Journal of Neurochemistry
Lippincott Williams & Wilkins, Inc., Philadelphia

Functional Role of Tryptophan Residues in the Fourth Transmembrane Domain of the CB_2 Cannabinoid Receptor

Man-Hee Rhee, Igal Nevo, Michael L. Bayewitch, Orna Zagoory, and Zvi Vogel

Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel

Abstract: Several tryptophan (Trp) residues are conserved in G protein-coupled receptors (GPCRs). Relatively little is known about the contribution of these residues and especially of those in the fourth transmembrane domain in the function of the CB_2 cannabinoid receptor. Replacing W158 (very highly conserved in GPCRs) and W172 (conserved in CB_1 and CB_2 cannabinoid receptors but not in many other GPCRs) of the human CB_2 receptor with A or L or with F or Y produced different results. We found that the conservative change of W172 to F or Y retained cannabinoid binding and downstream signaling (inhibition of adenylyl cyclase), whereas removal of the aromatic side chain by mutating W172 to A or L eliminated agonist binding. W158 was even more sensitive to being mutated. We found that the conservative W158F mutation retained wild-type binding and signaling activities. However, W158Y and W158A mutants completely lost ligand binding capacity. Thus, the Trp side chains at positions 158 and 172 seem to have a critical, but different, role in cannabinoid binding to the human CB_2 receptor. **Key Words:** Cannabinoids—CB_2 receptor—G protein—Site-directed mutagenesis—Adenylyl cyclase.
J. Neurochem. **75,** 2485–2491 (2000).

Relatively little is known about the structure–function relationship of the CB_2 cannabinoid receptor and about which amino acids in the various transmembrane (TM) domains are involved in ligand binding. However, progress is currently being made using site-directed mutagenesis of the CB_1 and CB_2 receptors (Chin et al., 1998; Tao and Abood, 1998; Tao et al., 1999). For example, it was reported that Lys^{192} in TM3 of CB_1 is crucial for receptor recognition by the tricyclic and bicyclic cannabinoids HU-210 and CP55,940, respectively, as well as by the endogenous cannabinoid anandamide but not by the aminoalkylindole cannabinoid WIN55,212-2 (Song and Bonner, 1996; Chin et al., 1998). Moreover, Chin et al. (1999) and Song et al. (1999) reported that Ser^{112} (TM3) and Phe^{197} (TM5) of CB_2 are crucial for the selectivity of WIN55,212-2 for this receptor. It was also shown that substitution of the highly conserved Asp residue in TM2 of CB_1 (Asp^{163}) and CB_2 (Asp^{80}) with Asn or Glu disrupts G protein coupling to both receptors (Tao and Abood, 1998).

Several Trp residues in various receptors were shown to play a role in receptor–ligand recognition (Wess et al., 1993; Matsui et al., 1995; Befort et al., 1996; Sautel et al., 1996; Roth et al., 1997). W158, located just below the middle of TM4 of the human CB_2 receptor (position 4.50 in the nomenclature of Ballesteros and Weinstein, 1995) (Fig. 1) is completely conserved in all currently known G protein-coupled receptors (GPCRs), suggesting that this residue may have an important role in receptor structure and function (Probst et al., 1992; Wess et al., 1993; Baldwin et al., 1997). Indeed, this Trp residue was shown to have an important role in ligand binding to various receptors, including the δ-opioid, 5-HT_{2A} serotonergic, m_1 and m_3 muscarinic, and human Y_1 receptors (Wess et al., 1993; Matsui et al., 1995; Befort et al., 1996; Sautel et al., 1996; Roth et al., 1997). Much less is known about W172 (at position 4.64, at the top of TM4 of the CB_2 receptor). This Trp is conserved in the CB_1 and CB_2 cannabinoid receptors of all species investigated (human and mouse CB_2; human, mouse, cat, and rat CB_1), as well as in several other GPCRs (including the β-adrenergic and m_1–m_5 muscarinic receptors), but is not conserved in most other known GPCRs (Probst et al., 1992; Baldwin et al., 1997; Horn et al., 1998), suggesting that it may have a role in the binding of specific ligands, e.g., cannabinoids, to their receptors. For example, substitution of this residue in the m_1 muscarinic receptor (W164A) led to a reduction in the binding affinity of acetylcholine to the receptor (Matsui et al., 1995).

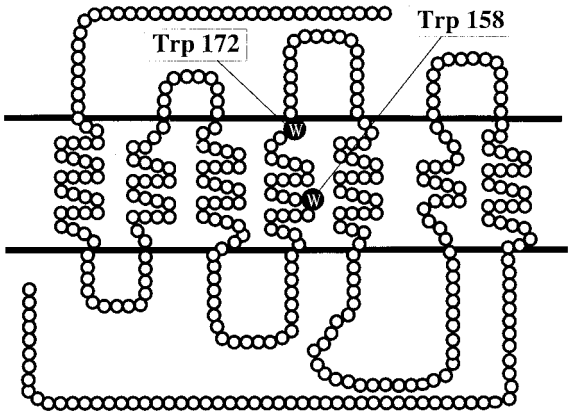
To examine the role of these Trp residues in CB_2 cannabinoid receptor binding and signaling [inhibition of adenylyl cyclase (AC)], we have mutated them into F, Y, or A and, in the case of W172, also to L. The results of these studies imply that both W158 and W172 have an

Received April 26, 2000; revised manuscript received July 13, 2000; accepted July 19, 2000.

