

Structure of a cannabinoid receptor and functional expression of the cloned cDNA

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MARIJUANA and many of its constituent cannabinoids influence the central nervous system (CNS) in a complex and dose-dependent manner^{1,2}. Although CNS depression and analgesia are well documented effects of the cannabinoids, the mechanisms responsible for these and other cannabinoid-induced effects are not so far known³. The hydrophobic nature of these substances has suggested that cannabinoids resemble anaesthetic agents in their action, that is, they nonspecifically disrupt cellular membranes. Recent evidence, however, has supported a mechanism involving a G protein-coupled receptor found in brain and neural cell lines, and which inhibits adenylate cyclase activity in a dose-dependent, stereoselective and pertussis toxin-sensitive manner⁴⁻⁷. Also, the receptor is more responsive to psychoactive cannabinoids than to non-psychoactive cannabinoids⁸. Here we report the cloning and expression of a complementary DNA that encodes a G protein-coupled receptor with all of these properties. Its messenger RNA is found in cell lines and regions of the brain that have cannabinoid receptors. These findings suggest that this protein is involved in cannabinoid-induced CNS effects (including alterations in mood and cognition) experienced by users of marijuana.

In our attempts to clone novel receptors, we isolated a cDNA (SKR6) from a rat cerebral cortex cDNA library, using an oligonucleotide probe derived from the sequence of bovine substance-K receptor⁹. The translated sequence of this cDNA identified its 473-amino-acid protein product as a member of the G protein-coupled family of receptors (Fig. 1). Seven hydrophobic domains, numerous residues that are highly conserved among G protein-coupled receptors and several potential glycosylation sites were apparent (Fig. 1). If glycosylated, the relative molecular mass of this receptor would therefore exceed that of 52,823 predicted from its amino-acid constituents. Despite its general similarity to other receptors in this family, the resemblance of SKR6 to the amino-acid sequence of any other receptor was not close enough to allow us to predict either the identity of the receptor's ligand or the coupling system responsible for its signal transduction processes in the cell. Before the identification of SKR6 as a cannabinoid receptor, therefore, many candidate ligands were examined.

Identification of the ligand for SKR6 initially involved screening either SKR6-transfected mammalian cells or *Xenopus* oocytes injected with RNA transcribed from the cDNA *in vitro*. Ligands for receptors that exist on cell lines in which SKR6 mRNA was also found (N18TG-2 or NG108-15 cells; Fig. 2a) were considered strong candidates^{5,10}. In addition, many substances were examined because their receptors and the distribution of SKR6 mRNA (L.A.M., T.I.B. and S.J.L., manuscript in preparation) displayed similar localization patterns in brain. In transfected cells, however, many substances failed to interact with the receptor in radiolabelled ligand binding assays (that is, bradykinin, angiotensin II, neurotensin, cholecystokinin, vasoactive intestinal peptide, adenosine analogues), as well as in assays designed to detect alterations in cyclic AMP production (that is, D-Ala-D-Leu enkephalin, somatostatin, secretin and others at 1 or 10 μ M). In addition, electrophysiological effects in oocytes due to receptor-mediated changes (including those due to increased phosphatidylinositol turnover) were not detected when tested with angiotensin II,

bradykinin, substance P, neuropeptide Y, neurotensin, vasopressin and other ligands at 1 or 10 μ M. Although this strategy for selecting candidate ligands is beset with limitations, the critical findings, which prompted us to examine cannabinoids as ligands for SKR6, included the presence of both cannabinoid receptors^{5,11} and SKR6 mRNA in the same cell lines (Fig. 2a) and the localization of both the receptor^{12,13} and SKR6 mRNA in similar brain areas (Fig. 2b; data not shown).

