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Molecular cloning of a human cannabinoid receptor which is also expressed in testis

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A cDNA clone encoding a receptor protein which presents all the characteristics of a guanine-nucleotide-binding protein (G-protein)-coupled receptor was isolated from a human brain stem cDNA library. The probe used (HGMP08) was a 600 bp DNA fragment amplified by a low-stringency PCR, using human genomic DNA as template and degenerate oligonucleotide primers corresponding to conserved sequences amongst the known G-protein-coupled receptors. The deduced amino acid sequence encodes a protein of 472 residues which shares 97.3% identity with the rat cannabinoid receptor cloned recently [Matsuda, Lolait, Brownstein, Young & Bronner (1990) Nature (London) **346**, 561–564]. Abundant transcripts were detected in the brain, as expected, but lower amounts were also found in the testis. The same probe was used to screen a human testis cDNA library. The cDNA clones obtained were partially sequenced, demonstrating the identity of the cannabinoid receptors expressed in both tissues. Specific binding of the synthetic cannabinoid ligand [^{3}H]CP55940 was observed on membranes from Cos-7 cells transfected with the recombinant receptor clone. In stably transfected CHO-K1 cell lines, cannabinoid agonists mediated a dose-dependent and stereoselective inhibition of forskolin-induced cyclic AMP accumulation. The ability to express the human cannabinoid receptor in mammalian cells should help in developing more selective drugs, and should facilitate the search for the endogenous cannabinoid ligand(s).

INTRODUCTION

Since the molecular cloning of the β-adrenergic receptor cDNA [1], a growing number of receptor genes for neurotransmitters and hormones shown to interact with guanine-nucleotide-binding proteins (G-proteins) have been cloned. All members of this superfamily share a similar overall structure, including an *N*-terminal extracellular domain (generally glycosylated), seven transmembrane segments and a *C*-terminal intracellular domain. The existence of conserved sequences amongst these receptors has led to the development of a low-stringency PCR method to selectively amplify cDNA coding for new members of the family using degenerate primers [2]. Four new receptors were cloned, two of them being recently identified as A_1 and A_2 adenosine receptors (F. Libert, unpublished work; [3]). Using the same strategy, eleven partial clones displaying sequence similarity with G-protein-coupled receptors were isolated from human genomic DNA [4]. One of these, HGMP09, corresponded to a follicle-stimulating hormone (FSH) receptor sequence and was used to clone dog and human thyroid-stimulating hormone (TSH) receptor cDNAs [4,5]. In the study of the ten remaining unidentified receptor clones, one of them, HGMP08, led to the isolation from a human brain stem cDNA library of a full-length clone with an open reading frame of 472 amino acids. This turned out to be the human homologue of the rat cannabinoid receptor [6].

Cannabinoids, the active chemicals in marijuana, have been used for centuries for their medicinal properties. However because of their side effects, their use as therapeutic agents has been strongly restricted. Thus the development of new cannabinoid analogues that are devoid of mood-altering effects would be highly beneficial. This should be facilitated by the availability of the recently identified cannabinoid receptor in a cloned form [7]. We report here the functional expression of the human recombinant receptor in mammalian cells. In addition, we provide evidence that an identical cannabinoid receptor is expressed in human testis.

MATERIALS AND METHODS

Library screening

Human brain stem and human testis cDNA libraries constructed in the λgt11 vector were screened at high stringency [8] with a 600 bp HGMP08 probe. The probe was obtained by PCR as described previously [2,4]. Positive clones were purified to homogeneity and their cDNA inserts were subcloned in pBluescript SK$^+$. Sequencing on both strands was performed by the dideoxynucleotide chain termination method [9] after partial subcloning in M13mp derivatives, using an automated DNA sequencer (Applied Biosystems 370A).

Northern blotting

Poly(A)$^+$ RNA was isolated from dog tissues either by the guanidium thiocyanate method followed by oligo(dT)–cellulose chromatography [10] or by using the Fast Track kit (Invitrogen).

