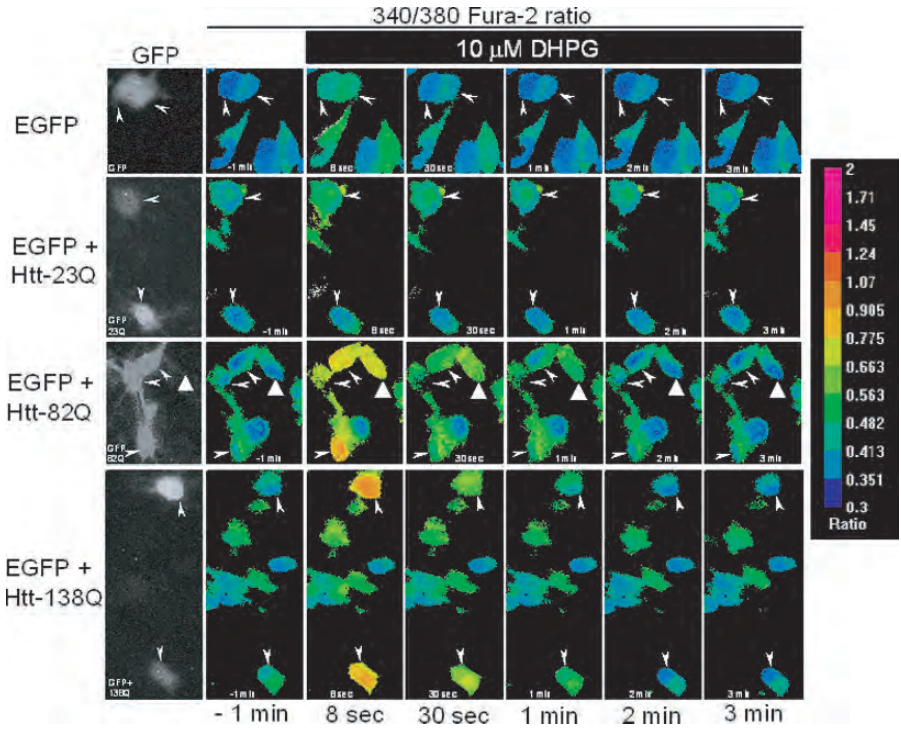

Plate 18. Htt^{exp} facilitates $InsP_3R1$-mediated Ca^{2+} release in cultured MSN.
The images show Fura-2 340/380 ratios in transfected rat MSN. Pseudocolor calibration scale for 340/380 ratios is shown on the right. The recordings are performed in Ca^{2+}-free ACSF containing 100 μM EGTA. GFP images (1st column) were captured before Ca^{2+} imaging to identify transfected cells (arrowheads). $InsP_3R1$-mediated Ca^{2+} release is initiated by addition of 10 μM DHPG, a specific mGluR1/5 agonist. Ratio recordings are shown for DHPG-induced Ca^{2+} transients in MSN neurons transfected with EGFP (first row), EGFP + Htt-23Q (second row), EGFP + Htt-82Q (third row), and EGFP + Htt-138Q (fourth row). The 340/380 ratio images are shown for MSN neurons 1 min before (2nd column), and 8 sec, 30 sec, 1 min, 2 min, and 3 min after application of 10 μM DHPG as indicated. Adapted from (Tang et al., 2003) (See Chapter 11 Figure 3, p. 326.)