In Chinese hamster ovary K1 cells stably transfected with SKR6, expression of a cannabinoid-responsive, G protein-coupled receptor was obtained. The major psychoactive cannabinoid found in marijuana (Δ^9 -tetrahydrocannabinol, Δ^9 -THC) and a synthetic analogue with potent analgesic properties (CP 55940) inhibited forskolin-stimulated accumulation of cAMP in a dose-dependent manner (Fig. 3a). In addition, the dose-response curves for the opposite (+) enantiomeric forms of these two cannabinoids indicated this effect was stereoselective. The effector concentration for half-maximum response (EC_{50}) of CP 55940 compared with that of its (+) enantiomer (CP 56667) revealed a >100-fold difference in potencies between these compounds. By contrast, the difference in EC_{50} observed between (+) and (-) Δ^9 -THC was only 50-fold. These data are in general agreement with data for N18TG-2 cell membranes, that is, that the degree of stereoselectivity between various cannabinoid analogues is greater with more potent compounds (such as CP 55940 compared with CP 56667) (ref. 14). As observed in neuroblastomas^{8,14}, none of the cannabinoids inhibited cAMP accumulation by 100 per cent but CP 55940 inhibited the accumulation of cAMP more than Δ^9 -THC. In addition, (-) Δ^8 -THC was less potent than (-) Δ^9 -THC yet affected cAMP to a similar extent (inhibition of 36 versus 39 per cent). Finally, in transfected cells, cannabinol produced only a slight effect on cAMP accumulation, whereas the non-psychoactive cannabinoid, cannabidiol, did not markedly alter cAMP (Fig. 3b).

In N18TG-2 neuroblastomas, the relative potencies of various cannabinoids that inhibit adenylate cyclase correlate well with those of the psychoactive cannabinoids in producing a 'high' in humans⁸. The rank order of potencies for several cannabinoid compounds in SKR6-transfected cells (Fig. 3b) was also similar to that for both the effects in N18TG-2 cell membranes and psychoactive effects in humans^{8,15}: 11-OH Δ^9 -THC > (-) Δ^9 -THC > (-) Δ^8 -THC > cannabinol > cannabidiol. In addition, nabilone, a synthetic cannabinoid analogue marketed for its anti-emetic effects also inhibited cAMP accumulation in SKR6-transfected cells. These cannabinoid-induced responses were probably mediated by the G protein, G_i (ref. 16), as the inhibition of cAMP accumulation was prevented by pretreatment with pertussis toxin (data not shown).

Clearly the dose-dependent, stereoselective and ligand-specific responses of SKR6-transfected cells were those that would be expected from a cannabinoid receptor. These data, along with the work of others, provide evidence for a receptor-mediated mechanism in the effects observed with cannabinoids. Nonetheless, given the substantial amount of research that has focused on the nonspecific actions of these compounds on cellular membranes^{17,18}, one might argue that cannabinoids could considerably compromise the ability of membrane-located receptors to respond correctly to their appropriate ligands. Cannabinoid-induced inhibition of adenylate cyclase activity might then seem to be receptor-mediated but would not be receptor-specific. The lack, however, of cannabinoid-induced inhibition of cAMP accumulation in nontransfected cells (data not shown) demonstrates that these compounds (Δ^9 -THC, 11-OH Δ^9 -THC, nabilone and CP 55940) failed to interact with the endogenous receptors present on CHO cells. Furthermore, when transfected into this same host (CHO cells), neither an α -adrenergic (M. Voigt and C. Felder, personal communication) nor muscarinic receptor⁹ responded to (-) Δ^9 -THC or CP 55940 (Table 1). Both these receptors, however, reduced cAMP

production in response to their respective agonists. As both the muscarinic and adrenergic receptors are G_i-coupled, the cannabinoid-induced inhibition of adenylate cyclase activity observed in SKR6-transfected cells was not due to the interaction of cannabinoids with this class of receptors and was clearly specific to SKR6.

Although the receptor-mediated actions of cannabinoids in N18TG-2 and SKR6-transfected cells help to define their biochemical and cellular effects, the physiological (increased heart rate, inhibition of vomiting, reduction of intraocular pressure),

behavioural (appetite stimulation, CNS depression), and psychoactive (hallucinations, memory deficits, altered time and space perception) effects of these compounds have traditionally been examined in humans and various animal models¹⁵. Data linking receptor-mediated responses in cultured cells with effects in animals or humans, therefore, are critical. The presence of SKR6-hybridizing signals of similar size (~6 kilobases (kb)) in northern blots of rat brain (data not shown) and neural cell-line RNAs (Fig. 2a) indicate that the receptor found in these cells is also present in brain. In addition, the degree of overlap