Address correspondence and reprint requests to Prof. Z. Vogel at Department of Neurobiology, Weizmann Institute of Science, Rehovot 76100, Israel. E-mail: zvi.vogel@weizmann.ac.il

Abbreviations used: AC, adenylyl cyclase; cAMP, cyclic AMP; GPCR, G protein-coupled receptor; HA, hemagglutinin; TM, transmembrane; wt, wild-type.

A. Topographical locations of W 158 and W 172 in CB2



B. Conservation of W at 4.50 and 4.64 positions

Position	4.50	4.64
Human CB2	IM W VL···MG W TC	
Mouse CB2	VM W VL···MG W TC	
Human CB1	LM W TI···LG W NC	
Mouse CB1	LM W TI···LG W NC	
Human m1	LA W LV···LF W QY	
Human m3	LA W VI···LF W QY	
Human β1 adrenergic	TV W AI···MH W WR	
Human α2 adrenergic	TV W VI···IS I EK	
Human D2 dopamine	IV W VL···FG L NN	
Human D3 dopamine	AV W VL···FG F NT	
Human neurokinin 1	VI W VL···GY Y ST	
Human δ opioid	CI W VL···MA V TR	

FIG. 1. Location of W158 and W172 in the CB_2 receptor and the alignment of the amino acid sequence of various GPCRs around these areas. **A:** General structure of the CB_2 receptor. **B:** Amino acid sequences around positions 4.50 and 4.64 of several GPCRs, as obtained from Attwood et al. (1991), Probst et al. (1992), and Horn et al. (1998).

important role in the binding of cannabinoid ligands to the human CB_2 receptor.

MATERIALS AND METHODS

Materials

[2-^{3}H]Adenine (18.0 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). The phosphodiesterase inhibitors 3-isobutyl-1-methylxanthine and Ro 20-1724 were from Calbiochem (La Jolla, CA, U.S.A.). Forskolin, cyclic AMP (cAMP), and fatty acid-free bovine serum albumin were from Sigma. The cannabinoid agonists [^{3}H]HU-243, HU-243, HU-210, CP55,940, and WIN55,212-2 were kindly provided by Dr. R. Mechoulam (Jerusalem, Israel). Tissue culture reagents were from Life Technologies (Gaithersburg, MD, U.S.A.).

Plasmids

Rabbit AC type V (AC-V) and β-galactosidase cDNAs in the pXMD1 vector (Wallach et al., 1994) were described previously (Avidor-Reiss et al., 1997; Rhee et al., 1998). The human CB_2 cDNA in pCDM8 was kindly provided by Dr. S. Munro (Cambridge, U.K.).

Construction of hemagglutinin (HA)-tagged human CB_2 in pcDNA3

The following oligonucleotide primers (P1 and P2) were synthesized and used to amplify (by PCR) a 1,100-bp fragment containing the entire human CB_2 coding sequence (using the human CB_2 cDNA in pCDM8 as a template): P1, 5′-GCGGATCCGAGGAATGCTGGGTG-3′ sense primer; P2, 5′-GCGCGGCCGCTCAGCAATCAGAGAG-3′ antisense primer. P1 is homologous to the cDNA sequence at the CB_2 coding start site and was engineered to contain a unique *Bam*HI site (underlined) for in-frame subcloning into a *Bgl*II site at the carboxy end of the HA sequence in HA-pcDNA3 (Coso et al., 1995). The P2 sequence was designed to introduce a unique *Not*I site (underlined) for ligation into the multiple cloning site of pcDNA3. The 1,100-bp PCR product (following 25 cycles of 1-min denaturation at 92°C, 1-min annealing at 45°C, and 1-min extension at 72°C) was digested with *Bam*HI and *Not*I and cloned into the *Bgl*II and *Not*I sites of HA-pcDNA3. The DNA sequence was confirmed by sequencing at the Weizmann Institute's DNA Sequencing Unit.

Preparation of point mutations in HA-CB_2

Mutations were introduced using the PCR-overlap extension method as previously described (Ho et al., 1989). In brief, two general primers were designed for PCR that cover the region in HA-CB_2 where the mutations were planned. The 5′ general primer 5′-GCCCTCATACCTGTTCATTGGC-3′ (complementing a coding sequence in CB_2 upstream of the area of the mutation) and the 3′ general primer 5′-GAGCTCTAGCATTTAGGTGACACTATAG-3′ (complementing part of the SP6 sequence of pcDNA3) were used in conjunction with internal sense and matching antisense primers that contained the desired mutation. Three PCR procedures were done: the first two providing the 5′ and 3′ ends of the mutated fragment and the third consecutive reaction joining the separate fragments. The final PCR product was cut with *Bst*EII and *Bgl*II (unique sites, present in HA-CB_2, before and beyond the area of the mutation), the mutated fragments were cloned into HA-CB_2, and the DNA sequences of the various HA-CB_2 mutants were confirmed.

