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The Difference between the CB_1 and CB_2 Cannabinoid Receptors at Position 5.46 Is Crucial for the Selectivity of WIN55212-2 for CB_2

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ABSTRACT

It has been reported that WIN55212-2, a prototypic aminoalkylindole, has higher affinity for CB_2 than for CB_1. To explain the selectivity of WIN55212-2 for CB_2, molecular modeling studies were performed to probe the interacting sites between WIN55212-2 and cannabinoid receptors. In TMH5 the position 5.46 is a Phe in CB_2 versus a Val in CB_1. Docking of WIN55212-2 into the models of CB_1 and CB_2 predicts that F5.46 will result in a greater aromatic stacking of CB_2 with WIN55212-2. Using site-directed mutagenesis, this hypothesis was tested by exchanging the amino acids at position 5.46 between CB_1 and CB_2. Two mutations, including a Phe to Val mutation at the position 5.46 in CB_2 (CB2F5.46V), and a corresponding Val to Phe mutation at the position 5.46 in CB_1 (CB_1V5.46F), were made. The mutant receptors were transfected into 293 cells, and stable cell lines expressing similar numbers of receptors as wild-type receptors were chosen for additional ligand binding and cAMP accumulation studies. In ligand- binding assays, the CB_2F5.46V mutation decreased the affinity of WIN55212-2 for CB_2 by 14-fold. In contrast, the CB_1V5.46F mutation increased the affinity of WIN55212-2 for CB_1 by 12-fold. However, these mutations did not change the affinity of HU-210, CP-55940, and anandamide for CB_1 and CB_2. In cAMP accumulation assays, the changes in EC_{50} values of WIN55212-2 were consistent with the changes in its binding affinity caused by the mutations. These results strongly support the hypothesis that the selectivity of WIN55212-2 for CB_2 over CB_1 is attributable to the change from Val in CB_1 at position 5.46 to Phe in CB_2.

Marijuana (*Cannabis sativa*), one of the oldest and most widely abused drugs, has also been used for medicinal purposes by various cultures. The primary psychoactive constituent of marijuana is a cannabinoid compound, Δ^9-tetrahydrocannabinol. During the past decade, a major investigative effort on the mechanisms of action of cannabinoids has been launched. Cannabinoids have been found to act through G protein-coupled receptors (GPCRs) on cell membranes (Devane et al., 1988). Several cDNAs and genes encoding cannabinoid receptors have been cloned, including CB_1 and CB_2 (Matsuda et al., 1990; Munro et al., 1993). The putative endogenous cannabinoid ligand anandamide has been isolated from the brain (Devane et al., 1992), and high-affinity cannabinoid mimetics with a variety of chemical structures have been synthesized (Musty et al., 1995).

Based on their chemical structures, cannabinoid agonists can be classified into at least four groups: classical cannabinoids, bicyclic or nonclassical cannabinoids, fatty acid amides and esters, and aminoalkylindoles (AAIs). AAIs are a class of cannabimimetics with structures entirely different from those of natural cannabinoids. AAIs have been shown to bind cannabinoid receptors and exhibit cannabinoid-like activities (Compton et al., 1992; Kuster et al., 1993).

Most cannabinoid agonists exhibit little or no subtype selectivity (Felder et al., 1995). Recent studies have demonstrated that WIN55212-2 (Fig. 1), a prototypic aminoalkylindole, has higher affinity for CB_2 than for CB_1, with a K_i ratio (CB_1:CB_2) of 19 (Felder et al., 1995). The molecular basis for the selectivity of WIN55212-2 for CB_2 is currently unknown. In the present study, to investigate the molecular basis for the CB_2 selectivity of WIN55212-2, models of the CB_1 and CB_2 transmembrane helix (TMH) bundles were first probed for possible AAI interaction sites.

In the cationic neurotransmitter GPCRs, a negatively charged residue (Asp at 3.32) is commonly hypothesized to serve as a counter-ion for protonated amine ligands (Dixon et al., 1987). This hypothesized interaction site can be used as an "anchor" point for the ligand, a point from which second-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; AAI, aminoalkylindole; TMH, transmembrane helix; SAR, structure-activity relationship; TRH, thyrotropin-releasing hormone.

ary interaction sites can be identified. Unlike the cationic neurotransmitters, cannabinoid ligands are uncharged. Furthermore, the cannabinoid receptors lack the negatively charged residue at 3.32 that is found in the cationic neurotransmitter GPCRs. The cannabinoid receptors possess, instead, a positively charged amino acid (Lys) at position 3.28, which is one turn up from position 3.32 toward the extracellular side of the TMH bundle. In rhodopsin, position 3.28 is of functional importance because Glu 3.28 (113) is an interaction site for the *cis*-retinal chromophore (Rao et al., 1994). The Lys at 3.28 in the cannabinoid receptors should be capable of hydrogen bonding with cannabinoid ligands. Position 3.28, therefore, would appear to be a logical interaction site for cannabinoid ligands. In fact, K3.28 has been proposed to be a primary interaction site for the nonclassical cannabinoid CP-55940 at the CB_1 (Tao et al., 1999).

