

Cloning and Pharmacological Characterization of the Rat CB₂ Cannabinoid Receptor¹

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ABSTRACT

Many of the pharmacological effects of Δ^9 -tetrahydrocannabinol are mediated through CB₁ and CB₂ cannabinoid receptors. However, with the discovery of endogenous cannabinoids, some discrepancies have arisen. Furthermore, unlike the CB₁ receptor, the sequences of the mouse and human CB₂ receptor are divergent, raising the possibility of species specificity. The gene for the rat CB₂ receptor was cloned, expressed, and its properties compared with those of mouse and human CB₂ receptors. Sequence analysis of the coding region of the rat CB₂ genomic clone indicates 90% nucleic acid identity (93% amino acid identity) between rat and mouse and 81% nucleic acid identity (81% amino acid identity) between rat and human. The rat CB₂ receptor was stably expressed in human embryonic kidney-293 cells to examine its pharmacology. The rat CB₂

showed low affinity for anandamide, an endogenous ligand shown to act at the CB₁ receptor. In contrast, high-affinity binding for SR144528 (CB₂-selective antagonist) as well as several cannabinoid receptor agonists was observed. Coupling to adenylate cyclase was observed. Aspects of the pharmacology of palmitoylethanolamide were also examined. It bound to CB₁ and CB₂ receptors with low affinity and stimulated GTP γ S binding in the cerebellum and CB₂-expressing cell lines with low potency. The data in this study suggest that the discrepancies in affinities between rat and human may represent species differences. The rat CB₂ receptor genomic clone will be a useful tool for studying the function and regulation of CB₂ in rats.

Marijuana, the common name for the plant *Cannabis sativa*, and its principal psychoactive ingredient, (–)- Δ^9 -tetrahydrocannabinol (THC) have prominent effects on the central nervous system as well as numerous peripheral effects. There are a number of well-documented and potential therapeutic effects of THC and related cannabinoids, including anti-emesis, analgesia, anticonvulsant action, immunomodulation, and lowered intraocular pressure (Hollister, 1986). However, the central effects and abuse potential of THC have discouraged its therapeutic use.

Two cannabinoid receptors have been identified to date; one is localized predominantly in the central nervous system (CB₁), whereas the other is located primarily in the immune system (CB₂). The CB₁ receptor cDNA was isolated from a rat brain library with its identity confirmed by transfecting the

clone into Chinese hamster ovary (CHO) cells and demonstrating cannabinoid-mediated inhibition of adenylyl cyclase (Matsuda et al., 1990). Shortly thereafter, the cloning of a human CB₁ receptor cDNA was reported (Gerard et al., 1991). There is an excellent correlation between binding affinities at the cloned CB₁ receptor and in brain homogenates using [³H](–)-3[2-hydroxyl-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxyl propyl] cyclohexan-1-ol (CP-55,940) as the radioligand (Felder et al., 1992). Furthermore, there is an excellent correlation between brain binding and potencies in several mouse behavioral measures including hypoactivity, antinociception, hypothermia, and catalepsy (Devane et al., 1988; Compton et al., 1993; Howlett, 1995). Thus, the CB₁ receptor appears to mediate many of the known psychoactive effects of cannabinoids.

In contrast, the physiological role of the CB₂ receptor is less well defined. The human CB₂ receptor was discovered by a polymerase chain reaction (PCR)-based strategy designed to isolate G-protein-coupled receptors (GCR) in differentiated myeloid cells (Munro et al., 1993). CB₂ receptor mRNA has also been found in the spleen and cells of the immune system

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ABBREVIATIONS: THC, (–)- Δ^9 -tetrahydrocannabinol; CB₁ and CB₂, cannabinoid receptors; PCR, polymerase chain reaction; CP-55,940, (–)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxyl propyl] cyclohexan-1-ol; SR141716A, [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidehydrochloride]; G418, geneticin; WIN-55212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone; CHO, Chinese hamster ovary; HEK, human embryonic kidney; PMSF, phenylmethylsulfonyl fluoride; PEA, palmitoylethanolamide; GCR, G-protein-coupled receptor.

(reviewed in Schatz et al., 1997; Klein et al., 1998). CB₂ has 44% amino acid identity with CB₁. Largely based on its tissue distribution, and previous literature on immune effects of cannabinoids, the putative role of CB₂ has been in immune modulation. Very low (nanomolar) concentrations of THC have been shown to induce human tonsillar B cell proliferation (Derocq et al., 1995). This effect has recently been shown to be due to activation of the CB₂ receptor, as it is inhibited by the recently identified selective CB₂ receptor antagonist, SR144528 (Rinaldi-Carmona et al., 1998). However, in general, high concentrations (micro- or millimolar) of THC and other psychoactive as well as nonpsychoactive cannabinoids have been required to produce immunosuppressive effects on lymphocyte and macrophage function (Kaminski et al., 1992; Friedman et al., 1995).

With the identification of endogenous ligands for the cannabinoid receptors, other opportunities and discrepancies have arisen. Anandamide, arachidonic acid ethanolamide, competes for binding to the cannabinoid receptor and inhibits electrically stimulated contractions of the mouse vas deferens in the same manner as THC (Devane et al., 1992). Further additional fatty acid ethanolamides, as well as a 2-arachidonyl glycerol, have been isolated and demonstrated to have cannabimimetic properties, suggesting the existence of a family of endogenous cannabinoids, which may interact with the CB₂ receptor or with additional cannabinoid receptor subtypes (Hanus et al., 1993; Mechoulam et al., 1995). Palmitoylethanolamide (PEA) has also been suggested as a possible endogenous ligand at the CB₂ receptor (Facci et al., 1995). Facci et al. found that although both anandamide and PEA were able to displace cannabinoid binding to a rat mast cell line (RBL-2H3) that expresses the CB₂ receptor, only PEA produced a functional response. This is in contrast to the finding that anandamide can inhibit adenylyl cyclase in CHO cells that have been transfected with the human CB₂ receptor (PEA was not examined in these studies by Felder et al., 1995)). However, we have found a discrepancy with respect to PEA, which was reported to displace (*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone (WIN-55,212-2) binding in mast cells isolated from rat (Facci et al., 1995). We found that it had a low affinity for the cloned human CB₂ receptor (Showalter et al., 1996). In this study, we have extended our previous investigation of PEA by examining its affinities in a number of cell lines and tissues and also by testing its ability to stimulate GTP γ S binding in these same membranes. The possibility exists that some of these discrepancies could be attributed to species differences with the CB₂ receptor, as has been found with other GCR (e.g., κ -opioid (Simonin et al., 1995) and bradykinin (Hess et al., 1994)). Therefore, we cloned the rat CB₂ receptor and compared its ligand binding and signal transduction properties to that of human and mouse CB₂ receptors.