Transient cell transfection

At 24 h before transfection, a confluent 10-cm-diameter plate of COS-7 cells in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37°C was trypsinized and split into five 10-cm-diameter plates. The cells were transfected, using the DEAE-dextran chloroquine method (Keown et al., 1990), with wild-type (wt) HA-CB_2 cDNA (2 μg per plate) or mutant cDNAs (4 μg per plate) and, where indicated, with AC-V cDNA (1.5 μg per plate). pXMD1-gal cDNA was used for mock DNA transfection. For binding studies, 72 h after transfection, the COS cells were washed twice with phosphate-buffered saline, scraped off, and centrifuged at 3,000 rpm for 10 min, and the cell pellets were stored at −70°C. For AC assay, 48 h after transfection, the COS cells were trypsinized and recultured in 24-well plates, and after an additional 24 h, the cells were assayed for AC activity as described below. Transfection efficiency was normally in the range of 40–80%, as determined by staining the cells for the activity of transfected β-galactosidase (Avidor-Reiss et al., 1996).

AC activity

The assay was performed in triplicate as described previously (Vogel et al., 1993; Rhee et al., 1998). In brief, cells cultured in 24-well plates were incubated for 2 h with 0.25 ml/well fresh growth medium containing 5 μCi/ml [2-^{3}H]adenine. This medium was replaced with Dulbecco's modified Eagle's medium containing 20 m*M* HEPES (pH 7.4) and the phosphodiesterase inhibitors Ro 20-1724 (0.5 m*M*) and 3-isobutyl-1-methylxanthine (0.5 m*M*). Cannabinoids diluted in 10 mg/ml fatty acid-free bovine serum albumin were then added. AC activity was stimulated in the presence or absence of cannabinoids by addition of 2 μ*M* forskolin. After 10 min at 37°C, the medium was removed, and the reaction was terminated by adding to the cell layer 1 ml of 2.5% perchloric acid containing 0.1 m*M* unlabeled cAMP. Aliquots of 0.9 ml of the acidic extract were neutralized with 100 μl of 3.8 *M* KOH and 0.16 *M* K_2CO_3 and applied to a two-step column separation procedure (Salomon, 1991). The [^{3}H]cAMP was eluted into scintillation vials and counted. Background levels (cAMP accumulation in the absence of stimulator) were subtracted from all values.

Binding of [^{3}H]HU-243

This assay was performed as described previously (Vogel et al., 1993; Rhee et al., 1997). Frozen pellets of transfected cells were homogenized in 2 ml of binding buffer per plate, and 100-μl aliquots of crude cell homogenates containing 15–20 μg protein [as determined by the method of Bradford (1976)] were mixed with 300 fmol of the high-affinity cannabinoid agonist [^{3}H]HU-243 in 1.5-ml Eppendorf tubes in a final volume of 1 ml of 50 m*M* Tris-HCl, 5 m*M* $MgCl_2$, 10 m*M* $CaCl_2$, 2.5 m*M* EDTA (pH 7.4), and 2 mg/ml fatty acid-free bovine serum albumin. The binding mixtures were incubated at 30°C for 1.5 h with gentle shaking and centrifuged at 14,000 rpm for 10 min. The bottoms of the 1.5-ml tubes were then cut and counted for radioactivity. Nonspecific binding determined in the presence of 1 μ*M* HU-210 was subtracted from all values. The K_i values for the cannabinoid agonists HU-210, WIN55,212-2, and CP55,940 were determined by adding increasing concentrations of these materials to the binding mixtures together with [^{3}H]HU-243 and applying the formula $K_i = IC_{50}/1 + ([[^3H]HU\text{-}243]/K_D)$ (see Rhee et al., 1997).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western immunoblotting

COS-7 cells transfected with the various HA-tagged CB_2 cDNAs in 10-cm-diameter culture plates were harvested with cold phosphate-buffered saline and spun down at 3,000 rpm (at 4°C for 10 min), and the cell pellets were mixed with 100 μl of Laemmli sample buffer per plate, sonicated, and frozen at −20°C. Samples of 10 μl (containing 30 μg of protein) were incubated for 5 min at 100°C with dithiothreitol (final concentration, 0.1 *M*) before loading onto 1.5-mm-thick 10% polyacrylamide gel. Following gel electrophoresis, proteins were blotted onto nitrocellulose membrane, and the blot was blocked in phosphate-buffered saline containing 5% fat-free milk and 0.5% Tween-20, followed by a 1.5-h incubation with anti-HA monoclonal antibody (HA.11; Boehringer Mannheim) diluted 1:1,000 in 5% fat-free milk and 0.5% Tween-20. Blots were washed three times with phosphate-buffered saline containing 0.3% Tween-20 and incubated for 1 h with horseradish peroxidase-coupled rat anti-mouse antibodies (Jackson Immunoresearch Laboratories) diluted 1:20,000 in 5% fat-free milk plus 0.5% Tween-20. The blot was extensively washed, and peroxidase activity was observed by the enhanced chemiluminescence (ECL) technique (Amersham).

Data analysis

Data are mean ± SEM values. Dose–response curves were plotted using the Sigma Plot version 4.11 program.

RESULTS

To study the role of W158 and W172 residues in binding and signaling of the CB_2 receptor, we replaced W158 with either Y or F (thus conserving the aromatic side chain, with or without a hydroxyl group) or with L (thus completely removing the aromatic side chain). A similar study was performed with W172, which was replaced with either F or Y or with A or L.

