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A Lysine Residue of the Cannabinoid Receptor Is Critical for Receptor Recognition by Several Agonists but not WIN55212–2

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SUMMARY

Lys^{192} in the third transmembrane domain of the human CB_1 cannabinoid receptor was converted to an alanine to study its role in receptor recognition and activation by agonists. HU-210, CP-55940, WIN55212–2, and anandamide, four cannabinoid agonists with distinct chemical structures, were used to characterize the wild-type and the mutant receptors. In human embryonal kidney 293 cells stably expressing the wild-type receptor, specific binding to [^{3}H]WIN55212–2 and inhibition of cAMP accumulation by cannabinoid agonists were demonstrated, with different ligands exhibiting the expected rank orders of potency and stereoselectivity in competition binding and functional assays. In cells expressing the mutant receptor, the binding affinity of the receptor for [^{3}H]WIN55212–2 was only slightly affected (the K_d for the mutant receptor was twice that of the wild-type), and the ability of WIN55212–2 to inhibit cAMP accumulation was unchanged. However, HU-210, CP-55940, and anandamide were unable to compete for [^{3}H]WIN55212–2 binding to the mutant receptor. In addition, the potencies of HU-210, CP-55940, and anandamide in inhibiting cAMP accumulation were reduced by >100-fold. These results demonstrate that Lys^{192} is critical for receptor binding by HU-210, CP-55940, and anandamide. Because Lys^{192} is not important for receptor binding and activation by WIN55212–2, WIN55212–2 must interact with the cannabinoid receptor through at least one point of interaction that is distinct from those of the three other agonists.

Even though marijuana (cannabis) has been used for thousands of years, receptor-mediated signal transduction mechanisms for cannabinoids were identified in only the past decade. The observation that cannabinoids bind to a brain membrane, pertussis toxin-sensitive, G protein-coupled receptor to inhibit adenylate cyclase was an important discovery (1, 2). The cloning and functional expression of the cDNA for the CB_1 cannabinoid receptor from brain provided information concerning the molecular nature of this receptor (3, 4). The recent isolation of an endogenous cannabinoid ligand, anandamide (5), and the cloning of the CB_2 cannabinoid receptor, which is distributed primarily in peripheral tissues (6), brought new hopes for a better medical use of cannabinoids.

Since the identification of Δ^9 THC as the major psychoactive component of cannabis (7), structure-activity relationships of cannabinoids have been studied through the use of medicinal chemical and pharmacological methods (8, 9, 10). Based on their structures, cannabinoid agonists can be classified into at least four groups: classic cannabinoids, bicyclic cannabinoids, aminoalkylindoles, and fatty acid amines and esters. Classic cannabinoids, such as Δ^9-THC (Fig. 1), have the tricyclic benzopyran structure as their skeletons. HU-210, which is among the most potent cannabinoid agonists reported, belongs to the group of classic cannabinoids (11, 12). Bicyclic cannabinoids were originally derived from the classic cannabinoid structure but they have no dimethyldihydropyran ring (13, 14). CP-55940, the first compound that was used to demonstrate a functional cannabinoid binding site in the brain, is a member of this group. Aminoalkylindoles are a new class of cannabimimetics with structures entirely different from those of natural cannabinoids (15, 16). WIN55212–2, a prototypic aminoalkylindole, has been shown to bind selectively to cannabinoid receptors and exhibit cannabinoid-like activities. Anandamide, the first cannabinoid receptor agonist to be isolated from animal tissues, is the ethanolamide of arachidonic acid (5). A glycerol ester of arachidonic acid as well as other fatty acid amines and esters may also function as endogenous agonists for the cannabinoid receptors (17, 18).

ABBREVIATIONS: Δ^9-THC, Δ^9-tetrahydrocannabinol; HU-210, (−)-11-hydroxy-Δ^8-tetrahydrocannabinol-dimethylheptyl; CP-55940, {(−)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol}; WIN55212–3, (*S*)(−)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone mesylate; WIN55212–2, (*R*)(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl)methanone mesylate; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride.

