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

Lisa A. Matsuda, Stephen J. Lollit, Michael J. Brownstein, Alice C. Young & Tom I. Bonner

Laboratory of Cell Biology, National Institutes of Mental Health, Bethesda, Maryland 20892, USA

MARIJUANA and many of its constituent cannabinoids influence the central nervous system (CNS) in a complex and dose-dependent manner. 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 known. The hydrophobic nature of these substances has suggested that cannabinoids resemble anesthetic agents in their action, that is, they nonspecifically disrupt cellular membranes. Recent evidence, however, has supported the involvement of 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 manner. Also, the receptor is more responsive to psychoactive cannabinoids than to non-psychoactive cannabinoids. 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 attempt to clone novel receptors, we isolated a cDNA (SKR6) from a rat cerebral cortex cDNA library, using a single-stranded probe derived from the sequence of bovine substance K receptor. 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 exceed that of the SK62 predicted from its amino-acid sequences. Despite its structural similarity to other receptors in this family, the sequence of SKR6 to the amino-acid sequence of any other receptor was not close enough to allow us to predict either the topology 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, 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. 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 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, somatostatin, vasopressin and other ligands at 1 or 10 μM. In addition, pharmacological effects in oocytes due to receptor-mediated including those due to increased phosphatidylinositol were not detected when tested with angiotensin II, bradykinin, substance P, neuropeptide Y, neurotensin, vasopressin and other ligands at 1 or 10 μM. 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 receptors5,6 and SKR6 mRNA in the same cell lines (Fig. 2a) and the localization of both the receptor7,8 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-sensitive, G protein-coupled receptor was obtained. The major psychoactive cannabinoid found in marijuana (Δ⁹-tetrahydrocannabinol, Δ⁹-THC) and a synthetic analogue with potent anagletic 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 (+) enantiomer of these two cannabinoids indicated this effect was stereoselective. The effector concentration for half-maximum response (EC₅₀) 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 EC₅₀ observed between (+) and (-) Δ⁹-THC was only 5-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 neuroblastomas4,13,14, none of the cannabinoids inhibited cAMP accumulation by 100 per cent but CP 55940 inhibited the accumulation of cAMP more than Δ⁹-THC. In addition, (-) Δ⁹-THC was less potent than (-) Δ⁹-THC yet affected cAMP to a similar extent (inhibition of 36 versus 39 per cent). Finally, in transfected cells, cannabinoid 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 humans. The rank order of potencies for several cannabinoid compounds in SKR6-transfected cells (Fig. 3b) was similar to that for both effects of the N18TG-2 cell membranes and psychoactive effects in humans4,15, 11-OH Δ⁹-THC > (-) Δ⁹-THC > (-) Δ⁹-THC > cannabidiol > (-) Δ⁹-THC. 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 (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 membranes15, one might argue that cannabinoids could considerably compromise the ability of membrane-located receptors to respond correctly to their appropriate ligands. A 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 (Δ⁹-THC, 11-OH Δ⁹-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 adrenergic (M. Vogt and C. Fedler, personal communication) nor muscarinic receptor responded to (-) Δ⁹-THC or CP 55940 (Table 1). Both these receptors, however, reduced cAMP
production in response to their respective agonists. As both the
muscarnic and adrenergic receptors are G-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 bio-
chemical and cellular effects, the physiological (increased heart
rate, inhibition of vomiting, reduction of intraocular pressure),
behavioural (appetite stimulation, CNS depression), and
psychostimulant (hallucinations, memory deficits, altered time
and space perception) effects of these compounds have traditionally
been examined in humans and various animal models. 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

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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 receptor, 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 proline22) or the mas oncogene
product (lacks the same cysteine residue23). Indeed, the homologous
cysteine is essential in functional modulations24. Potential N-linked glyco-
sylation sites are enclosed within boxes. The entire SKR6 cDNA (5.7 kb) includes
an additional ~4.100 bases 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 ~2900 bases shorter than that of SKR6. Comparison of the
sequences of these clones indicates that SKR14 was the product of a
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-labelled
expression libraries from mammalian brain (ref. 25). The sequence was
confirmed by automated sequencing and by DNA restriction
analysis of single-stranded DNA obtained from restriction fragments insert
into M13 mp18 or 19.
TABLE 1  Cyclic AMP accumulation

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Receptor/cDNA</th>
<th>Forskolin</th>
<th>Δ9THC (100 nM)</th>
<th>CP 55940 (10 nM)</th>
<th>Carbachol/clonidine</th>
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<tbody>
<tr>
<td>CHO</td>
<td>SKR6</td>
<td>100 ± 4</td>
<td>61 ± 5</td>
<td>—</td>
<td>—</td>
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<tr>
<td>CHO</td>
<td>SKR6</td>
<td>100 ± 5</td>
<td>—</td>
<td>44 ± 11</td>
<td>—</td>
</tr>
<tr>
<td>CHO</td>
<td>muscarinic m2</td>
<td>100 ± 5</td>
<td>104 ± 8</td>
<td>104 ± 10</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>CHO</td>
<td>adrenergic α2d</td>
<td>100 ± 8</td>
<td>96 ± 7</td>
<td>73 ± 5</td>
<td>—</td>
</tr>
<tr>
<td>N18TG-2</td>
<td>—</td>
<td>100 ± 4</td>
<td>61 ± 8</td>
<td>16 ± 2</td>
<td>—</td>
</tr>
<tr>
<td>NG108-15</td>
<td>—</td>
<td>100 ± 4</td>
<td>91 ± 7</td>
<td>57 ± 3</td>
<td>—</td>
</tr>
</tbody>
</table>

Effect of Δ⁹-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 ± 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 10⁶ 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 α-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 μM and 10 μM, 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 the 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.

