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Effects of D3.49A, R3.50A, and A6.34E mutations on ligand binding and activation of the cannabinoid-2 (CB2) receptor

Wenke Feng, Z.H. Song*

Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY 40292, USA

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Abstract

In several G protein-coupled receptors (GPCRs), the Asp-Arg-Tyr (DRY) motif at the bottom of third transmembrane domain and the amino acid at position 6.34 in the sixth transmembrane domain have been shown to play important roles in signal transduction. In this study, we propose that in the cannabinoid-2 (CB2) receptor, R3.50 in the DRY motif may be crucial for interacting with G proteins, and D3.49 and A6.34 may be important for constraining the receptor in an inactive conformation. To test our hypothesis, R3.50A, D3.49A, and A6.34E mutations of the human CB2 receptor were made by site-directed mutagenesis. These mutant receptors were stably transfected into human embryonic 293 cells, and their ligand binding and signal transduction properties were analyzed. Similar to other GPCRs, R3.50 of the CB2 receptor is crucial for signal transduction. Unlike other GPCRs, D3.49 and A6.34 of the CB2 receptor do not seem to be important for keeping the receptor in an inactive state. Furthermore, D3.49A and A6.34E mutations abolished ligand binding, and all three mutations abolished constitutive activity of the wild-type CB2 receptor.

Keywords: Marijuana; Cannabinoid receptor; G protein-coupled receptor; Site-directed mutagenesis; Ligand binding; Signal transduction

1. Introduction

Marijuana (cannabis) has been used for many centuries as both a recreational drug and a therapeutic agent. The major psychoactive component of cannabis is Δ^9-THC [1]. In 1990, the first cannabinoid receptor (CB1) was cloned from a rat brain cDNA library [2]. CB1 is located in the central nervous system as well as in peripheral systems [3–6]. In 1993, a second cannabinoid receptor (CB2) was cloned from a human promyelocytic cell line (HL-60), and showed 44% amino acid identity with CB1 [7]. The CB2 receptor has been found almost uniquely in immune cells [3,5–7]. This distribution suggests a possible role for the CB2 receptor in mediating the immunomodulatory, but not the psychoactive effects of cannabinoids. Both CB1 and CB2 receptors exert their functions via pertussis toxin-sensitive $G_{i/o}$ proteins [8,9].

Previous studies have indicated that intracellular regions of the GPCRs, particularly the second and third intracellular loops and the cytoplasmic tail, interact with G proteins [10–13]. It has been shown that agonist binding causes the movement of transmembrane helix 3 and 6, thereby inducing conformational changes in some GPCRs [14,15]. These agonist-induced conformational changes are crucial for signal transduction. At the junction of transmembrane helix 3 and the second intracellular loop of GPCRs, there is a highly conserved triplet of amino acids, DRY (Fig. 1). In several GPCRs, the highly conserved R3.50 of the DRY motif has been suggested to affect the interaction of receptors with G proteins [16–19]. Mutagenesis and molecular modeling studies have indicated that R3.50 in the DRY motif is buried in a highly conserved "polar pocket," which is essential to keep GPCRs in an inactive conformation [17,19]. Substitution of D3.49 in the DRY motif with alanine has been shown to

* Corresponding author. Tel.: +1-502-852-5160; fax: +1-502-852-7868.
E-mail address: zhsong@louisville.edu (Z.H. Song).

Abbreviations: GPCRs, G protein-coupled receptors; DRY, Asp-Arg-Tyr; Δ^9-THC, Δ^9-tetrahydrocannabinol; cAMP, cyclic AMP; HU-210, (−)-11-hydroxy-Δ^8-tetrahydrocannabinol-dimethylheptyl; SR144528, *N*-[(1*S*)-endo-1,3,3-trimethyl bicyclo[2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; WIN55212-2, (*R*)(+)-[2,3-dihydro-5-methyl-3-[(4-morpho-linyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate; HU-243, 5′-(1,1-dimethylheptyl)-7-hydroxyhexahydrocannabinol; and CP55940, (−)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol.

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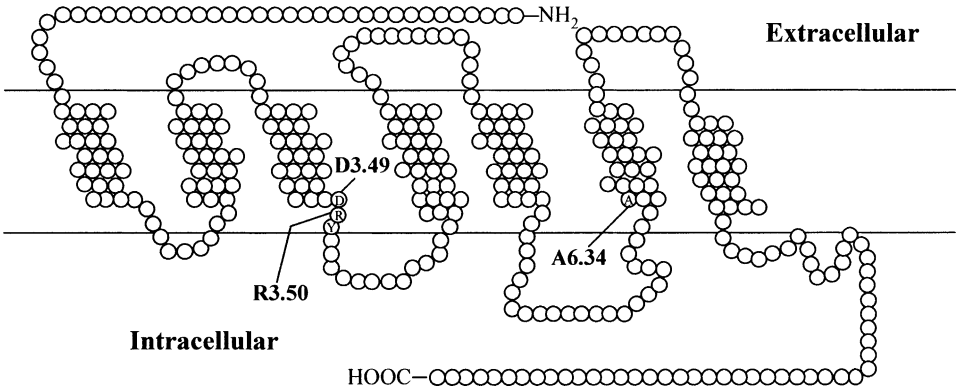