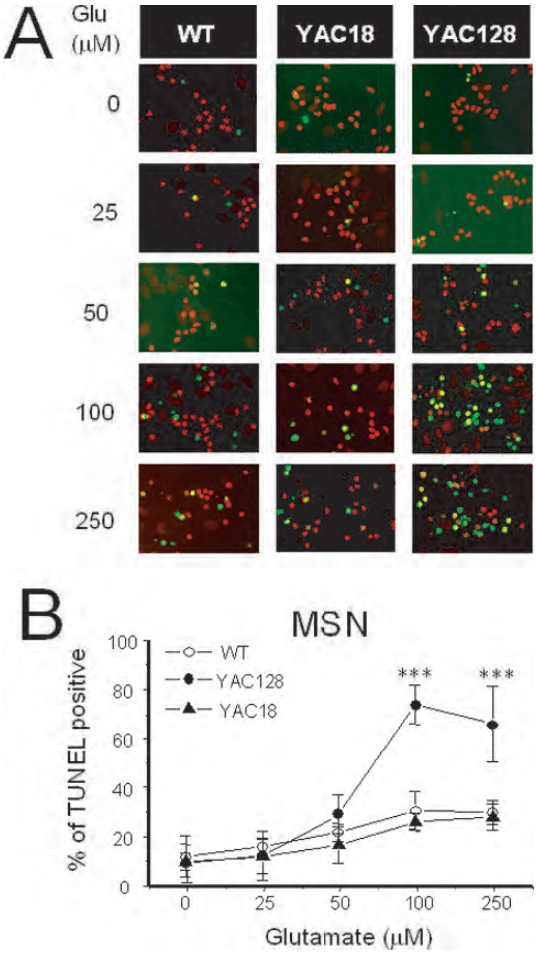

Plate 19. In vitro HD assay.
(A) 14 DIV MSN from wild type (WT), YAC18 and YAC128 mice were exposed to a range of glutamate concentrations for 8 h, fixed, permeabilized and analyzed by TUNEL staining (green) and propidium iodide counterstaining (PI). (B) The fraction of TUNEL-positive MSN nuclei was determined as shown on panel A and plotted against glutamate concentration for wild type (WT) (open circles), YAC128 (filled circles), and YAC18 (filled triangles) mice. At each glutamate concentration the data are shown as mean ± SD (n = 4–6 microscopic fields, 200–300 MSN per field). At 100 μM and 250 μM glutamate the fraction of TUNEL-positive MSN is significantly ($p < 0.05$) higher for YAC128 than for WT or YAC18. Similar results were obtained with 10 independent MSN preparations. Adapted from (Tang et al. 2005) (See Chapter 11 Figure 5, p. 330.)

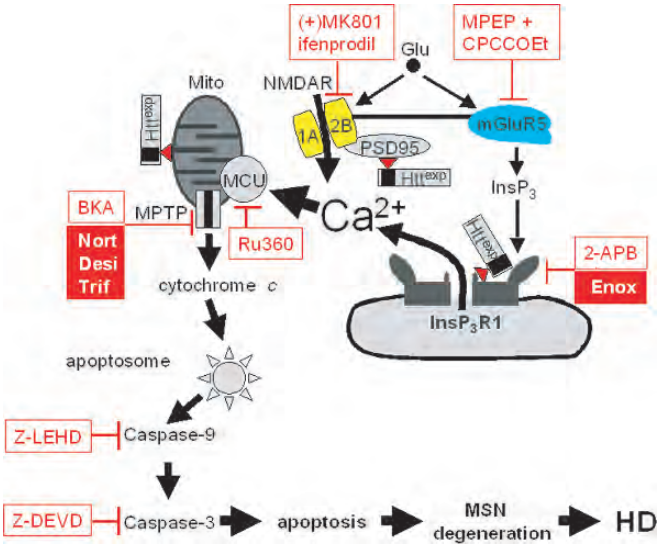

Plate 20. Ca^{2+} hypothesis of HD.
Glutamate released from corticostriatal projection neurons stimulates NR1A/NR2B NMDAR and mGluR5 receptors abundantly expressed in striatal MSN (Landwehrmeyer et al., 1995; Testa et al., 1995). Htt^{exp} affects Ca^{2+} signaling in HD MSN by sensitizing $InsP_3R1$ to activation by $InsP_3$ (Tang et al., 2003), stimulating NR2B/NR1 NMDAR activity (Chen et al., 1999; Sun et al., 2001; Zeron et al., 2002), and destabilizing mitochondrial Ca^{2+} handling (Panov et al., 2002; Choo et al., 2004). As a result, stimulation of glutamate receptors leads to supranormal Ca^{2+} responses in HD MSN and mitochondrial Ca^{2+} overload. Once mitochondrial Ca^{2+} storage capacity is exceeded, mitochondrial permeability transition pore (MPTP) opens, leading to release of cytochrome c into the cytosol and activation of caspases 9 and 3. Activation of caspase-3 leads to progression of apoptosis, MSN degeneration and HD. The model is supported by ability of blockers (shown in red) to reduce glutamate-induced apoptosis of YAC128 MSN to wild type levels in our experiments. The blockers which were effective in our experiments are: NMDAR blocker (+)MK801 and NR2B-specific blocker ifenprodil; mGluR1/5-specific blockers MPEP and CPCCOEt; membrane-permeable $InsP_3R1$ blockers 2-APB and Enoxaparin; MCU blocker Ru360, MPTP blockers BKA, Nortriptyline, Desipramine and Trifluoperazine, membrane-permeable caspase-9 blocker Z-LEHD-FMK and caspase-3 blocker Z-DEVD-FMK. Adapted from (Tang et al., 2005) (See Chapter 11 Figure 6, p. 331.)

