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Ligand Binding and Modulation of Cyclic AMP Levels Depend on the Chemical Nature of Residue 192 of the Human Cannabinoid Receptor 1

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Abstract: The human cannabinoid receptor associated with the CNS (CB1) binds Δ^9-tetrahydrocannabinol, the psychoactive component of marijuana, and other cannabimimetic compounds. This receptor is a member of the seven transmembrane domain G protein-coupled receptor family and mediates its effects through inhibition of adenylyl cyclase. An understanding of the molecular mechanisms involved in ligand binding and receptor activation requires identification of the active site residues and their role. Lys^{192} of the third transmembrane domain of the receptor is noteworthy because it is the only nonconserved, charged residue in the transmembrane region. To investigate the properties of this residue, which are important for both ligand binding and receptor activation, we generated mutant receptors in which this amino acid was changed to either Arg (K192R), Gln (K192Q), or Glu (K192E). Wild-type and mutant receptors were stably expressed in Chinese hamster ovary cells and were evaluated in binding assays with the bicyclic cannabinoid CP-55,940 and the aminoalkylindole WIN 55,212-2. We found that only the most conservative change of Lys to Arg allowed retention of binding affinity to CP-55,940, whereas WIN 55,212-2 bound to all of the mutant receptors in the same range as it bound the wild type. Analysis of the ligand-induced inhibition of cyclic AMP production in cells expressing each of the receptors gave an EC_{50} value for each agonist that was comparable to its binding affinity, with one exception. Although the mutant K192E receptor displayed similar binding affinity as the wild type with WIN 55,212-2, an order of magnitude difference was observed for the EC_{50} for cyclic AMP inhibition with this compound. The results of this study indicate that binding of CP-55,940 is highly sensitive to the chemical nature of residue 192. In contrast, although this residue is not critical for WIN 55,212-2 binding, the data suggest a role for Lys^{192} in WIN 55,212-2-induced receptor activation.
Key Words: Adenylyl cyclase—Aminoalkylindole—Bicyclic cannabinoid—Cannabinoid receptor—G protein-coupled receptor—Cyclic AMP.
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Δ^9-Tetrahydrocannabinol, the psychoactive component of marijuana, and other cannabinoid analogues produce a variety of effects including analgesia, inhibition of nausea, and lowering of intraocular pressure (Dewey, 1986). That these effects are receptor mediated was suggested by the identification of a receptor from rat brain that binds the cannabinoids with high affinity (Devane et al., 1988). The subsequent cloning of the cDNA encoding the cannabinoid receptor from rat brain (rat CB1; Matsuda et al., 1990), human brain (human CB1; Gérard et al., 1991), mouse spleen (CB2; Munro et al., 1993), and a mouse brain genomic library (Abood et al., 1997) has further helped to clarify the action of the cannabinoids at the cellular level. The amino acid sequences deduced from cDNA clones have confirmed that the cannabinoid receptors belong to the seven transmembrane (seven TM) domain, G protein-coupled receptor (GPCR) superfamily. Previous studies have shown that cannabinoid agonists inhibit adenylyl cyclase through coupling with a pertussis toxin-sensitive G_i protein (Howlett et al., 1986). It was also found that, in neuronal systems, cannabinoids alter the voltage-dependent potassium A-current (for review, see Deadwyler et al., 1995) and decrease N-type calcium channel conductance (Mackie and Hille, 1992), possibly through a separate G protein-mediated pathway.

Structure–activity relationships (SARs) of various cannabinoid ligands have been examined extensively using pharmacophore mapping with different classes of agonists, including the tricyclic and bicyclic cannabinoids and aminoalkylindoles (for review, see Martin et al., 1995; Xie et al., 1995, 1996). A "three-point requirement" for ligand–receptor interactions has

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Abbreviations used: BSA, bovine serum albumin; cAMP, cyclic AMP; CB1, cannabinoid receptor cloned from human brain; CHO, Chinese hamster ovary; GPCR, G protein-coupled receptor; PBS, phosphate-buffered saline; RT-PCR, reverse transcription–polymerase chain reaction; SAR, structure–activity relationship; TM, transmembrane; TME, 25 m*M* Tris-HCl, pH 7.5, 5 m*M* $MgCl_2$, 1 m*M* EDTA.

been proposed in which the geometric, electrostatic, and hydrophobic properties of prototypic compounds such as Δ^9-tetrahydrocannabinol and CP-55,940 are considered to contribute to the potency of their cannabimimetic effects. Despite their appreciable structural differences, some studies suggest that the binding site for the aminoalkylindoles and the cannabinoids may be overlapping although not likely identical (Martin et al., 1995; Song and Bonner, 1996). Anandamide, a fatty acid ethanolamide, is an endogenous agonist originally isolated from porcine brain (Devane et al., 1992). This new class of ligand is structurally very different, suggesting further complexities to SARs between the cannabinoid receptor and its various ligands. To explain fully the potency of various compounds, the binding sites of the ligands must be mapped and the contact points involved in ligand–receptor interactions delineated. Molecular modeling and sequence analysis of the receptor provide some first approximations regarding regions of the receptor likely involved in ligand binding.