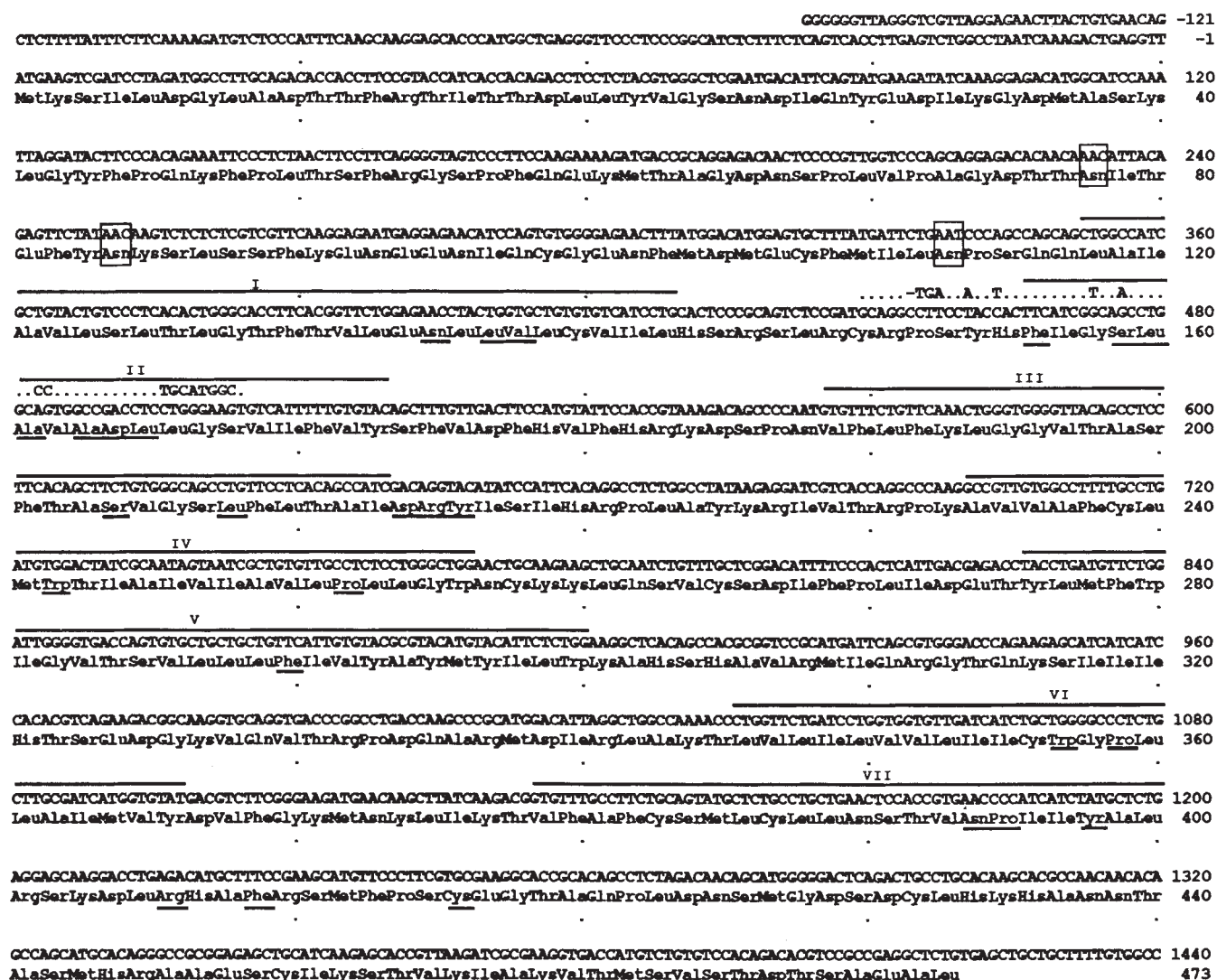


FIG. 1 Partial nucleotide sequence of SKR6 cDNA. Indicated above and below the sequence are the predicted hydrophobic domains (I-VII) and the translated primary structure of the receptor, respectively. The initial stretch of guanine nucleotides represent the G tail produced during cDNA synthesis. The 56-base probe sequence is indicated by dots (bases identical to SKR6) beginning at base number 449; nonidentical bases are provided above the cDNA sequence and a single nucleotide gap (hyphen) has been introduced to align the probe with the cDNA sequence. Although this oligonucleotide was derived from the nucleic acid sequence of the substance-K receptor¹⁹, less than 25% homology overall exists between the amino-acid sequences of SKR6 and the substance-K receptor. Underlined amino acids are those that are highly conserved among other G protein-coupled receptors. Notably absent from SKR6 is a proline residue in the fifth hydrophobic domain and a cysteine just before hydrophobic domain III. In terms of structure, these substitutions may indicate interesting similarities between SKR6 and the LH-CG receptor (lacks the corresponding proline^{20,21}) or the *mas* oncogene

