

Mutation of a Highly Conserved Aspartate Residue in the Second Transmembrane Domain of the Cannabinoid Receptors, CB1 and CB2, Disrupts G-Protein Coupling¹

QING TAO and MARY E. ABOOD

Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia

Accepted for publication January 21, 1998 This paper is available online at <http://www.jpet.org>

ABSTRACT

The cannabinoid receptors, CB1 and CB2, are members of the G-protein coupled receptor family and share many of this family's structural features. A highly conserved aspartic acid residue in the second transmembrane domain of G-protein coupled receptors has been shown for many of these receptors to be functionally important for agonist binding and/or G-protein coupling. To determine whether this residue is involved in cannabinoid receptor function, we used site-directed mutagenesis of receptor cDNA followed by expression of the mutant receptor in HEK 293 cells. Aspartate 163 (in CB1) and aspartate 80 (in CB2) were substituted with either asparagine or glutamate. Stably transfected cell lines were tested for radioligand binding and inhibition of cAMP accumulation. Binding of the cannabi-

noid receptor agonist [³H]CP-55,940 was not affected by either mutation in either the CB1 or CB2 receptor, nor were the affinities of anandamide or (-)- Δ^9 -tetrahydrocannabinol. Binding of the CB1-selective receptor antagonist SR141716A also was unaltered. However, the affinity of WIN 55,212-2 was attenuated significantly in the CB1, but not the CB2, mutant receptors. Studies examining inhibition of cAMP accumulation showed reduced effects of cannabinoid agonists in the mutated receptors. Our data suggest that this aspartate residue is not generally important for ligand recognition in the cannabinoid receptors; however, it is required for communication with G proteins and signal transduction.

The cannabinoid receptor is the initial site of action for the most widely abused street drug, marijuana. Marijuana has prominent effects on the central nervous system as well as numerous peripheral effects, including immunomodulation. The primary psychoactive constituent in marijuana, and prototypical cannabinoid compound is Δ^9 -THC. Δ^9 -THC and structurally related cannabinoids are extremely lipophilic molecules, and for many years it was difficult to prove that the unique profile of pharmacological effects produced by these drugs was receptor-mediated, for instance, by demonstration of high-affinity specific binding with Δ^9 -THC (reviewed in Martin, 1986). However, studies in neuroblastoma cells had shown an inhibition of adenylyl cyclase activity that was specific for psychoactive cannabinoids, implicating a GPCR-mediated process (Howlett and Fleming, 1984). The

development of [³H]CP-55,940, a highly potent synthetic analog of Δ^9 -THC, allowed the identification of specific cannabinoid binding sites in the brain (Devane *et al.*, 1988; Herkenham *et al.*, 1990). Then, a rat brain cDNA clone isolated by homology to GPCRs was identified as the cannabinoid receptor (CB1) by virtue of its ability to induce cannabinoid-mediated inhibition of cAMP accumulation in transfected cells and the similarities in its expression pattern to that of [³H]CP-55,940 binding sites (Matsuda *et al.*, 1990). Shortly thereafter, the cloning of a human CB1 receptor cDNA was reported (Gerard *et al.*, 1991). This CB1 receptor is one of the most abundantly expressed of the neuronal receptors. A second cannabinoid receptor subtype (CB2) was discovered by a PCR-based strategy designed to isolate GPCRs in differentiated myeloid cells (Munro *et al.*, 1993). The CB2 receptor, which has been found in the spleen and cells of the immune system, has 44% amino acid identity with the brain clones. The affinities for several cannabinoids at

Received for publication October 14, 1997.

¹This work was supported by National Institutes of Health grants DA-05274 and DA-09978 and the Council for Tobacco Research grant 4482.

ABBREVIATIONS: CB1, central cannabinoid receptor; CB2, peripheral cannabinoid receptor; GPCR, G-protein-coupled receptor; Δ^9 -THC, (-)- Δ^9 -tetrahydrocannabinol; CP-55,940, (-)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-[3-hydroxy propyl] cyclohexan-1-ol; WIN-55,212-2, (R)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone; SR141716A, [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride]; BSA, bovine serum albumin; TM2, transmembrane domain 2; DMEM, Dulbecco's modified Eagle's medium; CHO, Chinese hamster ovary; EDTA, ethylenediaminetetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; ANOVA, analysis of variance; PCR, polymerase chain reaction.; DMH, dimethylheptyl.

the CB2 receptor are distinct from that of the brain receptor (Showalter *et al.*, 1996). The CB2 receptor also mediates inhibition of cAMP accumulation (Felder *et al.*, 1995; Slipetz *et al.*, 1995).

A family of endogenous ligands has been identified for these receptors, of which arachidonic acid ethanolamide (anandamide) was the first (Devane *et al.*, 1992). The isolation of endogenous ligands has provided additional evidence supporting the role of cannabinoid receptors as important neurochemical and immune system modulators. In addition, the recent development of a selective antagonist to the CB1 receptor, SR141716A, provides a tool for determining the receptor-mediated *vs.* the non-receptor-mediated effects of the cannabinoids (Rinaldi-Carmona *et al.*, 1994).

In vitro mutagenesis of cloned cDNAs provides a means of examining the specific functions of the proteins they encode. Selected mutations can be introduced into regions of the receptor cDNAs believed to be critical to receptor recognition or second messenger function. The aspartic acid residue in the second transmembrane domain is highly conserved among GPCRs. Mutational studies have shown that this residue is important for ligand recognition, cation selectivity and/or coupling to G proteins in various receptors in this family (summarized in Ceresa and Limbird, 1994). To determine the role of this residue in the function of the human cannabinoid receptors (CB1 and CB2), aspartate 163 (CB1) and aspartate 80 (CB2) were replaced with glutamic acid or asparagine. Our data suggest that this aspartate residue is not generally important for ligand recognition in the cannabinoid receptors, but rather is involved in G-protein coupling and, thereby, signal transduction.