Experimental Procedures

Materials. [³H]CP-55,940 was purchased from DuPont-NEN (Wilmington, DE). THC, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidehydrochloride (SR141716A), and SR144528 were obtained from the National Institutes on Drug Abuse (Rockville, MD). CP-55,940 was synthesized by Dr. Larry Melvin (Pfizer, Groton, CT). WIN-55,212-2 was purchased

from Research Biochemicals (Natick, MA). PEA was purchased from Cayman Chemicals (Ann Arbor, MI). Palmitic acid and reagent grade chemicals were purchased from Sigma (St. Louis, MO). Anandamide was synthesized and provided by Dr. Raj Razdan (Organix, Woburn, MA). 2-Methyl-3-naphthoyl-*N*-propylindole (JWH-015) and 3-(1',1'-dimethylheptyl)-1-deoxy-11-hydroxy- Δ^8 -tetrahydrocannabinol (JWH-051) were synthesized and provided by Dr. John Huffman (Clemson University, Chapel Hill, SC). Dr. Sean Munro (Medical Research Center Laboratories, Cambridge, UK) generously provided the human CB₂ cDNA clone. Male ICR mice and Sprague-Dawley rats (Harlan, Dublin, VA) were used for sources of genomic DNA.

PCR. PCR was performed as follows: a cocktail containing 1 μ g of template DNA, 10 mM Tris, pH 8.3, 50 mM KCl, 2 mM MgCl₂, 1 mM each dATP, dCTP, dGTP, dTTP, 15 μ M designated primers, and 2.5 U of Vent polymerase (New England Biolabs, Beverly, MA) was denatured for 1 min at 95°C, cooled to 60°C, then heated for 4 min at 60°C (to allow optimal extension by the polymerase). This sequence was then repeated for 25 cycles with a 6-min 72°C extension at the final cycle.

Cloning of the Mouse and Rat CB₂ Genes. Oligonucleotide primers were designed to be homologous to the predicted translation initiation and termination sites of the mouse CB₂ gene (GenBank accession no. U21681). The 5' primer contained an *Eco*RI site and Kozak consensus sequence and corresponded to base pairs 535–550 of the mouse genomic sequence (GGAATTCGCCACCATGGAGG GATGCCGGGAGA). The 3' primer was designed to contain a *Xho*I site and corresponded to base pairs 1600–1626 on the opposite strand of mouse CB₂ (GCTCGAGTCAGCAGTTGGAGCAGCCTG). These were used in the PCR to generate a ~1.1-kb fragment in mouse and rat genomic DNA. Genomic DNA was isolated using standard methodology (Sambrook et al., 1989). The PCR products were gel-purified, digested with *Eco*RI and *Xho*I, and cloned into the pcDNA 3.1 expression vector (Invitrogen, San Diego, CA). The samples were sequenced via the dideoxy method (US Biochemical, Cleveland, OH) manually and confirmed by sequencing both strands (Retrogen, San Diego, CA).

Cell Culture and Transfection. Human embryonic kidney (HEK)-293 cells obtained from American Type Culture Collection (Manassas, VA) were maintained in Dulbecco's modified Eagle's medium with 10% fetal clone II (HyClone, Logan, UT) and 5% CO₂ at 37°C in a Forma incubator. Cell lines were created by transfection of CB₂ into HEK-293 cells by the Lipofectamine reagent (Life Technologies, Gaithersburg, MD). Stable transformants were selected in growth medium containing geneticin (G418, 1 mg/ml). Colonies of about 500 cells were picked (about 2 weeks post-transfection) and allowed to expand, then tested for expression of receptor mRNA by Northern blot analysis. Three cell lines containing moderate to high levels of receptor mRNA were tested for receptor binding and signal transduction properties. Each cell line had similar expression levels (about 20 pmol/mg protein), so one was chosen for further analysis. Transfected cell lines were maintained in Dulbecco's modified Eagle's medium with 10% fetal clone II (HyClone) plus 0.3 to 0.5 mg/ml G418 and 5% CO₂ at 37°C in a Forma incubator.

Cannabinoid Receptor Radioligand Binding Determinations. The assay has been previously described (Tao and Abood, 1998). Briefly, cells were harvested in PBS containing 1 mM EDTA and centrifuged at 500g. The cell pellet was homogenized and centrifuged three times at 1600g (10 min). The combined supernatants were centrifuged at 100,000g (60 min). The (P2 membrane) pellet was resuspended in 3 ml of buffer B (50 mM Tris-HCl, 1 mM EDTA, 3 mM MgCl₂, pH 7.4) to yield a protein concentration of ~1 mg/ml. The tissue preparation was divided into equal aliquots, frozen on dry ice, and stored at –70°C. Binding was initiated by the addition of 25 to 75 μ g membrane protein to silanized tubes containing [³H]CP-55,940 (102.9 Ci/mmol) and a sufficient volume of buffer C (50 mM Tris-HCl, 1 mM EDTA, 3 mM MgCl₂, and 5 mg/ml fatty acid-free BSA, pH 7.4) to bring the total volume to 0.5 ml. The addition of 1 μ M unlabeled CP-55,940 was used to assess nonspecific binding. Specific

binding averaged >50% of total binding at 1 nM [3 H]CP-55,940 in all cell lines used in the analysis. Following incubation (30°C for 1 h), binding was terminated by the addition of 2 ml of ice-cold buffer D (50 mM Tris-HCl, pH 7.4, plus 1 mg/ml BSA) and rapid vacuum filtration through Whatman GF/C filters (pretreated with polyethyleneimine (0.1%) for at least 2 h). Before radioactivity was quantitated by liquid scintillation spectrometry, filters were shaken for 1 h in 5 ml of scintillation fluid. CP-55,940 and all cannabinoid receptor analogs were prepared by suspension in assay buffer from a 1-mg/ml ethanolic stock without evaporation of the ethanol (final concentration of no more than 0.4%). In some assays with endogenous ligands, experiments were performed in the presence of phenylmethylsulfonyl fluoride (PMSF) (50 μ M). Saturation experiments were conducted with seven concentrations of [3 H]CP-55,940 ranging from 250 pM to 10 nM. Competition assays were conducted with 0.5 nM [3 H]CP-55,940 and six concentrations (0.1 nM to 10 μ M displacing ligands).