Song and Bonner's (1996) K3.28(192)A mutation study of the CB_1 revealed that K3.28(192) is crucial to the binding and activation produced by HU-210, a classical cannabinoid; CP-55940, a nonclassical bicyclic cannabinoid; and anandamide, an endogenous cannabinoid. However, the K3.28(192)A mutation did not affect the affinity or activity of WIN55212-2, an AAI at the CB_1. This result indicates a difference between the AAI binding site and that (those) of the other three cannabinoid ligand classes.

Recently, the carbonyl oxygen of AAIs (which could serve as a hydrogen bond acceptor) was shown to be unnecessary for cannabinoid receptor affinity and activity. Rigid AAI analogs, which lack this carbonyl group, the E naphthylidene indenes, have been reported to possess high affinity at both the CB_1 and CB_2 receptors (Reggio et al., 1998). Another possible hydrogen-bonding moiety in the AAIs is the morpholino ring. Huffman et al. (1994) have shown, however, that this morpholino ring can be replaced with an alkyl chain without loss of CB_1 affinity or efficacy. Taken together, both mutation and structure-activity relationship results suggest that hydrogen bonding may not be the primary interaction of AAIs at the CB_1.

Aromatic stacking interactions play important roles in protein stability and drug-receptor interactions (Burley and Petsko, 1985). Because WIN55212-2 is an aromatic ligand, we hypothesized that aromatic stacking interactions may be important to the interaction of WIN55212-2 with the CB_1 and CB_2 receptors. Our modeling studies identified an aromatic cluster in the TMH3-4-5 region as the most likely binding site for WIN55212-2. In this region, WIN55212-2 has greater aromatic interactions in CB_2 than in CB_1. Specifically, in CB_2, there is an aromatic interaction for WIN55212-2 with F5.46(197), a residue unique to CB_2. It is possible, therefore, that the presence of F5.46(197) in the CB_2 versus V5.46(282) in the CB_1 may result in the reported CB_2 selectivity of WIN55212-2. To test this hypothesis, a mutagenesis study at position 5.46 of the CB_1 and CB_2 receptors was undertaken.

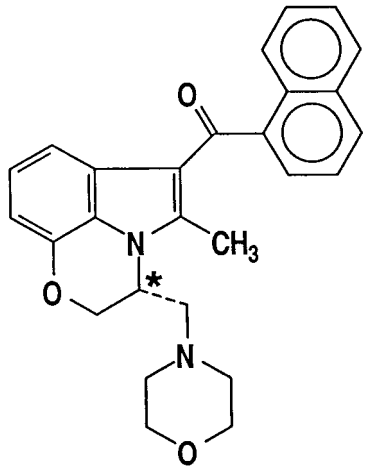

Fig. 1. The chemical structure of WIN55212-2.

Materials and Methods

Amino Acid Numbering System. The amino acid numbering system suggested by Ballesteros and Weinstein (1995) was used. Each amino acid identifier starts with the TMH number, followed by the amino acid position relative to a reference amino acid in that helix. This reference amino acid is the most highly conserved residue across GPCRs in that helix and is assigned a locant value of 0.50. The locant is followed by the sequence number of the amino acid residue in parentheses. This numbering system for the cannabinoid receptor has been described previously (Bramblett et al., 1995). As an example, the most highly conserved amino acid in TMH 2 is an aspartic acid. In the CB_1 sequence, this is residue 163. Using the proposed numbering system, this amino acid would be called D2.50(163). The amino acid preceding the aspartic acid, an Ala, would be called A2.49(162).

Model Construction and Refinement. Helix ends of the cannabinoid CB_1 and CB_2 receptors and the degrees of lipid exposure of each helix were determined using a Fourier-Transform Analysis of the periodicity in hydrophobicity and variability exhibited by the sequences of these receptors (Bramblett et al., 1995). The helices were constructed using the Chem Protein Module of Chem-X (Chemical Design Ltd., Chipping Norton, UK). A bend and twist of exposed faces of TMH 7 before and after the Pro kink produced by the NPXXY motif were introduced as described in Fu et al. (1996). Relative helix heights were checked and adjusted using the GPCR mutation literature (Rao et al., 1994; Zhou et al., 1994; Han et al., 1995; Nakanishi et al., 1995). Helix tilts were adjusted to be consistent with the 7.5Å resolution projection map of frog rhodopsin (Unger et al., 1997; Baldwin et al., 1997). Recent scanning cysteine accessibility method results for TM helices; 3, 5, 6, and 7 in the dopamine D_2 receptor (Javitch et al., 1995a,b, 1998; Fu et al., 1996) were used as a test of the cannabinoid receptor bundles. These studies have identified those residues that are accessible from within the binding site crevice of the D_2 receptor in the subject helices. The surface of this crevice is formed by residues that can contact specific ligands (agonists and/or antagonists) and by other residues that may play a structural role and affect binding indirectly . All residues identified as accessible by scanning cysteine accessibility method were found to be accessible from within the binding site crevice of the CB_1 and CB_2 receptor models.