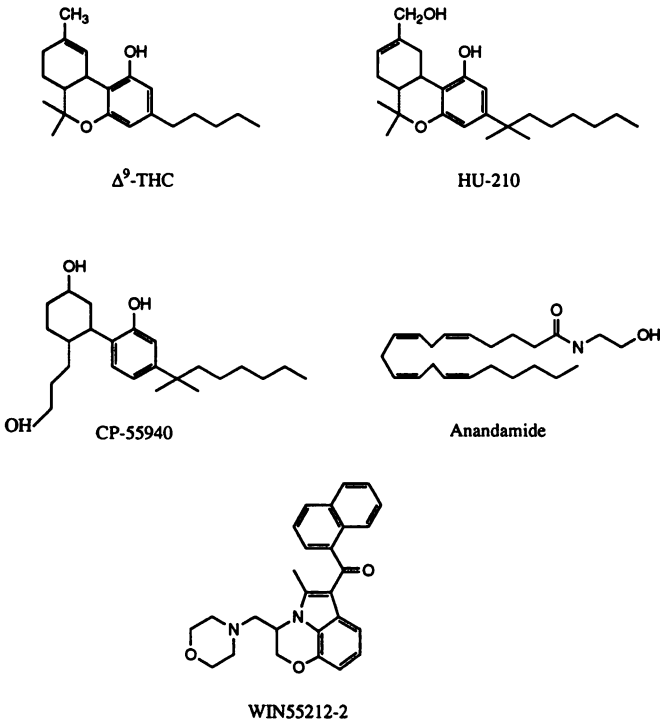

Fig. 1. The chemical structures of several cannabinoid agonists.

With the cloning of CB_1 and CB_2 cannabinoid receptors, the structural domains that are important for the functions of these receptors can be studied directly through molecular biological approaches, including mutagenesis of the receptors. Combined with three-dimensional molecular modeling and structural determination by biophysical methods, mutagenesis studies may reveal the molecular determinants of ligand binding, receptor activation, and ligand selectivity of the two cannabinoid receptors and lead to the design of new cannabinoid ligands with improved therapeutic activities and fewer side effects.

The cloned cannabinoid receptors belong to the family of seven-transmembrane-domain, rhodopsin-like G protein-coupled receptors. Previous mutagenesis studies on the members of this receptor family have revealed that amino acid residues within the transmembrane domains are often important for binding of small ligands (19). For example, a conserved aspartate in the third transmembrane domains of several biogenic amine receptors has been repeatedly demonstrated to be critical for ligand binding (19). Also, an aspartate at the analogous position of μ-opioid receptor has been shown to be involved in opioid binding (20). In the third transmembrane domains of the CB_1 and CB_2 cannabinoid receptors, there is a conserved lysine. By analogy with other G protein-coupled receptor, one could speculate that this lysine might be important for the binding of cannabinoids. As a first step in studying the functional domains of cannabinoid receptors by mutagenesis, we converted this lysine (Lys^{192}) in the human CB_1 cannabinoid receptor to an alanine. In this report, the ligand binding and signal transduction properties of this mutant receptor are compared with those of the wild-type cannabinoid receptor.

Experimental Procedures

Materials. Enzymes used for recombinant DNA experiments were obtained from New England Biolabs (Beverly, MA) or GIBCO-BRL (Gaithersburg, MD). Polymerase chain reaction reagents were purchased from Perkin Elmer Cetus (Norwalk, CT). Oligonucleotides were synthesized with a Cyclone Plus DNA synthesizer (MilliGen/Biosearch, Novato, CA). Tissue culture reagents were obtained from Biowhittaker (Walkersville, MD). Adenovirus-transformed 293 cells were obtained from American Type Culture Collection (Rockville, MD). Glass tubes used for diluting cannabinoid drugs and for all the assays were silanized through exposure to dichlorodimethylsilane (Sigma Chemical Co., St. Louis, MO) vapor while under vacuum for 3 hr.