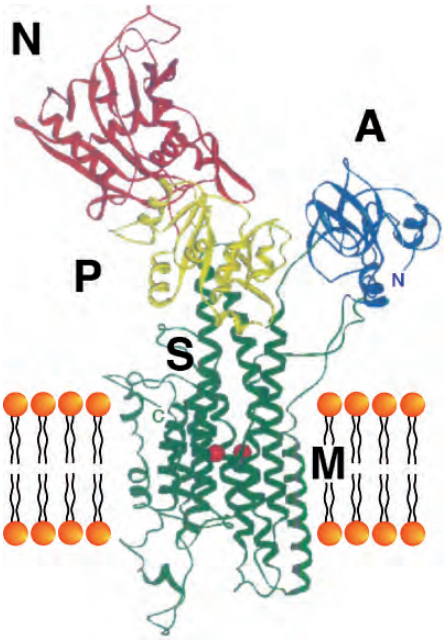

Plate 21. Ribbon diagram showing the different domains in the crystal structure of the rabbit skeletal muscle Ca^{2+}-ATPase (SERCA1) at 2.6 A resolution. The cytoplasmic region of the molecule is organized in three interacting domains: the nucleotide-binding (N) domain in red, which binds ATP; the phosphorylation (P) domain in yellow encompassing the phosphorylation site at Asp351, and the actuator (A) domain in blue. The transmembrane domain (M) in green comprises ten SER-transmembrane domains (M1–M10), four of which (M4, M5, M6 and 8) form the Ca^{2+} (red spheres) binding pocket. Stalk domains (S) link cytoplasmic domains to transmembrane domains. Major conformational changes occur during ATP-energized Ca^{2+} transport (Reprinted from (MacLennan et al., 2002) with permission from Elsevier) (See Chapter 12 Figure 2, p. 342.)