Fig. 1. Schematic depiction of the membrane topology of the CB2 receptor and locations of D3.49, R3.50, and A6.34.

shift the arginine out of the polar pocket, resulting in constitutive activity of the receptor. The amino acid at position 6.34 of transmembrane helix 6 (Fig. 1) has also been shown to interact with the R in the DRY motif. Mutating the A6.34 in the sixth transmembrane helix to other amino acids led to a progressive shift of R3.50 in the DRY motif out of the ''polar pocket,'' resulting in constitutive activation of the receptor [19,20]. These previous studies have established the importance of D3.49 and A6.34 in keeping R3.50 in the DRY motif in the ''polar pocket,'' thus keeping the receptor in an inactive state.

In this study, we propose that in the CB2 receptor, R3.50 in the DRY motif may also be crucial for interacting with G proteins, and D3.49 and A6.34 may be important for constraining the receptor in an inactive conformation. To test our hypothesis, R3.50, D3.49, and A6.34 of the human CB2 receptor were mutated to A3.49, A3.50, and E6.34, respectively. These mutant receptors were stably transfected into human embryonic 293 (HEK293) cells, and their ligand binding and signal transduction properties were analyzed.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin/streptomycin, L-glutamine, trypsin, and geneticin were from GIBCO-BRL. Enzymes and reagents used for recombinant DNA experiments were purchased from Promega. Adenovirus-transformed HEK293 cells were obtained from the American Type Culture Collection. Glass tubes used for diluting cannabinoid drugs, ligand binding, and cAMP accumulation assays were silanized through exposure to dichlorodimethylsilane (Sigma Chemical Co.) vapor under vacuum for 3 hr. Anandamide and WIN55212-2 were obtained from RBI. SR144528 was obtained from NIDA. CP55940, HU-210, and [^{3}H]HU-243 were purchased from Tocris. [^{3}H]WIN55212-2, [^{3}H]-CP55940, and [^{35}S]GTPγS were obtained from NEN Life Science Products.

2.2. Amino acid numbering system

An amino acid numbering system suggested previously [21] was used. Each amino acid identifier starts with the transmembrane helix number, followed by the amino acid position relative to a reference amino acid in that helix. This reference amino acid is the most conserved residue across GPCRs in that helix and is assigned a locant value of 0.50. This numbering system for the cannabinoid receptor has been described previously [22,23].

2.3. Mutagenesis

The 1.8-kb full-length human *CB2* gene was subcloned into pRC/CMV (Invitrogen) to construct the expression plasmid pHCB2-RC/CMV [23]. The GeneEditor *in vitro* site-directed mutagenesis system (Promega) was used to mutate the *CB2* gene. This system allows mutations to be made in any vector that contains ampicillin resistance as a selectable marker. Using this system, the desired mutation was produced by annealing a complementary mutagenic oligonucleotide as well as an oligonucleotide that encodes the resistance to the GeneEditor antibiotic selection mix. Subsequent synthesis and ligation of the mutant strand link the two oligonucleotides. The system uses the resistance to the GeneEditor antibiotic selection mix to facilitate the selection of the mutant DNA. The following mutagenic oligonucleotides were used: D3.49A, 5′-ACCGCCATT-GCCCGATACCTC-3′; R3.50A, 5′-TGACCGCCATTGA-CGCATACCTCTGCCTGC-3′; and A6.34E, 5′-GATGT-GAGGTTGGAGAAGACCCTAGGGC-3′. The presence of the mutation and the accuracy of the DNA sequences were confirmed by dideoxy sequencing.

2.4. Cell transfection and culture

Expression plasmids containing the wild-type and mutant cannabinoid receptors were purified with a Qiagen plasmid maxi kit (Qiagen Inc.), and then transfected into HEK293 cells. Transfected cells were selected in culture medium containing 500 μg/mL of geneticin, and

cell lines stably expressing wild-type and mutant cannabinoid receptors were established according to a method developed previously [23,24]. Cells were grown as monolayers in DMEM containing 10% (w/v) fetal bovine serum, 2 mM glutamine, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 400 μg/mL of geneticin in a humidified atmosphere consisting of 5% CO_2 and 95% air, at 37°.

2.5. Ligand binding assays

Ligand binding assays were performed as described previously [9,23] with slight modifications. For membrane preparations, cells were washed twice with cold PBS and scraped off the tissue culture plates. Subsequently, the cells were homogenized in binding buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, 2.5 mM EDTA, pH 7.4) with a Polytron homogenizer. After the homogenate was centrifuged at 32,000 *g* for 20 min at 4°, the pellet was resuspended in binding buffer and stored at −80°. Protein concentrations were determined using a bicinchoninic acid protein reagent kit (Pierce).