product (lacks the same cysteine residue²²). Indeed, the homologous cysteine is essential in functional rhodopsin²³. Potential N-linked glycosylation sites are enclosed within boxes. The entire SKR6 cDNA (5.7 kb) includes an additional ~4,100 bases 3' of the given sequence. In addition to SKR6, a second clone (SKR14) was isolated whose coding region, although incomplete, was identical to SKR6. The 3' untranslated sequence of SKR14, however, was ~2,900 bases shorter than that of SKR6. Comparison of the sequences of these clones indicates that SKR14 was the product of an alternatively polyadenylated mRNA.

METHODS. SKR6 was isolated from a rat cerebral cortex cDNA library constructed in the mammalian expression vector pCD (ref. 24). Screening was as described previously for cloning muscarinic receptor subtype cDNAs⁹. Nucleic acid sequence was determined by dideoxynucleotide chain termination of single-stranded DNA obtained from restriction fragments inserted into M13 mp 18 or 19.

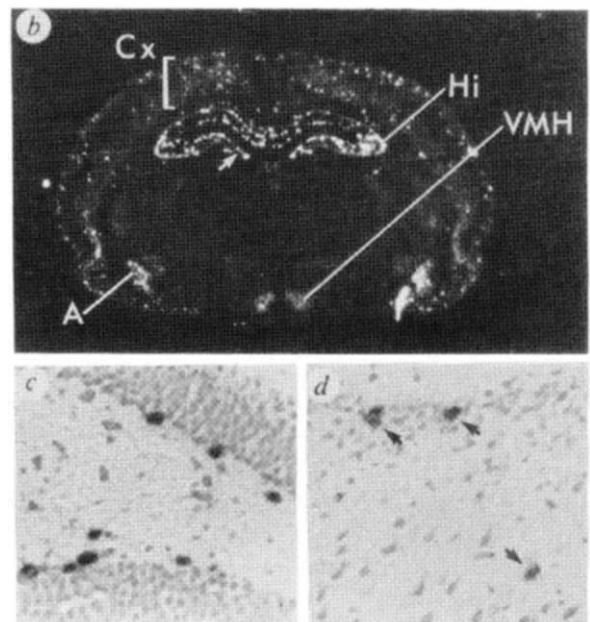
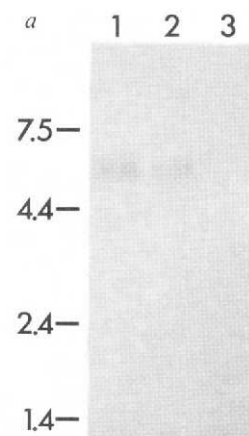
TABLE 1 Cyclic AMP accumulation

Cell line	Receptor/cDNA	Forskolin	Δ^9 THC (100 nM)	CP 55940 (10 nM)	Carbachol/clonidine
CHO	SKR6	100 \pm 4 (12.6 \pm 0.5)	61 \pm 5	—	—
CHO	SKR6	100 \pm 5 (12.1 \pm 1.4)	—	44 \pm 11	—
CHO	muscarinic m2	100 \pm 5 (18.0 \pm 0.9)	104 \pm 8	104 \pm 10	8 \pm 1
CHO	adrenergic α 2d	100 \pm 4 (13.9 \pm 0.5)	100 \pm 4	96 \pm 7	73 \pm 5
N18TG-2	—	100 \pm 10 (44.4 \pm 4.3)	61 \pm 8	16 \pm 2	—
NG108-15	—	100 \pm 4 (320.5 \pm 11.7)	91 \pm 7	57 \pm 3	—