Role of W158 in CB_2 binding and signaling

Figure 2A demonstrates by western blot analysis (using antibodies against the HA tag) that all the W158 mutants were efficiently expressed, although, in general, the wt receptor was more highly expressed than the mutated forms (by up to threefold). Two immunoreactive bands could be observed. The molecular size of the lower immunoreactive band is 41 kDa, which is consistent with the amino acid composition of the HA-tagged receptor and with the molecular mass obtained for the expressed human CB_2 receptor protein (Nowell et al., 1998). The somewhat higher molecular mass of the second immunoreactive band (≈43 kDa) could represent a glycosylated form of the receptor. However, we cannot rule out the presence of small amounts of more highly glycosylated forms of the receptor.

Using homologous competition binding of HU-243 to determine the binding properties and possible changes in B_{max} and K_D of the mutants, we found (Fig. 2B) that W158A did not bind [^{3}H]HU-243. It is interesting that the replacement of this W residue with Y also led to a complete loss of binding activity. On the other hand, the conservative substitution of W158F retained most of its binding capacity. A small reduction in the B_{max} of the binding of HU-243 and a very small reduction in binding affinity were observed for W158Y versus the wt receptor.

We then analyzed the capacity of HA-CB_2 (wt) and the W158 mutants to inhibit AC activity. The COS cells were cotransfected with rabbit AC-V together with either HA-CB_2 (wt) or the mutated receptors, and the effect of increasing concentrations of HU-210 or WIN55,212-2 on forskolin-stimulated AC activity was determined. The results (Fig. 2C and D) show that, in agreement with the results of the binding studies, W158A and W158L showed only a very slight inhibition of AC activity in response to relatively high (1 μ*M*) concentrations of HU-210 or WIN55,212-2. On the other hand, the W158F mutant showed an identical AC inhibition pattern to that obtained with HA-CB_2 (wt). The EC_{50} values calculated for the inhibition of AC by W158F and HA-CB_2 (wt) were 1.2 ± 0.2 versus 1.0 ± 0.3 n*M* for HU-210 and 2.7 ± 0.8 versus 1.9 ± 0.9 n*M* for WIN55,212-2. In simi-

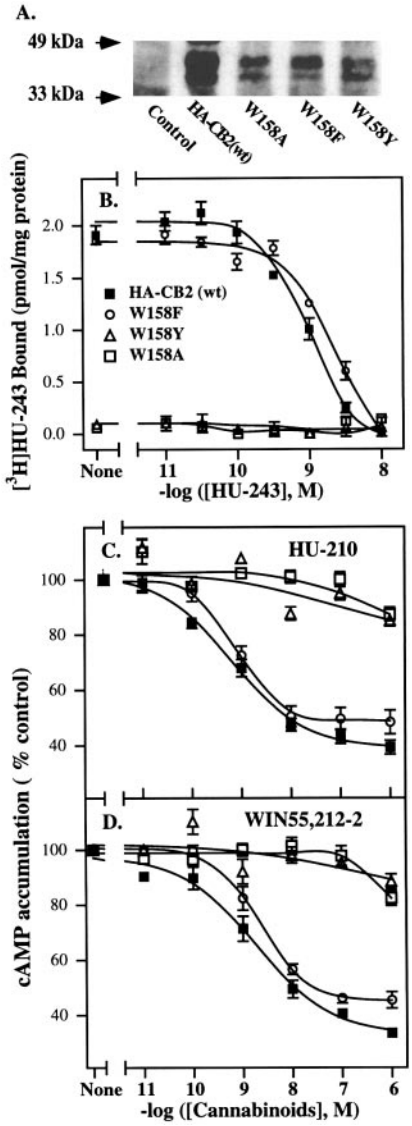


FIG. 2. Expression and binding of [^{3}H]HU-243 and AC inhibition by HA-CB_2 (wt) and W158 mutants. COS cells were transfected with the cDNAs of HA-CB_2 (wt) or the indicated mutants. **A:** Western blot analysis shows expression of the various W158 mutants. Control represents mock-transfected cells. The ratio of the expression of mutants versus wt receptor varied by a factor of up to threefold in different experiments. **B:** Homologous competition binding curves of [^{3}H]HU-243 to COS cell homogenates containing wt or W158 mutated CB_2 receptor. Data (in pmol of [^{3}H]HU-243/mg of protein) are mean ± SEM (bars) values of two experiments. **C** and **D:** COS cells transfected as in A and B but also cotransfected with AC-V were assayed for the effects of various concentrations of HU-210 and WIN55,212-2, respectively, on forskolin-stimulated AC activity. Data are mean ± SEM (bars) values of three experiments.

larity with these results, obtained with exogenous cannabinoids, the application of 2-arachidonoylglycerol (in the presence of phenylmethylsulfonyl fluoride to protect it from degradation) led to similar AC inhibition following activation of either W158F or the wt receptor, with EC_{50} values of 350 ± 160 and 230 ± 80 n*M*, respectively, and it had no effect on the W158A mutant (data not shown).

Role of W172 in CB_2 binding and signaling

Subsequently, the role of W172 in CB_2 binding and signaling was investigated. Figure 3A shows that all the W172 mutants examined were efficiently expressed, reaching levels similar to that of HA-CB_2 (wt). Figure 3B shows that the W172L mutant does not bind [^{3}H]HU-243 and that W172A binds [^{3}H]HU-243 at <10% of the levels observed for HA-CB_2 (wt). On the other hand, the W172F and W172Y mutants retained almost normal specific binding, at 80 and 68% of wt values, respectively. These results imply the involvement of the aromatic side chain at this position in the binding of tricyclic cannabinoids by the receptor.