Side chain torsion angles were set to literature values for transmembrane proteins (McGregor et al., 1987). Each helix was capped by acetamide at its N terminus and *N*-methylamide at its C terminus. Ionizable residues in the helices were considered to be uncharged if they faced lipid.

WIN55212-2 was first docked in an s-*trans* conformation into the TMH 3-4-5 of each bundle using interactive computer graphics. The TMH bundle, complexed with ligand, was then energy-minimized using AMBER 4.0 with its all-atom force field parameters (Weiner et al., 1986). A protocol was used that alternated three runs (2000 steps conjugate gradient) in which the helix backbones were constrained, with three runs (50 steps steepest descent) in which the helix backbones were relaxed. The last step was a run of conjugate gradient for 2000 steps. A distance-dependent dielectric and a 20Å cut-off for nonbonded interactions was used. Docking studies of an s-cis con-

former of WIN55212-2, as well as of the S stereoisomer of WIN55212, i.e., WIN55212-3 were also performed.

Structure-Activity Relationship (SAR) Criteria Used to Test the TMH3-4-5 Aromatic Cluster Binding Site for WIN55212-2. Several criteria from aminoalkylindole SAR were used to test the proposed TMH 3-4-5 aromatic cluster-binding site for WIN55212-2 (see Discussion). First, Pachecho et al. (1991) have shown that the R stereoisomer of WIN55212 (called WIN55212-2), but not the S stereoisomer of WIN55212 (called WIN55212-3), produces effects at the cannabinoid receptor. Second, Reggio et al. (1998) have shown that the C-2 methyl rigid AAI analogs, the E-aminoalkylindenes (analogous to the s-*trans* conformer of WIN55212-2) have significantly higher affinity and efficacy at the CB_1 and CB_2 than do the *Z*-aminoalkylindenes (analogous to the s-*cis* conformer of WIN55212-2). Finally, WIN55212-2 has been shown to be 19-fold selective for the CB_2 (Felder et al., 1995).

Distance and Angle Measurements of Aromatic Stacking Interactions. Centroids for each ring were generated using the Chem-X molecular modeling suite of programs. For fused ring aromatic moieties (i.e., naphthyl and indole rings), a centroid for each ring was generated. Distances between centroids were measured using Chem-X. The distances reported here are the shortest centroid–centroid distances between the two moieties. A normal vector to the plane of each aromatic system also was generated, and the angle between the normal vectors of two interacting rings α then was evaluated using Chem-X.

Reagents. Enzymes and reagents used for recombinant DNA experiments were purchased from GIBCO-BRL (Gaithersburg, MD), or Promega (Madison, WI). Oligonucleotides were synthesized by the Gene Technology Laboratory of the Texas A&M University. Tissue culture reagents were obtained from Biowhittaker (Walkersville, MD). Adenovirus-transformed 293 cells were from American Type Culture Collection (Rockville, MD). Glass tubes used for diluting cannabinoid drugs and for ligand-binding assays were silanized through exposure to dichlorodimethylsilane (Sigma Chemical Co., St. Louis, MO) vapor while under vacuum for 3 h.

Anandamide and WIN55212-2 were purchased from Research Biochemicals Inc. (Natick, MA). CP-55940 was provided by Dr. Lawrence Melvin (Pfizer Inc., Groton, CT). HU-210 was obtained from Tocris (Ballwin, MO).

Expression of Recombinant Cannabinoid Receptors. A 1.5-kb *Sst*I/*Xba*I fragment of the human *CB1* gene containing the entire coding region was subcloned into expression vector pRC/CMV (Invitrogen, San Diego, CA) to construct the expression plasmid $pHCB_1$-RC/CMV. A 1.8-kb full-length human CB_2 cDNA was subcloned into pRC/CMV to construct the expression plasmid $pHCB_2$-RC/CMV.

Expression plasmids containing the wild-type and mutant cannabinoid receptors were purified with Qiagen plasmid kits (Chatsworth, CA). The plasmids were transfected into human embryonic kidney 293 cells with the use of a calcium phosphate precipitation method (Chen and Okayama, 1987). Transfected cells were selected in culture medium containing 500 μg/ml geneticin (G418), and cell lines stably expressing wild-type and mutant cannabinoid receptors were established (Chen and Okayama, 1987). Cells were grown at 37°C in a humidified 5% CO_2 incubator in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Site-Directed Mutagenesis. Mutations were introduced through the GeneEditor in vitro site-directed mutagenesis system (Promega). This system allows mutations to be made in any vector that contains ampicillin resistance as a selectable marker. Using this system, the selection oligonucleotide, which encodes the resistance to the GeneEditor antibiotic selection mix, is annealed to DNA template at the same time as a mutagenic oligonucleotide. Subsequent synthesis and ligation of the mutant strand link the two oligonucleotides. The system uses the resistance to the GeneEditor antibiotic selection mix to facilitate the selection of the mutant DNA. For CB1V5.46F, the mutagenic oligonucleotide 5′-TGGATCGGGTTC ACCAGCG-3′ was used. For CB2F5.46V, the mutagenic oligonucleotide 5′-CTGGCTCCTG*G*TCATCGCCTTC-3′ was used. The presence of the mutation and the accuracy of the DNA sequences were verified with dideoxy sequencing.