Effect of Δ^9 -THC and CP 55940 on forskolin-stimulated accumulation of cAMP in CHO-K1 cells transfected with SKR6, muscarinic and α -adrenergic receptor cDNAs. Values represent the average accumulation of cAMP \pm s.e.m. as per cent of forskolin-stimulated controls. In each cell line, the effects of the various agonists were examined in three to five experiments (each performed in triplicate). Numbers in parentheses are the absolute values of cAMP as determined by radioimmunoassay (pmol cAMP per 10^6 cells per 5 min). Final concentrations of forskolin were 500 nM for all cell lines except NG108-15; forskolin concentration for this cell line was 250 nM. The muscarinic and adrenergic receptor-transfected cells were assayed under conditions identical to those routinely used to test the SKR6-transfected cells (see Fig. 3). Final concentrations of carbachol (agonist for muscarinic receptors) and clonidine (agonist for α -adrenergic receptors) were 100 μ M and 10 μ M, respectively. Clearly, the extent to which a receptor can inhibit cAMP accumulation varies considerably across different cell lines. The moderate effect of clonidine to inhibit cAMP accumulation reported here is lower than normally observed in this transfected cell line (inhibits cAMP accumulation to 50–25% of forskolin-stimulated control). This difference is due to the bovine serum albumin included in our assay.

FIG. 2 Presence of SKR6 mRNA in cell lines and its localization in rat brain. *a*, Northern analysis of total RNA from N18TG-2 (lane 1), NG108-15 (lane 2) and C6BU-1 (lane 3) cell lines. N18TG-2 and C6BU-1 cells are the neuroblastoma and glioma parents of the NG108-15 hybrid cell line, respectively. The single hybridizing bands present in lanes 1 and 2 are \sim 6 kb. Size markers (kb), on the left. Northern analysis was also performed on both total (10 μ g) and poly(A)⁺ RNA (5 μ g) prepared from several peripheral tissues (data not shown). But using conditions in which the SKR6 message is readily detected in rat brain RNAs, we saw no hybridizing signal in rat heart, liver, kidney, spleen, thymus, small intestine, testes and ovary RNAs. These data do not prove the absence of cannabinoid receptors in these tissues as they may be present at considerably lower abundance than in brain. *b*, Low-magnification photograph of an *in situ* hybridization histochemical autoradiogram. In this negative image of a coronal rat brain section, the silver grains appear white. Very high levels of SKR6 mRNA are expressed in isolated cells of the hippocampus and cerebral cortex. In the hippocampus, the strongly labelled cells include granule cells in the dentate gyrus (arrow) as well as cells in both the pyramidal and molecular layers of Ammon's horn. Similarly, in the cortex, layers II, V and VI contain a moderate number of cells expressing very high levels of SKR6 mRNA. These layers also appear to contain many cells that have a much lower message level. Control sections (hybridized under the same condition with a 48-base probe that corresponds to no known message and that gives no signal on northern blots) give a low level, uniform signal (not shown). Cx, cerebral cortex; Hi, hippocampal formation; VMH, ventromedial hypothalamic nucleus; A, amygdaloid nuclei. *c*, Bright-field photomicrograph of the hilar region (see arrow in *b*) of the dentate gyrus (\times 250). Three heavily labelled cells are shown lining the innermost edge of the granule cell layer of the external limb. An additional five cells with high levels of SKR6 mRNA are associated with the internal limb. *d*, Bright-field photomicrograph of the superficial layers of the cerebral cortex (\times 300) showing cells expressing high levels (arrows) of SKR6 mRNA. In this same brain region, cells that express less message are readily seen when a dark-field condenser is used (image similar to that seen in *b*); these less intensely labelled cells, however, are not easily discernible in bright-field photomicrographs.