Sequence alignment, homology analysis, and computer modeling have revealed that the cannabinoid receptor belongs to a distinct subclass of GPCRs (Mountjoy et al., 1992; Baldwin, 1993). The main characteristic of this subclass of receptors is the lack of several normally conserved residues that are believed to play important structural roles, such as a Pro in helix 5 and a Cys in the extracellular loop adjacent to helix 3. This suggests subtle differences in the arrangement of the seven TM helices of this receptor subclass from the rest of the GPCR family. In addition to the above variations, there are several other residues in the TM region that are typically conserved in the GPCR superfamily but not in the cannabinoid receptor. The corresponding residues in the cannabinoid receptor CB1 include the following: Glu^{133}, Gly^{157}, Lys^{192}, Thr^{210}, and Tyr^{292}. The Glu^{133} is found in other members of the subfamily, however. Indeed, of the three charged residues presumed to be located in the TM domain, Glu^{133}, Asp^{163}, and Lys^{192}, only the Lys is unique to both the subfamily and GPCRs in general. Furthermore, Lys^{192} is noteworthy because it is predicted to be located in the extracellular half and interior of the helical bundle in TM region 3 (TM3). In mammalian opsin, the corresponding residue, Glu^{113}, acts as a counterion to the protonated retinal Schiff base (Sakmar et al., 1989), and in cationic neurotransmitter receptors, an Asp in a similar position is the key residue that interacts with the positively charged ligand (Fraser et al., 1989). Its position and its charge suggest that Lys^{192} is likely to be involved in the interactions with cannabinoid ligands, thus providing a key contact point for a polar substituent of the ligand.

Extensive SARs on classical and nonclassical cannabinoids and modeling studies have suggested that the phenolic hydroxyl is one of the key moieties conferring cannabimimetic activity (Razdan, 1986; Reggio, 1987; Semus and Martin, 1990). In contrast, a recent study using several cannabinoid analogues has indicated that a hydroxyl at the C-11 position or the pyran oxygen in tricyclic cannabinoids may also be involved in hydrogen bonding with the receptor even in the absence of the phenolic hydroxyl group (Huffman et al., 1996). Regardless, the terminal amino group of Lys^{192} on the receptor is a good candidate for interaction with any of these groups.

While our study was in progress, a mutational study involving replacement of Lys^{192} for Ala was reported (Song and Bonner, 1996). The results indicated that the Ala substitution disrupted binding to several agonists including the bicyclic (CP-55,940) and tricyclic (HU-210) cannabinoids, and anandamide, but not to an aminoalkylindole (WIN 55,212-2). To investigate the sensitivity of ligand binding to the nature of the amino acid side chain at this position, including side chain length, charge, and hydrogen bonding potential, we have mutated Lys^{192} of the human CB1 receptor to Arg, Gln, and Glu. This series of mutant cannabinoid receptors was stably expressed in Chinese hamster ovary (CHO) cells and evaluated for ligand binding and modulation of cellular cyclic AMP (cAMP) levels. The results presented here demonstrate the necessity for a basic residue at position 192 for binding to CP-55,940 but a relative lack of sensitivity to the nature of the side chain for binding to WIN 55,212-2. Furthermore, the results suggest that Lys^{192} of the cannabinoid receptor not only plays a role in the interaction with ligands but may also be involved in activation of the signal transduction pathway.