The $B_{\max}$ and K_d values obtained from Scatchard analysis of saturation binding curves (Scatchard, 1951) were determined by the KELL package of binding analysis programs for the Macintosh computer (Biosoft, Milltown, NJ). Displacement IC_{50} values were originally determined by unweighted least-squares nonlinear regression of log concentration-percent displacement data and then converted to K_i values using the method of Cheng and Prusoff (1973).

cAMP Accumulation Assay. Intracellular cAMP levels were measured with a competitive protein binding assay (Diagnostic Products, Los Angeles, CA) (Tao and Abood, 1998). Cells were harvested at 70 to 90% confluency in PBS containing 1 mM EDTA and counted with a hemacytometer. After pelleting at 500g, the cell pellet was resuspended at a concentration of 1×10^6 cells/ml in Dulbecco's modified Eagle's medium containing 20 mM HEPES pH 7.3, 0.1 mM RO-20-1724, and 1 mM isobutylmethylxanthine, and incubated for 30 min at 37°C. Aliquots of cells (90 μ l) were added to polypropylene microfuge tubes containing 1.0 μ M forskolin, compound, and 1 mg/ml fatty acid-free BSA in a final volume of 100 μ l and incubated for 5 min at 37°C. Because the compounds tested were dissolved in ethanol, all tubes contained an equivalent amount of ethanol (0.5%). The reactions were terminated by boiling for 4 min, followed by centrifugation and removal of 50 μ l of the supernatant, which was assayed for cAMP levels. The results are expressed as percent inhibition of forskolin-stimulated cAMP accumulation. EC_{50} curves were generated with the use of the GraphPad Prism program (GraphPad, San Diego, CA).

Stimulation of GTP γ S Binding. Rat cerebella were dissected on ice from three fresh male Sprague-Dawley rats. The tissue was then homogenized in centrifugation buffer (50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, pH 7.4), and the homogenate was centrifuged at 48,000g for 20 min at 4°C. The pellet was then resuspended in assay buffer (50 mM Tris-HCl, 9 mM $MgCl_2$, 0.2 mM EGTA, 150 mM NaCl, pH 7.4), homogenized, and centrifuged as previously. The final pellet was then resuspended in assay buffer, homogenized, and diluted to a concentration of $\sim 2 \mu$ g/ μ l with assay buffer. Membrane homogenates to be used for radioligand binding experiments were resuspended in buffer A (50 mM Tris-HCl, 1 mM EDTA, 3 mM $MgCl_2$, 1 mg/ml fatty acid-free BSA, pH 7.4). For experiments using transfected cells, membranes were prepared as described above. Aliquots were then stored at -80°C.

The methods for measuring agonist-stimulated [35 S]GTP γ S binding were adapted from those of Sim et al. (1995). Rat cerebellar membranes (10 μ g) were incubated in assay buffer, containing 0.1% BSA with GDP 100 μ M, [35 S]GTP γ S 0.05 nM, and cannabinoids/ethanol control in siliconized glass tubes. The assay buffer also contained 50 μ M PMSF unless otherwise stated. The total assay volume was 0.5 ml, which was incubated at 30°C for 30 min. The reaction was terminated by addition of 2 ml ice-cold wash buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4) followed by rapid filtration under vacuum through Whatman GF/C glass-fiber filters using a 12-well sampling manifold. The tubes were washed once with 2 ml of

ice-cold wash buffer, and the filters were washed twice with 4 ml of ice-cold wash buffer. Filters were placed into 7-ml plastic scintillation vials (Research Products International, Mount Prospect, IL). Bound radioactivity was determined by liquid scintillation spectrophotometry after extraction in 5 ml BudgetSolve scintillation fluid, having been shaken for 1 h. Nonspecific binding was determined using 10 μ M GTP γ S. Basal binding was assayed in the absence of agonist and in the presence of GDP. The stimulation by agonist was defined as a percentage increase above basal levels (i.e., [(dpm (agonist) - dpm (no agonist))/dpm (no agonist)] $\times$ 100). GTP γ S binding experiments using the human CB $_2$ -293 cells or rat CB $_2$ -293 cells were adapted from the conditions of MacLennan et al. (1998). Membranes (40 μ g) were incubated (1 h) in cell assay buffer containing 10 mM HEPES, 100 mM NaCl, 32 mM $MgCl_2$, 320 μ M GDP, 0.1% BSA, and 0.5 nM [35 S]GTP γ S in a final volume of 500 μ l. The cell assay buffer also contained 10 mM captopril and 0.1 mM PMSF. Filtration and counting methods were identical with those used for brain tissues. Data are reported as means $\pm$ S.E. of four to eight experiments performed in triplicate.

Statistical Analyses. The K_i , EC_{50} values in the mutant versus wild type cell lines, and significance of stimulation of GTP γ S binding over basal binding in rat cerebellum and in transfected cells were compared using ANOVA. The cAMP concentration-response curves were also analyzed using ANOVA. Bonferroni-Dunn post hoc analyses were conducted when appropriate. $P < .05$ defined statistical significance.

Results

Oligonucleotide primers were designed to be homologous to the predicted translation initiation and termination sites of the mouse CB $_2$ gene (GenBank accession no. U21681). The 5' primer contained an *Eco*RI site and Kozak consensus sequence and corresponded to base pairs 535–550 of the mouse genomic sequence. The 3' primer was designed to contain a *Xho*I site and corresponded to base pairs 1600–1626 on the opposite strand of mouse CB $_2$. These were used in the PCR to generate a ~ 1.1 -bp fragment in mouse and rat genomic DNA. These fragments were gel-purified, digested with *Eco*RI and *Xho*I, and cloned into the pcDNA3.1 expression vector for further analysis. Sequence analysis of the rat CB $_2$ genomic clone is shown (Fig. 1) (GenBank accession no. AF176350). The transmembrane domains and putative glycosylation and protein kinase C phosphorylation sites are indicated. The mouse CB $_2$ genomic clone (obtained from the ICR strain of mice) was identical with previous sequences [X86405 (Shire et al., 1996) and U21681]. Rat CB $_2$ is similar to the mouse (90% nucleic acid identity, 93% amino acid identity) and human (Munro et al., 1993) (81% nucleic acid identity, 81% amino acid identity). A sequence alignment is presented in Fig. 2. The three sequences share the most identity in the putative transmembrane domains, and the largest divergence is located in the C terminus. In particular, the rat and human sequences are the same length (360 amino acids) and the mouse sequence is considerably shorter (348 amino acids).