Abbreviations used: G-protein, guanine-nucleotide-binding protein; Δ8- and Δ9-THC, Δ8- and Δ9-tetrahydrocannabinol respectively; FSH, follicle-stimulating hormone; TSH, thyroid-stimulating hormone; KRH, Krebs–Ringer/Hepes; PBS, phosphate-buffered saline (137 mM-NaCl/8 mM-Na_2HPO_4/3 mM-KCl/1.5 mM-KH_2PO_4, pH 7.5).

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The nucleotide sequence data reported here will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X54937.

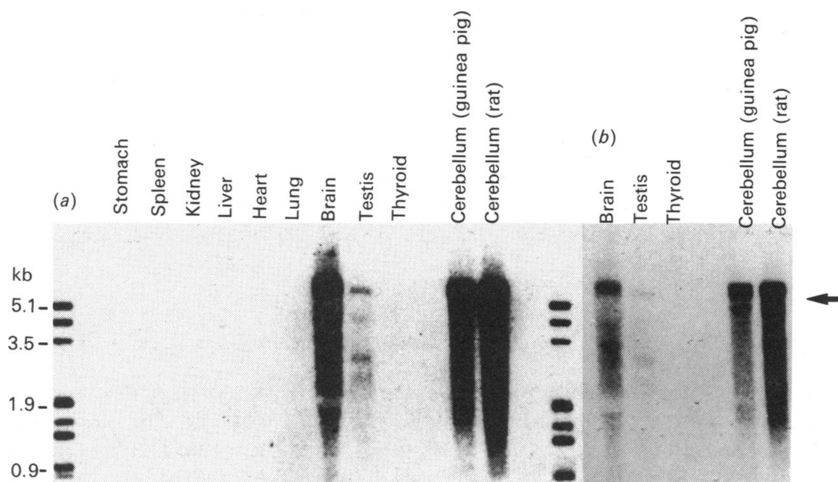

Fig. 1. Tissue distribution of transcripts corresponding to the HGMP08 clone

Distribution was determined by Northern blotting of poly(A)$^+$ RNA prepared from nine dog tissues (10 μg per lane) and guinea pig and rat cerebella (5 μg per lane). The blot was autoradiographed for 1 day (*b*) and 3 days (*a*). the arrow indicates the position of the 6 kb transcript detected in dog brain and testis and in cerebella from rat and guinea pig. The sizes of the labelled DNA markers are indicated.

Glyoxal-treated RNA samples (10 μg) were separated on 1% agarose gels in 10 mM-phosphate buffer (pH 7) and blotted on to nitrocellulose (Schleicher & Schuell). After baking, the filters were prehybridized and hybridized as described previously [11], washed with 0.1 × SSC/0.1% SDS at 55 °C (1 × SSC = 0.15 M-NaCl/0.015 M-sodium citrate) and autoradiographed with intensifying screens on to Kodak X-AR5 films for 1–3 days at −70 °C.

Membrane preparation

Cos-7 cell membranes were prepared 72 h after transfection. Cells were rinsed once with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). After low-speed centrifugation, the cell pellet was resuspended in 25 mM-Tris/HCl (pH 7.5)/1.5 mM-$MgCl_2$/0.5 mM-EDTA (TME buffer), freeze–thawed twice and homogenized in a glass homogenizer. The crude membrane fraction was collected by centrifugation at 40000 *g* for 30 min, washed once, resuspended in TME buffer and rapidly frozen. Protein content was assayed by the Lowry method [12].

Saturation binding assay

Experiments were performed in glass tubes treated with dimethyldichlorosilane solution (BDH) in order to decrease non-specific adsorption of ligands to wall tubes. The incubation mixture (1 ml) contained 100 μg of transfected or control Cos cell membranes, increasing amounts of [^{3}H]CP55940 (Pfizer) and 1 mg of fatty acid-free BSA/ml in TME buffer. Non-specific binding was assayed in the presence of 10 μM-Δ9-THC (Δ9-tetrahydrocannabinol) or 1 μM-CP55940. After 1 h of incubation at 30 °C, membranes were rapidly filtered on glass fibre discs (Whattman GF/C) and rinsed with 3 × 4 ml of TME buffer containing 5 mg of BSA/ml. Filters were air-dried at room temperature and counted for radioactivity in 8 ml of Instagel 2 (Packard).