MATERIALS AND METHODS

DNA constructs and transfection

A 1.5-kb *Hin*dIII–*Eco*RI fragment of the human CB1 gene containing the entire coding region was cloned into the corresponding sites of pAlter-I (Promega, Madison, WI, U.S.A.) for site-directed mutagenesis. Mutations were introduced with oligonucleotides following the manufacturer's protocol and were confirmed by DNA sequencing (Sanger et al., 1977). The insert was then subcloned into pcDNA3 (Invitrogen, San Diego, CA, U.S.A.) for expression in mammalian cells. The resulting DNA constructs were stably transfected into CHO cells using Lipofectamine (Life Technologies). Transfected cells were selected by the addition of 0.5 mg/ml Geneticin (Life Technologies). Individual clones were isolated by limiting dilution in 96-well culture plates and screened for the level of mRNA by reverse transcription–polymerase chain reaction (RT-PCR). CHO cells were cultured in minimum essential medium with Earle's salt and L-glutamine supplemented with 5% fetal bovine serum and 0.1 m*M* nonessential amino acids or F-12 plus 10% fetal bovine serum.

RT-PCR

Total RNA was isolated from stably transfected CHO cells using a Rapid RNA purification kit (Amresco, Solon, OH, U.S.A.). RNA samples were treated with DNase I (Life Technologies) for 15 min before RT-PCR. Reverse transcription was performed by using murine leukemia virus reverse transcriptase (Perkin-Elmer) with ~1 μg of total

RNA and incubated for 30 min at 45°C. PCR amplification was performed subsequently using an amplification cycle of 95°C (30 s), 55°C (30 s), 72°C (1 min). A pair of primers specific to the human CB1 gene were used in the reaction to amplify a 1.4-kb product from the cDNA of CB1 gene. No PCR product was detected when reverse transcriptase was omitted.

Ligand binding studies

Transfected CHO cells in monolayers were washed with phosphate-buffered saline (PBS), and membranes were prepared by scraping cells into hypotonic buffer (5 m*M* Tris-HCl, pH 7.4, 2 m*M* EDTA) and then rupturing the cells by sonication. The cell lysate was spun at 1,000 *g* for 5 min at 4°C. The pellet was discarded and the membrane was recovered from the supernatant by further centrifugation at 100,000 *g* for 1 h at 4°C, resuspended in 25 m*M* Tris-HCl, pH 7.4, 5 m*M* $MgCl_2$, 1 m*M* EDTA (TME) with 200 m*M* sucrose, frozen quickly on dry ice, and stored at −70°C. Protein concentration was determined by the method of Bradford (1976).

Saturation binding to the cannabinoid receptor was performed as previously described (Abadji et al., 1994). In brief, ~50 μg of crude membrane was incubated in TME buffer containing 0.1% bovine serum albumin (BSA) along with the varying concentrations of radioligand ([^{3}H]CP-55,940 or [^{3}H]WIN 55,212-2) to a final volume of 0.2 ml. Nonspecific binding was determined in the presence of 1 μ*M* unlabeled ligand. Reactions were incubated at 30°C for 1 h and terminated by the addition of 250 μl of TME buffer containing 5% BSA. The reaction mixture was filtered on a Brandell cell harvester using GF/C filters. The filters were subsequently washed four times with TME buffer. Bound radioactivity was determined by scintillation counting.

cAMP determination

CHO cells (1.8×10^5 per well) were seeded in 24-well plates. When the cell number reached ~$4–5 \times 10^5$ cells per well, the media were removed and cells were rinsed with PBS. A mixture (100 μl) containing growth medium without serum, 0.2 m*M* Ro 20-1724, 0.25% BSA, and 20 m*M* HEPES was added. cAMP accumulation was stimulated by the addition of 1 μ*M* forskolin. The appropriate cannabimimetic ligand was then added, incubated for 20 min at 37°C, and the reaction was stopped by the addition of 1.2 *M* HCl to give a final HCl concentration of 0.1 *M*. Cells were frozen at −80°C. On thawing they were neutralized with 2 *M* HEPES, pH 7.5, and 50-μl aliquots were removed and assayed for cAMP content using the cAMP ^{3}H assay system (Amersham).

Data analysis

Data obtained from saturation binding assays and cAMP assays were analyzed by nonlinear regression analysis using GraphPad Prism computer software (GraphPad Software, San Diego, CA, U.S.A.). Data were also analyzed by Hill transformation and were all found to have Hill coefficients very close to 1. Binding curves were best fit to a one-site binding model.

RESULTS

Expression of wild-type CB1 and mutant Lys^{192} receptors in stable CHO cell lines

Expression plasmids carrying either the wild-type or mutant CB1 genes were used to transfect CHO cells.