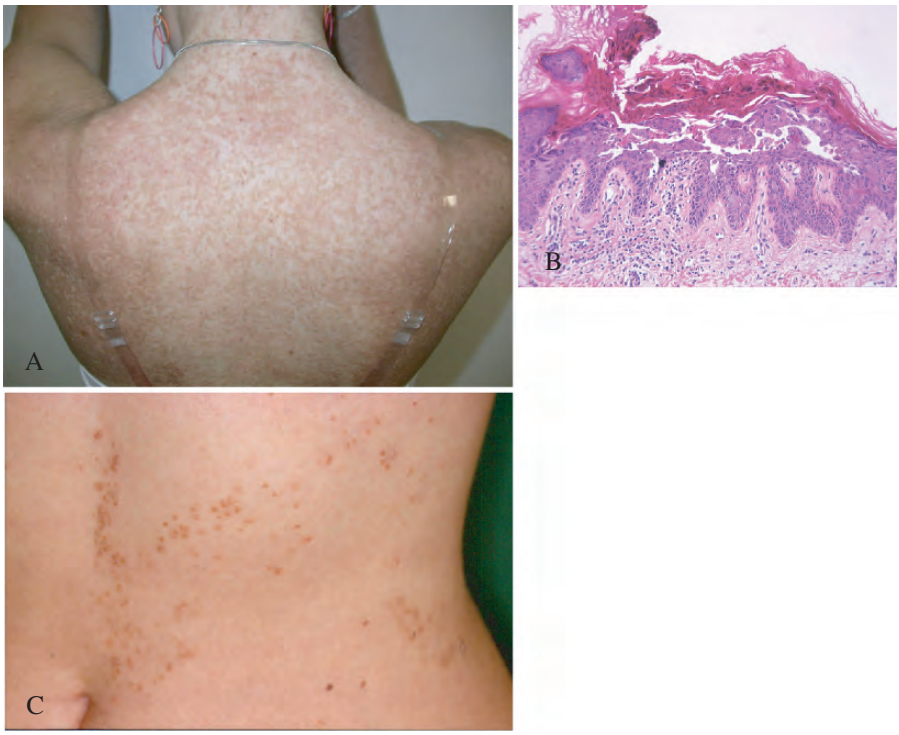

Plate 22. **a**, Clinical aspect of Darier disease showing extensive and confluent keratotic and inflammatory papules on the back of a 27 years old patient; **b**, Histology of affected skin in Darier disease showing suprabasal cleft of the epidermis containing acantholytic cells, associated with hyperkeratosis and rounded dyskeratotic cells (« corps ronds » or « grains »); **c,** Acantholytic epidermal naevi (segmental Darier disease). Limited and unilateral distribution of keratotic papules following Blaschko lines on the lower abdominal region. These lesions correspond to somatic mosaicism for an ATP2A2 mutation. (Courtesy of Dr Susan Burge, Department of Dermatology, Churchill hospital, Oxford, UK) (See Chapter 12 Figure 4, p. 349.)