For binding assays, cannabinoid ligand dilutions were made in binding buffer containing 0.5 mg/mL of bovine serum albumin and then added to the assay tubes. [^{3}H]HU-243 (2 nM) was used as a labeled ligand for competition binding assays. Nonspecific binding was determined in the presence of 1 μM unlabeled HU-210. Binding assays were performed in 0.5 mL of binding buffer containing 0.1 mg/mL of bovine serum albumin for 60 min at 30°. Free and bound radioligands were separated by rapid filtration through polyethylenimine-treated GF/B filters (Whatman International). The filters were washed three times with 3 mL of cold wash buffer (50 mM Tris–HCl, pH 7.4, containing 1 mg/mL of bovine serum albumin). The bound [^{3}H]HU-243 was determined by liquid scintillation counting after overnight equilibration in 5 mL of scintillation fluid (Hydrofluor, National Diagnostics). The assays were performed in duplicate, and the results represent the combined data from three independent experiments.

Saturation binding of [^{3}H]HU-243 was performed by incubating different concentrations (0.025 to 5 nM) of the radioligand with the membranes under the conditions described above for competition assays. Nonspecific binding was determined in the presence of 1 μM HU-210.

2.6. Assay of cAMP accumulation

cAMP accumulation assays were performed using a method described previously [9,23]. Briefly, cells were grown to confluence, washed twice in PBS containing 0.5 mM EDTA, and then collected in DMEM containing 0.2% (w/v) bovine serum albumin. Subsequently, cells were centrifuged at 500 *g* for 5 min at 25°, and resuspended in 10 mL of DMEM containing 0.2% (w/v) fatty acid free bovine serum albumin. To prevent the hydrolysis of cAMP, the cells were incubated with 0.2 mM Ro20-1724, a phosphodiesterase inhibitor. Aliquots of cells were added to tubes containing forskolin with or without cannabinoids and incubated for 10 min at 37°. The reaction was stopped with the addition of 0.1 N HCl, after which 50 μL was removed for cAMP radioimmunoassay, using a kit from DuPont-NEN.

2.7. Constitutive activation

Constitutive activity was measured as the ability of the receptor to inhibit forskolin-stimulated cAMP accumulation in the absence of agonists. The procedures for cell collection and preparation were described in the previous section. After incubating with different concentrations of forskolin for 10 min at 37°, the cells were assayed for cAMP levels. The results are expressed as percent of basal cAMP accumulation, which was measured in the absence of forskolin.

2.8. [^{35}S]GTPγS binding

Cannabinoid stimulation of [^{35}S]GTPγS binding to membranes was performed as described with slight modifications [25]. Briefly, membranes (10 μg) prepared from cells expressing wild-type, D3.49A, R3.50A, and A6.34E mutant CB2 receptors were suspended in the assay buffer (25 mM Tris, pH 7.4, 2.5 mM $MgCl_2$, 1.25 mM EDTA, 150 mM NaCl) and incubated with 1 μM HU-210, 1 μM WIN55212-2, or 3 μM anandamide in the presence of 0.5 nM [^{35}S]GTPγS, 0.25% bovine serum albumin, and 10 μM GDP in a total volume of 0.5 mL. After a 1-hr incubation at 30°, the reaction was stopped by rapid filtration under vacuum through GF/B filters. The filters were washed three times with 3 mL of cold wash buffer (25 mM Tris–HCl, pH 7.4, 100 mM NaCl containing 1 mg/mL of bovine serum albumin). The bound [^{35}S]GTPγS was determined by liquid scintillation counting after overnight equilibration in 5 mL of scintillation fluid.

2.9. Immunofluorescent microscopy

HEK293 cells stably expressing wild-type and mutant CB2 receptors were grown on glass coverslips (Fisher Scientific Inc.). Cells were washed twice with 0.1 M PBS, pH 7.4, fixed with 4% (v/v) paraformaldehyde for 15 min, and then washed twice again with PBS. Next they were incubated with PBS containing 5% (v/v) normal goat serum (NGS) for 1 hr at room temperature, and then incubated with anti-CB2 antibody (Cayman) for 2 hr at room temperature. After washing three times with PBS containing 5% (v/v) NGS for 10 min each time, cells were incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG (Zymed) for 1 hr at room temperature. Subsequently, the coverslips were washed four times with PBS,

mounted with Vectashield (Vector Laboratories), and viewed with an Olympus IX50 fluorescence microscope.

2.10. Data analysis

Data from ligand binding and cAMP accumulation assays were analyzed, and curves were generated by using the GraphPad Prizm program (GraphPad Software). The IC_{50} and EC_{50} values were determined through nonlinear regression analysis performed with Prizm. K_d and B_{max} values were calculated from saturation binding experiments through nonlinear regression analysis. The K_i values were calculated based on the Cheng–Prusoff equation: $K_i = IC_{50}/(1 + L/K_d)$ [26].

3. Results

3.1. Expression of wild-type and mutant CB2 receptors

An immunofluorescent microscopy study was performed to examine the expression of wild-type and mutant CB2 receptors. Using an anti-CB2 primary antibody, which is directed against the extracellular N-terminus of the CB2 receptor, positive immunofluorescent staining signals were shown with nonpermeabilized HEK293 cells stably transfected with D3.49A, R3.50A, and A6.34E, as well as with wild-type CB2 receptors (Fig. 2). These data indicate that these receptors were properly targeted into the plasma membranes.