Methods

Materials. [³H]CP-55,940 and [³H]WIN-55,212-2 were purchased from DuPont-NEN (Wilmington, DE). [³H]SR141716A was purchased from Amersham (Arlington Heights, IL). Δ⁹-THC and anandamide were obtained from the National Institutes on Drug Abuse (Rockville, MD). CP-55,940 initially was provided by Dr. Larry Melvin (Pfizer Inc., Groton, CT). SR141716A was synthesized by Dr. John Lowe (Pfizer Inc., Groton, CT). WIN-55,212-2 originally was provided by Dr. Susan Ward (Sterling-Winthrop Research Institute, Rensselaer, NY). 11-Hydroxy-Δ⁸-THC-dimethylheptyl was provided generously by Dr. Raphael Mechoulam (Hebrew University, Jerusalem, Israel). Human embryonic kidney HEK 293 cells were obtained from American Type Culture Collection. The human CB1 cDNA was provided by Dr. Marc Parmentier (Universite Libre de Bruxelles, Belgium). The human CB2 cDNA was provided by Dr. Sean Munro (MRC, Cambridge, England).

Mutagenesis. The Altered Sites (Promega Corp., Madison, WI) *in vitro* mutagenesis system was used to mutate the CB1 receptor. The human CB1 cDNA was subcloned into the pALTER phagemid, and with the helper phage R408, single-stranded templates were produced. The desired mutation was produced by annealing a complementary mutagenic oligonucleotide as well as an oligonucleotide which confers ampicillin resistance to the single-stranded template followed by elongation with T4 DNA polymerase and ligation. The heteroduplex DNA was used to transform the repair-minus *Escherichia coli* strain BMH 71-18 mut S and the cells grown in the presence of ampicillin. A second round of transformation in JM109 ensured proper segregation of mutant and wild-type plasmids. The mutations were confirmed by sequencing, and the mutated cDNA subcloned into the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA) for expression. To mutate D163 to E163 of CB1, the mutagenic oligonucleotide (5' CGG TGG CAG AAC TCC TGG

GGA) containing the desired mutation (GAC to GAA) was used. The entire cDNA insert was then sequenced to confirm the absence of additional mutations. To make the D163N mutation, the mutagenic oligonucleotide (5' CGG TGG CAA ACC TCC TGG GGA) containing the desired mutation (GAC to AAC) was used.

Mutations of the CB2 receptor were introduced with the QuikChange site-directed mutagenesis kit (Stratagene, LaJolla, CA) (Papworth *et al.*, 1996). This method allows mutagenesis to be performed in any vector, hence we used human CB2 which we had subcloned into pcDNA3 (Showalter *et al.*, 1996). Oligonucleotide primers, each complementary to opposite strands of the sequence to be altered were annealed and extended during 12 cycles of temperature cycling by means of *Pfu* DNA polymerase (which replicates both strands with high fidelity and without displacing the mutant oligonucleotide primers). The product was treated with *DpnI*, which digests methylated and hemimethylated DNA (the parental, nonmutated DNA), then the remainder (containing nicked, double-stranded mutant DNA) transformed into *E. coli*. The DNAs were sequenced to confirm mutation in the desired regions only. To make the D80E mutation, the primers GCT GGG GCT GAA TTC CTG GCC (forward) and GGC CAG GAA TTC AGC CCC AGC (reverse) containing the desired mutation (GAC to GAA) were used. To make the D80N mutation, the primers GCT GGG GCT AAC TTC CTG GCC (forward) and GGC CAG GAA GTT AGC CCC AGC (reverse) containing the desired mutation (GAC to AAC) were used.

Cell culture and transfection. Human embryonic kidney 293 cells were maintained in DMEM 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 wild-type or mutant CB1pcDNA3 or CB2pcDNA3 into 293 cells by the Lipofectamine reagent (Life Technologies, Gaithersburg, MD). Stable transformants were selected in growth medium containing geneticin (1 mg/ml, reagent, Life Technologies, Gaithersburg, MD). 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. Cell lines containing moderate to high levels of receptor mRNA were tested for receptor-binding properties. Transfected cell lines (including CB2-CHO, previously described in Showalter *et al.*, 1996) were maintained in DMEM with 10% fetal clone II plus 0.3 to 0.5 mg/ml geneticin and 5% CO₂ at 37°C in a Forma incubator.

[³H]Cannabinoid binding in cells. The current assay is a modification of Compton *et al.* (1993). Cells were harvested in phosphate-buffered saline containing 1 mM EDTA and centrifuged at 500 × *g*. The cell pellet was homogenized in 10 ml of solution A (50 mM Tris-HCl, 320 mM sucrose, 2 mM EDTA, 5 mM MgCl₂, pH 7.4). The homogenate was centrifuged at 1,600 × *g* (10 min), the supernatant saved and the pellet washed three times in solution A with subsequent centrifugation. The combined supernatants were centrifuged at 100,000 × *g* (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 approximately 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 40 to 50 μg membrane protein to silanized tubes containing [³H]CP-55,940 (102.9 Ci/mmol), [³H]WIN-55,212-2 (45.5 Ci/mmol) or [³H]SR141716A (55 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 μM unlabeled CP-55,940 was used to assess nonspecific binding. After incubation (30°C for 1 hr), 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 hr]. Tubes were rinsed with 2 ml of ice-cold buffer D, which was also filtered, and the filters subsequently rinsed twice with 4 ml of ice-cold buffer D. Before radioactivity was quantitated by liquid scintillation spectrometry, filters were shaken for 1 hr in 5 ml of scintillation fluid.