Stable cell lines were established in HEK-293 cells that expressed the rat, mouse, or human CB $_2$ receptor. No specific binding to HEK-293 cells was found before transfection (data not shown). Specific binding in the transfected cell lines was found to be linear at protein concentrations between 10 and 60 μ g/ml, but dropped off at protein concentrations over 60 μ g/ml (data not shown). Thus, 10 μ g/ml of membrane protein was used in subsequent assays, where specific binding aver-

1	GGAATTGCCACCATGGAGGGATGCCGGGAGCTGGAGTTGACCAACGGCTCCAATGGCGG	60
1	M E G C R E L E L T N* G S N G G	16
61	CTTGGAGTTCAACCCTATGAAGGAGTACATGATCTTGAGTGATGCGCAGCAGATCGCTGT	120
17	L E F N P M K E Y M I L S D A Q Q I A V	36
121	GGCGGTGCTGTGTACCCTGATGGGGCTGCTGAGTGCCTGGAGAACGTGGCTGTGCTCTA	180
37	<u>A V L C T L M G L L S A L E N V A V L Y</u>	56
181	TCTCATCTGTCTCGCAGCGCTCCGCAGGAAGCCCTCGTACCTGTTTCATCGGCAGCTT	240
57	<u>L I L S S Q R L R R K P S Y L F I G S L</u>	76
241	GGCCGGAGCTGACTTCCTGGCCAGCGTGATCTTTGCCTGCAACTTCGTATCTTCCATGT	300
77	<u>A G A D F L A S V I F A C N F V I F H V</u>	96
301	CTTTCACGGTGTGGACTCCAGGAATATCTTCTGTTGAAGATCGGCAGCGTGACCATGAC	360
97	<u>F H G V D S R N I F L L K I G S V T M T</u>	116
361	CTTCACGGCCTCTGTGGGCAGCCTGCTGCTGACTGCTGTTGACCGATACCTATGTCTGTG	420
117	<u>F T A S V G S L L L T A V D R Y L C L C</u>	136
421	CTACCCACCTACCTACAAAGCTCTCGTCACCCGTTGGGAGGGCACTGGTGGCCCTTGGTGT	480
137	<u>Y P P T† Y K A L V T R G R A L V A L G V</u>	156
481	CATGTGGGTCTCTCGGCGTTGATCTCTACCTACCGCTCATGGGTGGACTTGTGTGCC	540
157	<u>M W V L S A L I S Y L P L M G W T C C P</u>	176
541	TAGTCCCTGTTCTGAACTTTTCCCCCTGATCCCCAACGACTACCTCCTGGGCTGGCTTCT	600
177	<u>S P C S E L F P L I P N D Y L L G W L L</u>	196
601	TTTCATTGCCATCTCTTTTCTGGCATCATCTATACCTATGGGTATGTCCTCTGGAAAGC	660
197	<u>F I A I L F S G I I Y T Y G Y V L W K A</u>	216
661	ACACCAACATGTAGCCAGCTTGACTGAGCACCTGGACAGGCAGGTGCCTGGGATAGCTCG	720
217	H Q H V A S L T E H L D R Q V P G I A R	236
721	GATGCGGCTAGACGTGAGGTTGGCCAAGACTCTGGGCTGGTCATGGCTGTTCTGCTCAT	780
237	<u>M R L D V R L A K T L G L V M A V L L I</u>	256
781	ATGCTGGTTCCTGCACTGGCTCTCATGGGCCATAGCCTGGTCACCACACTGAGTGACAA	840
257	<u>C W F P A L A L M G H S L V T T L S D K</u>	276
841	GGTCAAGGAGGCCTTCGCCTTCTGCTCCATGCTGTGCCTTGTTAACTCCATGGTCAATCC	900
277	<u>V K E A F A F C S M L C L V N S M V N P</u>	296
901	TATCATTTATGCCCTGCGGAGTGAGAGATCCGCTCTGCTGCCAGCACTGCCTGACAGG	960
297	<u>I I Y A L R S G E I R S A A Q H C L T G</u>	316
961	CTGGAAGAAATATCTCCAGGGCTCGGATCTGAGGGGAAAGAAGCCCCAAAGTCCTC	1020
317	<u>W K K Y L Q G L G S E G K E E A P K S S</u>	336
1021	AGTTACAGAGACAGAGGCTGAGGTGAAAACACCACAGGGCCGGGATCCAGAACACCAGG	1080
337	<u>V T E T E A E V K T T T G P G S R T P G</u>	356
1081	CTGCTCCAACCTGCTGACTCG	1100
357	C S N C *	361

Fig. 1. Sequence of rat CB₂. The transmembrane domains are underlined according to Bramblett et al. (1995). The putative glycosylation and protein kinase C phosphorylation sites are indicated by * and †, respectively. The GenBank accession number for rat CB₂ is AF176350.

aged 68% at a radioligand concentration of 500 pM. Saturable, high-affinity binding was obtained with membranes prepared from the transfected cells, compatible with a single site. Using [³H]CP-55,940 as a radioligand, K_d values of 0.64 ± 0.05 nM and B_{max} values of 27.4 ± 6.15 pmol/mg were determined for rat CB₂-293 cells. For mouse CB₂-293 cells, the K_d was calculated to be 0.73 ± 0.20 nM and the B_{max} to be 9.9 ± 1.60 pmol/mg protein. For human CB₂-293 cells the K_d was calculated to be 0.87 ± 0.08 nM and the B_{max} 5.8 ± 0.67 pmol/mg protein.