Anandamide was kindly provided by Dr. William Devane (National Institute of Mental Health, National Institutes of Health, Bethesda, MD); CP-55940 by Dr. Lawrence Melvin (Pfizer Inc., Groton, CT); HU-210 and HU-211 by Dr. Raphael Mechoulam (Hebrew University of Jerusalem, Jerusalem, Israel); and WIN55212–2 and WIN55212–3 by Dr. Susan Ward (Sterling-Winthrop Research Institute, Rensselaer, NY). [^{3}H]WIN55212–2 was purchased from New England Nuclear (Boston, MA).

Expression and mutagenesis of the cannabinoid receptor gene. A 1.5-kb *SstI/Xba*I fragment of the human CB_1 cannabinoid receptor gene containing the entire coding region was subcloned into pCD-PS to place a *Kpn*I site adjacent to the *Sst*I site. The resulting *Kpn*I/*Xba*I fragment was ligated with the 0.76-kb *Xba*I/*Eco*RI and the 4.6-kb *Eco*RI/*Kpn*I fragments of pRC/CMV (Invitrogen, San Diego, CA) to construct the expression plasmid pHCB1-RC/CMV.

A 0.7-kb *Pst*I fragment of the human CB_1 cannabinoid receptor gene was subcloned into pUC18 to use as a template for making mutations. A lysine-to-alanine mutation at the position 192 of the receptor ($K^{192}A$) was introduced through a PCR-based method (21). The two pairs of primers used for PCR were $K^{192}A_1$ (5′-GCT CTA GAA GAC NNC GCA CTG GGT GGG GTC ACG GCC TCC T-3′) plus pUCfor2 (5′-GTG AAA TAC CGC AGA GAT GCG-3′) and $K^{192}A_2$ (5′-GCT CTA GAA GAC NNT GCG AAC AGA AAC ACG TTG CGG CTA T-3′) plus pUC18rev2 (5′-ACA CAG GAA ACA GCT ATG ACC ATG ATT ACG AA-3′). The two PCR fragments were digested with *Bbs*I plus *Eco*RI and *Bbs*I plus *Hin*dIII, respectively; ligated together; and inserted into pUC18 using the *Eco*RI and *Hin*dIII sites. The *Bbs*I digestion eliminates the *Bbs*I site, and the mutation occurs in the sticky end generated by the *Bbs*I digestion (21). The presence of the mutation as well as the accuracy of the DNA sequences derived from PCR were verified with dideoxy sequencing. A 0.5-kb *PmlI/Apa*I fragment that contained the mutation was subcloned into pHCB1-RC/CMV. The presence of the $K^{192}A$ mutation in the expression plasmid and the proper reconstitution of the receptor were confirmed through sequencing.

Expression plasmids containing the wild-type and mutant cannabinoid receptor genes were purified with the Qiagen plasmid Maxi kit (Qiagen Inc, Chatsworth, CA) and transfected into 293 cells with the use of a calcium phosphate precipitation method (22). Cell lines stably expressing wild-type and mutant cannabinoid receptors were established as previously described (22). Cells were grown at 37° in a humidified 5% CO_2 incubator in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Ligand binding and cAMP accumulation assays. To prepare membranes for binding assays, confluent cells were washed twice with cold phosphate-buffered saline and scraped off in ice-cold binding buffer (50 mM Tris, 5 mM $MgCl_2$, 2.5 mM EDTA, pH 7.4) plus 200 mM sucrose. The cells were homogenized with a Tekmar Tissumizer (setting 80, 2 × 30 sec), followed by centrifugation at 32,000 × *g* for 20 min. The pellet was resuspended in binding buffer and stored at −80° until use. Membrane protein concentrations were determined with a bicichoninic acid protein reagent kit (Pierce, Rockford, IL).

Ligand binding assays were carried out as previously described (23), with the following modifications. Cannabinoid ligands were

diluted in binding buffer containing 25 mg/ml bovine serum albumin and then added to assay tubes; the final bovine serum albumin concentration was 5 mg/ml. [^{3}H]WIN55212–2 (2 nM) was used for competition binding studies. Nonspecific binding was defined as binding in the presence of 1 μM unlabeled WIN55212–2. Specific binding was 70–80% of the total binding. Free and bound radioligands were separated by rapid filtration through polyethylenimine-treated GF/B filters (Whatman International, Maidstone, UK) with a Brandel cell harvester. The filters were washed three times with 3 ml cold 50 mM Tris, pH 7.4, and then soaked overnight in a scintillation cocktail (Hydrofluor, National Diagnostics, Manville, NJ). Radioactivity was determined with a liquid scintillation counter.