3.2. Ligand binding

As shown in Fig. 3A, in saturation binding experiments, specific, high-affinity binding of [^{3}H]HU-243 was demonstrated with the wild-type CB2 receptor, with K_d and B_{max} values of 0.38 ± 0.02 nM and 2.32 ± 0.05 pmol/mg protein, respectively. The R3.50A mutant receptor demonstrated [^{3}H]HU-243 binding characteristics similar to those of the wild-type receptor, with K_d and B_{max} values of 0.48 ± 0.03 nM and 3.68 ± 0.09 pmol/mg protein, respectively (Fig. 3B). These values were not significantly different from those of the wild-type CB2 receptor ($P > 0.05$, Student's t-test). No specific [^{3}H]HU-243 binding was observed for the D3.49A and A6.34E mutant receptors (data not shown). For both the wild-type and R3.50A mutant CB2 receptors, competition of [^{3}H]HU-243 binding was observed with three cannabinoid agonists—HU-210, WIN55212-2, and anandamide—with the rank order of potency being HU-210 > WIN55212-2 > anandamide (Fig. 4A–C). In addition, [^{3}H]HU-243 binding to wild-type and R3.50A mutant CB2 receptors can be competed by the CB2 receptor antagonist SR144528 (Fig. 4D). The K_i values for competition binding experiments are summarized in Table 1. For all four ligands, there were no significant differences between the K_i values of the wild-type and R3.50A mutant CB2 receptors ($P > 0.05$, Student's t-test). Two other radioligands, [^{3}H]WIN55212-2 and [^{3}H]CP55940, were used to confirm ligand binding properties of D3.49A and A6.34E mutant receptors. These two radioligands exhibited binding affinity similar to that of their unlabeled forms in wild-type cannabinoid receptors [23]. Similar to binding experiments performed with

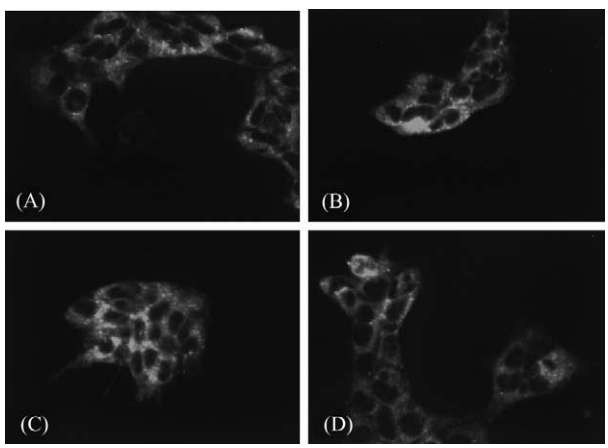

Fig. 2. Immunofluorescent staining of HEK293 cells expressing wild-type and mutant CB2 receptors. (A) Wild-type CB2 receptor; (B) D3.49A mutant; (C) R3.50A mutant; and (D) A6.34E mutant.

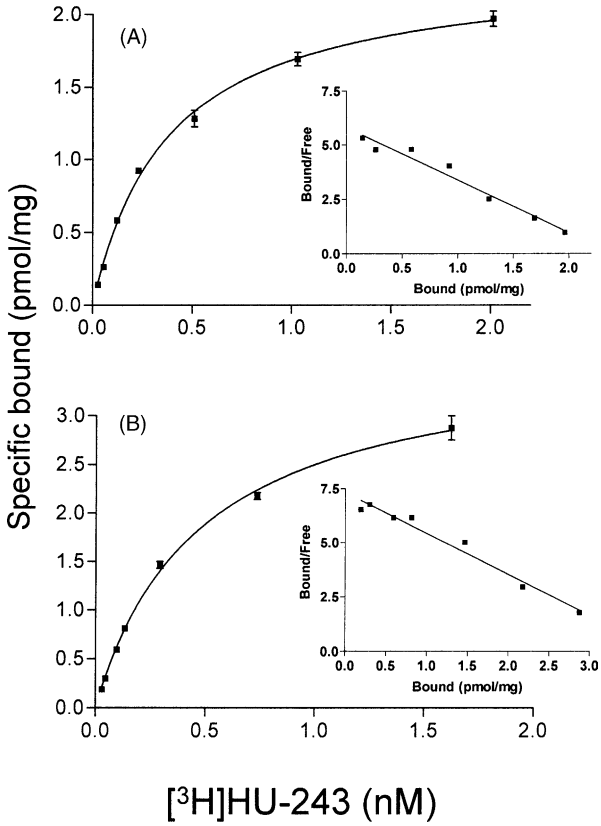

Fig. 3. Saturation binding of [^{3}H]HU-243 to wild-type (A) and R3.50A mutant (B) CB2 receptors. Insets: Scatchard plot of the data. One representative experiment out of three is shown. Curves were generated as described in Section 2.