Ligand Binding. For membrane preparations, confluent cells were washed twice with cold PBS and scraped off the tissue culture plates. Cells were homogenized in membrane buffer (50 mm Tris–HCl, 200 mM sucrose, 5 mM $MgCl_2$, 2.5 mM EDTA, pH 7.4) with a Tekmar Tissumizer. The homogenate was centrifuged at 32,000*g* for 20 min at 4°C. The pellet was resuspended in binding buffer (50 mm Tris–HCl, 5 mM $MgCl_2$, 2.5 mM EDTA, pH 7.4) and stored at −80°C until use. Protein concentrations were determined by the use of a bicichoninic acid protein reagent kit (Pierce, Rockford, IL).

For binding assays, cannabinoid ligands were diluted in binding buffer containing 25 mg/ml BSA and then added to assay tubes. [^{3}H]WIN55212-2 (2 nM) or [^{3}H]CP-55940 (1 nM) was used as labeled ligand for competition binding assays. Nonspecific binding was defined as binding in the presence of 1 μM unlabeled WIN55212-2 or CP-55940. The final volume of the assay was 0.5 ml, and the final BSA concentration was 5 mg/ml. With 20 μg of membrane protein, binding assays were performed at 30°C for 60 min. Free and bound radioligands were separated by rapid filtration through polyethylenimine-treated GF/B filters (Whatman International, Maidstone, UK) with a Brandel cell harvester (Gaithersburg, MD). The filters were washed three times with 3 ml of cold 50 mM Tris–HCl, pH 7.4, and equilibrated overnight in a scintillation cocktail (Hydrofluor, National Diagnostics, Manville, NJ), and radioactivity was determined with a liquid scintillation counter.

cAMP Accumulation. cAMP accumulation assays were performed using a method published previously (Song and Bonner, 1996). Briefly, using silanize test tubes, cannabinoid ligands were diluted with Eagles #2 buffer containing 50 mg/ml fatty acid-free BSA, and drugs were added to tubes in a volume of 25 μl. Forskolin or buffer also was added to the test tubes in a volume of 25 μl. Confluent cells were lifted from culture plates with Eagle's 2 buffer containing 0.5 mm EDTA, washed twice with Eagle's 2 buffer, and incubated with phosphodiesterase inhibitor RO20-1724 for 10 min. The stimulation was initiated by adding cells to test tubes containing forskolin and cannabinoids and incubated for 5 min at 37°C. The final assay volume was 250 μl with 1×10^6 cells per tube. The reaction was stopped with the addition of an equal volume of 0.1 N HCl, after which 50 μl was removed for cAMP radioimmunoassay, using a kit from DuPont-NEN (Wilmington, DE).

Data Analysis. Data from ligand-binding cAMP accumulation assays were analyzed and curves generated with the use of the Prism program (GraphPad Software, San Diego, CA). IC_{50} and EC_{50} values were determined through nonlinear regression analysis performed with Prism. K_d and B_{max} values were estimated from competition binding experiments with the following equations: $K_d = IC_{50} - L$ and $B_{max} = (B_0 IC_{50})/l$, where l is the concentration of free radioligand, and B_0 is specifically bound radioligand (DeBlasi et al., 1989). The K_i values were calculated with the Cheng-Prusoff equation: $K_i = IC_{50}/(1 + L/K_d)$ (Cheng and Prusoff, 1973).

Results

Molecular Modeling.

Aromatic Stacking Interactions. Two regions of the cannabinoid receptor-binding site crevice are rich in aromatic amino acids. One region is an intrahelix aromatic stack formed by F2.57, F2.61, F2.64, and F2.67. A second region rich in aromatic amino acids is formed by residues on TMH 3, 4, and 5. This second region was deemed the more likely interaction region, because there is an upper (extracellular side) aromatic stack formed by F3.25, W4.64, W5.43, and a lower aromatic stack with F3.36 when WIN55212-2 is docked in this region. In this docking position, WIN55212-2 would

create a continuous aromatic stack over several turns of helices 3, 4, and 5 that is likely to be energetically favored. Binding in this region of the cannabinoid receptors is supported by chimera studies of the CB_1 and CB_2 reported by Shire et al. (1997), which indicated that the region delimited by the fourth and fifth transmembrane regions contain amino acids important for WIN55212-2 binding to the CB_2.