According to a previously described method (23), cAMP accumulation assays were performed on whole cells, and cAMP levels were detected with radioimmunoassay.

Data from ligand binding and cAMP accumulation assays were analyzed, and curves were generated with use of the InPlot 4.0 program (GraphPad Software, San Diego, CA). The binding curves were fitted to a single-site model with no evidence for a significant amount of a second class of binding sites with different affinities. IC_{50} and EC_{50} values were determined through nonlinear regression analysis performed with InPlot 4.0 (24). Fits for both binding and cAMP accumulation were unconstrained for all data involving wild-type receptors and for WIN55212–2 on the K[192]A receptors. Except as noted in the figure legend, fits for the other compounds on the K[192]A receptors were constrained to the same minimum value as observed for each compound on the wild-type receptor. K_d and B_{max} values were estimated from competition binding experiments with the following equations: $K_d = IC_{50} - L$ and $B_{max} = (B_o IC_{50})/L$, where L is the concentration of free radioligand, and B_o is specifically bound radioligand (25). The K_i values were calculated with the Cheng-Prusoff equation: $K_i = IC_{50}/(1 + L/K_d)$ (26).

Results

Expression of the wild-type human CB_1 cannabinoid receptor. Radioligand binding assays were performed to characterize cannabinoid receptor 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 the wild-type human CB_1 cannabinoid receptor gene. The K_d and the B_{max} values were 11.9 ± 1.9 nM and 1217.6 ± 221.9 fmol/mg protein, respectively. No specific binding of [^{3}H]WIN55212–2 was detected with untransfected 293 cell membranes.

HU-210, CP-55940, WIN55212–2, and anandamide, four cannabinoid ligands with distinct structures (Fig. 1), were tested for their ability to compete for the specific binding of [^{3}H]WIN55212–2 to the wild-type receptor. As shown in Table 1, the rank order of potency for these ligands to compete for the binding of [^{3}H]WIN55212–2 was HU-210 > CP-55940 > WIN55212–2 > anandamide. To demonstrate the stereoselectivities of cannabinoid ligand binding, HU-211, the inactive enantiomer of HU-210, and WIN55212–3, the inactive enantiomer of WIN55212–2, were used. Compared with their active enantiomers, HU-211 and WIN55212–3 were much less potent in competing for [^{3}H]WIN55212–2 binding (Table 1).

Functional expression of wild-type cannabinoid receptors 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 cannabinoid receptor gene (Table 1). The rank order of potency was HU-210 > CP-55940 > WIN55212–2 > 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. Also, stereoselectivities between HU-210 and HU-211 and between WIN55212–2 and WIN55212–3 were displayed in the cAMP accumulation assays (Table 1).

TABLE 1

Parameters of radioligand binding and functional assays for the wild-type cannabinoid receptor

Values are presented as mean ± standard error of three separate experiments.

Ligand	Competition of [^{3}H]WIN55212–2 binding, K_i	Inhibition of cAMP accumulation, EC_{50}
	nM	nM
HU-210	0.26 ± 0.06	0.19 ± 0.03
HU-211	1523.3 ± 437.3	53.5 ± 11.5
CP-55940	4.6 ± 1.2	3.1 ± 1.7
Anandamide	115.6 ± 36.0	81.8 ± 18.4
WIN55212–2	11.9 ± 1.9[a]	7.6 ± 2.8
WIN55212–3	>1000[b]	>1000[c]

[a] K_d value in nM.
[b] Twenty percent competition of specific [^{3}H]WIN55212–2 binding was seen at 10,000 nM.
[c] No inhibition of cAMP accumulation was seen at 1000 nM.