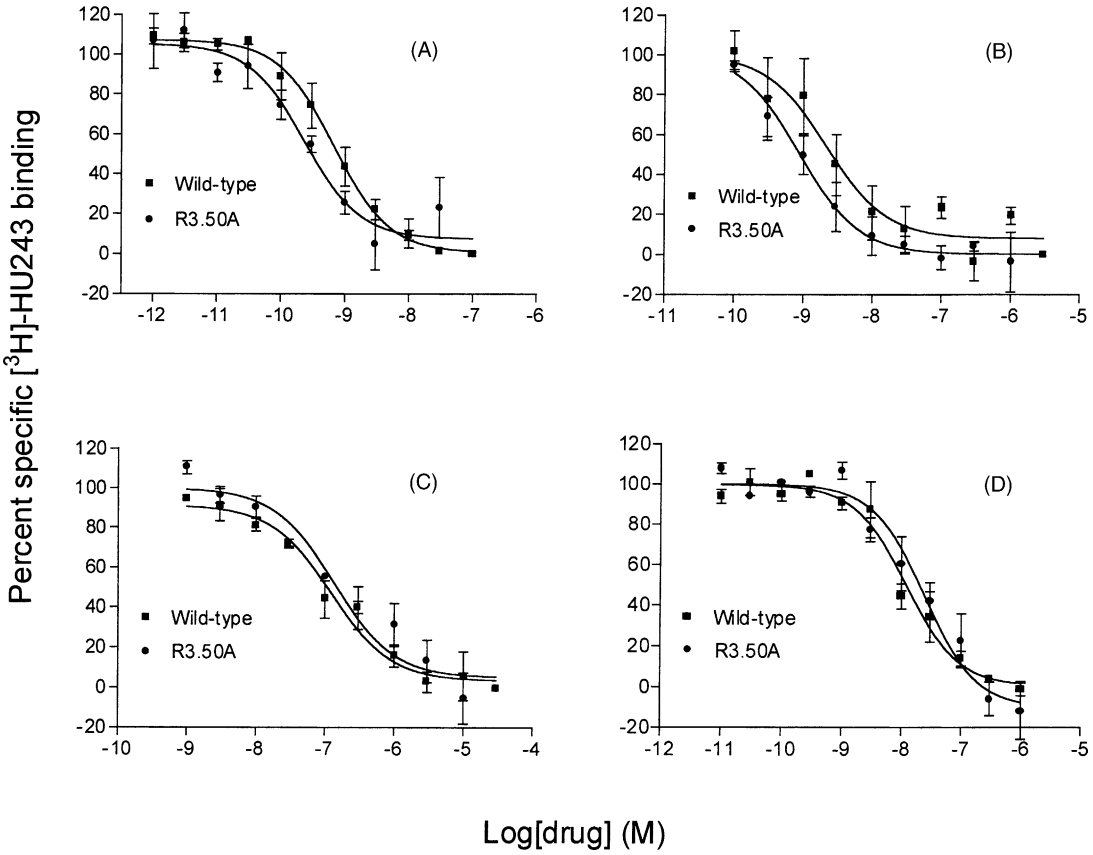

Fig. 4. Comparison of wild-type and mutant CB2 receptors for cannabinoid ligand binding. Three cannabinoid receptor agonists [(A) HU-210; (B) WIN55212-2; (C) anandamide] and SR144528, a CB2 antagonist/inverse agonist (D) were used for competition binding on membranes prepared from HEK293 cells expressing the wild-type and R3.50A mutant CB2 receptors. Data shown represent the means ± SEM of at least three independent experiments performed in duplicate. Curves were generated as described in Section 2.

[^{3}H]HU-243, D3.49A and A6.34E mutant receptors did not show any specific binding for either [^{3}H]WIN55212-2 or [^{3}H]CP55940 (data not shown).

3.3. Coupling to adenylate cyclase

To compare the functions of wild-type and mutant receptors, the abilities of cannabinoid agonists to inhibit forskolin-stimulated cAMP accumulation were determined. In cells expressing the wild-type CB2 receptor, three structurally distinct cannabinoid agonists inhibited forskolin-stimulated cAMP accumulation in a concentration-dependent manner (Fig. 5). The EC_{50} values for HU-210, WIN55212-2, and anandamide were 0.87 ± 0.23, 2.33 ± 0.21, and 194.55 ± 54.95 nM, respectively. At the highest concentrations used on the transfected cells, none of these cannabinoid agonists inhibited cAMP accumulation in mock-transfected cells (data not shown). Thus, all of the inhibition of cAMP accumulation shown is receptor-mediated. In cells expressing the A6.34E mutant receptor, the ability of HU-210 and WIN55212-2 to inhibit forskolin-stimulated cAMP accumulation was reduced severely, and the ability of anandamide to inhibit forskolin-stimulated cAMP accumulation was abolished (Fig. 5). Furthermore, in cells expressing D3.49A and R3.50A mutant receptors, the ability of the three agonists to inhibit forskolin-stimulated cAMP accumulation was also abolished completely (Fig. 5).

Table 1
Ligand binding affinity of wild-type and R3.50A mutant CB2 receptors

Ligand	K_i (nM)	
	Wild-type	R3.50A
HU210	0.47 ± 0.06	0.24 ± 0.07
WIN55212-2	3.41 ± 1.04	1.60 ± 0.16
Anandamide	314.5 ± 64.8	261.1 ± 53.6
SR144528	12.27 ± 2.93	19.20 ± 0.12

K_i values were measured in competition experiments using [^{3}H]HU-243 as a radioligand. Data are the means ± SEM of 3–5 experiments performed in duplicate.