Burley and Petsko (1985) have reported that aromatic–aromatic interactions in proteins operate at distances (d) of 4.5 to 7.0Å between ring centroids. The angle α between the normal vectors of interacting aromatic rings typically is between 30 and 90°, producing a "tilted T" or "edge-to-face" arrangement of the interacting rings. Using this range of distances and angles, aromatic amino acids were identified that interact directly with WIN55212-2 in the energy minimized CB_1 and CB_2 TMH bundles. In the energy-minimized CB_1 bundle (Fig. 1a), the naphthyl ring of the s-*trans* conformer of WIN55212-2 interacts with F3.25(189) (d = 6.17Å, α = 88°) and W5.43(279) (d = 5.18Å, α = 65°). The indole ring of the WIN55212-2 s-*trans* conformer interacts with F3.36(200) (d = 5.49Å, α = 77°).

WIN55212-2 has been reported to have 19 times higher affinity for the CB_2 (Felder et al., 1995). A recent report by Shire and coworkers (1997) has revealed that the region delimited by the TMH 4-5 region contains amino acids important for the binding of WIN55212-2. The major change in the TMH 4–5 region between the CB_1 and CB_2 bundles involves two aromatic amino acids on helix 5. In CB_1, position 5.42(278) is aromatic. There is, however, no direct interaction of the WIN55212-2 s-*trans*-conformer with F5.42(278), because this residue is too far from the ligand. In the CB_2 receptor, position 5.42 is nonaromatic [S5.42(193)], whereas position 5.46 becomes aromatic [F5.46(197)]. F5.46(197) is positioned to interact directly with the indole ring of the WIN55212-2 s-*trans*-conformer (Fig. 1) in the CB_2 receptor (d = 5.49Å, α = 78°). The WIN55212-2 s-*trans*-conformer also is positioned in CB_2 such that its indole ring interacts with F3.36(117) (d = 5.17Å, α = 53°) and W5.43(194) (d = 6.15Å, α = 86 °), whereas its naphthyl ring interacts with F3.25(106) (d = 5.57Å, α = 83°) and W5.43(194) (d = 5.07Å, α = 46°).

In summary, the CB_1 and CB_2 interaction sites for the s-*trans*-conformer of WIN55212-2 share three amino acids in common: F3.25, F3.36, and W5.43. In CB_2, there is a fourth aromatic interaction for WIN55212-2 with F5.46, a residue unique to CB_2. It is possible, therefore, that the presence of F5.46 in the CB_2 bundle may result in the reported CB_2 selectivity of WIN55212-2.

Consistency of Proposed Binding Site with AAI SAR. SAR data from amino-alkylindenes indicate that only the s-*trans*-conformer of C2 methyl AAIs has significant affinity for CB_1 and CB_2 (Reggio et al., 1998). When an s-*cis*-conformer of WIN55212-2 was docked in the receptor model, the ligand was found to have steric clashes with Val 3.32, in both the CB_1 and CB_2 bundles. This steric clash resulted in no aromatic stacking interactions in the TMH 3-4-5 region of CB_1 and CB_2 for the s-*cis*-conformer.

Only the *R*-stereoisomer of WIN55212 (i.e., WIN55212-2) has been shown to bind to the cannabinoid receptors (Pachecho et al., 1991). Consistent with this SAR, in the ligand docking position within the CB_1 and CB_2 receptors identified here, the opposite (*S*)-stereoisomer WIN55212-3 would not fit at the binding site because of severe steric clashes with helix 5.

Expression of the Wild-Type CB_1 and CB_2 Cannabinoid Receptors. Radioligand-binding assays were conducted to characterize CB_1 and CB_2 expression in 293 cells. Specific high-affinity binding of cannabinoid ligand [^{3}H]WIN55212-2 was observed with membranes prepared from 293 cells stably transfected with pHCB1-RC/CMV or pHCB2-RC/CMV (Fig. 3, Table 1). For CB_1, the K_d and B_{max} values were determined to be 11.9 ± 1.9 nM and 1217.6 ± 221.9 fmol/mg protein, respectively. For CB_2, the K_d and B_{max} values were determined to be 0.76 ± 0.04 nM and 1172.7 ± 184.9 fmol/mg protein, respectively. To determine the stereospecificity of ligand binding, WIN55212-3, the inactive enantiomer WIN55212-2, was tested. WIN55212–3