Expression in cell lines

A *Sac*I–*Bam*H1 fragment of clone BS08 containing the whole coding region was cloned in the corresponding sites of the eukaryotic expression vector pSVL (Pharmacia). The resulting construct was transfected in Cos-7 cells using a modification of the DEAE-Dextran method [13], and co-transfected with the antibiotic-resistant plasmid pSV2neo in the CHO-K1 cell line using a modified calcium phosphate precipitate method as described [14], except that no carrier DNA was present. At 24 h after transfection CHO-K1 cells were trypsin-treated and diluted 1:10. The following day, the selection for transfectants was initiated by the addition of 400 μg of G418/ml (Gibco, Paisley, Scotland, U.K.). After 10 days, individual resistant clones were isolated and cultured separately. CHO-K1 and Cos-7 cells were cultured respectively in Ham's F12 medium and Dulbecco's modified Eagle's medium (Gibco) supplemented with 1 mM-sodium pyruvate, 100 units of penicillin/ml, 100 μg of streptomycin/ml, 2.5 μg of amphotericin B/ml (Flow Laboratories, Irvine, Scotland, U.K.) and 10% foetal calf serum.

Assays of cyclic AMP

When needed, sterile glass tubes were seeded with 100 μl of culture medium containing 10^5 trypsin-treated CHO cells. At 24 h later, cells were rinsed with 1 ml of KRH (Krebs–Ringer/Hepes: 5 mM-KCl, 1.25 mM-$MgSO_4$, 124 mM-NaCl, 25 mM-Hepes, 8 mM-glucose, 1.25 mM-KH_2PO_4, 1.5 mM-$CaCl_2$, 0.5 mg of BSA/ml) and drugs were added as a 200 μl solution in KRH containing 0.1 mM-Ro20-1724 (gift from Hoffmann-La Roche, Nutley, N.J., U.S.A.) and 0.1 mM-3-isobutyl-1-methylxanthine (Sigma, St. Louis, MO, U.S.A.). Cyclic AMP accumulation was stimulated by the addition of 500 nM-forskolin. After a 20 min incubation at 37 °C cyclic AMP accumulation was stopped by the rapid addition of 400 μl of boiling water. Tubes were further boiled for 10 min and then vacuum-dried overnight (Speedvac). The cyclic AMP concentration was measured by radioimmunoassay [15].

RESULTS AND DISCUSSION

Eleven partial clones encoding new G-protein-coupled receptors were obtained by selective amplification of human genomic DNA by low-stringency PCR [4]. The receptor tissue distribution was determined by Northern blot analysis on poly(A)$^+$ RNA prepared from a variety of dog tissues and from rat and guinea pig cerebella. Fig. 1 shows that the mRNA corresponding to one of these clones, HGMP08, was present as a 6 kb transcript in dog brain and in rat and guinea pig cerebella, indicating a significant expression in the central nervous system. Interestingly, a weaker signal of similar size was also detected in

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        human   -63  GATTGCCCCCTGTGGGTCACTTTCTCAGTCATTTTGAGCTCAGCCTAATCAAAGACTGAGGTT
        rat          .GG.T...T.TCG.CA..T............CC.....TCTG....................

      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
   1  MetLysSerIleLeuAspGlyLeuAlaAspThrThrPheArgThrIleThrThrAspLeuLeuTyrValGlySerAsnAspIleGlnTyr
   1  ATGAAGTCGATCCTAGATGGCCTTGCAGATACCACCTTCCGCACCATCACCACTGACCTCCTGTACGTGGGCTCAAATGACATTCAGTAC
      ..........................C..........T..........A.......C..........G.............T

      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
  31  GluAspIleLysGlyAspMetAlaSerLysLeuGlyTyrPheProGlnLysPheProLeuThrSerPheArgGlySerProPheGlnGlu
  91  GAAGACATCAAAGGTGACATGGCATCCAAATTAGGGTACTTCCCACAGAAATTCCCTTTAACTTCCTTTAGGGGAAGTCCCTTCCAAGAG
      .....T.......A..................A...................C.........C.....T.............A

      . . . . . . . SerPro . . . . Gly . ThrThr . . . . . . . . . . . . .
  61  LysMetThrAlaGlyAspAsnProGlnLeuValProAla---AspGlnValAsnIleThrGluPheTyrAsnLysSerLeuSerSerPhe
 181  AAGATGACTGCGGGAGACAACCCCCAGCTAGTCCCAGCA---GACCAGGTGAACATTACAGAATTTTACAACAAGTCTCTCTCGTCCTTC
      ........C..A........T...C.T.G.........GGA...ACAACA..........G..C..T...............G...