To inhibit the metabolic hydrolysis of anandamide, 100 μM PMSF, an amidase inhibitor, was included in the receptor binding and cAMP accumulation assays. In the presence of PMSF, the K_i and EC_{50} values (shown in Table 1) for anandamide were 10- and 2-fold lower than in the absence of PMSF, respectively. Therefore, although PMSF has been reported to have no effect on anandamide binding to cloned cannabinoid receptors expressed in L−M(TK−) and AtT-20 cells (27), it does have a significant effect on anandamide binding to receptors expressed in 293 cells.

Comparison of the mutant and wild-type cannabinoid receptors. Radioligand binding and cAMP accumulation experiments were carried out to compare the pharmacological profiles of the K[192]A mutant cannabinoid receptor with those of wild-type cannabinoid receptor. Specific, high affinity binding of [^{3}H]WIN55212–2 was observed in membranes prepared from 293 cells stably transfected with the K[192]A mutant cannabinoid receptor gene. The K_d and B_{max} values were 20.2 ± 5.8 nM and 1202.2 ± 288.1 fmol/mg protein, respectively. This K_d value is approximately twice that of the wild-type cannabinoid receptor (11.9 ± 1.9 nM). This B_{max} value is not significantly different from that of the wild-type (1217.6 ± 221.9 fmol/mg protein). Although the ability of unlabeled WIN55212–2 to compete for [^{3}H]WIN55212–2 binding was only slightly affected by the K[192]A mutation, HU-210, CP-55940, and anandamide failed to compete for [^{3}H]WIN55212–2 binding to the mutant cannabinoid receptor (Fig. 2).

Consistent with the data from radioligand binding experiments, the abilities of HU-210, CP-55940, and anandamide to inhibit forskolin-stimulated cAMP accumulation were severely reduced in cells expressing the mutant cannabinoid receptor versus cells expressing the wild-type cannabinoid receptor (Fig. 3). For the highest concentrations of anandamide and CP-55940, there is only a hint of inhibition with the mutant receptor, but at micromolar concentrations of HU-210, there is clear inhibition. An unconstrained fit to the data suggests a reduced efficacy (~50% of that of the wild-type