3.4. Agonist-stimulated [^{35}S]GTPγS binding

To confirm the functional properties of the wild-type and R3.50A mutant CB2 receptors, agonist-stimulated [^{35}S]GTPγS binding assays were conducted. As shown in Fig. 6A, three CB2 agonists—HU-210, WIN55212-2, and anandamide—stimulated [^{35}S]GTPγS binding by

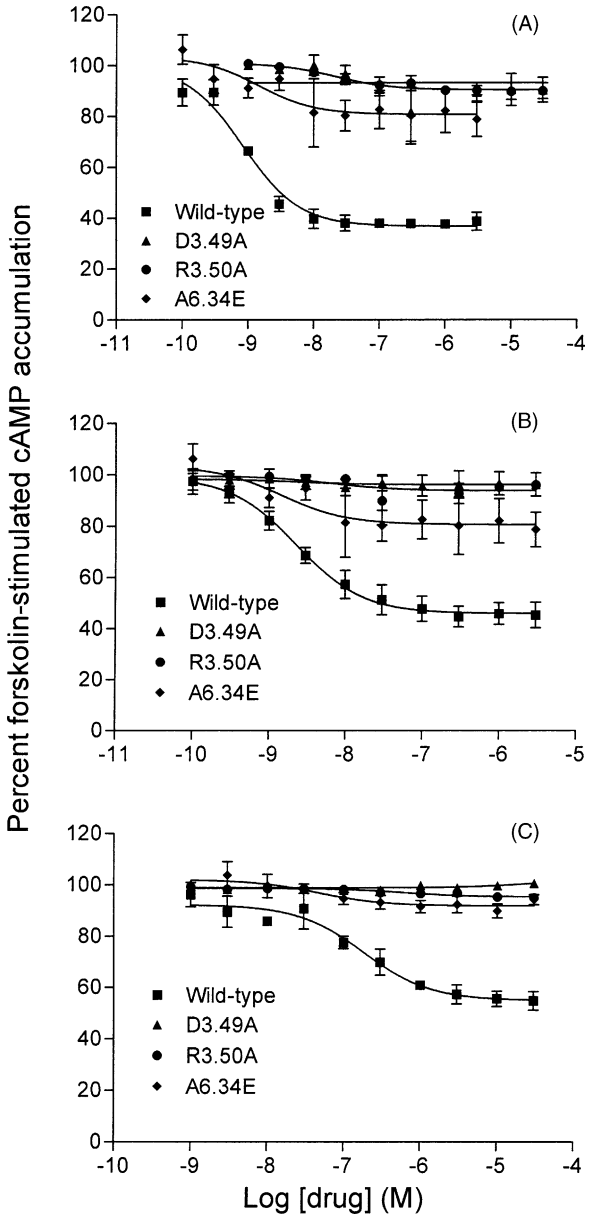

Fig. 5. Comparison of wild-type and mutant CB2 receptors for agonist-induced inhibition of forskolin-stimulated cAMP accumulation. The concentration of forskolin used was 1 μM. Three cannabinoid ligands [(A) HU210; (B) WIN55212-2; and (C) anandamide] were used for competition binding on membranes prepared from HEK293 cells expressing wild-type, D3.49A, R3.50A, and A6.34E mutant CB2 receptors. Data shown represent the means ± SEM of at least three independent experiments performed in duplicate. Curves were generated as described in Section 2.

about 25% in membranes prepared from cells expressing the wild-type receptor. However, no stimulation of [^{35}S]GTPγS binding was observed with membranes prepared from cells expressing the R3.50A receptor (Fig. 6B).

3.5. Constitutive activation

To study the possible constitutive activation of wild-type and mutant CB2 receptors, we tested the abilities of these