Figure 3 shows representative radioligand displacement experiments conducted in the rat CB₂ cell line, a summary of which is included in Table 1 along with the affinities of a range of cannabinoid receptor ligands in each of the cell lines. These particular ligands were chosen as they represent members of each of the different structural classes of cannabinoid receptor compounds and also two of the more CB₂-selective ligands available (JWH-015 and JWH-051) (Showalter et al., 1996; Huffman et al., 1996). The affinities of several of the compounds at rat and mouse CB₂ receptors differed signifi-

	1				50
ratcb2	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
mousecb2	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
hcb2	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
hcb1	MKSILDGLAD	TTFRTITTDL	LYVGSNDIGY	EDIKGDMAK	LGYPQKFPL
					100
ratcb2	~~~~~	~~~~~	~~~~~	~~~MEGCREL	ELTNGSNGGL
mousecb2	~~~~~	~~~~~	~~~~~	~~~MEGCRET	EVTNGSNGGL
hcb2	~~~~~	~~~~~	~~~~~	~~~MEECWVT	EIANGSKDGL
hcb1	TSFRGSPFQE	KMTAGDNPQL	VPADQVNITE	FYNKSLSSFK	ENEENIQCGE
					150
ratcb2	EFNPMKEYMI	LSDAQQIAVA	VLCTLMGLLS	ALENAVLYL	ILSSQRLRRK
mousecb2	EFNPMKEYMI	LSSGQQIAVA	VLCTLMGLLS	ALENMAVLYI	ILSSRRVRRK
hcb2	DSNPMKDYMI	LSGPQKTAVA	VLCTLLGLLS	ALENAVLYL	ILSSHQLRRK
hcb1	NFMDIECFMV	LNPSQQLAIA	VLSLTLTGFT	VLENLLVLCV	ILHSRSLRCR
					200
ratcb2	PSYLFIGSLA	GADFLASVIF	ACNFVIFHVF	HGVDSRNIFL	LKIGSVTMTF
mousecb2	PSYLFISSLA	GADFLASVIF	ACNFVIFHVF	HGVDSNAIFL	LKIGSVTMTF
hcb2	PSYLFIGSLA	GADFLASVVF	ACSFVNFHVF	HGVDSKAVFL	LKIGSVTMTF
hcb1	PSYHFIGSLA	VADLLGSVIF	VYSFIDFHVF	HRKDSRNVFL	FKLGGVTASF
					250
ratcb2	TASVGSLLLT	AVDRYLCLCY	PPTYKALVTR	GRALVALGVM	WVLSALISYL
mousecb2	TASVGSLLVT	AVDRYLCLCY	PPTYKALVTR	GRALVALCVM	WVLSALISYL
hcb2	TASVGSLLLT	AIDRYLCLRY	PPSYKALLTR	GRALVTLGIM	WVLSALVSYL
hcb1	TASVGSFLFT	AIDRYISIHR	PLAYKRIVTR	PKAVVAFCLM	WTIAIVIAVL
					300
ratcb2	PLMGWTC..C	PSPCSELFPL	IPNDYLLGWL	LFIAILFSGI	IYTYGYVLWK
mousecb2	PLMGWTC..C	PSPCSELFPL	IPNDYLLGWL	LFIAILFSGI	IYTYGYVLWK
hcb2	PLMGWTC..C	PRPCSELFPL	IPNDYLLSWL	LFIAFLFSGI	IYTYGHVLWK
hcb1	PLLGWNCEKL	QSVCSDFPH	IDETYLFWI	GVTSVLLFI	VYAYMYILWK
					350
ratcb2	AHQHVASL..		.TEHQDRQVP	GIARMRLDVR	LAKTLGLVMA
mousecb2	AHRHVATL..		.AEHQDRQVP	GIARMRLDVR	LAKTLGLVLA
hcb2	AHQHVASL..		.SGHQDRQVP	GMARMRLDVR	LAKTLGLVLA
hcb1	AHSHAVRMIQ	RGTQKSIIH	TSEDGKVQVT	RPDQARMDIR	LAKTLVLILV
					400
ratcb2	VLLICWFPAL	ALMGHSLVTT	LSDKVKEAFA	FCSMLCLVNS	MVNPIIYALR
mousecb2	VLLICWFPAL	ALMGHSLVTT	LSDQVKEAFA	FCSMLCLVNS	MVNPIIYALR
hcb2	VLLICWFPVL	ALMAHSLATT	LSDQVKKAF	FCSMLCLINS	MVNPVIYALR
hcb1	VLIICWGPLL	AIMVYDVFGK	MNKLIKTVFA	FCSMLCLLNS	TVNPIIYALR
					450
ratcb2	SGEIRSAAQH	CLTGWKKYLQ	GLGSEGKEEA	PKSSVTETEA	EVKTTTGPGS
mousecb2	SGEIRSAAQH	CLIGWKKYLQ	GLGPEGKEEG	PRSSVTETEA	DVKTT*~~~~
hcb2	SGEIRSSAHH	CLAHWKKCVR	GLGSEAKEEA	PRSSVTETEA	DGKITPWPDS
hcb1	SKDLRHAFFRS	MFPSCEGTAQ	PLDNSMGDSD	CLHKHANNA	SVHRAAESCI
					473
ratcb2	RTPGCSNC*~	~~~~~	~~~~~	~~~~~	~~~~~
mousecb2	~~~~~	~~~~~	~~~~~	~~~~~	~~~~~
hcb2	RDLDSLDC*~	~~~~~	~~~~~	~~~~~	~~~~~
hcb1	KSTVKIAKVT	MSVSTDTSAE	AL*		

Fig. 2. Comparison of the sequences of mouse, rat, and human cannabinoid receptors. The predicted amino acid sequences were aligned using the PILEUP program (Genetics Computer Group, Madison, WI). Amino acids common to the four receptors are shown in bold. Underlined residues are unique to rat CB₂. Dots indicate gaps introduced to produce the alignment.

cantly from their affinities at the human CB₂ receptor. These included WIN-55212-2 and anandamide. The differences are reasonably large, with the affinity of WIN-55212-2 and anandamide reduced 10- and ~30-fold, respectively, from the human to the rat.

The expressed rat CB₂ receptor also coupled to adenylate cyclase with WIN-55212-2 and CP-55,940 both inhibiting forskolin-stimulated cAMP accumulation with $E_{\max}$ values of 32 and 17%, respectively, at concentrations of 10 μ M. The concentration-response curves were analyzed (one-way ANOVA, Dunnett's post hoc ($P < .05$)) and only WIN-55212-2 was found to cause a significant concentration-related inhi-

bition of cAMP accumulation. The EC₅₀ value was 325.1 nM (WIN-55212-2) (Fig. 4).