[Image: A northern analysis of total RNA from N18TG-2 (lane 1), NG108-15 (lane 2) and C68B-1 (lane 3) cell lines. N18TG-2 and C68B-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 ~6 kb. Size markers (kb) on the left. Northern analysis was also performed on both total (10 μg) and poly(A)⁺ RNA (5 μg) prepared from several peripheral tissues (data not shown). But using conditions in which the SKR6 message was 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

[Image: a) Bright-field photomicrograph of the hiric region (see in b) of the dentate gyrus (×250). Three heavily labelled cells are present in the innermost edge of the granule cell layer of the external molecular layer. b) Bright-field photomicrograph of the superficial layers of the CA3 region (×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 in b); these less intensely labelled cells, however, are not easily discernible in bright-field photomicrographs.

[Image: METHODS. Northern analysis. RNAs were isolated from cultured cells using the guanidinium thiocyanate method as described previously and loaded 10 μg per lane) into a 1% agarose/formaldehyde gel. After electrophoresis and electrophoretic transfer the filter was hybridized to a nick-translated EcoRV-XbaI fragment (bases 97–1271) of the SKR6 cDNA, washed (0.1 × SSPE buffer, 1% sodium dodecyl sulphate (SDS), 60 °C) and exposed to X-ray sensitive film for 6 days (–80 °C). In situ hybridization histochemistry, the brain from male Sprague-Dawley rat (200–250 g) was sectioned and the 12-μm sections were thaw-mounted to gelatin-coated slides. In situ hybridization histochemistry was as described previously. An 3²P-labelled 48-base oligonucleotide (SKR6-1) complimentary to bases 349–396) was used to localize the SKR6 mRNA. Under similar hybridization conditions, this oligonucleotide hybridized to a single ~6 kb band in preparations of rat cerebral cortex, striatum, hippocampus and cerebellar RNA (data not shown). Similar hybridizations were also observed in brain sections hybridized with another probe SKR6-2 (complementary to bases 451–501, data not shown).]
FIG. 3 Cannabinoid-induced inhibition of forskolin-stimulated cAMP production in SKR6-transfected CHO-K1 cells. a. Stereoselective inhibition by Δ9-THC and CP 55940. b. Dose-response curves of various cannabinoids and cannabimimetics. Data represent the average ± S.E.M. of cAMP accumulation in duplicate experiments performed in triplicate. Curves were generated using the Graph-Pad InPlot nonlinear regression analysis program. Cannabinoids did not significantly inhibit cAMP accumulation in nontransfected cells (data not shown). IC50 values (mean ± S.E.M.) for the inhibition of stimulated cAMP accumulation were: Δ9-THC: 1.3 ± 0.7 nM, CP 55940: 96.3 ± 1.1 nM, CP 56677: 8.9 ± 1.8 nM, CP 56677-THC: 16.6 ± 4.9 nM, nabilone: 27.4 ± 8.4 nM, 3Δ-THC: 16.0 ± 3.2 nM. 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 56677 are synthesized by Pfizer. Nabilone is produced by Lilly Research laboratories. Other cannabinoids are distributed by the National Institute of Drug Abuse. CBN6L cannabidiol. CBN6L cannabidiol.

METHODS. Transfection and selection of cells were performed as described previously. A monoclonal line expressing the SKR6 cDNA was obtained by limiting dilution cloning of cells expressing the corresponding mRNA determined by northern blot analysis. Methods used for measurements of cAMP were similar to those of Howlett et al. 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) in culture medium (37°C) containing HEPES buffer (20 mM) and RO-20-1724 (20.5 mM). Cells were incubated (0.4 ml) in silanized glass tubes and the assay initiated with the addition of forskolin (0.1 μM), 3Δ-THC (20 μM), and IBMX (0.5 mM), followed by addition of radioactive [3H]cAMP (0.25 μCi). Final ethanol concentrations were less than 0.1% (w/v). 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 — 20-fold above basal concentrations: absolute values in forskolin-stimulated controls ranged from 9.5 to 17.7 pmol cAMP per 10^6 cells per 5 min. In experiments involving pertussis toxin, subconfluent cultures of cells were grown in the presence of the holoenzyme (1 mg ml−1) for 24 hours before treatment with forskolin.

between the relative amounts of SKR6 mRNA and cannabinoid receptors in individual brain areas is substantial. High levels of both SKR6 message and cannabinoid receptors (localized by [3H]-labeled CP 55940 autoradiography; ref. 12) are found in the dentate gyrus, hippocampal formation and the cerebral cortex (Fig. 2b, 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. 2c, d). Although protein expression is proportional to message levels, these cells probably account for the very high density of cannabinoid receptor reported previously. Although more diffuse, there were moderate to high amounts of message in the hypothalamus and amygdala. Although receptors in these regions are relatively sparsely distributed, 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 endogenous ligand should increase our understanding of not only the actions of the cannabinoids but also the CNS.

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