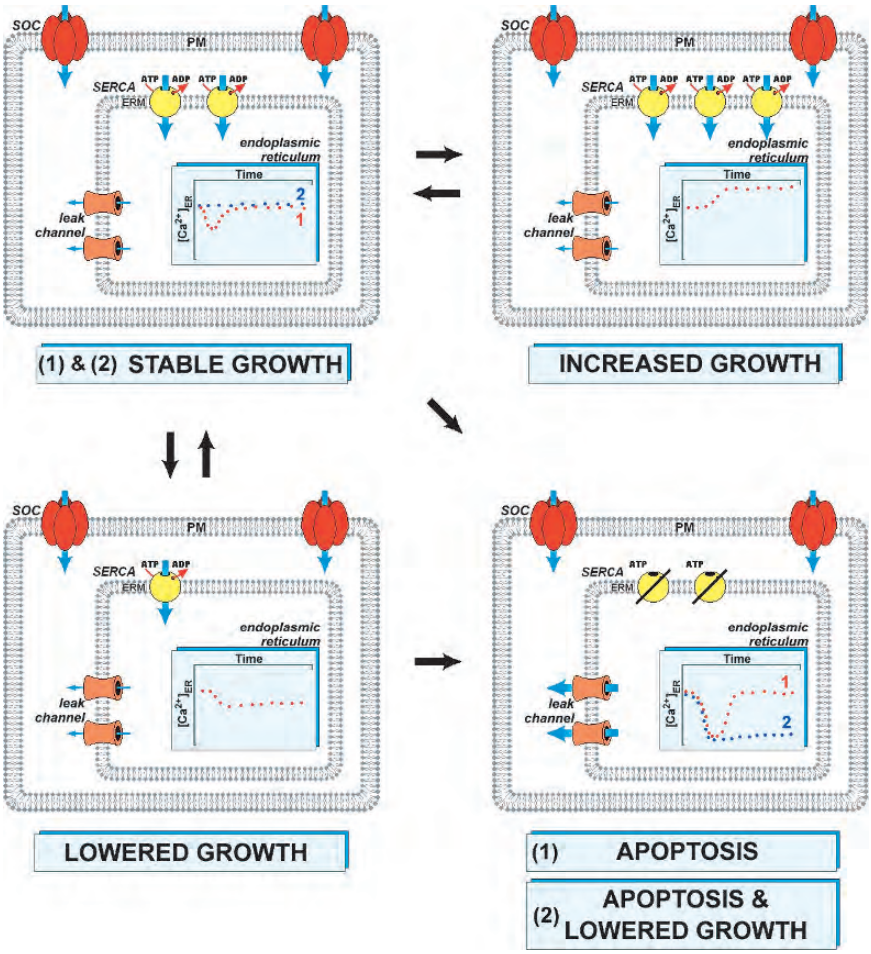

Plate 23. Endoplasmic reticulum Ca^{2+} pool as a key determinant of cell destiny. Each panel shows cellular plasma membrane (PM) and endoplasmic reticulum membrane (ERM) incorporating key Ca^{2+}-handling molecules: store operated channels (SOC), SERCA pump and ER leak channels. The inset graphs show ER calcium concentration ($[Ca^{2+}]_{ER}$) characteristic of each depicted state. **Stable growth** (upper left panel) is characterized by balanced expression of the SERCA pump and ER leak channel that provide optimal basal calcium filling of the ER (blue symbols marked by "2" in the inset graph). Transient decreases in the $[Ca^{2+}]_{ER}$ caused by physiological stimuli (red symbols marked by "1" in the inset graph) do not impair stable growth. **Increased growth** (upper right panel) is characterized by sustained elevation in $[Ca^{2+}]_{ER}$ (red symbols in the inset graph) mainly due to SERCA pump overexpression; the result is stimulated cell proliferation. **Lowered growth** (lower left panel) is characterized by a small decrease in the basal $[Ca^{2+}]_{ER}$ (red symbols in the inset graph) due to SERCA pump underexpression, which slows cell proliferation, but does not induce apoptosis yet. **Apoptosis** (lower right panel) can be induced by massive reduction in $[Ca^{2+}]_{ER}$ (red symbols marked by "1" in the inset graph); big long-lasting reduction in $[Ca^{2+}]_{ER}$ (blue symbols marked by "2" in the inset graph) due to substantial decrease in SERCA pump expression (marked by slash) and increased leak (marked as thiker arrows through the channels) shifts the whole balance towards growth arrest and enhanced programmed death. Black arrows indicate possible transitions between the states (See Chapter 15 Figure 1, p. 414.)

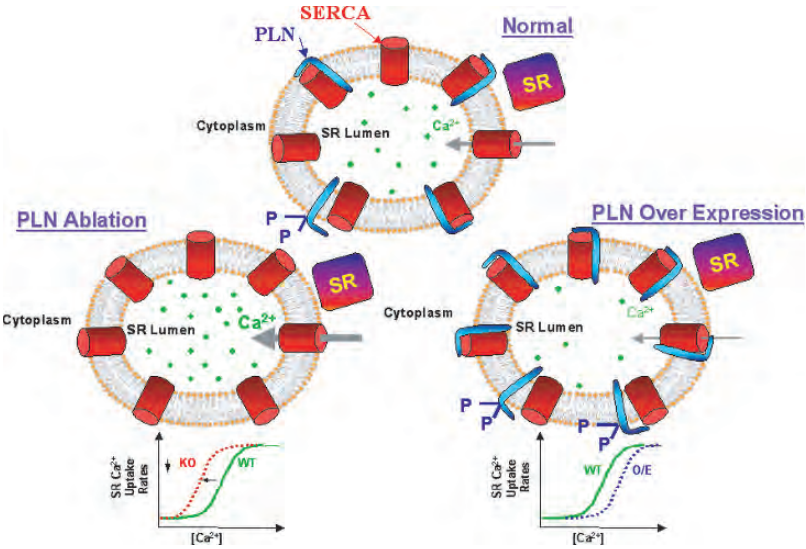

Plate 24. Schematic representation of PLN Regulation of the SR Ca-ATPase Ca-affinity, which reflects altered SR Ca-load (green dots in SR) (See Chapter 20 Figure 1, p. 526.)

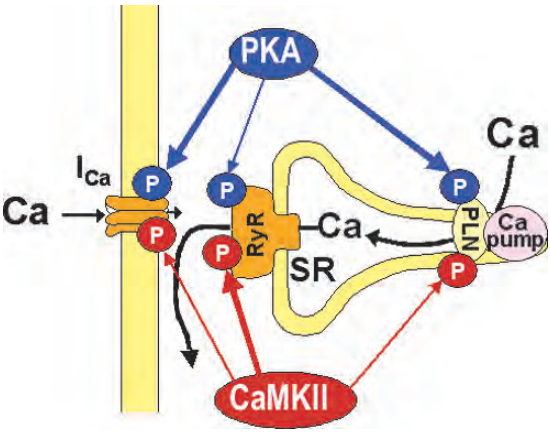

Plate 25. Both PKA and CaMKII have common molecular targets in E-C Coupling (ICa, RyR and PLN), The specific amino acids that are targets for phosphorylation (P) differ between PKA and CaMKII, as well as the intensity of functional regulation (indicated by arrow thickness). Both kinases may bind directly to the RyR and L-type Ca channel (via an anchoring protein for PKA) (See Chapter 20 Figure 2, p. 526.)