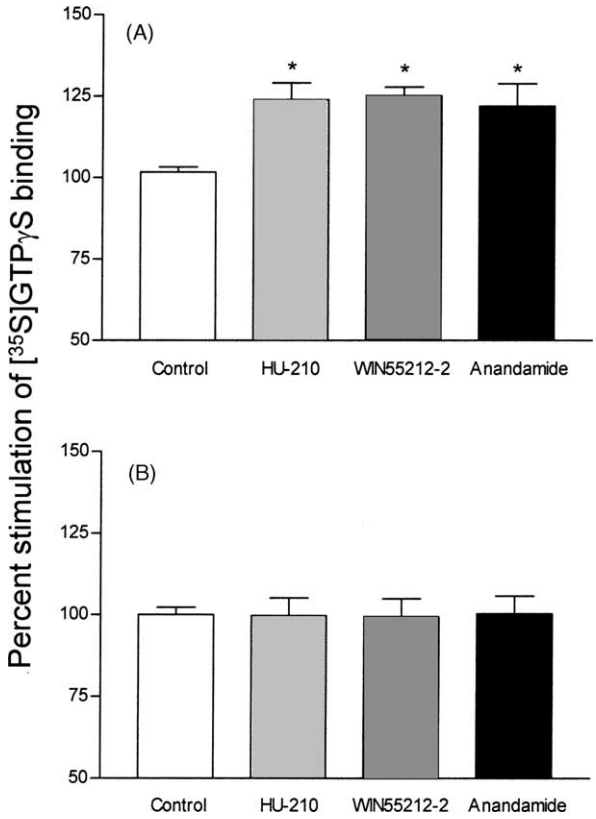

Fig. 6. Comparison of wild-type and mutant CB2 receptors for agonist-stimulated [^{35}S]GTPγS binding. Membranes prepared from HEK293 cells expressing (A) wild-type and (B) R3.50A mutant CB2 receptors were incubated with 0.5 nM [^{35}S]GTPγS and 10 μM GDP in the presence or absence of 1 μM HU-210, 1 μM WIN55212-2, or 3 μM anandamide. Data shown represent the means ± SEM of at least three independent experiments performed in duplicate. Key: (*) denotes a significant difference from control ($P < 0.05$, Student's t-test).

receptors to inhibit forskolin-induced cAMP accumulation in the absence of cannabinoid ligands. In both HEK293 cells transfected with wild-type CB2 receptors and mock-transfected HEK293 cells, forskolin stimulated cAMP accumulation in a concentration-dependent manner (Fig. 7A). However, at concentrations over 0.5 μM, the level of forskolin-induced cAMP accumulation in wild-type CB2 transfected cells was significantly lower than that of the mock-transfected cells (Fig. 7A). In HEK293 cells transfected with D3.49A, R3.50A, and A6.34E mutant CB2 receptors, forskolin-stimulated cAMP accumulation returned to the level observed in the mock-transfected cells (Fig. 7B). Because of the poor solubility of forskolin, a complete stimulation curve for this drug cannot be generated.

The lowered forskolin-stimulated cAMP production in wild-type CB2 receptor-transfected HEK293 cells was blocked by pretreatment with the CB2 inverse agonist SR144528 (Fig. 7A). In other words, SR144528 enhanced forskolin-stimulated cAMP accumulation in wild-type CB2 receptor-transfected HEK293 cells. However, in mutant receptor-transfected or mock-transfected HEK293 cells, SR144528 did not enhance forskolin-stimulated cAMP accumulation (data not shown).

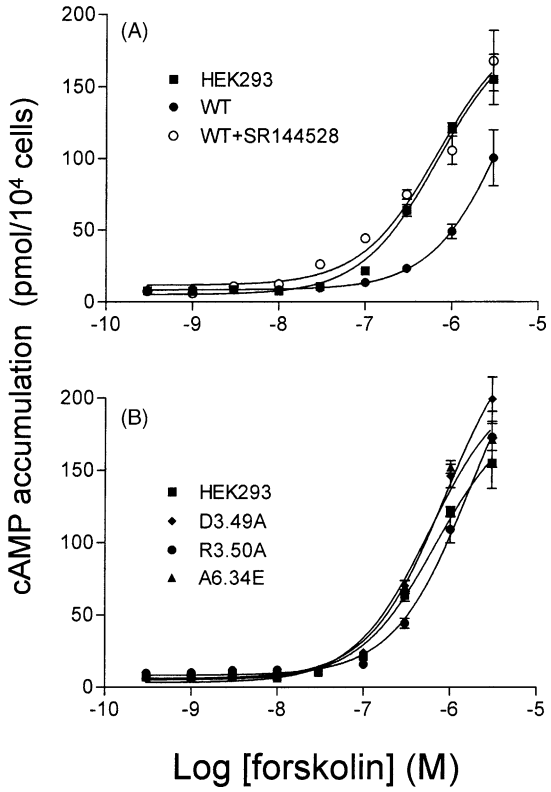

Fig. 7. Constitutive activation of the CB2 receptor. Mock-transfected HEK293 cells and cells expressing wild-type and mutant receptors were assayed for cAMP accumulation in response to various concentrations of forskolin. (A) Comparison between cells expressing wild-type receptor and mock-transfected HEK293 cells. For SR144528 experiments, the cells were pretreated with this inverse agonist for 15 min prior to stimulation with forskolin. (B) Comparison between cells expressing D3.49A, R3.50A, and A6.34E mutant CB2 receptors and mock-transfected HEK293 cells. Data shown represent the means ± SEM of at least three independent experiments performed in duplicate. Curves were generated as described in Section 2.

4. Discussion

In this study, we hypothesized that in the CB2 receptor, R3.50 in the DRY motif may be crucial for signal transduction, and D3.49 in the DRY motif and A6.34 in transmembrane helix 6 may be important for constraining the receptor in an inactive conformation. To test our hypothesis, the R3.50A, D3.49A, and A6.34E mutant CB2 receptors were made and examined. All three mutations produced mutant receptors that were correctly targeted into plasma membrane.