To determine whether the reduction in binding is due to changes in B_{max} or binding affinity, we analyzed the homologous competition binding of [^{3}H]HU-243 in homogenates of cells transfected with the various W172 mutants. We found (Fig. 4A) that W172L and W172A did not show any significant binding. On the other hand, W172Y and W172F showed binding values close to those obtained with HA-CB_2 (wt). Moreover, the binding affinities of W172Y and W172F did not appreciably differ from that of the wt receptor. Heterologous competitive binding studies showed that the W172F and W172Y mutants bound the tricyclic cannabinoid agonist HU-210 (a compound very similar in structure to HU-243) with affinities essentially equivalent to that observed with the wt receptor (Table 1). The binding affinity (K_i) values for HU-210 were as follows: 190 ± 40

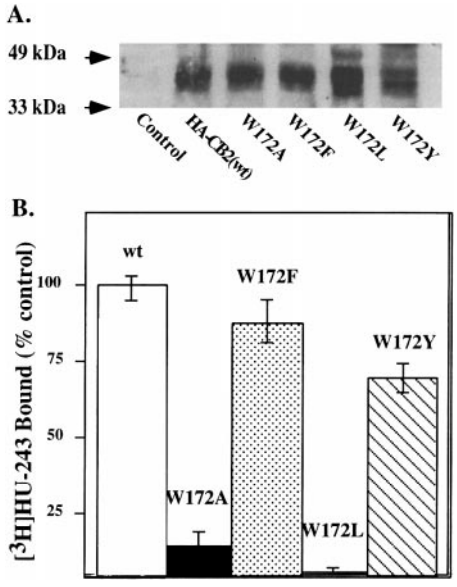


FIG. 3. Expression and binding of [^{3}H]HU-243 to HA-CB_2 (wt) and W172 mutants. **A:** Western blot analysis shows expression of the various W172 mutants. **B:** Binding of [^{3}H]HU-243 to equivalent amounts of cell homogenates. Binding to HA-CB_2 (wt) is defined as 100%. Data are mean ± SEM (bars) values of three experiments.

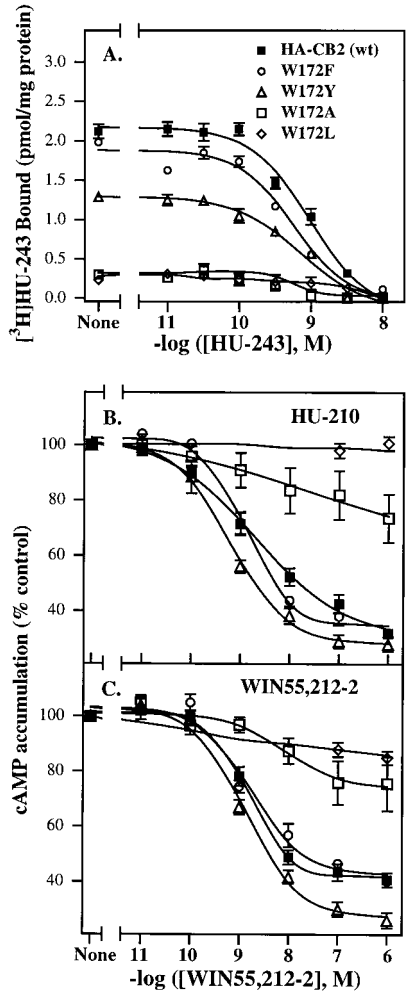


FIG. 4. Binding of [^{3}H]HU-243 and agonist-induced AC inhibition by various W172 CB_2 mutants. **A:** Homologous competition binding curves of [^{3}H]HU-243 to homogenates of cells transfected with W172 mutants. **B** and **C:** Inhibition of forskolin-stimulated AC-V activity by (B) HU-210 and (C) WIN55,212-2. For additional details, see the legend of Fig. 2.

and 140 ± 20 p*M* versus 160 ± 70 p*M* for the wt receptor. Similarly, the binding of the bicyclic cannabinoid CP55,940 was not affected by these mutations, showing binding affinities of 6.4 ± 1.7 and 6.9 ± 0.9 n*M* versus 5.9 ± 1.0 n*M* for the wt receptor. On the other hand, the binding of the aminoalkylindole WIN55,212-2 was slightly reduced by the mutations, demonstrating a K_i of 3.6 ± 0.5 and 3.6 ± 1.2 n*M* for W172F and W172Y mutants versus 1.0 ± 0.3 n*M* for the wt receptor.

The analysis of the capacity of the W172 mutants to inhibit AC activity is shown in Fig. 4B and C. We found that, in complete agreement with the binding studies, agonist activation of W172L did not inhibit and that of W172A only negligibly inhibited AC activity. On the other hand, using HU-210, WIN55,212-2, or 2-arachidonoylglycerol, we found that the W172F and W172Y mutants showed similar AC inhibition patterns to that observed with the wt receptor. The EC_{50} values calculated for the inhibition of AC by W172F, W172Y, and HA-CB_2 (wt) were 1.4 ± 0.3, 1.3 ± 0.6, and 2.0 ± 0.6 n*M* for HU-210 and 2.0 ± 0.3, 1.3 ± 0.2, and 1.7 ± 0.3 n*M* for WIN55,212-2, respectively.