CP-55,940 and all cannabinoid analogs were prepared by suspen-

sion in assay buffer from a 1 mg/ml ethanolic stock without evaporation of the ethanol (final concentration of no more than 0.4%). When anandamide was used as a displacing ligand, experiments were performed in the presence of phenylmethylsulfonyl fluoride (50 μ M). Saturation experiments were conducted with seven concentrations of [3 H]CP-55,940 ranging from 250 pM to 5 nM. Competition assays were conducted with 1 nM [3 H]CP-55,940 or 1 nM [3 H]SR141716A and six concentrations (0.1 nM to 10 μ M displacing ligands). For the allosteric regulation studies, the addition of 150 mM NaCl or 0.1 to 1 mM Gpp(NH)p (Calbiochem, La Jolla, CA) was assessed with a 1 nM concentration of [3 H]CP-55,940 or [3 H]WIN-55,212-2.

The B_{max} and K_d values obtained from Scatchard analysis of saturation binding curves (Rosenthal, 1967; Scatchard, 1951) were determined by the KELL package of binding analysis programs for the Macintosh computer (Biosoft, Milltown, NJ). Displacement IC_{50} values originally were determined by unweighted least-squares linear regression of log concentration-percent displacement data and then converted to K_i values by the method of Cheng and Prusoff (1973).

cAMP accumulation assay. Intracellular cAMP levels were measured with a competitive protein binding assay (Diagnostic Products, Inc., Los Angeles, CA) (Abood and Tao, 1995). Cells were harvested at 70 to 90% confluence by mechanical disruption in phosphate-buffered saline containing 1 mM EDTA and counted with a hemacytometer. After centrifugation at $500 \times g$, the cell pellet was resuspended at a concentration of 1×10^6 cells/ml in DMEM 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 0.5 μ M forskolin \pm cannabinoids + 5 mg/ml fatty acid-free BSA, in a final volume of 100 μ l and incubated for 5 min at 37°C. Because the cannabinoids 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. The levels of forskolin-stimulated cAMP accumulation (expressed in pmol/ 10^6 cells/min) for the cell lines tested were: 7.22 ± 1.66 (CB1); 9.39 ± 1.44 (D163E); 5.50 ± 1.05 (D163N); 8.55 ± 2.78 (CB2); 6.38 ± 0.83 (D80E); 9.72 ± 2.50 (D80N). EC_{50} curves were generated with the use of the GraphPad Prism program (GraphPad, San Diego, CA).

Statistical analysis. Statistical analysis of binding and EC_{50} data were compared by ANOVA or the student's t test, where suitable. Bonferroni *post hoc* analyses were conducted when appropriate. Statistical significance was defined as $P < .05$.

Results

Expression of the wild-type human CB1 and CB2 receptors. Stable transformants of 293 cells were established which expressed the human CB1 or CB2 receptors. No specific [3 H]CP 55,940 or [3 H]SR141716A cannabinoid binding to 293 cells was found before transfection (data not shown). With [3 H]CP 55,940 as a radioligand in the cell line expressing wild-type CB1, K_d and B_{max} values of 1.21 ± 0.27 nM and 0.95 ± 0.16 pmol/mg protein, respectively, were obtained (fig. 1, table 1). These values are similar to a CB1-expressing CHO cell line described previously ($K_d = 0.65 \pm 0.09$ nM; $B_{max} = 0.83 \pm 0.16$ pmol/mg protein, table 1) (Showalter *et al.*, 1996). In the 293 cell line stably expressing the CB2 receptor, K_d and B_{max} values of 0.88 ± 0.09 nM and 1.55 ± 0.39 pmol/mg protein, respectively, were obtained (table 1). These values are similar to a CB2-expressing CHO cell line described previously ($K_d = 0.61 \pm 0.14$ nM; $B_{max} =$

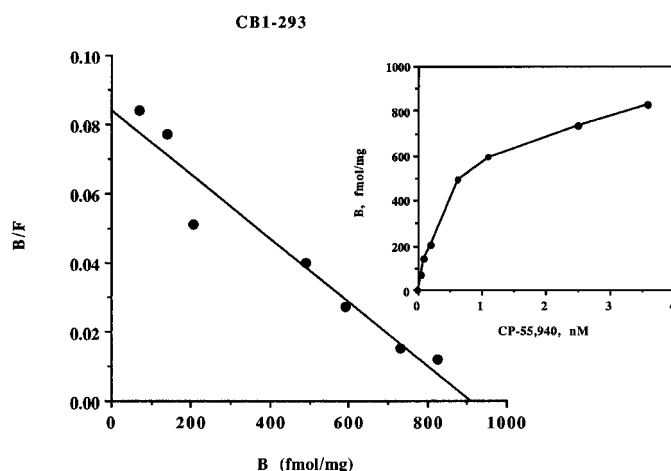


Fig. 1. Representative Scatchard and saturation (inset) analysis of [3 H]CP-55,940 binding to membranes prepared from transfected 293 cells expressing human CB1. In this experiment, K_d and B_{max} values were 1.02 nM and 898 fmol/mg protein for CB1. These data are representative of three experiments performed in triplicate.