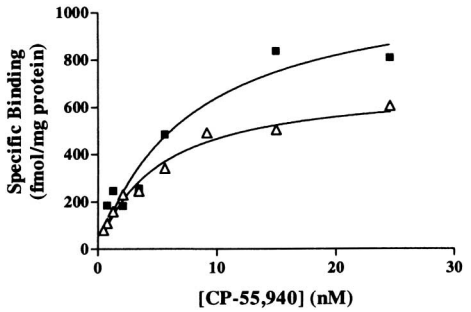

FIG. 1. Binding of [^{3}H]CP-55,940 to CB1 wild-type (■) and K192R (△) receptors. The experiments were performed at 30°C at the indicated [^{3}H]CP-55,940 concentrations using membrane preparations as described in Materials and Methods. Specific binding of [^{3}H]CP-55,940 is the difference between the binding in the absence and presence of 1 μ*M* unlabeled CP-55,940. Saturation binding isotherms were analyzed by nonlinear regression analysis (GraphPad Prism) and represent mean values of three independent experiments, each performed in duplicate.

Cell lines stably expressing the receptor were initially screened using RT-PCR as described in Materials and Methods, and receptor expression was verified by ligand binding. Expression levels ranged from 1,300 to 450 fmol/mg of membrane protein, with the wild type exhibiting the highest receptor densities. RNA from untransfected CHO cells did not produce a fragment corresponding to the CB1 gene as determined by RT-PCR, and no specific ligand binding was found with untransfected CHO cells.

High-affinity [^{3}H]CP-55,940 binding and receptor activation require a basic residue at position 192

Membranes from CHO cells expressing the wild-type or K192R, K192Q, or K192E receptors were analyzed for saturation binding with [^{3}H]CP-55,940. Nonspecific binding ranging from 30 to 60% was observed and was dependent on the concentration of radioligand and the level of receptor expression. Specific binding of [^{3}H]CP-55,940 to the wild-type cannabinoid receptor was saturable (Fig. 1). Nonlinear regression analysis yielded a K_D value of 7.7 ± 3.5 n*M* and a B_{max} value of 1,136.0 ± 215.5 fmol/mg (Table 1). This is consistent with values reported for [^{3}H]CP-55,940 binding to membranes from rat cerebellum (K_D = 2.3 n*M*, B_{max} = 2.5 pmol/mg; Felder et al., 1992) and with 293 cell culture lines transfected with CB1 (K_i = 4.6 n*M*; Song and Bonner, 1996).

Substitution of K192 in CB1 with Arg produced little change in the binding isotherm with [^{3}H]CP-55,940 relative to the wild-type receptor (Fig. 1). A K_D value of 4.9 ± 2.2 n*M* and a B_{max} value of 696.7 ± 127.8 fmol/mg were determined (Table 1). In contrast, no binding isotherm with [^{3}H]CP-55,940 in the same concentration range was observed with the mutant K192Q and K192E receptors. For these receptors, <10% specific binding was detected using up to 75 n*M* ligand. These results underscore the sensitivity of

TABLE 1. *Parameters of saturation binding and cAMP assays with CP-55,940 for CB1 wild-type and mutant receptors*

	[^{3}H]CP-55,940 binding		Inhibition of cAMP accumulation	
Receptor	K_D (n*M*)	B_{max} (fmol/mg)	EC_{50} (n*M*)	% of max inhibition
CB1 WT	7.7 ± 3.5	1,136.0 ± 215.5	4.0 ± 1.9	62.0 ± 9.9
CB1 K192R	4.9 ± 2.2	696.7 ± 127.8	6.9 ± 2.5	37.1 ± 3.4
CB1 K192Q	No binding up to 75 n*M*		ND	ND
CB1 K192E	No binding up to 75 n*M*		ND	ND

Binding data are presented as mean ± SE values from at least three experiments performed in duplicate. The data for cAMP accumulation are based on two experiments performed in duplicate and are presented as mean ± SE values. WT, wild type; ND, not determined.

[^{3}H]CP-55,940 binding to the nature of the residue at position 192 and suggest a requirement for a basic residue at this location. Other amino acids, including Gln with high hydrogen bonding potential, do not permit binding with this compound.

It has been shown that CB1 is coupled to an inhibitory G protein and, on receptor activation by agonists, this coupling leads to a decrease in intracellular cAMP in a concentration-dependent manner (Howlett et al., 1986; Matsuda et al., 1990). To evaluate G protein coupling with the K192R receptor, the inhibition of forskolin-stimulated cAMP by CP-55,940 was examined (Fig. 2). The efficacy of CP-55,940 to inhibit cAMP accumulation in cells expressing the wild-type and K192R receptors correlated well with their binding affinities to this ligand. The EC_{50} values were 4.0 ± 1.9 and 6.9 ± 2.5 n*M*, respectively (Table 1), indicating that the change of Lys^{192} to Arg has little or no effect on the activation of the receptor by CP-55,940. As indicated in Fig. 2 and Table 1, the maximum effect of CP-55,940 on the K192R receptor was ~1.7-fold lower than on the wild type (37 vs. 62% inhibition), consistent with the variation in receptor cell density (see below).