To investigate the pharmacological properties of PEA at the two cannabinoid receptors, its ability to displace [³H]CP-55,940 from CB₁ receptors in the presence and absence of PMSF (50 μ M) and from cells transfected with human or rat CB₂ receptors was examined. The cerebellum was chosen as a source of CB₁ receptors as it is known to contain a large number of these receptors, but thought to lack CB₂ (Herkenham et al., 1991; Griffin et al., 1999). The results are shown in Table 2. At concentrations up to 100 μ M, PEA only marginally displaced [³H]CP-55,940 (~50% for both the rat and

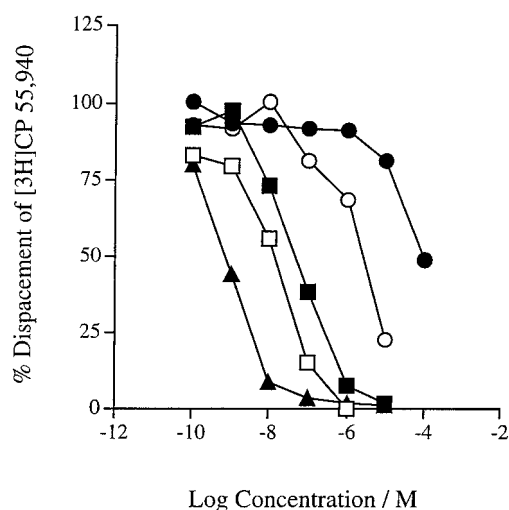


Fig. 3. Ability of five cannabinoid receptor ligands to displace [³H]CP-55,940 from membranes prepared from the rat CB₂ cell line. The ligands used were WIN-55212-2 (□), THC (■), anandamide (○), PEA (●), and SR144528 (▲). Points indicate a % displacement of [³H]CP-55,940 from a representative experiment for each ligand.

TABLE 1

Binding profile of rat, mouse, and human CB₂ cell lines

Inhibition constants were obtained from experiments using stably transfected 293 cells (see *Experimental Procedures*). Data shown are the means $\pm$ S.E. of at least two experiments performed in triplicate.

Ligand	Rat K_i	Mouse K_i	Human K_i
	nM	nM	nM
WIN-55212-2	10.4 $\pm$ 2.30*	9.46 $\pm$ 0.65*	1.19 $\pm$ 0.05
CP-55,940	0.64 $\pm$ 0.06	0.73 $\pm$ 0.20	0.88 $\pm$ 0.09
Δ^9 -THC	28.3 $\pm$ 0.06	27.3 $\pm$ 3.5	44.9 $\pm$ 21.8
Anandamide	>10 μ M*	1480 $\pm$ 134*	306 $\pm$ 48
SR144528	0.30 $\pm$ 0.14	0.05 $\pm$ 0.02*	0.32 $\pm$ 0.01
JWH-015	269 $\pm$ 46.1	431 $\pm$ 28.2*	98.4 $\pm$ 8.90
JWH-051	3.16 $\pm$ 0.52*	0.72 $\pm$ 0.23	0.33 $\pm$ 0.02

*, statistically significant differences from human CB₂ ($P < .05$).

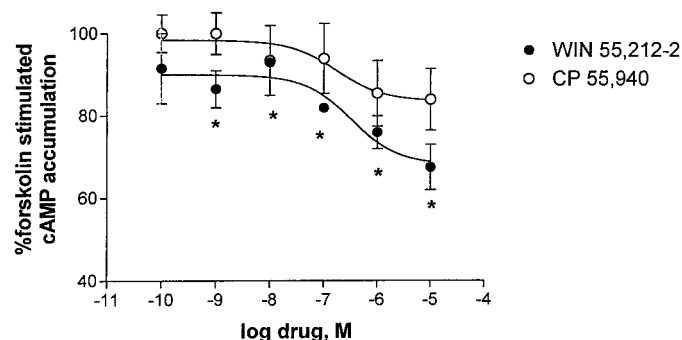


Fig. 4. Ability of WIN-55212-2 (filled symbols) and CP-55,940 (open symbols) to inhibit forskolin-stimulated cAMP accumulation in HEK-293 cells transfected with the cloned rat CB₂ receptor. The concentration of forskolin used in these experiments was 1 μ M. Basal cAMP levels were 4.4 ± 1.7 pmol/10⁶ cells/5 min. Points indicate mean $\pm$ S.E. percentage of forskolin-stimulated cAMP calculated from three experiments, each performed in triplicate. Asterisks represent points on the WIN-55212-2 concentration-response curve that differ significantly from control ($P < .05$).

human CB₂ receptors and 20% in the cerebellar membranes). PMSF had no significant effect on the ability of PEA to displace [³H]CP-55,940 in the cell lines or in the cerebellum. Because significant displacement of the radioligand only occurred at the highest concentration of PEA used (100 μ M)

TABLE 2

Binding affinity of PEA in a variety of membrane preparations

Inhibition constants were obtained from experiments using stably transfected 293 cells or from rat cerebellar membranes (see *Experimental Procedures*).

Tissue	% Displacement of Bound [³ H]CP55,940 (at 100 μ M)
Cerebellum (with PMSF)	23.2
Cerebellum (without PMSF)	19.2
Human CB ₂ -293 cells	50.2
Rat CB ₂ -293 cells	46.2

due to its very low affinity within any of the membrane preparations used, displacement curves could not be constructed and hence the expression of the results as a percentage displacement at 100 μ M rather than as K_i values. Furthermore, to assess whether or not this binding resulted in functional activation of either CB₁ or CB₂ receptors, the ability of PEA to stimulate GTP γ S binding in rat cerebellar or transfected cell membrane preparations was investigated. It was found that PEA caused a concentration-dependent stimulation of GTP γ S binding in cerebellar membranes at concentrations from 10 to 100 μ M. This stimulation (maximum of 18.73% at 100 μ M) was antagonized by the CB₁ receptor antagonist, SR141716A (Fig. 5), suggesting an activation of CB₁ receptors. PEA stimulated GTP γ S binding in human and rat CB₂-transfected cells at a concentration of 100 μ M, although the level of stimulation was greater than in the cerebellum (Fig. 6). Interestingly, the level of stimulation of GTP γ S binding was greater in the human CB₂-transfected cells than in the rat. The maximal level of stimulation of GTP γ S binding by PEA in the human CB₂ cell line was similar to that obtained with 1 μ M CP-55,940 (105 $\pm$ 12%) in the same cell line, under identical conditions, a value which correlates closely with that previously found by MacLennan et al. (1998).