TABLE 1

Scatchard analysis of [3 H]CP-55,940 binding

The wild-type CB1 receptor was expressed in CHO (CB1-CHO) and 293 (CB1-293) cells. Similarly, the wild-type CB2 receptor was expressed in CHO (CB2-CHO) and 293 (CB2-293) cells. The mutant CB1 (D163N, D163E) and CB2 (D80N, D80E) receptors were expressed in 293 cells. The data shown are the means \pm S.E. of at least three experiments performed in triplicate.

Cell Line	K_d	B_{max}
	nM	pmol/mg protein
CB1-CHO	0.65 ± 0.09	0.83 ± 0.16
CB1-293	1.21 ± 0.27	0.95 ± 0.16
D163N	2.83 ± 0.63	0.99 ± 0.34
D163E	0.81 ± 0.17	0.76 ± 0.18
CB2-CHO	0.61 ± 0.14	3.10 ± 0.90
CB2-293	0.88 ± 0.09	1.55 ± 0.39
D80N	0.48 ± 0.09	1.76 ± 0.68
D80E	0.60 ± 0.20	1.65 ± 0.29

3.1 ± 0.9 pmol/mg protein, table 1) (Showalter *et al.*, 1996). The binding characteristics of the cannabinoid receptors are the same whether they are expressed in 293 cells or in CHO cells (tables 1-3 and Showalter *et al.*, 1996).

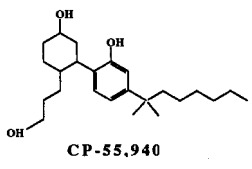
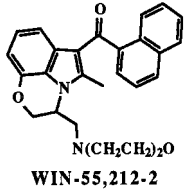
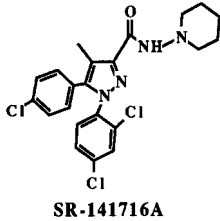
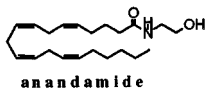
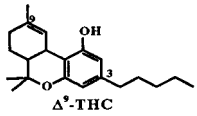
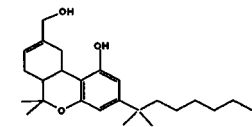
To establish the pharmacological profile of the wild-type CB1 and CB2 receptors expressed in 293 cells, several representative cannabinoid ligands were tested for inhibition of [3 H]CP-55,940 binding. The K_i values, shown in table 2, demonstrate that the binding profile with these compounds in the wild-type CB1-transfected cells is similar to binding in the brain (a rich source of CB1 receptors). The data in table 3 show that binding in the wild-type CB2-transfected cells also parallels that obtained in mouse spleen (a predominantly CB2-expressing tissue; Schatz *et al.*, 1997). The only compound that exhibited significantly different K_i values in the transfected cells *vs.* native tissues was WIN 55,212-2. The reported K_i value for WIN 55,212-2 in brain (1.89 ± 0.09 nM; Kuster *et al.*, 1993 and table 1) was lower than that obtained in the CB1-transfected cell line (17.4 ± 6.2 nM, table 1). Conversely, the K_i value for WIN 55,212-2 in spleen (6.8 ± 0.6 nM; Schatz *et al.*, 1997 and table 3) was 23-fold higher than that found in the CB2-transfected cells (0.28 ± 0.16 nM, table 1, $P < .05$, ANOVA).

The CB1- and CB2-transfected 293 cells also demonstrated functional coupling to G proteins, as measured by inhibition

TABLE 2

Comparison of receptor affinities: CB1

Values from competition assays with 1 nM [³H]CP-55,940 are presented as mean ± S.E. of three experiments.

	K_i			
	CB1 ^a	CB1-293	D163N	D163E
	<i>nM</i>			
 CP-55,940	0.92 ± 0.14 ^b	1.21 ± 0.28	2.83 ± 0.63	0.81 ± 0.17
 WIN-55,212-2	1.89 ± 0.09 ^c	17.4 ± 6.2	781 ± 226*†	148 ± 11.5*†
 SR-141716A	0.16 ± 0.01 ^d	0.68 ± 0.12	0.435 ± 0.03	0.31 ± 0.11
 anandamide	89 ± 10 ^e	321 ± 85	450 ± 87†	146 ± 49
 Δ⁹-THC	40.7 ± 1.7 ^b	33.6 ± 9.5	37.2 ± 7.3	13.3 ± 9.1
 (-)-11-OH-Δ⁸-THC-DMH	0.73 ± 0.11 ^b	0.44 ± 0.16	2.1 ± 0.37*	

^a CB1 binding data in the brain.^b From Compton *et al.* (1993).^c From Kuster *et al.* (1993).^d From Abood *et al.* (1997).^e From Adams *et al.* (1995).* Different from CB1-293 ($P < .05$).† Different from brain ($P < .05$).

of forskolin-stimulated cAMP accumulation (fig. 2). EC_{50} values were 1.64 ± 1.5 nM for WIN-55,212-2 and 4.17 ± 3.1 nM for CP-55,940 in the CB1-293 cells, with maximal inhibition of 63% (fig. 2A). EC_{50} values were 12.3 ± 3.3 nM for WIN 55,212-2 and 52.1 ± 6.2 nM for CP-55,940 for the CB2 cell line, with a maximal inhibition of 80% (fig. 2B). The lower EC_{50} value for WIN 55,212-2 than for CP-55,940 in the

CB2-transfected cells parallels the binding affinities for these compounds in the transfected cells.