[^{3}H]WIN 55,212-2 binds to the wild-type and Lys^{192} mutants with comparable affinity

The ability of [^{3}H]WIN 55,212-2 to bind to the wild-type and K192R, K192Q, and K192E receptors was examined using saturation binding assays. Nonspecific binding in the range of 50–60% was observed, consistent with the particularly hydrophobic nature of this compound. Specific binding with membrane preparations of each of the receptors examined was saturable as shown in Fig. 3. Nonlinear regression analysis of the binding data from the wild-type receptor gave values of K_D = 16.2 ± 7.4 n*M* and B_{max} = 1,265.0 ± 274.5 fmol/mg (Table 2). The K_D is comparable to that observed for [^{3}H]WIN 55,212-2 binding to CB1 from rat brain (Jansen et al., 1992) and is consistent with a K_i = 11.9 n*M* reported for competition binding with CB1-transfected 293 cells (Song and Bonner, 1996).

As anticipated, the K192R receptor had a similar binding affinity to [^{3}H]WIN 55,212-2 (Fig. 3B) as the wild type (K_D = 12.3 ± 4.8 n*M*, B_{max} = 782.3 ± 126.5 fmol/mg). However, unlike binding to CP-55,940, mutation of Lys^{192} to Gln had little effect on the binding of the receptor with WIN 55,212-2. As shown in Fig. 3C, the K192Q receptor retained specific, high-affinity binding to WIN 55,212-2 with a K_D value of 27.1 ± 8.6 n*M* and a B_{max} value of 617.0 ± 155.0 fmol/mg. Furthermore, the K192E receptor also bound [^{3}H]WIN 55,212-2 (Fig. 3D) with a K_D = 37.6 ± 12.1 n*M* and a B_{max} = 553.5 ± 80.9 fmol/mg. Thus, the K192Q and K192E receptors, with which no appreciable [^{3}H]CP-55,940 binding was detected, bound [^{3}H]-WIN 55,212-2 with K_D values just slightly different from the wild type (Table 2).

The high affinity binding with [^{3}H]WIN 55,212-2 that was retained by the mutants suggests that none of the Lys^{192} substitutions generated pronounced perturbations in the overall structural integrity of the receptor. It is remarkable that the Lys^{192} to Glu substitution had only a marginal effect on [^{3}H]WIN 55,212-2 binding. Barring an unusually high pK_a due to the specific microenvironment at this location, this substitution introduces a negatively charged side chain with no natural counterpart available for salt bridge formation. Apparently, the receptor can make accommodations for the Glu to achieve WIN 55,212-2 binding. However, an adverse effect on G protein coupling was observed as described below.

The Lys^{192} mutants vary in WIN 55,212-2-induced modulation of cAMP levels

To compare the binding affinity and receptor activation profiles for the Lys^{192} mutants, we examined the inhibitory effect of WIN 55,212-2 on the forskolin-stimulated cAMP production. As shown in Fig. 4A,

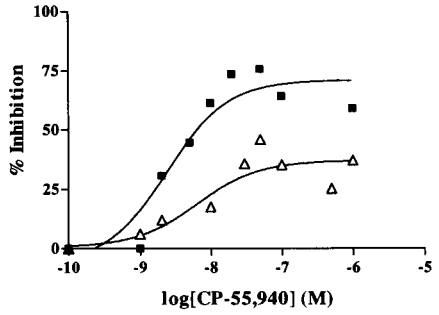

FIG. 2. Effect of CP-55,940 on forskolin-stimulated cAMP accumulation in CHO cells expressing the CB1 wild-type (■) and K192R (△) receptors. Data are presented as percentages of cAMP accumulation in the presence of forskolin plus ligand relative to that in the presence of forskolin alone. Basal levels of cAMP (in the absence of forskolin) were <0.04 pmol/1 × 10^5 cells, and the forskolin-stimulated levels of cAMP were ~4 pmol/1 × 10^5 cells.