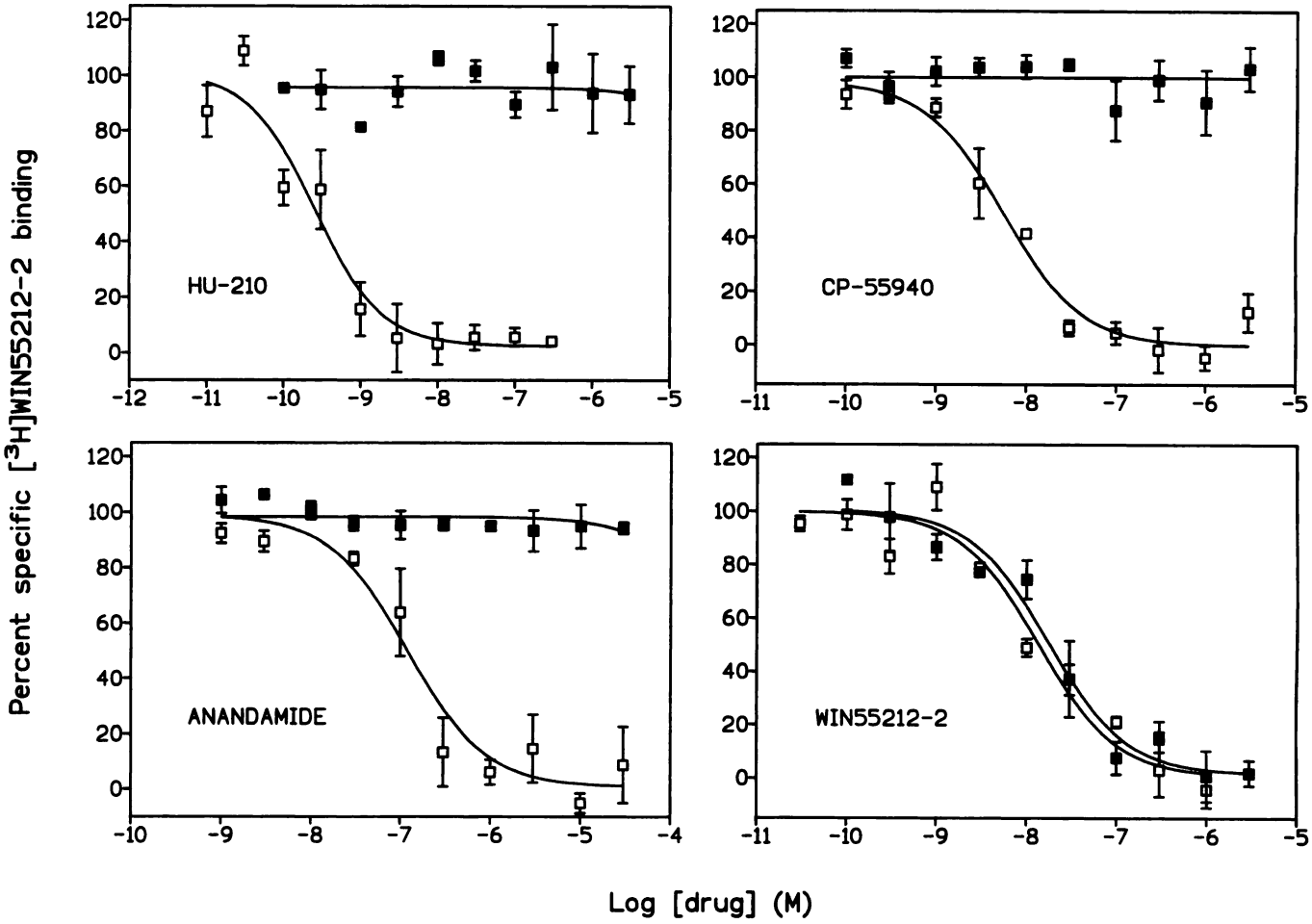

Fig. 2. Comparison between the wild-type and the $K^{192}A$ mutant cannabinoid receptors for cannabinoid ligand binding. Competition binding assays were performed on membranes prepared from 293 cells stably expressing wild-type (□) or $K^{192}A$ mutant (■) receptor genes. *Points*, mean ± standard error of three independent experiments performed in duplicate. Curves were generated as described in Experimental Procedures.

receptor) and an EC_{50} of 24.1 ± 3.5 nM. However, the data do not seem to be sufficient to exclude the possibility that there is an EC_{50} in the micromolar range and no reduction in efficacy. Regardless of which method is used to fit the data, it is clear that the EC_{50} is reduced by >100-fold. In contrast, the potency for WIN55212–2 to inhibit cAMP accumulation remained the same (EC_{50} = 8.2 ± 2.0 nM for the $K^{192}A$ mutant receptor and 7.6 ± 2.8 nM for the wild-type receptor) (Fig. 3). The mutation did not alter the stereoselectivity of WIN55212–2 versus WIN55212–3 in binding and cAMP accumulation assays (data not shown).

Discussion

Previous site-directed mutagenesis studies have shown that small molecular weight ligands bind to the hydrophobic cores of G protein-coupled receptors, and amino acid residues at the outer portions of the third transmembrane domains seem to be important for ligand binding (19). By analogy with other G protein-coupled receptors, we hypothesized that the conserved lysine in the third transmembrane domain of cannabinoid receptors might be important for ligand recognition. Although the $K^{192}A$ mutation had little effect on receptor binding and activation by WIN55212–2, it led to an apparently complete loss of binding for HU-210, CP-55940, and anandamide and a large reduction in their potencies for inhibition of cAMP accumulation. The inhibition of cAMP accumulation for the mutant receptor did not reach a clear maximum at the highest practical concentrations of HU-210, and there were only hints of cAMP inhibition at the highest concentrations of CP-55940 and anandamide. Therefore, it is difficult to quantify the reductions in potency for these three ligands. The increase in their EC_{50} values, obtained by assuming that the curves can be extrapolated to the same maximal inhibition as for the wild-type receptor, is >100-fold for anandamide and >1000-fold for HU-210 and CP-55940.