Comparison of the mutant and wild-type receptors. Radioligand binding and cAMP accumulation experiments were performed with transfected cells expressing mutated CB1 or CB2 receptors. The mutation was a single base change that altered an aspartate residue in the second trans-

TABLE 3

Comparison of receptor affinities: CB2

Values from competition assays with 0.5 nM [³H]CP-55,940 are presented (means ± S.E. from three experiments).

	K_i			
	CB2 ^a	CB2	D80N	D80E
	nM			
CP-55,940	1.9 ± 0.7	0.88 ± 0.09	0.48 ± 0.04	0.60 ± 0.20
WIN-55,212-2	6.8 ± 0.6	0.14 ± 0.01†	0.48 ± 0.09†	0.19 ± 0.06†
Δ ⁹ -THC	11.8 ± 0.7	44.9 ± 12.6	16.8 ± 1.3	25.6 ± 11.2
(-)-11-OH-Δ ⁸ -THC-DMH		0.38 ± 0.01	0.49 ± 0.21	0.25 ± 0.05

^a CB2 binding in spleen from Schatz *et al.* (1997).† Different from spleen ($P < .05$).

membrane domain of CB1 (D163) or CB2 (D80) to asparagine (N) or glutamate (E). The D to N mutation removes a positive charge and has been shown in several GPCRs to be important for receptor G-protein signaling. The D to E mutant retains the charge but introduces an additional methyl group that may result in conformational changes.

The K_d and B_{max} values obtained for the mutant receptors with [³H]CP-55,940 as a radioligand were not significantly different from wild-type receptors (table 1). Mutant receptor-expressing cell lines used had B_{max} values similar to wild-type cell lines so that alterations observed could not be attributed to different receptor expression levels.

Displacement curves conducted with several representative cannabinoids revealed that the mutated receptors exhibited wild-type affinities for most of the ligands tested (tables 2 and 3). The affinities of Δ⁹-THC and 11-OH-Δ⁸-THC-DMH were unaffected by the mutations in both the CB1 and CB2 mutant cell lines (tables 2 and 3) as were the affinities of anandamide and the (CB1 receptor) antagonist SR141716A in the CB1 receptor mutants (table 2). WIN 55,212-2 was the exception in the CB1 mutant receptors. The asparagine mutant (D163N) had a 45-fold lower affinity for WIN 55,212-2 than the wild-type receptor ($P < .05$, ANOVA), whereas the glutamate mutant (D163E) exhibited 8.5-fold lower affinity ($P < .05$, ANOVA, table 2). The affinity of WIN 55,212-2 in the mutated CB2 receptors was not significantly different from the wild-type CB2 cell line (table 3).

As in several other GPCRs, the aspartate to asparagine mutation resulted in an attenuated signaling response. In contrast to the dose-responsive inhibition of forskolin-stimulated cAMP accumulation seen with the wild-type receptors, no significant inhibition was observed in the D163N CB1 line with either WIN 55,212-2 or CP-55,940 (fig. 3, A and B). Similarly, no dose-responsive inhibition of forskolin-stimulated cAMP accumulation was seen in the D80N cell line (fig. 3, C and D). The aspartate to glutamate mutants also showed reduced efficacy in this measure of coupling. Neither WIN 55,212-2 nor CP-55,940 produced significant inhibition of forskolin-stimulated cAMP accumulation in the D163E cell line (fig. 3, A and B). Some inhibition was observed in the CB2 D80E cell line; however, the maximal inhibition of forskolin-stimulated cAMP accumulation obtained with the D80E mutant was 45% at 1 μM WIN 55,212-2 as compared with 73% inhibition in wild-type cells (fig. 3C) and 35% at 1 μM CP-55,940 as compared with 80% in wild-type cells (fig. 3D).

Allosteric regulation studies. The functional uncoupling of the mutant receptors also was seen as a loss of the

ability of Gpp(NH)p to reduce agonist binding (table 4). In the wild-type CB1- and CB2-receptor expressing cell lines, inhibition of specific binding was observed in the presence of Gpp(NH)p. Addition of 1 mM Gpp(NH)p produced greater than a 38% decrease in percent specific binding of 1 nM [³H]CP 55,940 ($P < .05$). Mutation of the D to N resulted in disruption of the regulation by Gpp(NH)p (table 4). In the D to E mutants of the CB1 and CB2 receptors, a reduced inhibition by Gpp(NH)p also was observed (table 4).

Mutation of the conserved aspartate did not always result in a loss of high-affinity agonist binding in the cannabinoid receptors. In many other GPCRs, sodium and other monovalent cations reduce high-affinity agonist binding. Mutation of the conserved aspartate (or glutamate) in these receptors seems to mimic the effect of NaCl. The CB1 receptor in brain previously has been shown to be relatively insensitive to NaCl (Herkenham *et al.*, 1991), as has the cloned CB2 receptor (Showalter *et al.*, 1996). For both the wild-type CB1- and CB2-expressing 293 cells, specific [³H]CP-55,940 binding was reduced slightly in the presence of 150 mM NaCl (29–36%, table 5). The effects of NaCl on [³H]CP-55,940 binding were statistically significant in all the receptors except the CB1 D163N mutant (table 5). We did not observe inhibition of specific binding of [³H]WIN-55,212-2 in the CB1 cell lines by 150 mM NaCl. Furthermore, we were unable to achieve sufficient specific binding with [³H]WIN 55,212-2 in the D163N cell line to accurately assess the role of sodium in this mutant receptor (note the low affinity for WIN 55,212-2 in table 2).