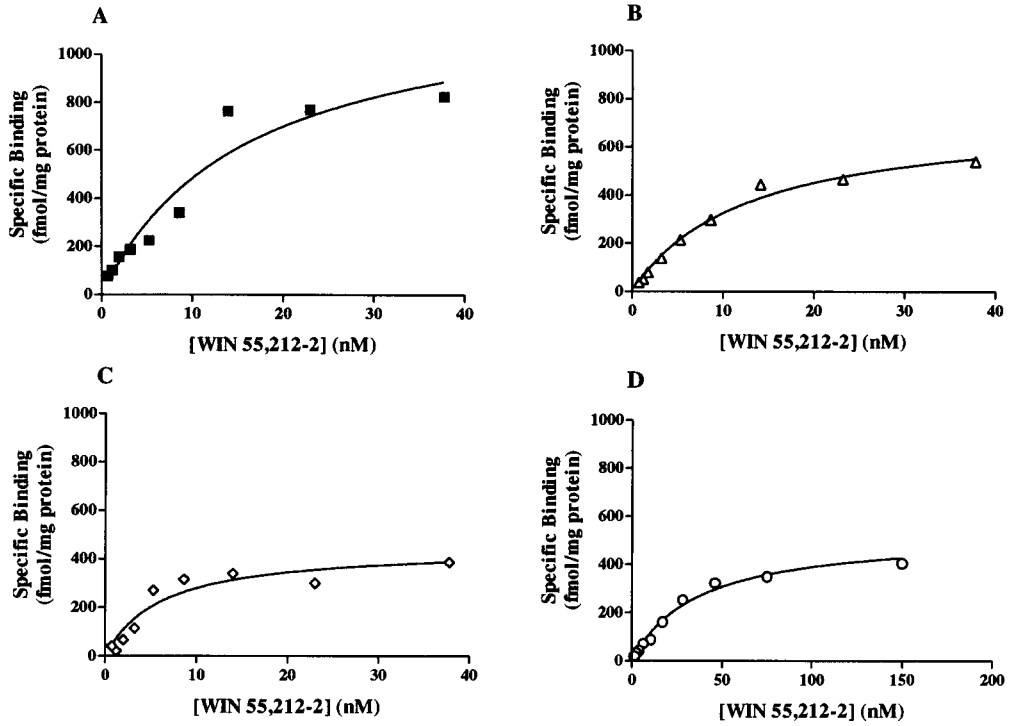

FIG. 3. Binding of [^{3}H]WIN 55,212-2 to CB1 wild-type (**A;** ■), K192R (**B;** △), K192Q (**C;** ◇), and K192E (**D;** ○) receptors. Saturation binding analysis was performed and analyzed, as described in Fig. 1, using [^{3}H]WIN 55,212-2 as the radioligand and 1 μM unlabeled WIN 55,212-2 to define nonspecific binding. Data presented are mean values of three independent experiments each performed in duplicate.

the wild-type and K192R receptors responded to WIN 55,212-2 similarly, with EC_{50} values of 31.0 ± 1.5 and 40.9 ± 3.0 nM, respectively. Although slightly higher, these values are well within the range of the binding constants determined with this compound for these receptors. Furthermore, the extent of maximum inhibition of cAMP production corresponded to the expression level of these receptors.

It is noteworthy that attenuation of receptor activation by WIN 55,212-2 with the K192E receptor was observed. The EC_{50} value was 483.2 ± 96.0 nM, which is about one order of magnitude higher than the EC_{50} values determined for the wild-type and K192R receptors. This value is also more than an order of magnitude higher than the K_D determined for WIN 55,212-2 binding to the same receptor. This indicates that although the Glu substitution does not have a significant effect on WIN 55,212-2 binding, it has changed the effectiveness of the receptor in the signal transduction process.

Also shown in Fig. 4 are the results from analysis of cAMP levels on WIN 55,212-2 treatment of the K192Q receptor. Due to the low receptor density, the EC_{50} value was not determined.

TABLE 2. *Parameters of saturation binding and cAMP assays with WIN 55,212-2 for CB1 wild-type and mutant receptors*

	[^{3}H]WIN 55,212-2 binding		Inhibition of cAMP accumulation	
Receptor	K_D (nM)	B_{max} (fmol/mg)	EC_{50} (nM)	% of max inhibition
CB1 WT	16.2 ± 7.4	1,265.0 ± 274.5	31.0 ± 1.5	75.1 ± 14.2
CB1 K192R	12.3 ± 4.8	782.3 ± 126.5	40.9 ± 3.0	53.3 ± 8.8
CB1 K192Q	27.1 ± 8.6	450.7 ± 79.3	ND	25.0 ± 3.6
CB1 K192E	37.6 ± 12.1	553.5 ± 80.9	483.2 ± 96.0	78.3 ± 3.2

Binding data are presented as mean ± SE values from at least three experiments performed in duplicate. The data for cAMP accumulation are based on two experiments performed in duplicate and are presented as mean ± SE values. WT, wild type; ND, not determined because of low receptor density.