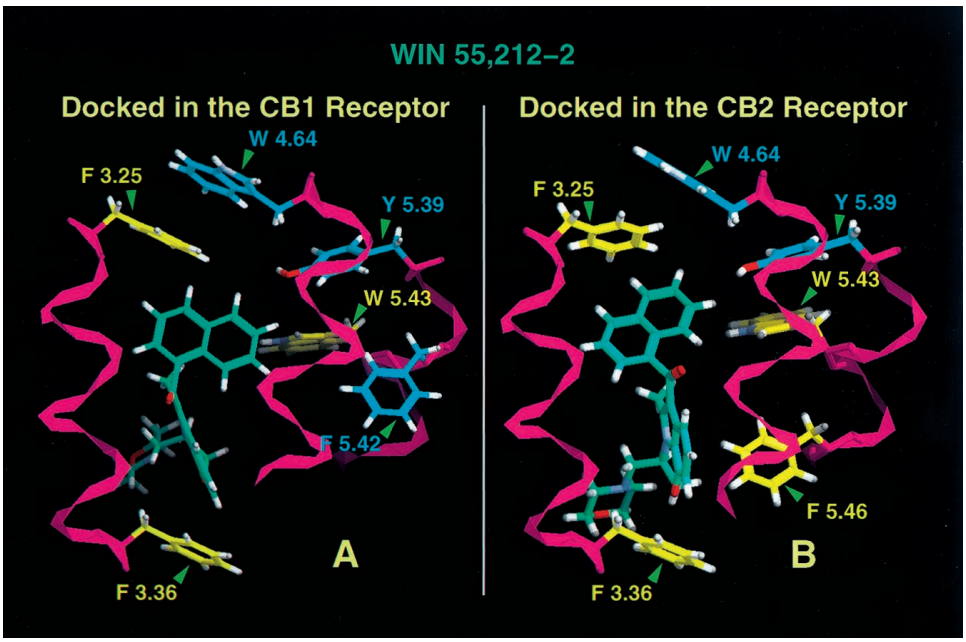

Fig. 2. WIN55212-2 in an s-*trans*-conformation is shown docked here in an energy minimized CB1 (A) or CB2 (B) TMH bundle. The aromatic residues with which WIN55212–2 directly interacts are shown in yellow. Residues which are part of the TMH 3-4-5 aromatic cluster, but do not directly interact with WIN55212-2 are shown in cyan. The view is inside the membrane, looking from outside the bundle toward helix 4.

was inactive in competing for [^{3}H]WIN55212-2 binding (data not shown).

HU-210, CP-55940, WIN55212-2, and anandamide, four cannabinoid ligands with distinct structures, were tested for their ability to compete for the specific binding of [^{3}H]CP-55940 to CB_1 and CB_2. For CB_1, the rank order of potency of these ligands to compete for the binding of [^{3}H]CP-55940 was HU-210 > CP-55940 > WIN55212-2 > anandamide (Table 2). For CB_2, the rank order of potency of these ligands to compete for the binding of [^{3}H]CP-55940 was HU-210>WIN55212–2> CP-55940>anandamide (Table 2). HU-210, CP-55940, and anandamide demonstrated no selectivity between CB_1 and CB_2 (Table 2). In contrast, WIN55212-2 was CB_2 selective, with a K_i ratio CB_1:CB_2 of 25.4:1.6 = 15.9 (Table 2).

Functional expression of CB_1 and CB_2 in 293 cells was confirmed with the use of cAMP accumulation assays. In a concentration-dependent manner, the four cannabinoid agonists inhibited forskolin-stimulated cAMP accumulation in 293 cells stably transfected with CB_1or CB_2 cDNA (Table 3). For CB_1, the rank order of potency was HU-210 > WIN55212-2 > CP-55940 > WIN55212-2 > anandamide. For CB_2, the rank order of potency was HU-210 > WIN55212-2 > CP- 55940 > anandamide. At the highest concentrations used on transfected cells, none of these cannabinoid ligands inhibited cAMP accumulation in untransfected 293 cells. Thus, all of the inhibition of cAMP accumulation shown is receptor-mediated. Consistent with data from ligand-binding assays, HU-210, CP-55940, and anandamide demonstrated no selectivity between CB_1 and CB_2 in cAMP accumulation assays (Table 3). On the contrary, WIN55212-2 was CB_2 selective, with an EC_{50} ratio CB_1:CB_2 of 10.3:0.52 = 19.8 (Table 3).

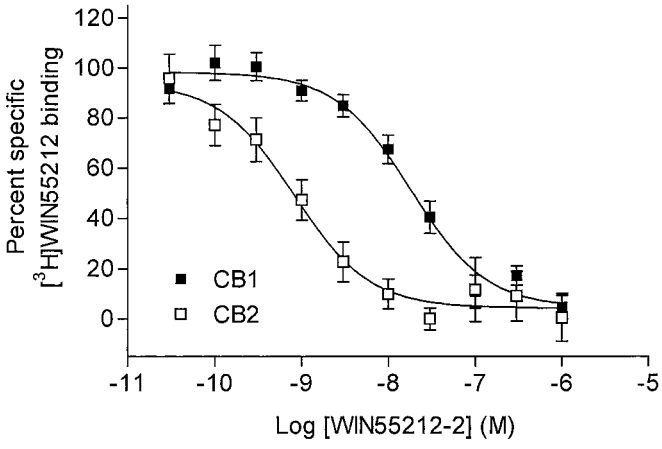

Fig. 3. Comparison of [^{3}H]WIN55212-2 binding to human CB1 and CB2. Competition binding assays were performed on membranes prepared from 293 cells stably expressing CB1 (■) or CB2 (□). Points, mean ± SE of three independent experiments performed in duplicate. Curves were generated as described in *Materials and Methods*.