Cannabinoid receptors, both CB₁ and CB₂, are members of the superfamily of GCR. It is also well established that many GCR, including α_2A - and β_2 -adrenergics and D₁ dopamine receptors (Kennedy and Limbird, 1984; O'Dowd et al., 1989; Ng et al., 1994) are subject to regulation of activity by palmitoylation of the receptor by the binding of palmitic acid to the receptor. As it is feasible that one of the breakdown products of PEA is palmitic acid, preliminary experiments were conducted to investigate whether palmitic acid bound to and/or activated either the CB₁ or CB₂ receptor and if this may account for the apparent activity of PEA. It was found that palmitic acid displaced [³H]CP-55,940 with approximately the same affinity as PEA, 30% displacement at 100 μ M (CB₁ receptors), and 25% at 100 μ M (CB₂ receptors), but did not, at concentrations up to 100 μ M, produce significant effects on GTP γ S binding at either receptor (results not shown). These results suggest that although a limited binding of palmitic acid may occur, it is not accountable for the activation of the cannabinoid receptors observed with PEA.

Discussion

In this study, the successful cloning of the rat CB₂ receptor is reported. Comparison of the receptor with the previously reported mouse and human CB₂ receptor sequences reveals a 90% nucleic acid and 93% amino acid identity with the mouse and a 81% nucleic acid and amino acid identity with the

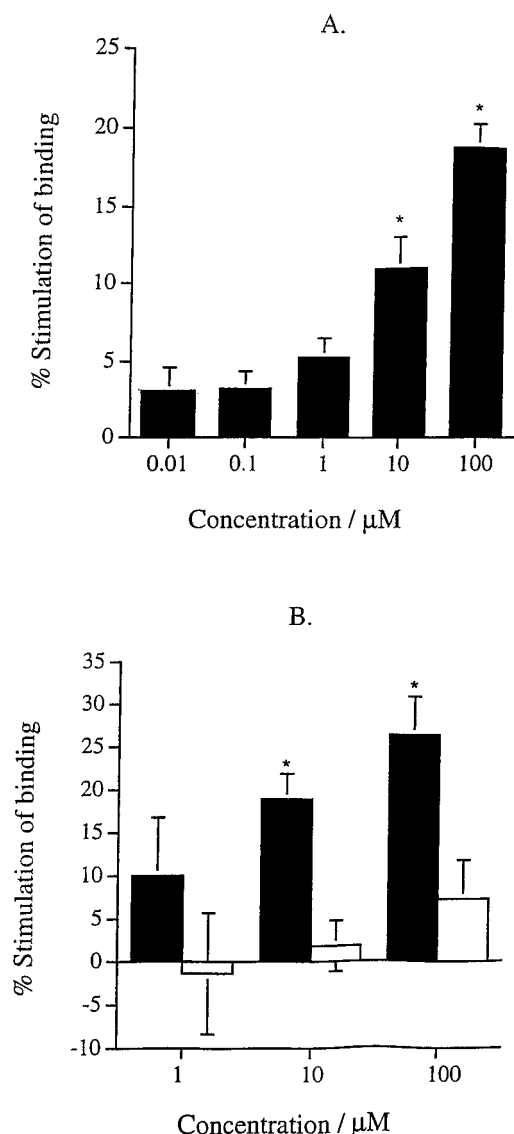


Fig. 5. Ability of PEA to stimulate GTP γ S binding in rat cerebellar membranes. A, PEA concentration response data. B, effect of SR141716A, 3 nM (white columns), on the PEA concentration response curve. Bars indicate mean $\pm$ S.E. percentage stimulation over basal levels calculated from four to eight experiments, each performed in triplicate. The asterisk represents statistical significance ($P < .05$; one-way ANOVA, Dunnett's post hoc).

human. Unlike the CB₁ receptor, which is highly conserved across the three species, the CB₂ receptor is much more divergent. This observation may have implications for future pharmacological studies involving the CB₂ receptor. It is possible that these differences may be reflected in differing pharmacological profiles of receptor ligands depending on the particular species in which the receptor is studied. Indeed, this may already be reflected in the literature to date with several discrepancies being reported. An example of this would be from the study of Berglund et al. (1998) using anandamide analogues in mouse spleen membranes and human tonsillar preparations, both rich in CB₂ receptors. The study found altered affinities and selectivities of methanandamide analogues at human, compared with mouse, CB₂ receptors. Although the authors noted several differences

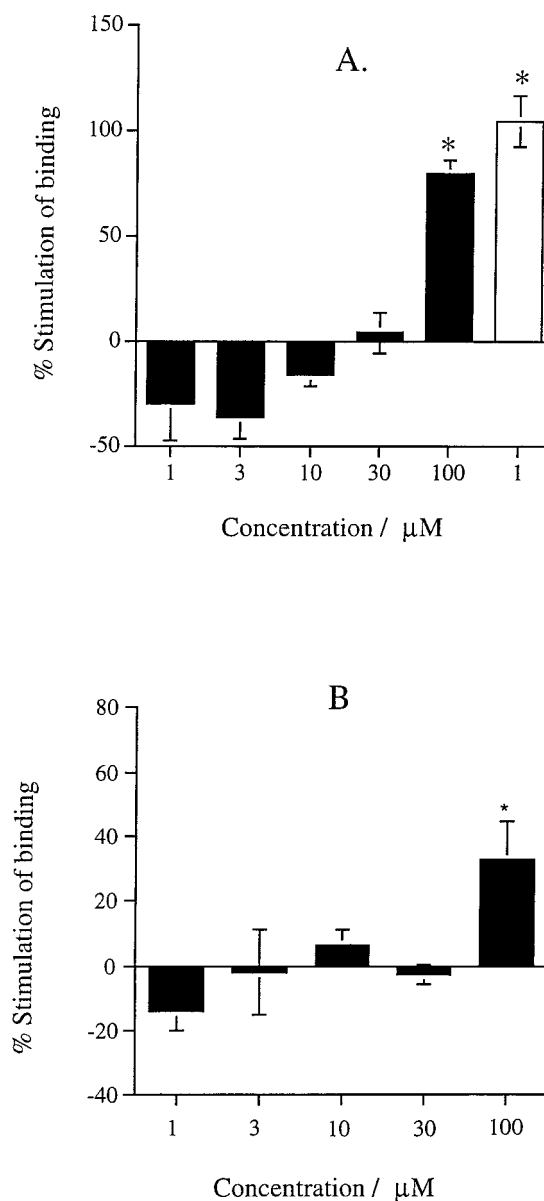


Fig. 6. Ability of PEA to stimulate GTP γ S binding in membranes prepared from HEK-293 cells transfected with human CB₂ receptors (A) or rat CB₂ receptors (B). The effect of 1 μ M CP-55,940 is also shown in A (■). Bars indicate mean $\pm$ S.E. percentage stimulation over basal levels calculated from five to six experiments, each performed in triplicate. The asterisk represents statistical significance ($P < .05$; one-way ANOVA, Dunnett's post hoc).

between the two studies, it is also possible that the differences reflected a species difference.