      . . . . . . . . . . . . . . . . . . . Met . . . . Ile . . . . . . . . . .
  90  LysGluAsnGluGluAsnIleGlnCysGlyGluAsnPheMetAspIleGluCysPheMetValLeuAsnProSerGlnGlnLeuAlaIle
 268  AAGGAGAATGAGGAGAACATCCAGTGTGGGGAGAACTTCATGGACATAGAGTGTTTCATGGTCCTGAACCCCAGCCAGCAGCTGGCCATT
      ......................................T........G.....C..T...A.T.....T...................C

                                      I
      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
 120  AlaValLeuSerLeuThrLeuGlyThrPheThrValLeuGluAsnLeuLeuValLeuCysValIleLeuHisSerArgSerLeuArgCys
 358  GCAGTCCTGTCCCTCACGCTGGGCACCTTCACGGTCCTGGAGAACCTCCTGGTGCTGTGCGTCATCCTCCACTCCCGCAGCCTCCGCTGC
      ..T..A..........A................T...........A..........T.......G...........T.....A...

                                           II
      . . . . . . . . . . . . . . . . . . . . . . . . . Val . . . .
 150  ArgProSerTyrHisPheIleGlySerLeuAlaValAlaAspLeuLeuGlySerValIlePheValTyrSerPheIleAspPheHisVal
 448  AGGCCTTCCTACCACTTCATCGGCAGCCTGGCGGTGGCAGACCTCCTGGGGAGTGTCATTTTTGTCTACAGCTTCATTGACTTCCACGTG
      ..............................A.....C...........A.............G........TG..........T..A

                                                        III
      . . Pro . . . . . . . . . . . . . . . . . . . . . . . . . .
 180  PheHisArgLysAspSerArgAsnValPheLeuPheLysLeuGlyGlyValThrAlaSerPheThrAlaSerValGlySerLeuPheLeu
 538  TTCCACCGCAAAGATAGCCGCAACGTGTTTCTGTTCAAACTGGGTGGGGTCACGGCCTCCTTCACTGCCTCCGTGGGCAGCCTGTTCCTC
      ........T.....C....C...T.........................T..A..........A..T..T.................

      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
 210  ThrAlaIleAspArgTyrIleSerIleHisArgProLeuAlaTyrLysArgIleValThrArgProLysAlaValValAlaPheCysLeu
 628  ACAGCCATCGACAGGTACATATCCATTCACAGGCCCCTGGCCTATAAGAGGATTGTCACCAGGCCCAAGGCCGTGGTGGCGTTTTGCCTG
      ...............................T................C..................T.....C.........

                IV
      . . . . . . . . . . . . . . . . . . . . Lys . . . . . . . . . .
 240  MetTrpThrIleAlaIleValIleAlaValLeuProLeuLeuGlyTrpAsnCysGluLysLeuGlnSerValCysSerAspIlePhePro
 718  ATGTGGACCATAGCCATTGTGATCGCCGTGCTGCCTCTCCTGGGCTGGAACTGCGAGAAACTGCAATCTGTTTGCTCAGACATTTTCCCA
      ........T..C..A..A..A.....T...T.....................A....G...............G...........

                                           V
      Leu . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
 270  HisIleAspGluThrTyrLeuMetPheTrpIleGlyValThrSerValLeuLeuLeuPheIleValTyrAlaTyrMetTyrIleLeuTrp
 808  CACATTGATGAAACCTACCTGATGTTCTGGATCGGGGTCACCAGCGTACTGCTTCTGTTCATCGTGTATGCGTACATGTATATTCTCTGG
      .T......C..G....................T.....G.....T..G.....G.......T.....C...........C.........