DISCUSSION

All exogenous cannabinoid ligands (classical tricyclic cannabinoids, bicyclic cannabinoids, and aminoalkylindoles) have an aromatic ring and/or a long hydrophobic side chain, which were shown to have an important role in receptor binding and signaling (Howlett, 1995). For example, it was suggested that the aminoalkylindole WIN55,212-2 interacts with both CB_1 and CB_2 by aromatic stacking (Reggio et al., 1998). The replacement of the pentyl group with a dimethylheptyl group at position 3 of the cyclic and bicyclic cannabinoids was found to increase ligand affinity to CB_1 and CB_2. It was therefore inferred that a hydrophobic region in both receptors accommodates this hydrophobic side chain (Rhee et al., 1997; Howlett, 1998).

Using a chimeric construct of the CB_1 and CB_2 receptors and site-directed mutagenesis, Shire et al. (1999) have shown that the region consisting of TM4–extracellular loop 2–TM5 of both CB_1 and CB_2 contains residues that are critical for binding of cannabinoid agonists and antagonists. The aromatic side chain of the Trp residue is known to be involved in aromatic–aromatic interactions, cation–π interactions, and amino group–aromatic interactions (Waksman et al., 1992; Dougherty, 1996). W172, located at the top of TM4, and the conserved W158, just below the middle of TM4, are thus good candidates for having a role in cannabinoid ligand–receptor interactions.

Various aromatic amino acids in the fourth and other TM domains have been studied for ligand binding and signaling in a large variety of GPCRs, including the m_1 and m_3 muscarinic, 5-HT_2 serotonergic, NK_2 tachykinin, and δ-opioid receptors (Wess et al., 1992, 1993; Choudhary et al., 1993; Matsui et al., 1995; Befort et al., 1996; Roth et al., 1997; Renzetti et al., 1999*a*,*b*). In similarity to the data presented here, Befort et al. (1996) found that W173 of the δ-opioid receptor (in an analogous position to W158 of CB_2) is involved in receptor–

TABLE 1. K_i *values for HU-210, WIN55,212-2, and CP55,940 binding to HA-CB_2 (wt) and mutants*

	K_i (n*M*)		
	HU-210	CP55,940	WIN55,212-2
HA-CB_2 (wt)	0.16 ± 0.07	5.9 ± 1.0	1.0 ± 0.3
W172A	ND	ND	ND
W172F	0.19 ± 0.04	6.4 ± 1.7	3.6 ± 0.5[a]
W172Y	0.14 ± 0.02	6.9 ± 0.9	3.6 ± 1.2[a]

Data are mean ± SEM values of three experiments. ND, not detected.

[a] $p < 0.05$ versus wt receptor.

ligand recognition and that mutation of this amino acid reduced the receptor's binding affinity to many δ ligands. Similar results were obtained with the cognate W163A mutation of the human Y_1 receptor (Sautel et al., 1996) and with the W200A mutation of the 5-HT_{2A} receptor. It is interesting that Wess et al. (1993) reported that mutating W192 of the m_3 muscarinic receptor to F only slightly reduced agonist binding affinity and signaling. In line with this result, we show here that although W158 in CB_2 is very important for receptor activity, the conservative W158F mutation has little or no effect on cannabinoid binding and signaling. This result suggests that the hydrophobic interaction of W158 either with the ligand or with an appropriate area of the receptor is critical for receptor function and is conserved in W158F but not when this residue is altered into an aliphatic amino acid or even into Tyr. The fact that Tyr at this site interferes with receptor function could be due to interference by the OH group or by the change in π electron distribution compared with that present in Trp or Phe.

Both the W172A and W172L mutants were well expressed. However, W172A showed almost no apparent binding and relatively low inhibition of AC, and W172L did not bind HU-243 and did not show any AC inhibition. This result is in line with the finding that the W164A mutation in the m_1 muscarinic receptor significantly affected agonist binding (Matsui et al., 1995). Taken together, these results suggest that the Trp residue at this position is involved in ligand binding. However, we cannot exclude the possibility that this amino acid, rather than directly interacting with the cannabinoid ligand, could also affect the conformation of the binding pocket. Some of the reduction in the apparent binding of [^{3}H]HU-243 to either the W158Y and W158A, or the W172L and W172A mutants, could be explained by a reduction in receptor–G protein coupling as it is known that agonists bind with lesser affinity to G protein-uncoupled receptors. These experiments should therefore be verified as soon as a labeled CB_2 antagonist becomes available. However, the fact that, as described above, cognate mutations in other receptors showed a reduction in receptor–ligand recognition suggests that the observed reduction in binding described above is due to an actual reduction in binding.

It is of interest to note that although both W158 and W172 of the CB_2 receptor seem to have an important role in receptor–ligand interaction, W172 shows a less stringent structural requirement as this residue could be replaced with either F or Y, whereas W158 could only be replaced with F. This result is in agreement with the complete conservation of the W residue at position 4.50 of almost all GPCRs, whereas the W at position 4.64 is not generally conserved.