Discussion

The present studies demonstrate that the conserved aspartate residue in the second transmembrane domain of the cannabinoid receptors, CB1 and CB2, is required for efficient signal transduction, but generally does not disrupt high-affinity agonist binding. These findings distinguish the cannabinoid receptors from other GPCRs, especially those in which inhibition of adenylyl cyclase is a predominant second messenger response. In several other GPCRs, the aspartate to asparagine mutation resulted in a loss of high-affinity agonist binding (*e.g.*, Ceresa and Limbird, 1994; Kong *et al.*, 1993A). We found that the binding of several cannabinoid ligands was not affected in either the CB1 or CB2 receptor mutations, with the exception of WIN-55,212-2 in the CB1 D163E and D163N mutants.

For several other GPCRs, the aspartate to asparagine mutation also was associated with a loss of cation-sensitivity for agonist (*e.g.*, Ceresa and Limbird, 1994; Kong *et al.*, 1993a). Previous reports have indicated that sodium does not substantially reduce high-affinity agonist binding in the cannabinoid receptors (Herkenham *et al.*, 1991; Showalter *et al.*, 1996). In the present report, the addition of NaCl to the binding buffer resulted in a slight, but significant, reduction of agonist binding in all cell lines except the CB1 D163N mutant. [³H]WIN-55,212-2 binding was not altered significantly by the addition of sodium. Sodium ions have been shown to stabilize "empty", uncoupled, receptors (Costa *et al.*, 1989), which may explain why the effect of sodium was retained in some of the mutant cannabinoid receptors. This was a preliminary attempt to address the regulation by sodium in the mutant receptors and was conducted simply by addition of 150 mM NaCl to the binding buffer. Further

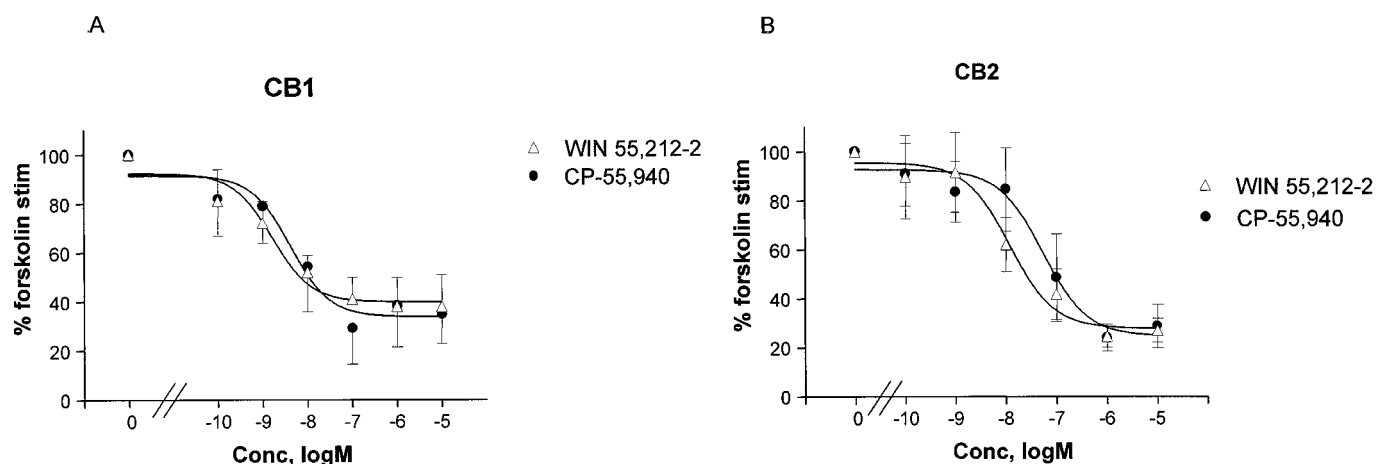


Fig. 2. Agonist-induced inhibition of cAMP accumulation in wild-type CB1 (A) and CB2 (B) receptor-expressing cell lines. EC_{50} values were 1.64 ± 1.5 nM for WIN-55,212-2 and 4.17 ± 3.1 nM for CP-55,940 for CB1-293 cells (A). EC_{50} values were 12.3 ± 3.3 nM for WIN-55,212-2 and 52.1 ± 6.2 nM for CP-55,940 for the CB2 cell line (B). Data are expressed as percent forskolin stimulation *versus* log molar drug concentration. Points, mean \pm S.E. of three or more independent experiments performed in triplicate. Curves were generated as described under "Methods."

studies (*e.g.*, controlling for ionic strength, competition curves assessing presence of multiple affinity states) are warranted to determine the role of sodium in binding to the D163N mutant and to determine the sensitivity of WIN-55,212-2 to sodium.

The cannabinoid receptors showed greatly reduced G-protein coupling, both as measured by inhibition of adenylyl cyclase activity, as well as in the ability of guanine nucleotides to reduce agonist binding. In the *alpha-2a* adrenergic, SSTR2 somatostatin and *delta* opioid receptors, the D to N mutant retained its ability to inhibit forskolin-stimulated cAMP accumulation (Ceresa and Limbird, 1994; Kong *et al.*, 1993a, b). On the other hand, mutation of the conserved aspartate in the dopamine D2 receptor to alanine or glutamate resulted in a loss of inhibition of adenylyl cyclase activity (Neve *et al.*, 1991). Also, the ability of Gpp(NH)p to inhibit agonist binding was abolished in the *alpha-2a* adrenergic receptor D79N mutation as was functional coupling as assessed by loss of receptor-activated potassium currents (Ceresa and Limbird, 1994; Surprenant *et al.*, 1992). Furthermore, when the *alpha-2a* adrenergic receptor D79N mutation was introduced into the genome of mice, *alpha-2* adrenergic agonist mediated hypotension was abolished, which indicates that receptor-activated processes were absent in the gene-targeted mice (MacMillan *et al.*, 1996).