The lack of effect of the $K^{192}A$ mutation on receptor binding and activation by WIN55212–2 implies that the loss of binding by the other three agonists is not simply due to a general conformational change in the receptor induced by the mutation. In addition, the magnitude of the effect of the $K^{192}A$ mutation on the EC_{50} for activation of the receptor by HU-210, CP-55940, and anandamide is in the range expected for the elimination of a hydrogen bond between the agonist and the charged lysine of the receptor (28, 29). The loss of such a hydrogen bond would be expected to have a much larger effect than the elimination of a hydrogen bond between uncharged partners. Although we cannot rule out some impairment of receptor activation since we cannot measure the maximal response to HU-210, CP-55940, and anan-

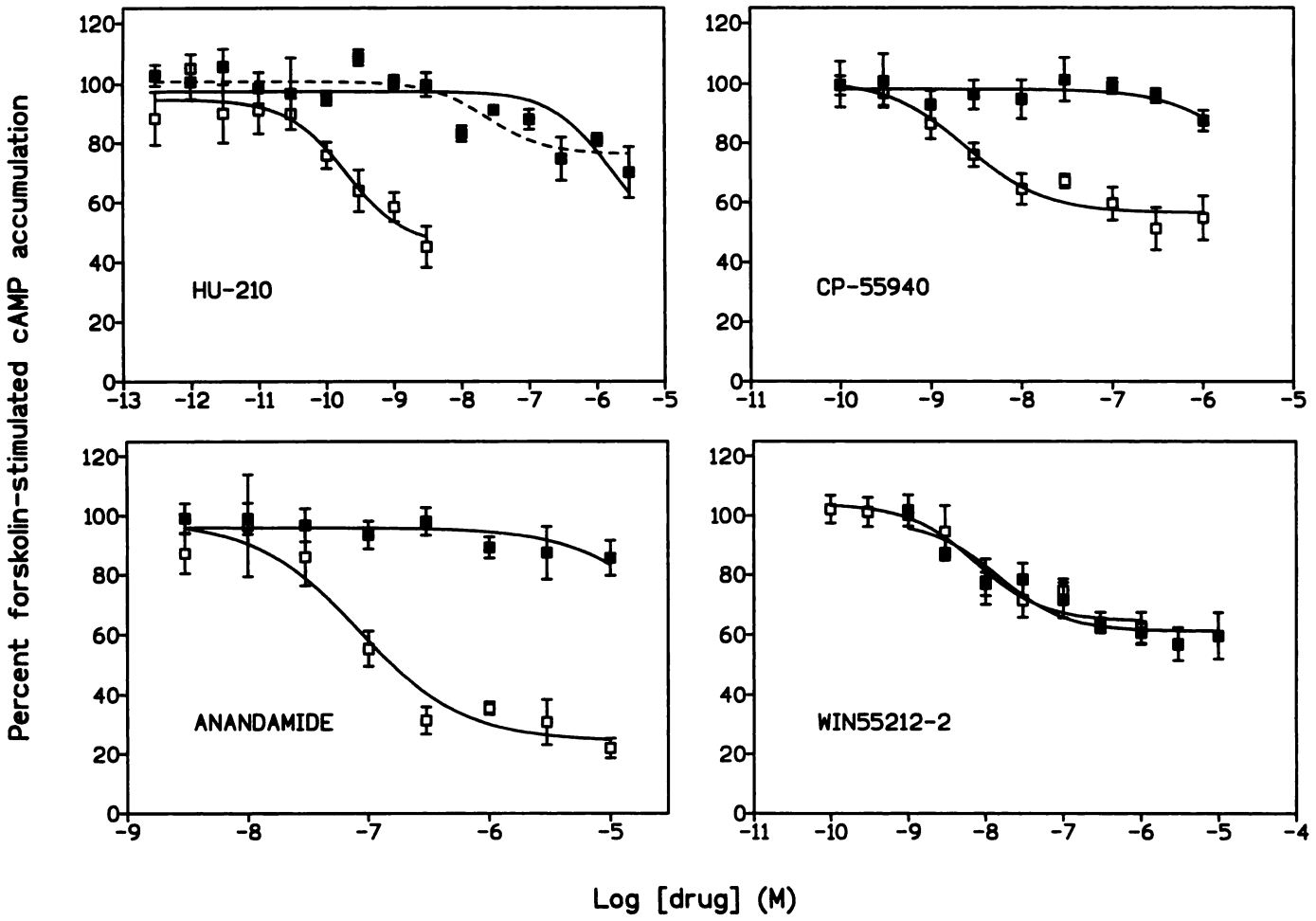

Fig. 3. Comparison between the wild-type and the $K^{192}A$ mutant cannabinoid receptors for agonist-induced inhibition of cAMP accumulation. cAMP accumulation assays were performed on 293 cells stably expressing wild-type (□) or $K^{192}A$ mutant (■) receptor genes. *Points,* mean ± standard error of three independent experiments performed in triplicate. Curves were generated as described in Experimental Procedures, except the *dashed curve* for HU-210 is an unconstrained single-site fit to the $K^{192}A$ data.

damide on the mutant receptor, it seems that the simplest interpretation of these results is that the effect of mutation is primarily due to a decrease in binding affinity brought about by the loss of a highly stabilizing hydrogen bond between these ligands and the receptor.

Previous structure-activity relationship studies have indicated that the phenolic hydroxyl on classic cannabinoid ligands is essential for their activity *in vivo* (8, 9) and that it acts as a hydrogen bond acceptor rather than as a donor (30). Because Lys^{192} is critical for the binding of the structurally related ligands HU-210 and CP-55940 and would be expected to be a hydrogen donor (31), it would be reasonable to hypothesize that there is a hydrogen bond between the phenolic hydroxyl of these ligands and Lys^{192}. However, this hypothesis seems to be inconsistent with the observation of Melvin *et al.* (14) that removal of the phenolic hydroxyl on CP-55940 caused only a 35-fold drop in receptor binding affinity *in vitro* and a 200-fold decrease in potency *in vivo*. Less is known about the structure-function relationships of anandamide in terms of suggesting which functional group interacts with Lys^{192}. Because replacement of the hydroxyl group on anandamide by a methyl group produced a 3-fold increase in affinity for the CB_1 cannabinoid receptor (32), it would not be reasonable to assume that it forms a hydrogen bond with Lys^{192}.