METHODS. Northern analysis: RNAs were isolated from cultured cells using the guanidinium thiocyanate method as described previously²⁵ and loaded (10 μ g per lane) into a 1% agarose-formaldehyde gel. After electrophoresis and electrotransfer the filter was hybridized to a nick-translated EcoRV-XbaI fragment (bases 97–1,271) of the SKR6 cDNA, washed (0.1 \times SSPE buffer, 0.1% sodium dodecyl sulphate (SDS), 60 $^{\circ}$ C) and exposed to X-ray-sensitive film for 6 days (-80° C). *In situ* hybridization histochemistry: the brain from a male, Sprague-Dawley rat (200–250 g) was sectioned and the 12- μ m slices were thaw-mounted to gelatin-coated slides. *In situ* hybridization histochemistry was as described previously^{26,27}. An ³⁵S-labelled 48-base oligonucleotide (SKR6-1, complementary to bases 349–396) was used to probe the section. Under similar hybridization conditions, this oligonucleotide probe hybridized to a single \sim 6 kb band in preparations of rat cerebral cortex, hippocampal and cerebellar RNA (data not shown). Similar hybridization patterns were also observed in brain sections hybridized with another 48-base probe SKR6-2 (complementary to bases 4–51, data not shown);



b was produced by placing this section against X-ray-sensitive film (25 $^{\circ}$ C) for 16 days. The hybridized sections were then dipped in NBT-2 emulsion (Kodak), exposed for 21 or 28 days (4 $^{\circ}$ C), developed, stained with 0.1% toluidine blue and a coverslip applied, to produce the images shown in *c* and *d*.