Acknowledgment: This work was supported by the Israel Science Foundation. Z.V. is the incumbent of the Ruth and Leonard Simon Chair for Cancer Research. We thank Dr. Raphael Mechoulam, Hebrew University, Jerusalem, Israel, for the cannabinoid agonists; Dr. Sean Munro, Medical Research Council, Cambridge, U.K., for the plasmid encoding the human CB_2 receptor; and Dr. Thomas Pfeuffer, Heinrich-Heine University, Dusseldorf, Germany, for the plasmids encoding AC-V and β-galactosidase. We thank Dr. J. Barg for helping with the K_i determinations.

REFERENCES

Attwood T. K., Eliopoulos E. E., and Findlay J. B. C. (1991) Multiple sequence alignment of protein families showing low sequence homology: a methodological approach using database pattern-matching discriminators for G-protein-linked receptors. *Gene* **98,** 153–159.

Avidor-Reiss T., Nevo I., Levy R., Pfeuffer T., and Vogel Z. (1996) Chronic opioid treatment induces adenylyl cyclase V superactivation. Involvement of $G_{\beta\gamma}$. *J. Biol. Chem.* **271,** 21309–21315.

Avidor-Reiss T., Nevo I., Levy R., Bayewitch M. L., and Vogel Z. (1997) Opioid-induced adenylyl cyclase superactivation is isozyme-specific. *J. Biol. Chem.* **272,** 5040–5047.

Baldwin J. M., Schertler G. F. X., and Ungar V. M. (1997) An alpha-carbon template for the transmembrane helices in the rhodopsin family of G-protein-coupled receptors. *J. Mol. Biol.* **272,** 144–164.

Ballesteros J. A. and Weinstein H. (1995) Integrated methods for G-protein coupled receptors, in *Methods in Neuroscience, Vol. 25* (Sealfon S. C., ed), pp. 366–428. Academic Press, San Diego.

Befort K., Tabbara L., Kling D., Maigret B., and Kieffer B. L. (1996) Role of aromatic transmembrane residues of the δ-opioid receptor in ligand recognition. *J. Biol. Chem.* **271,** 10161–10168.

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72,** 248–254.

Chin C.-N., Lucas-Lenard J., Abadji V., and Kendall D. A. (1998) Ligand binding and modulation of cyclic AMP levels depend on the chemical nature of residue 192 of the human cannabinoid receptor 1. *J. Neurochem.* **70,** 366–373.

Chin C.-N., Murphy J. W., Huffman J. W., and Kendall D. A. (1999) The third transmembrane helix of the cannabinoid receptor plays a role in the selectivity of aminoalkylindoles for CB2, peripheral cannabinoid receptor. *J. Pharmacol. Exp. Ther.* **291,** 837–844.

Choudhary M. S., Craigo S., and Roth B. L. (1993) A single point mutation ($Phe^{340} \rightarrow Leu^{340}$) of a conserved phenylalanine abolishes 4-[^{125}I]iodo-(2,5-dimethoxy)phenylisopropylamine and [^{3}H]mesulergine but not [^{3}H]ketanserin binding to 5-hydroxytryptamine receptors. *Mol. Pharmacol.* **43,** 755–761.

Coso O. A., Chiariello M., Yu J.-C., Teramoto H., Crespo P., Xu N., Miki T., and Gutkind J. S. (1995) The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signalling pathway. *Cell* **81,** 1137–1146.

Dougherty D. A. (1996) Cation–π interactions in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp. *Science* **271,** 163–168.

Ho S., Hunt H., Horton R., Pullen J., and Pease L. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77,** 51–59.

Horn F., Weare J., Beukers M. W., Hörsch S., Bairoch A., Chen W., Edvardsen Ø., Campagne F., and Vriend G. (1998) GPCRDB: an information system for G protein-coupled receptors. *Nucleic Acids Res.* **26,** 275–279.

Howlett A. C. (1995) Pharmacology of cannabinoid receptors. *Annu. Rev. Pharmacol. Toxicol.* **35,** 607–634.

Howlett A. C. (1998) The CB1 cannabinoid receptor in the brain. *Neurobiol. Dis.* **5,** 405–416.

Keown W. A., Campbell C. R., and Kucherlapati R. S. (1990) Methods for introducing DNA into mammalian cells. *Methods Enzymol.* **185,** 527–537.

Matsui H., Lazareno S., and Birdsall N. J. M. (1995) Probing of the location of the allosteric site on the m1 muscarinic receptors by site-directed mutagenesis. *Mol. Pharmacol.* **47,** 88–98.

Nowell K. W., Dove Pettit D., Cabral W. A., Zimmerman J. H. W., Abood M. E., and Cabral G. A. (1998) High-level expression of

the human CB2 cannabinoid receptor using a baculovirus system. *Biochem. Pharmacol.* **55,** 1893–1905.

Probst W. C., Snyder L. A., Schuster D. I., Brosius J., and Sealfon S. C. (1992) Sequence alignment of the G-protein coupled receptor superfamily. *DNA Cell. Biol.* **11,** 1–20.

Reggio P. H., Basu-Dutt S., Barnett-Norris J., Castro M. T., Hurst D. P., Seltzman H. H., Roche M. J., Gilliam A. F., Thomas B. F., Stevenson L. A., Pertwee R. G., and Abood M. E. (1998) The bioactive conformation of aminoalkylindoles at the cannabinoid CB1 and CB2 receptors: insights gained from (*E*)- and (*Z*)-naphthylidene indenes. *J. Med. Chem.* **41,** 5177–5187.