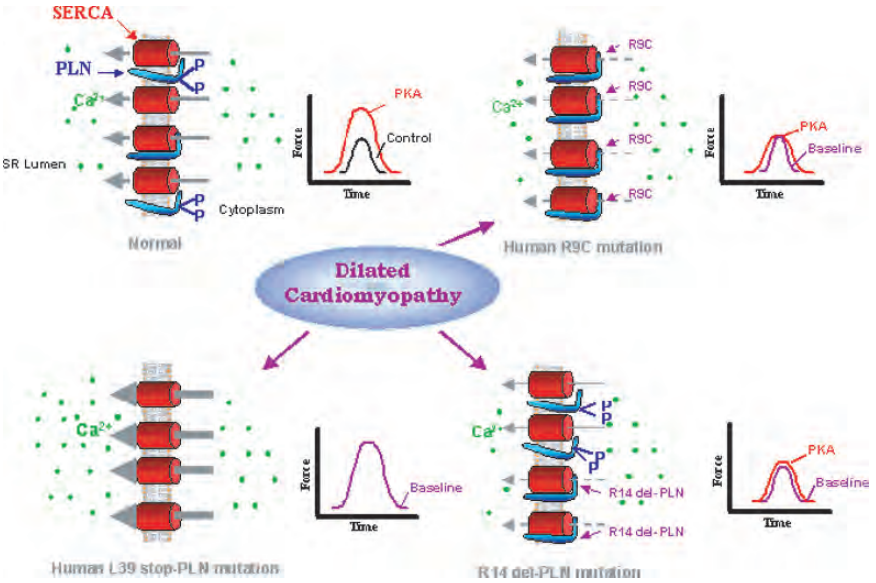

Plate 26. Schematic representation of the effects of Human PLN Mutations on the SR Ca-ATPase Activity and contractility under basal and isoproterenol (PKA)-stimulated conditions (See Chapter 20 Figure 3, p. 532.)

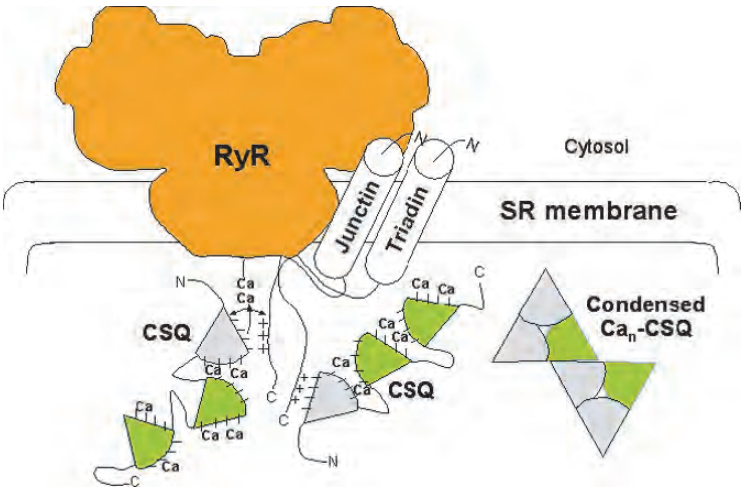

Plate 27. Calsequestrin (CSQ) is the main intra-SR Ca buffering protein, and its structure and interaction with other CSQ and partner proteins (triadin and junctin) is influenced by intra-SR [Ca]. In addition to its role as a low affinity Ca buffer, CSQ may also regulate RyR gating via its interaction with triadin and junctin (See Chapter 20 Figure 4, p. 532.)