Linear correlation between B_{max} and the maximum, ligand-induced inhibitory effect on cAMP production

As shown in Tables 1 and 2, values for the B_{max} and the maximal inhibitory effect of the ligands on cAMP accumulation varied among the various receptor clones. It is interesting that a clear linear correlation between these two parameters was observed, as demonstrated in Fig. 5. The exception involves the clone expressing the K192E receptor, for which it was not possible to do the binding (and thus B_{max} determination) and cAMP assays in tandem, and the receptor density fell during the intervening passages of the clone. The overall trend indicates that there is close

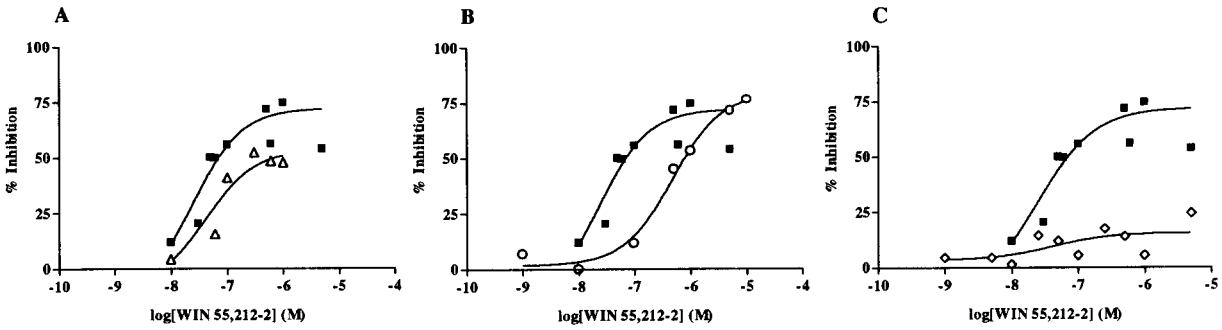

FIG. 4. WIN 55,212-2 induced inhibition of forskolin-stimulated cAMP accumulation in cells expressing the CB1 wild-type (■) and K192R (△) (**A**), CB1 wild-type (■) and K192E (○) (**B**), and CB1 wild-type (■) and K192Q (◇) (**C**) receptors. The inhibitory effect of WIN 55,212-2 was measured as described in Fig. 2. Data presented are mean values of two or three experiments each performed in duplicate.

coupling between the specific action of the ligand on CB1 and the observed effect on cAMP levels; interactions with other receptors are not contributing to the response; and we have not overexpressed the receptor relative to G_i levels. A similar correlation has been observed for the lutropin receptor and maximal stimulation of cAMP production (Kosugi et al., 1996).

DISCUSSION

In this study we demonstrated that residue Lys^{192} in TM3 of the cannabinoid receptor is important for ligand binding and agonist-induced TM signaling. First, substitution of Lys^{192} to Glu or Gln abolished binding to the bicyclic cannabinoid CP-55,940 but not to the aminoalkylindole WIN 55,212-2, whereas substitution of Lys^{192} to Arg produced a receptor that still retained high affinity for these two ligands. Second, although mutations of Lys^{192} did not substantially change the binding affinity of the receptor for WIN 55,212-2, the change of Lys^{192} to Glu greatly impaired the ability of this ligand to activate the receptor. This was reflected in an EC_{50} that was >10-fold higher than the K_D for WIN 55,212-2.

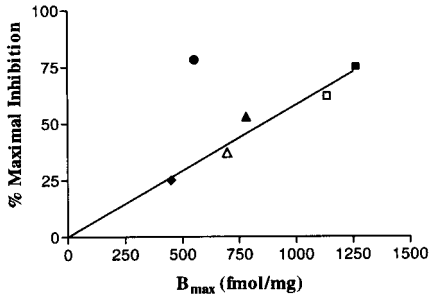

FIG. 5. The correlation of B_{max} and the maximum level of ligand-induced inhibition of cAMP accumulation in forskolin-stimulated cells. The B_{max} values were determined by [^{3}H]CP-55,940 (open points) or [^{3}H]WIN 55,212-2 (closed points) binding to CB1 wild-type (■, □), K192R (▲, △), and K192Q (◆) receptors. The linear regression analysis showed a value of $R^2 = 0.98$. The data from K192E (●) were omitted in the linear regression analysis because the ligand binding and cAMP studies were done at significantly different times with cells having different receptor densities.