Renzetti A. R., Catalioto R.-M., Carloni C., Criscuoli M., Cucchi P., Giolitti A., Zappitelli S., Rotondaro L., and Maggi C. A. (1999*a*) Effects of tyrosine289phenylalanine mutation on binding and functional properties of the human tachykinin NK2 receptor stably expressed in Chinese hamster ovary cells. *Biochem. Pharmacol.* **57,** 899–906.

Renzetti A. R., Catalioto R.-M., Criscuoli M., Cucchi P., Ferrer C., Giolitti A., Guelfi M., Rotondaro L., Warner F. J., and Maggi C. A. (1999*b*) Relevance of aromatic residues in transmembrane segments V to VII for binding of peptide and nonpeptide antagonists to the human tachykinin NK2 receptor. *J. Pharmacol. Exp. Ther.* **290,** 487–495.

Rhee M.-H., Vogel Z., Barg J., Bayewitch M. L., Levy R., Hanus L., Breuer A., and Mechoulam R. (1997) Cannabinoid derivatives: binding to cannabinoid receptors and inhibition of adenylyl cyclase. *J. Med. Chem.* **40,** 3228–3233.

Rhee M.-H., Bayewitch M. L., Avidor-Reiss T., Levy R., and Vogel Z. (1998) Cannabinoid receptor activation differentially regulates the various adenylyl cyclase isozymes. *J. Neurochem.* **71,** 1525–1534.

Roth B., Shoham M., Choudharty M., and Khan N. (1997) Identification of conserved aromatic residues essential for agonist binding and second messenger production at 5-hydroxytryptamine$_{2A}$ receptors. *Mol. Pharmacol.* **52,** 259–266.

Salomon Y. (1991) Cellular responsiveness to hormones and neurotransmitters: conversion of [^{3}H]adenine to [^{3}H]cAMP in cell monolayers, cell suspensions and tissue slices. *Methods Enzymol.* **195,** 22–28.

Sautel M., Rudolf K., Wittneben H., Herzog H., Martinez R., Munoz M., Eberlein W., Engel W., Walker P., and Beck-Sickinger A. G. (1996) Neuropeptide Y and the nonpeptide antagonist BIBP 3226 share an overlapping binding site at the human Y_1 receptor. *Mol. Pharmacol.* **50,** 285–292.

Shire D., Calandra B., Bouaboula M., Barth F., Rinaldi-Carmona M., Casellas P., and Ferrara P. (1999) Cannabinoid receptor interactions with the antagonists SR 141716A and SR 144528. *Life Sci.* **65,** 627–635.

Song Z. H. and Bonner T. I. (1996) A lysine residue of the cannabinoid receptor is critical for receptor recognition by several agonists but not WIN55212-2. *Mol. Pharmacol.* **49,** 891–896.

Song Z. H., Slowey C.-A., Hurst D. P., and Reggio P. H. (1999) The difference between the CB1 and CB2 cannabinoid receptors at position 5.46 is crucial for the selectivity of WIN55212-2 for CB2. *Mol. Pharmacol.* **56,** 834–840.

Tao Q. and Abood M. E. (1998) Mutation of a highly conserved aspartate residue in the second transmembrane domain of the cannabinoid receptors, CB1 and CB2, disrupts G-protein coupling. *J. Pharmacol. Exp. Ther.* **285,** 651–658.

Tao Q., McAllister S. D., Andreassi J., Nowell K. W., Cabral G. A., Hurst D. P., Bachtel K., Ekman M. C., Reggio P. H., and Abood M. E. (1999) Role of a conserved lysine residue in the peripheral cannabinoid receptor (CB2): evidence for subtype specificity. *Mol. Pharmacol.* **55,** 605–613.

Vogel Z., Barg J., Levy R., Saya D., Heldman E., and Mechoulam R. (1993) Anandamide, a brain endogenous compound, interacts specially with cannabinoid receptors and inhibits adenylate cyclase. *J. Neurochem.* **61,** 352–355.

Waksman G., Kominos D., Robertson S. C., Pant N., Baltimore D., Birge R. B., Cowburn D., Hanafusa H., Mayer B. J., Overduin M., Resh M. D., Rios C. B., Silverman L., and Kuriyan J. (1992) Crystal structure of the phosphotyrosine recognition domain SH2 of v-src complexed with tyrosine-phosphorylated peptides. *Nature* **358,** 646–653.

Wallach J., Droste M., Kluxen F. W., Pfeuffer T., and Frank R. (1994) Molecular cloning and expression of a novel type V adenylyl cyclase from rabbit myocardium. *FEBS Lett.* **338,** 257–263.

Wess J., Maggio R., Palmer J. R., and Vogel Z. (1992) Role of conserved threonine and tyrosine residues in acetylcholine binding and muscarinic receptor activation. *J. Biol. Chem.* **267,** 19313–19319.

Wess J., Nanavati S., Vogel Z., and Maggio R. (1993) Functional role of proline and tryptophan residues highly conserved among G protein-coupled receptors studied by mutational analysis of the m3 muscarinic receptor. *EMBO J.* **12,** 331–338.