The R3.50A mutant receptor bound to HU-210, WIN55212-2, and anandamide with high affinity that was comparable to that of the wild-type CB2 receptor. Among these ligands, HU-210 and WIN55212-2 are synthetic, whereas anandamide is a putative endogenous cannabinoid [27,28]. The results are consistent with a previous report showing that membranes prepared from COS-7 cells transiently transfected with an HA-tagged R3.50A mutant CB2 receptor bind to HU-210 with an affinity comparable to that of the wild-type CB2 receptor [29]. However, in the current study, the R3.50A mutation resulted in an abolishment of cannabinoid agonist-induced inhibition of forskolin-stimulated cAMP accumulation. The functional loss of the CB2 receptor caused by R3.50A mutation was confirmed further by agonist-stimulated [^{35}S]GTPγS binding assays, in which membrane made from R3.50A mutant receptor expressing cells failed to respond to three agonists. These data are different from the report by Rhee *et al.* [29], in which they showed that the R3.50A mutation receptor only weakly affected cannabinoid agonist-induced inhibition of forskolin-stimulated cAMP accumulation. The reasons behind this discrepancy are currently unknown. Among the possible reasons are the differences in cell lines and constructs used. Importantly, our observation on the R3.50A CB2 mutant receptor is in agreement with studies of the mutant R3.50A α_{1b}-adrenergic receptor [19] and the m1 muscarinic receptor [16]. These studies have shown that the R3.50A mutation severely impairs agonist-induced responses. Therefore, similar to the results obtained with other GPCRs, our data support the hypothesis that the R3.50 in the CB2 receptor plays a key role in signal transduction.

In the current study, both D3.49A and A6.34E mutations resulted in a complete abolishment of ligand binding, and a severe impairment of agonist-induced inhibition of forskolin-stimulated cAMP accumulation. Previous studies have suggested that the activation of GPCRs is accompanied by rigid domain motions of certain helices in the transmembrane bundle and the cytoplasmic ends of TM3 and TM6 are in the proximity. Previously, it has been shown that the ligand binding pocket of the CB2 receptor is located at the upper part of the transmembrane domains [23]. Based on the locations of D3.49 and A6.34 in the CB2 receptor, which are at the cytoplasmic end of TM3 and TM6, respectively (Fig. 1), the decreases in agonist binding caused by the two mutations are most likely due to conformational changes, rather than direct changes in ligand–receptor interactions. The loss of cannabinoid agonist binding by the D3.49A mutation is in agreement with the observation by others for this mutation in the CB2 receptor [29]. The data are also consistent with a report on the m1 muscarinic receptor [30], which showed that replacement of the aspartate with alanine leads to loss of agonist binding. However, our data are different from the studies with the α_{1b}-adrenergic receptor [19] and the β_2-adrenergic receptor [31]. In these studies, increased agonist affinity was found with the mutant D3.49A receptor and/or the A6.34E receptor. Taken together, this suggests that the functional roles for D3.49 and A6.34 seem to be receptor-specific.

In this study, the level of forskolin-stimulated cAMP accumulation was lower in cells expressing the wild-type CB2 receptor than in the mock-transfected HEK293 cells. Furthermore, the lowered level of forskolin-stimulated cAMP accumulation was enhanced by pretreatment with the CB2 receptor inverse agonist SR144528. These data strongly suggest that the wild-type CB2 receptor expressed in HEK293 cells is constitutively active. This is consistent with previous observations that wild-type CB2 receptors

stably transfected into CHO cells are constitutively active [32,33]. Previous mutagenesis and molecular modeling studies have suggested that both D3.49 and A6.34 are crucial for keeping the R3.50 in a "polar pocket" and constraining the receptor in an inactive state [19,20]. The D3.49A and A6.34E mutations have been shown to cause constitutive activation by perturbing the polar pocket and exposing R3.50. However, in the current study, neither D3.49A nor A6.34E mutations caused constitutive activation of the CB2 receptor. These data are against the hypothesis that D3.49 and A6.34 in the CB2 receptor are important for keeping the receptor in an inactive conformation. These results suggest that there are substantial differences in receptor structures and activation mechanisms between the CB2 receptor and previously studied biogenic amine receptors, including the α_{1b}-adrenergic receptor [19,20] and the β_2-adrenergic receptor [31].

An interesting finding in this study was that the constitutive inhibition of adenylate cyclase activity by the wild-type CB2 receptor in HEK293 cells was abolished by R3.50A, D3.49A, and A6.34E mutations. It is generally believed that the reason for constitutive activity is that the receptor can adopt a ligand-independent active conformation as a result of either overexpression or mutation. It has also been established that inverse agonists can shift the constitutively activated receptor to an inactive conformation [33–35]. Our data suggest that the R3.50A, D3.49A, and A6.34E mutations somehow changed the conformation of constitutively activated wild-type CB2 receptors to the inactive state. These results are consistent with the report of inactivating mutations that disrupt the constitutive activity of the α_{1b}-adrenergic receptor [35].

In summary, using site-directed mutagenesis, we have established the functional importance of R3.50, D3.49, and A6.34 in the CB2 receptor. The conclusions are: (i) similar to other GPCRs, R3.50 of the CB2 receptor is crucial for signal transduction; (ii) unlike other GPCRs, D3.49 and A6.34 of the CB2 receptor do not seem to be important to keep the receptor in an inactive state; and (iii) D3.49A and A6.34E mutations abolished ligand binding, and all three mutations abolished constitutive activity of the wild-type CB2 receptor.

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