The highly conserved aspartate residue in the second transmembrane domain of many GPCR apparently is associated closely with an asparagine residue in the seventh transmembrane domain. Mutations of one of these residues often disrupt functional coupling, whereas reciprocal mutations restore function (Sealfon *et al.*, 1995; Suryanarayana *et al.*, 1992; Zhou *et al.*, 1994). The cannabinoid receptors also contain an asparagine residue in the seventh transmembrane region. Molecular modeling studies have implicated a hydrogen bonding network which could be involved in receptor activation by agonists (Sealfon *et al.*, 1995; Zhou *et al.*, 1994). Furthermore, when the analogous aspartate residue in the serotonin 5-HT_{2A} receptor was mutated to asparagine, this resulted in a loss of G-protein coupling, but had no effect on binding of a wide range of ligands (Sealfon *et al.*, 1995; Zhou *et al.*, 1994). Modeling results presented by Sealfon

et al. (1995) demonstrated that the mutant receptor undergoes a conformational change (in helices 5 and 6) upon agonist binding, but in the opposite direction seen with the wild-type receptor. They suggest that this conformational change still may produce a high-affinity state for ligand binding (and thus result in receptor affinities essentially unchanged from those of the wild-type receptor), but that the resulting helix arrangements may not support coupling to the appropriate G protein. A similar mechanism may arise in the cannabinoid receptors in response to agonists such as CP-55,940.

However, a different situation must arise in the CB1 receptors on binding WIN 55,212-2. Although the mutations did not eliminate high-affinity binding for most ligands tested, they did exert differential effects on WIN-55,212-2 binding. The affinity of WIN 55,212-2 but not CP-55,940 was reduced in the CB1 receptor mutants, whereas neither was reduced in the CB2 receptor mutants. WIN 55,212-2 also discriminated between receptor expression in the cell lines and the native tissues. A previous mutation study on the CB1 receptor revealed that the binding site for WIN 55,212-2 was distinct from that of other cannabinoid ligands (Song and Bonner, 1996). Mutation of a lysine residue in the third transmembrane domain of the CB1 receptor resulted in a loss of binding for Δ^9 -THC, CP-55,940 and anandamide, but not WIN 55,212-2 (Song and Bonner, 1996). Conversely, WIN 55,212-2 may bind in a less energetically favorable site in the D163N and D163E mutant cell lines. It is also possible that WIN 55,212-2 still may bind in the same site in the CB1 mutants as in the wild-type receptor, but it cannot produce the conformational change which results in the high-affinity state.

The mutated receptors were analyzed by creating stably transfected cell lines. Our previous experience in radioligand binding with the cannabinoid receptor has demonstrated the need to express the receptor in excess of 0.5 pmol/mg protein to obtain reasonable specific binding (Abood *et al.*, 1997). One concern may be that overexpression in different cell lines may lead to altered ligand affinities. However, the data showing that similar K_d values were obtained between 293 cell lines expressing CB1 or CB2 receptors as compared with CB1- or CB2-CHO cell lines demonstrate that this is not a