It seems paradoxical that in cells expressing the mutant receptor, HU-210 inhibits cAMP accumulation at high concentrations, whereas no competition of [^{3}H]WIN55212–2 binding was detected. The observation that HU-210 inhibits cAMP accumulation indicates that it binds to the mutant receptor, although with much lower affinity than for the wild-type receptor. The explanation presumably lies in the fact that the cAMP accumulation assay measures the function of only G protein-coupled (i.e., high affinity state) receptors, whereas the binding assay measures the sum of both coupled and uncoupled (low affinity state) receptors. Because the receptor has been expressed at fairly high levels in a cell in which it is not normally expressed, it is quite possible that only a small proportion of the receptors are coupled to appropriate G proteins and the vast majority of the receptors measured in the binding assay are in the uncoupled state.

In the wild-type cannabinoid receptor, [^{3}H]WIN55212–2 binding is fully competed by HU-210, CP-55940, and anandamide. Therefore, in pharmacological terms, WIN55212–2 binds to the same site as other cannabinoid agonists. However, from a more molecular point of view, the binding of a

ligand to a receptor must occur through multiple points of interaction, and these points of interaction need not to be the same for each agonist that binds to the receptor as long as there is sufficient spatial overlap between the bound agonists for their binding to be mutually exclusive. Our data indicate that Lys^{192} is a critical point of interaction on the cannabinoid receptor for HU-210, CP-55940, and anandamide but not for WIN55212–2. One explanation would be that the points of interaction are mostly shared but that WIN55212–2 interacts not with Lys^{192} but instead with another nearby amino acid residue. However, considering that the chemical structure of WIN55212–2 is very different from that of the three other ligands, it is possible that WIN55212–2 uses substantially different points of interaction and perhaps even a different mechanism of achieving the conformational change that activates the cannabinoid receptor.

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