Our results are consistent with the work of Song and Bonner (1996) who found that substitution of Lys^{192} to Ala abolished receptor binding to three types of cannabinoid agonists, CP-55,940, HU-210, and anandamide, but not to WIN 55,212-2. Our data further demonstrate that CP-55,940 binding is particularly sensitive to the chemical nature of the residue at 192; only the most conservative substitution of one basic residue for another permitted retention of high-affinity binding and receptor activation by CP-55,940. The bulky nature of the Arg side chain relative to Lys had no apparent effect. In contrast, other modifications in the length, degree, or sign of the charge of residue 192 resulted in a complete loss of binding. In addition to the K192Q and K192E receptors, a K192L receptor made in our laboratory was also found not to bind CP-55,940 although it responded to WIN 55,212-2 (data not shown).

The potential role of the Lys^{192} side chain in CP-55,940 binding has been debated, and a few different possibilities are compatible with our findings. Although we cannot rule out a problem due to the shorter length of the Gln side chain, the complete loss of binding affinity with this residue points toward a requirement for positive charge. Based on traditional SAR and molecular modeling studies, the phenolic hydroxyl group at C-1 of the ligand has been implicated in hydrogen bonding with the receptor (Reggio, 1987; Semus and Martin, 1990). In combination with mutational analysis (Song and Bonner, 1996), these studies suggested the possibility that Lys^{192} acts as a hydrogen bond donor in an interaction with the oxygen of the phenolic hydroxyl. Our results are consistent with this possibility particularly because Gln would not be expected to function as well in this type of interaction. Huffman et al. (1996) have raised the possibility that Lys^{192} could hydrogen bond with other oxygen atoms,

such as the C-11 hydroxyl group or the pyran oxygen of the ligand. The lengthy side chain of Lys is thought to provide the flexibility to achieve these types of interactions with different cannabinoid analogues in the ligand binding pocket. Other studies have suggested that the C-1 hydroxyl group serves as a hydrogen bond donor in interacting with the receptor. Our data argue to the contrary in terms of the residue at 192, because the positively charged Lys or Arg species would not serve as a hydrogen bond acceptor for the phenolic proton. It is also possible that the $\delta(+)$ amino group of Lys or Arg could interact with the $\delta(-)$ π electrons of the phenolic ring of CP-55,940. This type of amino–aromatic interaction has been reported for the binding of several small molecular ligands with other GPCRs, such as the neurokinin-1 receptor (Fong et al., 1994) and the angiotensin type I receptors (Noda et al., 1995). Finally, it is possible that the requirement for a Lys or Arg residue reflects a role in the proper positioning of TM3 rather than a direct interaction with the ligand. A positively charged residue sitting near the membrane surface may function to inhibit this region of TM3 from penetrating too deeply into the bilayer.

In accordance with previous reports (Jansen et al., 1992; Bouaboula et al., 1995), WIN 55,212-2 displayed a lower affinity to the CB1 receptor than CP-55,940. However, replacement of Lys^{192} with either Arg, Gln, or Glu had, at most, a marginal affect on the binding affinity of WIN 55,212-2 to the receptor relative to the wild type. This is consistent with the notion that the two ligands bind to different, although perhaps overlapping, sites. Using a computer model of CB1, Bramblett et al. (1995) found that aminoalkylindole docking could involve an aromatic stacking interaction with residues not likely to interact with CP-55,940.

Although WIN 55,212-2 had a similar affinity for the wild-type and K192E receptors, an unexpected 10-fold decrease in the EC_{50} for WIN 55,212-2-induced cAMP inhibition with K192E was observed. This may reflect a structural perturbation due to the inclusion of the negatively charged residue. Apparently, the perturbation can be sufficiently compensated for to permit productive folding for WIN 55,212-2 binding. However, either directly or due to the compensatory alteration, the association with G_i is no longer as tightly coupled. In terms of the two-state model proposed by Lefkowitz and co-workers (Samama et al., 1993) involving the inactive (R) and active (R*) forms of the receptor, substitution of Lys with Glu may either shift the equilibrium away from the R* form or directly impair complex formation between ligand-R* and G_i. Clarification of these possibilities will require more detailed analysis when an inverse agonist for the cannabinoid receptor becomes available.

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