TABLE 1
Parameters of [^{3}H]WIN55212-2 binding
Values are presented as mean ± S.E. of three separate experiments.

	CB_1	CB_1V5.46F	CB_2	CB_2F5.46V
B_{max}[a]	1217.6 ± 221.9	1064.1 ± 147.2	1172.7 ± 184.8	1265.3 ± 114.5
K_d[b]	11.9 ± 1.9	1.0 ± 0.19	0.76 ± 0.04	10.9 ± 2.4

[a] B_{max} value in fmol/mg protein.
[b] K_d value in nM.

Comparison of the Mutant and Wild-Type Cannabinoid Receptors. Radioligand-binding assays were performed to compare the pharmacologic profiles of the CB1V5.46F and CB2F5.46V mutant cannabinoid receptors with those of wild-type CB_1 and CB_2. Specific high-affinity binding of [^{3}H]WIN55212-2 was observed in membranes prepared from 293 cells stably transfected with the mutant cannabinoid receptor DNAs. The B_{max} values for CB1V5.46F and CB2F5.46V were 1064.0 ± 147.2 and 1265.3 ± 114.5 fmol/mg proteins, respectively. These B_{max} values are not significantly different from those of the wild-type CB_1 and CB_2 (1217.6 ± 221.9 and 1172.7 ± 184.8 fmol/mg protein, respectively).

As shown in Tables 1 and 2, the mutation CB1V5.46F did not change the affinity of HU-210, CP-55940, and anandamide for CB_1, and the mutation CB2F5.46V did not change the affinity of these ligands for CB_2. In contrast, the CB1V5.46F mutation increased the affinity of WIN55212-2 for CB_2 by 12- to 13-fold, and the CB2F5.46V mutation decreased the affinity of WIN55212-2 for CB_2 by 14-fold.

cAMP accumulation assays were performed to compare the functional properties of the CB1V5.46F and CB2F5.46V mutant cannabinoid receptors with those of wild-type CB_1 and CB_2. Consistent with the data from radioligand-binding experiments, the mutation CB1V5.46F did not change significantly the EC_{50} values of HU-210, CP-55940, and anandamide for CB_1, and the mutation CB2F5.46V did not change significantly the EC_{50} values of these ligands for CB_2 (Table 3). In contrast, the CB1V5.46F mutation increased the EC_{50} value of WIN55212-2 for CB_2 by 12- to 13-fold, and the CB_2F5.46V mutation decreased the EC_{50} value of WIN55212-2 for CB2 by 15-fold. For all four cannabinoid agonists, the maximum inhibition (40–60%) of forskolin-stimulated cAMP accumulation was unchanged by these mutations.

Discussion

A fundamental hypothesis underlying the modeling studies reported here is that aromatic stacking interactions are important to the WIN55212–2/cannabinoid receptor complexes. Aromatic stacking interactions have been shown to play important roles in protein stability and drug-receptor

TABLE 2
Parameters of [^{3}H]CP-55940 binding
Values are presented as mean ± SE of three separate experiments.

Ligand	CB_1	CB_1V5.46F	CB_2	CB_2F5.46V
HU-210[b]	0.21 ± 0.02	0.24 ± 0.05	0.19 ± 0.07	0.16 ± 0.03
CP-55940[a]	2.2 ± 0.3	2.2 ± 0.6	2.4 ± 0.4	2.9 ± 0.7
Anandamide[b]	76.2 ± 11.1	80.8 ± 6.0	131.2 ± 14.1	123.4 ± 12.1
WIN55212-2[b]	25.4 ± 5.9	1.9 ± 0.4	1.6 ± 0.5	22.9 ± 2.7

[a] K_d value in nM.
[b] K_i value in nM.

TABLE 3
EC_{50} values for the functional inhibition of forskolin-stimulated cAMP accumulation
Values are presented as mean ± S.E. of three separate experiments.

Ligand	CB_1	CB_1V5.46F	CB_2	CB_2F5.46V
HU-210[a]	0.18 ± 0.04	0.13 ± 0.05	0.19 ± 0.04	0.15 ± 0.02
CP-55940[a]	2.4 ± 0.8	2.7 ± 0.8	3.4 ± 1.2	2.5 ± 0.4
Anandamide[a]	74.2 ± 17.7	94.7 ± 35.5	113.4 ± 34.3	127.2 ± 47.8
WIN55212-2[a]	10.3 ± 2.1	0.82 ± 0.22	0.52 ± 0.11	7.9 ± 1.7

[a] EC_{50} value in nM.

interactions (Burley and Petsko 1985). Aromatic clusters such as the CB_1/CB_2 TMH 3–4-5 aromatic cluster discussed in this article have been proposed to form the binding site of other GPCRs. For example, Colson et al. (1998) have proposed that the thyrotropin-releasing hormone (TRH) binding site at the TRH receptor is an aromatic (hydrophobic) cluster on TMH 5 and 6. Through mutagenesis studies, these investigators showed that F5.46(199), W6.48(279), and Y6.51(282) constrain the TRH receptor in an inactive conformation. In the dopamine D_2 receptor, four highly conserved aromatic residues F6.44 (382), W6.48(386), F6.51(389), and F6.52(390), along with a histidine (H6.5(393)), are clustered together on the water-accessible surface of the D_2 binding site crevice. These residues have been suggested to define an interconnected aromatic cluster that may be involved in ligand binding and receptor activation (Javitch et al., 1998).