 300  LysAlaHisSerHisAlaValArgMetIleGlnArgGlyThrGlnLysSerIleIleIleHisThrSerGluAspGlyLysValGlnVal
 898  AAGGCTCACAGCCACGCCGTCCGCATGATTCAGCGTGGCACCCAGAAGAGCATCATCATCCACACGTCTGAGGATGGGAAGGTACAGGTG
      .................G..................G...........................A..A..C..C.....G......

                                                                  VI
      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
 330  ThrArgProAspGlnAlaArgMetAspIleArgLeuAlaLysThrLeuValLeuIleLeuValValLeuIleIleCysTrpGlyProLeu
 988  ACCCGGCCAGACCAAGCCCGCATGGACATTAGGTTAGCCAAGACCCTGGTCCTGATCCTGGTGGTGTTGATCATCTGCTGGGGCCCTCTG
      ........T.....................C.G.....A.......T...................................

                                                                                       VII
      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
 360  LeuAlaIleMetValTyrAspValPheGlyLysMetAsnLysLeuIleLysThrValPheAlaPheCysSerMetLeuCysLeuLeuAsn
1078  CTTGCAATCATGGTGTATGATGTCTTTGGGAAGATGAACAAGCTCATTAAGACGGTGTTTGCATTCTGCAGTATGCTCTGCCTGCTGAAC
      .....G............C.....C................T..C............C.......................
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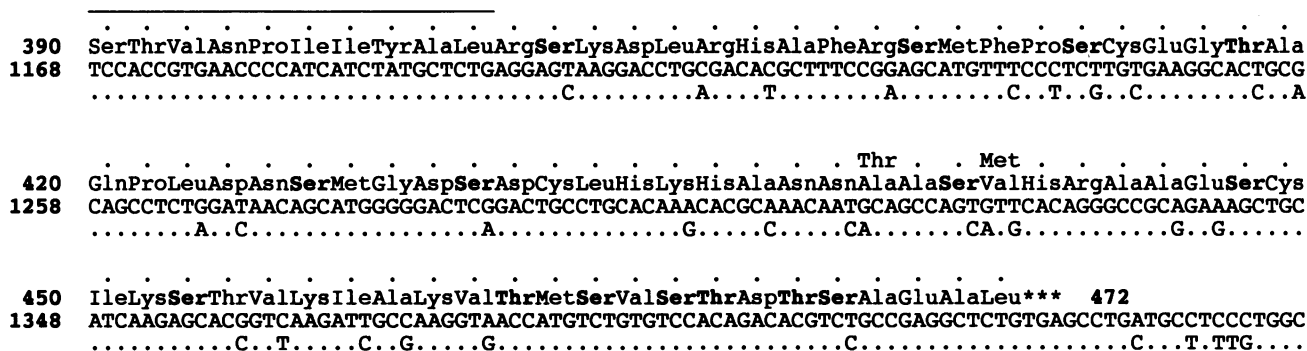

Fig. 2. Partial nucleotide sequence of the BS08 clone and deduced amino acid sequence of the human cannabinoid receptor

Divergent amino acids and nucleotides of the corresponding rat sequences are indicated respectively above and below the human sequences. Asparagine residues constituting potential acceptor sites of *N*-linked glycosylation are represented in bold characters. Putative transmembrane segments are overlined and numbered I–VII. The partial nucleotide sequence obtained from human testis clones is represented in bold.

dog testis. A human brain stem cDNA library was screened with the HGMP08 probe. Three overlapping 1.5 kb cDNA clones were isolated and sequenced entirely on both strands. One of them, BS08, displayed an open reading frame of 472 amino acids. This was defined from the first ATG codon following an in-frame stop codon (Fig. 2). The amino acid sequence of the predicted protein (calculated molecular mass 52.8 kDa) presented 97.3 % identity with that encoded by the rat cannabinoid receptor cDNA recently isolated [6]. Alignment of the amino acid sequence of the cannabinoid receptor from both species indicated an exceptionally high similarity (Fig. 2). Only slight differences were observed in the extracellular parts and the *C*-terminus of the protein: 12 replacements, generally conservative, and loss of a glycine residue. Sequences of the transmembrane segments, which are known in other G-protein-coupled receptors to interact with ligands, are identical. Similarly, the intracellular loops, implicated in the interaction with G-proteins, are perfectly conserved. The *N*-terminal extracellular and *C*-terminal intracellular domains generally exhibit the highest interspecies variability. Unexpectedly, the first half of the relatively long *N*-terminal domain (116 amino acids) is identical in rat and human. This could indicate a conserved role for this part of the protein, such as in ligand binding, as described for glycoprotein receptors [4,16]. This extracellular domain also displayed three conserved *N*-glycosylation acceptor sites. The high conservation of the cannabinoid receptor indicates that the mechanism of action as well as the ligand have been conserved among species, and suggests a crucial role in the nervous system. As an example, the identity between the human receptor and its mammalian orthologues is 87 % for the β_2-adrenergic and TSH receptors, 90 % for the MAS oncogene, 96 % for the dopamine D2 receptor and 93–97 % for the muscarinic acetylcholine receptor subtypes.