To compare the cloned rat CB₂ receptor with the human and rat variations, the binding of a number of cannabinoid receptor ligands was compared in HEK-293 cells transfected with either rat, mouse, or human CB₂ receptors. Interestingly, a number of compounds exhibited significant differences in affinity at rat CB₂ (and/or mouse) compared with the human CB₂ receptor. The affinity of anandamide was reduced at rat (~30-fold) and mouse (9-fold) CB₂ receptors compared with human CB₂ receptors. Similarly, the affinity of WIN-55212-2 was reduced 10-fold by the same comparisons. The observed difference with anandamide is particularly noteworthy as anandamide has previously been shown

to exhibit different pharmacological properties depending on the species used. Furthermore the tritiated form of WIN-55212-2 is frequently used for CB₂ binding studies as it has a higher CB₂ selectivity than the other more commonly used cannabinoid radioligands (Showalter et al., 1996). However, the degree of its selectivity may also be dictated by the species in which the receptor is being studied. The extent to which these differences in cannabinoid receptor ligand affinity transfer to pharmacological differences in vivo and in vitro remains to be established.

PEA has been proposed to act as an endogenous CB₂ receptor ligand. There are similarities between this compound and a previously described endogenous cannabinoid, anandamide. Both are structurally similar ethanolamide derivatives of membrane lipids, palmitic acid and arachidonic acid, and both are thought to be inactivated by the same enzyme (Di Marzo et al., 1998). We have previously reported that PEA was unable to displace [³H]CP-55,940 from human CB₂ receptors (Showalter et al., 1996), a finding in contradiction to Facci et al. (1995), who reported high-affinity binding to rat CB₂ receptors. To test the possibility that this difference was as a result of a species difference, we tested the affinity of PEA at the cloned rat CB₂ receptor. PEA only significantly displaced [³H]CP-55,940 at a concentration of 100 μ M, a very much lower affinity than previously reported. Similarly, using the GTP γ S binding assay and rat CB₂-293 cells, only this highest concentration of PEA (100 μ M) was able to produce a significant stimulation of binding. Likewise, when using cerebellar membranes (a tissue proposed to contain only CB₁ receptors), PEA (100 μ M) produced only about 20% displacement of [³H]CP-55,940. Binding of PEA in cells and cerebellum was unaffected by the presence of the nonspecific amidase inhibitor, PMSF, which has previously been demonstrated to increase the affinity of anandamide in certain tissues (Abadji et al., 1994). Similarly, using the GTP γ S binding assay and cerebellar membranes, significant stimulation of binding was only seen at high ligand concentrations. Furthermore, this stimulation was susceptible to antagonism by the CB₁-selective antagonist SR141716A (Rinaldi-Carmona et al., 1994) at a concentration that would indicate functional antagonism of CB₁ receptors. The results with the GTP γ S experiments depicted in Fig. 5 (A and B) raise the interesting possibility that the lower concentrations of PEA (1.3 and 10 μ M in the human cell line and 1 μ M in the rat) are having an inverse, albeit nonconcentration-dependent effect on GTP γ S binding. However, the only value that is significantly different from 0 is that of 3 μ M in the human cell line ($P < .05$, one-way ANOVA, Dunnett's post hoc). The lack of a concentration dependence of effect suggests that this is most likely not a significant pharmacological property of PEA and may simply be experimental variation. Overall, these results would suggest that PEA, rather than being an endogenous CB₂ receptor ligand, is capable of binding to and activating both CB₁ and CB₂ receptors in the rat, although whether the concentrations of PEA required to do this are physiologically relevant remains to be established.

The rat CB₂ receptor also demonstrated coupling to G-proteins and to adenylate cyclase by its activity in two different functional assays, stimulation of GTP γ S binding and inhibition of forskolin-stimulated cAMP accumulation. The EC₅₀ value of WIN-55212-2 in the cAMP assay is in line with both affinity at the receptor and with previously published

data. However, the $E_{\max}$ value is significantly less than similar results using human CB₂-expressing cell lines (Tao and Abood, 1998). Similarly, PEA produced a lower level of stimulation of GTP γ S binding in the rat CB₂ cell line when compared with the human. Whether this truly reflects a reduced coupling efficiency of this species receptor or is simply a result of the difference in receptor expression levels (27.4 ± 6.15 pmol/mg for the rat CB₂-293 cells and 5.8 ± 0.67 pmol/mg protein for the human equivalent) remains to be established.

Interestingly, the maximal levels of stimulation of GTP γ S binding observed varied between the CB₁ and CB₂ receptors. Although it is not possible to directly compare the two due to the differences in the assay conditions, the activity of PEA may be compared with other cannabinoid receptor agonists tested under the same conditions. In the rat cerebellum, PEA produces about 20 to 25% of the maximal stimulation produced by a full agonist, WIN-55212-2 (Griffin et al., 1998), whereas in the human CB₂-293 cells, PEA produces about 90% of the stimulation of a full agonist, CP-55,940. However, it is premature to draw conclusions from the data presented in this study regarding the efficacy of PEA at CB₁ and CB₂ receptors due to the incomplete concentration-response curves constructed (as a result of the very low potency of PEA), but it may give a preliminary indication of its activity at the two receptors.

The results of the experiments with palmitic acid suggest that although the role of, and extent of, palmitoylation of the cannabinoid receptors may or may not be a relevant physiological phenomenon, the breakdown of PEA to palmitic acid and a possible subsequent activity of the metabolite is not accountable, at least under the conditions being measured in this study, for the apparent ability of PEA to cause activation of cannabinoid receptors. Nonetheless, the possibility of other active breakdown products giving rise to an activity of PEA cannot be discounted.

In summary, we report the cloning of the rat CB₂ receptor, the isolation and expression of which allows further opportunity to probe the structure and regulation of this receptor. The difference in ligand recognition between rat mouse and human CB₂ receptors suggest species specificity that may account for previously reported pharmacological differences.

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