In the energy-minimized model of the WIN55212-2-CB_1 complex (Fig. 1a), the indole ring of WIN55212-2 interacts with F3.36(200) on helix 3. The naphthyl ring of WIN55212-2 interacts with helix 3 [(F3.25(189)] and helix 5 [W5.43(279)]. In the energy-minimized model of the WIN55212-2/CB_2 complex (Fig. 1b), the indole ring of WIN55212-2 is positioned to have a strong aromatic interaction with F5.46(197), an aromatic amino acid unique to CB_2. In addition, aromatic interactions for the indole ring with two other aromatic amino acids occur [F3.36(117), W5.43(194)]. For the naphthyl moiety of WIN55212-2, the energy-minimized WIN55212-2–CB_2 complex reveals aromatic stacking interactions with F3.25(106) and W5.43(194).

If the selectivity of a ligand for a particular receptor subtype is attributable to a favorable interaction with a single amino acid, then introduction of this functional group via mutagenesis into the receptor subtype lacking this amino acid in an analogous position would be predicted to increase the affinity of the ligand for the latter receptor. Furthermore, the removal of this amino acid (by replacement with some other amino acid) from the wild-type receptor for which the ligand is selective should result in decreased affinity. In the experimental part of this study, the selectivity of WIN55212-2 for CB_2 was first confirmed in our system. In ligand-binding assays, WIN55212-2 exhibited 15- to 16- fold higher affinity for CB_2 (Fig. 3; Tables 1 and 2). Most important, mutagenesis studies showed that the CB1V5.46F mutation increased the affinity of WIN55212-2 for CB_1 by 12- to 13-fold, and the CB2F5.46V mutation decreased the affinity of WIN55212-2 for CB_2 by 14-fold. In contrast, these mutations did not affect the affinity of three other cannabinoid agonists for either CB_1 or CB_2. In cAMP accumulation assays, the maximum inhibition (40–60%) of forskolin-stimulated cAMP accumulation by cannabinoid agonists was unchanged on these mutant receptors. The changes in EC_{50} values of WIN55212-2 were consistent with the changes of its binding affinity caused by the mutations. These data strongly support the hypothesis that the selectivity of WIN55212-2 for CB_2 over CB_1 is caused by the change from Val in CB1 at position 5.46 to Phe.

Residue 5.46 has been shown to be important for ligand-binding and subtype selectivity in other GPCRs as well. In the β-adrenergic receptor, S5.46 (along with S5.42) has been proposed to interact with the catechol hydroxyl groups of β-adrenergic ligands (Strader et al., 1989). Position 5.46 has been proposed to be the major contributor to the pharmacologic differences between human (S5.46) and rat (A5.46) 5-Hydroxytryptamine $(5\text{-HT})_2$ receptors (Kao et al., 1992). The ergolines, mesulergine (5-HT_{2C} selective), and *d*-lysergic acid diethylamide (5-HT_{2A} selective) have been shown to reverse their relative affinities with the mutations 5-HT_{2A} [S5.46(242)A] and 5-HT_{2C} [A5.46(222)S], supporting a direct role of this locus in the selectivity of these ligands (Almaula et al., 1996).

Marijuana and its active component cannabinoids have a number of potential therapeutic properties, such as for the treatment of inflammation and autoimmune diseases (Lyman et al., 1989; Mazzari et al., 1996). However, one of the major problems for cannabinoids as therapeutic agents is their severe psychoactive side effect. The recent cloning of CB_2, a subtype of cannabinoid receptors, brought us new hope for a better medical use of cannabinoids (Munro et al., 1993). Because CB_2 is predominantly located in the immune system, and rarely in the brain, CB_2 selective compounds may prove to be potential therapeutic agents for inflammation and autoimmune diseases. CB_2 selective drugs should retain immunomodulatory effects, but be devoid of the psychoactive effects of marijuana.

By combining the approaches of molecular modeling and site-directed mutagenesis, this study has identified an amino acid residue that is important for the selectivity of WIN55212-2 for CB_2. Results described in this article suggest that cannabinoid agonists designed to have direct interactions with F5.46, in the CB_2 receptor, as well as with other aromatic amino acids in the TMH 3-4-5 cluster, may prove to be useful lead compounds for the design of more potent CB_2-selective agonists.

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