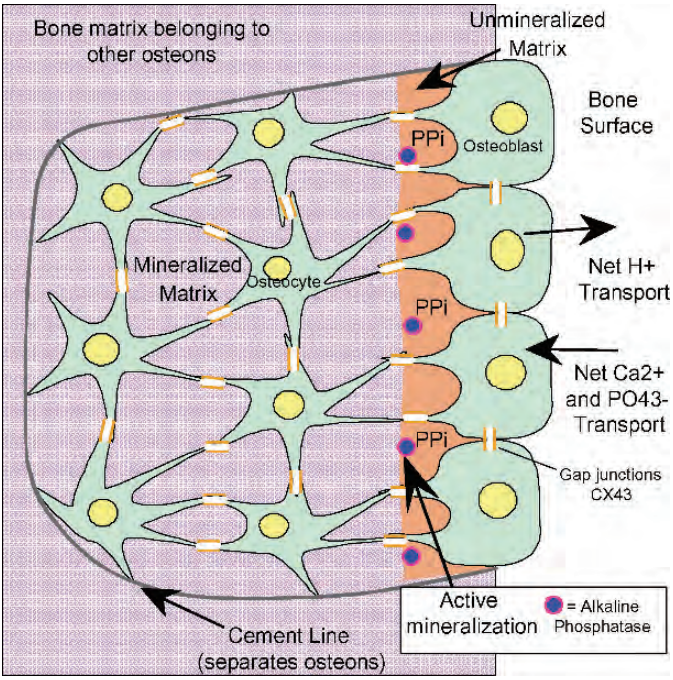

Plate 28. Bulk calcium transport by the osteoblast. While chondrocytes are individual cells embedded in an acellular matrix, and calcify the matrix focally by producing high local concentrations of phosphate, osteoblasts are arrayed in a three-dimensional organized matrix that allows calcium to be deposited in an efficient site-directed mechanism. The osteoblasts are connected into sheets of cells at the surface of bone by gap junctions containing connexin-43. The osteoblasts secrete an organic matrix comprised mainly of type I collagen, which is oriented in layers alternately along the axis of stretch of the bone and orthogonal to this axis. There are also minor proteins, including the calcium binding low molecular weight protein osteocalcin, which facilitate mineral deposition. Mineral deposition is driven by alkaline phosphatase activity which degrades pyrophosphate. Pyrophosphate can be transported either by membrane transporters including ANKH, or may be produced locally by nucleoside pyrophosphatase (PC-1) activity. The high phosphate produced is balanced by calcium transport and by alkalinization of the mineralization site, which are required for continuing mineral deposition, but the specific transporters involved in these activities are unclear (See Chapter 21 Figure 1, p. 542.)

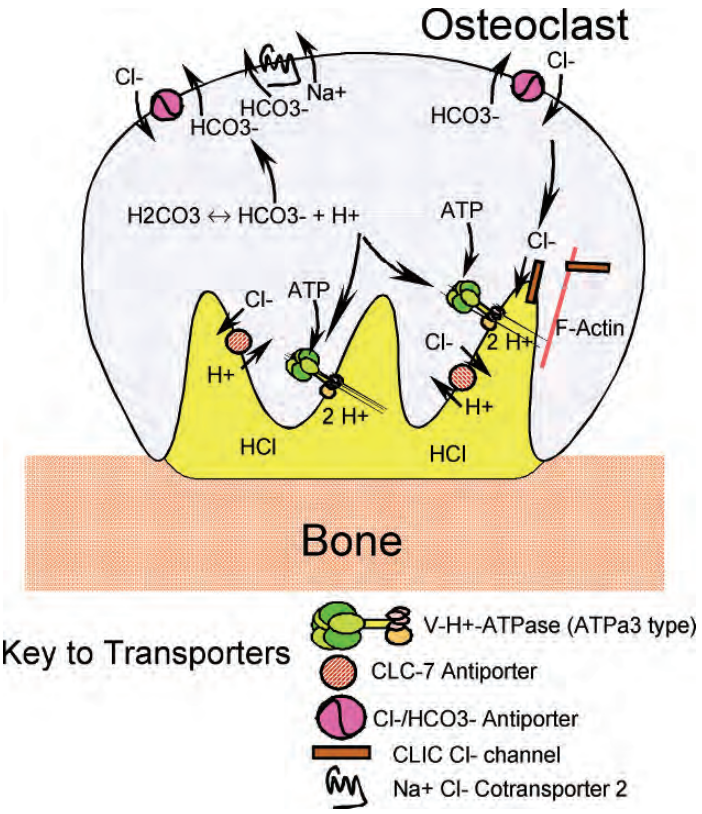

Plate 29. Bulk calcium transport by the osteoclast. Net acid transport is driven by the vacuolar-type H^+-ATPase with a specialized large membrane subunit. Transport is balanced by chloride transport, probably involving both a chloride channel (CLIC-5) and a chloride bicarbonate antiporter (CLCN7). Supporting transport processes include chloride-bicarbonate exchange. Insertion of transporters is specific for subcellular locations and involves interaction of transporters with specific cytoskeletal components, including actin (See Chapter 21 Figure 2, p. 552.)