In the light of the Northern blotting results (Fig. 1), it was of interest to determine whether the cannabinoid receptor transcripts detected in the testis could represent a subtype distinct from the brain receptor. Using the same HGMP08 probe, a human testis cDNA library was screened. Three incomplete overlapping clones were isolated and a partial sequence was determined. With the 680 bp sequenced, a perfect identity with BS08 DNA (positions 373–1053) was observed, suggesting that brain and testis receptors are identical (Fig. 2). No testicular transcripts were observed by Matsuda *et al.* [6] in their Northern blot analysis. Future studies will show whether this reflects a true species divergence or differences in experimental conditions. Various effects of cannabinoids have been reported in the human, affecting the cardiovascular, respiratory, immune and reproductive systems. The presence of cannabinoid receptors in human testis could be correlated with the depression of reproductive functions observed as a side effect of marijuana addiction [17].

There is a long-standing interest in the understanding of the cellular mechanisms by which cannabinoids, the psychoactive substances of one of the oldest and most widely used drugs in the world, marijuana, exert their mind-altering effects. Because of their high hydrophobicity, cannabinoids have long been considered as substances which alter membrane properties 'non-specifically' without the need for a membrane receptor. A battery of analogues to the major psychoactive chemical of cannabis, (−)-Δ9-THC, have been synthesized [18,19]. Using these new compounds, it was established [20] that cannabinoids act by inhibiting cyclic AMP synthesis in neuroblastoma cell lines by a mechanism sensitive to pertussis toxin [21]. As the specific and saturable binding obtained on rat cerebral tissue was inhibited by guanine nucleotides [22], it was concluded that cannabinoids interact with a G_i-protein-coupled type of receptor.

In order to test the binding characteristics of the human cannabinoid receptor, the coding sequence of BS08 was inserted into the mammalian expression vector pSVL and the resulting construct was transfected into Cos-7 cells. At 3 days after

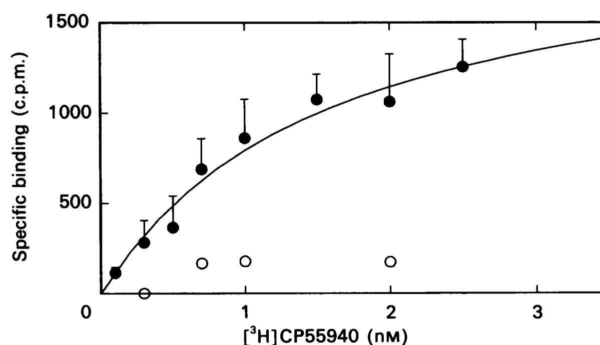

Fig. 3. Comparison of specific binding of [^{3}H]CP55940 to membranes prepared from COS-7 cells transfected (●) or not (○) with the PSVL/BS08 construct

Each point of the binding curve with transfected COS-7 cells is representative of the mean of three independent experiments performed in duplicate. Non-specific binding (radioactivity bound in the presence of 1 μM-CP55940) was deducted for each experimental point. Experimental data were computer-analysed using a non-linear least-squares method on the basis of a single binding site model.

transfection, membranes were prepared and saturation binding experiments were performed using the potent synthetic cannabinoid agonist [^{3}H]CP55940 as radioligand. In spite of a high (70%) non-specific binding (which is often observed with cannabinoid ligands), membranes from transfected Cos cells clearly exhibited a saturable and specific binding activity ($B_{max.} \approx 200$ fmol/mg of protein; $K_d \approx 1$ nM) compared with non-transfected cells (Fig. 3). However, the affinity of the recombinant receptor is slightly lower than that of the native form [22–25].