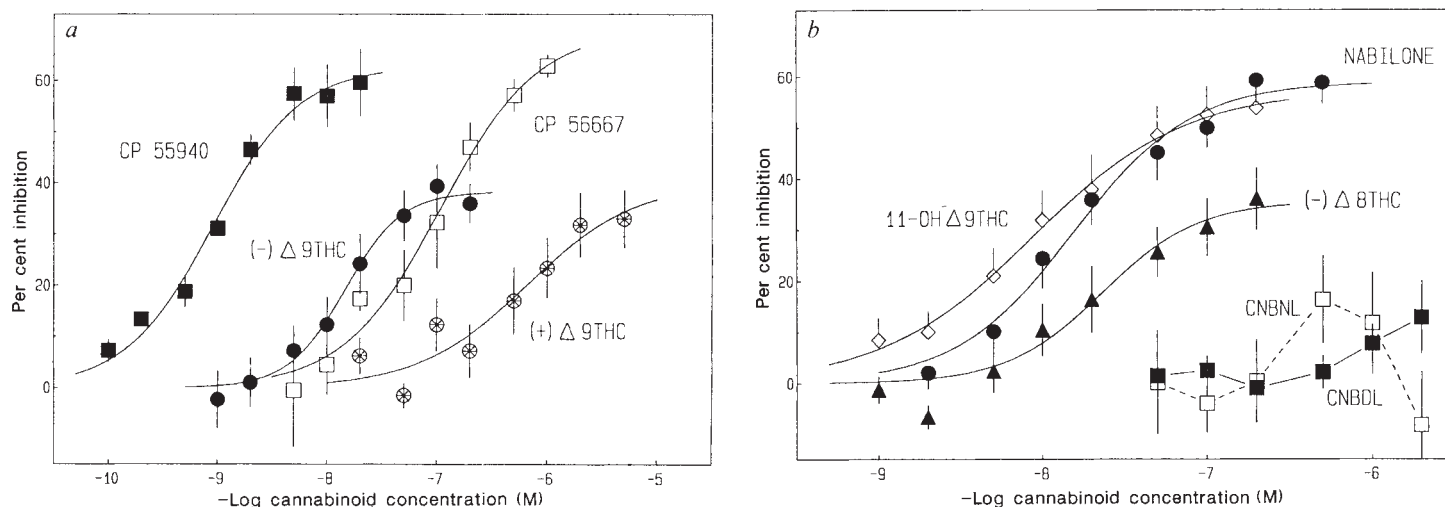


FIG. 3 Cannabinoid-induced inhibition of forskolin-stimulated cAMP production in SKR6-transfected CHO-K1 cells. *a*, Stereoselective inhibition by Δ^9 -THC and CP 55940. *b*, Dose-response curves of various cannabinoids and cannabinoid analogues. Data represent the average per cent inhibition \pm s.e.m. of cAMP accumulation for three to five experiments, each performed in triplicate. Curves were generated using the Graph-Pad InPlot nonlinear regression analysis programme. Cannabinoids did not significantly inhibit cAMP accumulation in nontransfected cells (data not shown). EC_{50} values (mean nM \pm s.e.m.) for the inhibition of stimulated cAMP accumulation were: 13.5 ± 2.7 , (-) Δ^9 -THC; 773 ± 187 , (+) Δ^9 -THC; 0.87 ± 0.20 , CP 55940; 96.3 ± 7.1 , CP 56667; 8.9 ± 1.8 , 11-OH Δ^9 -THC; 16.6 ± 4.9 , nabilone; 27.4 ± 8.4 , Δ^8 -THC. Cannabinoid-induced inhibition of cAMP accumulation was also observed in transfected cells in which cAMP production was stimulated by the peptide hormone, calcitonin gene-related peptide, instead of forskolin. CP 55940 and CP 56667 are synthesized by Pfizer. Nabilone is produced by Lilly Research laboratories. Other cannabinoids are distributed by the National Institute of Drug Abuse. CNBNL, cannabinol; CNBDL, cannabidiol. METHODS. Transfection and selection of cells were performed as described previously²⁸. A monoclonal line expressing the SKR6 cDNA was obtained by

between the relative amounts of SKR6 mRNA and cannabinoid receptors¹² in individual brain areas is substantial. High levels of both SKR6 message and cannabinoid receptors (localized by ³H-labelled CP 55940 autoradiography; ref. 12) are found in the dentate gyrus, hippocampal formation and the cerebral cortex (Fig. 2*b*, and ref. 12). A striking feature of the SKR6 message, in these areas, is the presence of many isolated cells expressing very high levels of receptor message (Fig. 2*c*, *d*). Assuming that protein expression is proportional to message levels, these cells probably account for the very high density of cannabinoid receptor reported previously¹². Although more diffuse, there were moderate to high amounts of message in the hypothalamus and amygdala. Although receptors in these regions are relatively

limiting-dilution cloning of cells expressing the corresponding mRNA as determined by northern blot analysis. Methods used for measurements of cAMP were similar to those of Howlett *et al.*⁶. Transfected cells were grown to confluence and released with 0.5 mM EDTA in PBS. Washed cells were resuspended (1.25×10^6 cells ml⁻¹) in culture media (37 °C) containing HEPES buffer (20 mM) and RO-20 1724 (0.25 mM). Cells were aliquoted (0.4 ml) into silanized glass tubes and the assay initiated with the addition (0.1 ml) of forskolin (0.1 ml, 0.5 μ M, final) \pm cannabinoids in media containing fatty acid-free BSA (0.25%). Final ethanol concentrations were less than or equal to 0.2%. Cells were incubated (37 °C) for 5 min and the reaction terminated with the addition of 0.1 N HCl, 0.1 mM CaCl₂. Samples were frozen at -20 °C and thawed just before determination of cAMP by radioimmunoassay (refs 29, 30). Forskolin increased cAMP \sim 20-fold above basal concentrations; absolute values in forskolin-stimulated controls ranged from 9.5 to 17.7 pmole cAMP per 10⁶ cells per 5 min. In experiments involving pertussis toxin, subconfluent cultures of cells were grown in the presence of the holoenzyme (1 ng ml⁻¹) for 24 hours before treatment with forskolin \pm cannabinoids.

sparsely distributed¹², these data support the notion that cannabinoid-induced effects in the brain are mediated by the same receptor as found in neural cell lines and in cell lines expressing the SKR6 cDNA.

Our data do not eliminate the possibility that other mechanisms also contribute to various cannabinoid-induced effects. Assuming there is an endogenous 'cannabinoid,' SKR6-transfected cell lines can be used to facilitate its identification and purification. These cell lines should prove particularly valuable, as an antagonist for this receptor is not so far available. Addressing the physiological significance of both this receptor and its endogenous ligand should increase our understanding of not only the actions of the cannabinoids but also the CNS. □

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