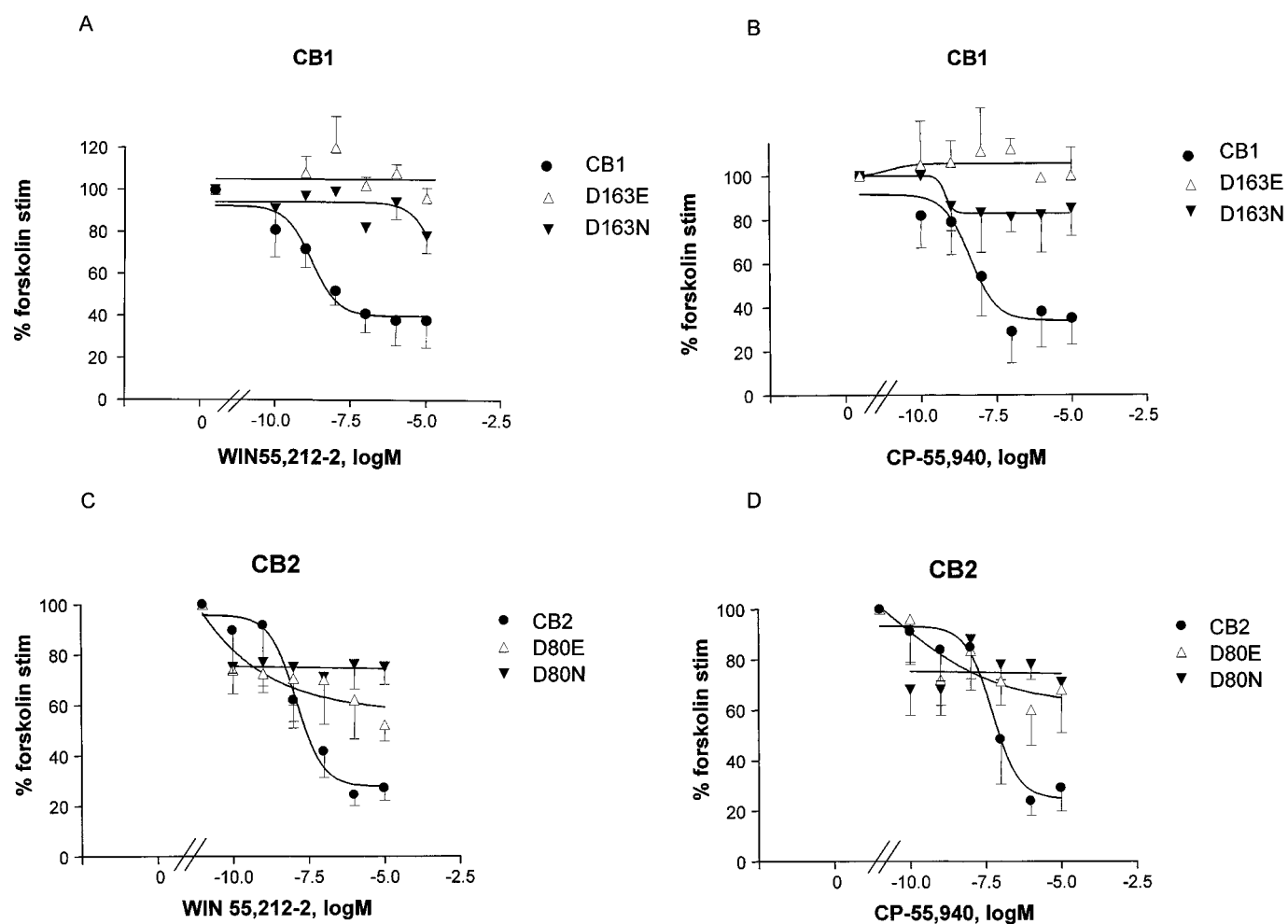


Fig. 3. Comparison between the wild-type and the mutant cannabinoid receptors for agonist-induced inhibition of cAMP accumulation. (A) Effect of WIN 55,212-2 on wild-type CB1 (●) and mutant D163E (△) and D163N (▼) receptor-expressing cell lines. (B) Effect of CP-55,940 on wild-type CB1 (●) and mutant D163E (△) and D163N (▼) receptor-expressing cell lines. (C) Effect of WIN 55,212-2 on wild-type CB2 (●) and mutant D80E (△) and D80N (▼) receptor-expressing cell lines. (D) Effect of CP-55,940 on wild-type CB2 (●) and mutant D80E (△) and D80N (▼) receptor-expressing cell lines. Data are expressed as percent forskolin stimulation *versus* log molar drug concentration. Points, mean \pm S.E. of three or more independent experiments performed in triplicate. Curves were generated as described under "Methods."

TABLE 4

Regulation of agonist binding by Gpp(NH)p

Competition of a 1 nM concentration of [3 H]CP-55,940 by 100 μ M and 1 mM Gpp(NH)p. Data are expressed as percent specific binding. Data shown are the means \pm S.E. of at least three experiments performed in triplicate.

[GppNHp]	CB1	D163E	D163N	CB2	D80E	D80N
0	100	100	100	100	100	100
100 μ M	77 \pm 6	95 \pm 17	101 \pm 2	87 \pm 6	95 \pm 6	99 \pm 6
1 mM	62 \pm 8*	82 \pm 10	87 \pm 7	60 \pm 3*	90 \pm 1	91 \pm 7

* Statistically significant differences from control ($P < .05$).

TABLE 5

Regulation of agonist binding by NaCl

Presented is the percent specific binding of a 1 nM concentration of [3 H]CP-55,940 or [3 H]WIN-55,212-2 in the presence of 150 mM NaCl. Data shown are the means \pm S.E. of three experiments performed in triplicate.

Radioligand	CB1	D163E	D163N	CB2	D80E	D80N
[3 H]CP-55,940	64 \pm 8*	91 \pm 2.5*	90 \pm 10	71 \pm 7*	66 \pm 12*	88 \pm 0.1*
[3 H]WIN 55,212-2	111 \pm 6	97 \pm 3.5				

* Statistically significant differences ($P < .05$) from binding in the absence of sodium which was defined as 100%.

concern. In addition, the similar affinities found when comparing the stably transfected cell lines with native tissues strengthens the use of transfected cells as model systems. Another concern with transfected cell lines is that receptor

coupling with G proteins may be altered because of overexpression of receptors in excess of G proteins. Our data demonstrating the ability of Gpp(NH)p to reduce agonist binding in the wild-type receptors indicate that the cannabinoid re-

ceptors expressed in 293 cells are regulated by G proteins. Furthermore, the results of the cAMP accumulation studies in the wild-type receptor cell lines indicating efficient adenylyl cyclase inhibition both in terms of EC_{50} values and E_{max} demonstrate appropriate signal transduction.

The overall aim of this mutagenesis research is to elucidate important molecular components of the cannabinoid pharmacophore. This knowledge may lead to the design of more specific cannabinoid ligands, which could offer increased therapeutic activity and decreased side effects. Additionally, as knowledge emerges regarding the role of the cannabinoid receptor in normal physiological function, identification of aberrations in receptor-effector coupling may be critical in treating conditions arising from disorders of the cannabinoid system. Mutation of the highly conserved aspartate residue in the second transmembrane domain of the CB1 and CB2 receptors provided a separation of ligand binding from signal transduction in both subtypes. Other amino acids presumably are involved in discrimination of ligands between the receptor subtypes and are the targets for future research.

Acknowledgments

The authors thank Melissa Noel for technical assistance in the early stages of this work.

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Send reprint requests to: Dr. Mary E. Abood, Department of Pharmacology and Toxicology, Virginia Commonwealth University, P.O. Box 980524, Richmond, VA 23298-0524.