Inhibition by cannabinoids of forskolin-stimulated cyclic AMP production was assayed in CHO-K1 cells stably transfected with the pSVL/BS08 construct. Data presented in Fig. 4 show that cannabinoid agonists induced a 40–80% inhibition of cyclic AMP accumulation. In control cells transfected by PSV2neo alone, minor (10–20%) inhibition was observed only for high concentrations (10 μM) of CP55940. The rank order potency (where EC_{50} = drug concentration causing half-maximal effect) of the various ligands was: CP55244 (EC_{50} = 0.128 nM) > CP55940 (EC_{50} = 0.99 nM) > Δ9-THC (EC_{50} = 13 nM) > Δ8-THC (EC_{50} = 82 nM) > CP56667 (EC_{50} = 346 nM) > CP55243 ($EC_{50} \gg 10$ μM), with CP55244 and CP55940 producing maximal inhibition (80%).

Inhibition of the cyclic AMP accumulation is stereoselective, since CP55940 and CP55244 are respectively 1000 and 100000 times more potent than their respective enantiomers CP56667 and CP55243. These results correlate well with binding properties and adenylate cyclase inhibition curves described previously for non-recombinant receptor preparations [20–25].

Until now, the therapeutic uses of cannabinoids as analgaesic or anti-emetic agents have been limited because of their undesirable side effects. The molecular cloning of the human cannabinoid receptor and the availability of mammalian cellular systems expressing it will greatly facilitate the search for more selective agonists or antagonists. Perhaps more importantly, one may predict that the availability of the recombinant human cannabinoid receptor will be instrumental in the discovery of its endogenous ligand(s), promoting it from its role as a plant substance receptor to that of an important component of the neurotransmitter network.

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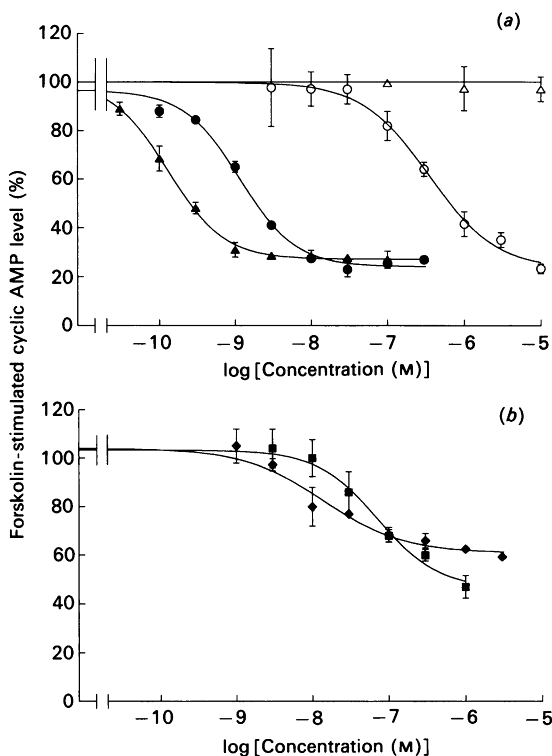

Fig. 4. Inhibition by cannabinoid agonists of the forskolin-stimulated cyclic AMP accumulation in a CHO-K1 cell line stably expressing the human cannabinoid receptor

After transfection of the pSVL/BS08 construct, 24 independent clones were selected and tested for cannabinoid expression by binding studies and inhibition of cyclic AMP accumulation. The clone displaying the highest activity was used for the present experiments. Results are expressed as a percentage of the maximal cyclic AMP production obtained with 500 nM-forskolin (basal cyclic AMP level in CHO-K1 cells was 924 ± 55 fmol/10^5 cells; forskolin-stimulated cyclic AMP level was 9871 ± 1654 fmol/10^5 cells, $n = 3$). (*a*) ▲, CP55244; ●, CP55940; ○, CP556667; △, CP55243. (*b*) ◆, Δ9-THC; ■, Δ8-THC.

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