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A cannabinoid receptor 1 mutation proximal to the DRY motif results in constitutive activity and reveals intramolecular interactions involved in receptor activation

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Abstract

Activation of a G-protein-coupled receptor involves changes in specific microdomain interactions within the transmembrane region of the receptor. Here, we have focused on the role of L207, proximal to the DRY motif of the human cannabinoid receptor 1 in the interconversion of the receptor resting and active states. Ligand binding analysis of the mutant receptor L207A revealed an enhanced affinity for agonists (three- to six-fold) and a diminished affinity for inverse agonists (19- to 35-fold) compared to the wild-type receptor, properties characteristic of constitutive activity. To further examine whether this mutant adopts a ligand-independent, active form, treatment with GTP γ S was used to inhibit G protein coupling. Under these conditions, the L207A receptor exhibited a 10-fold increase in affinity for the inverse agonist SR141716A, consistent with a shift away from an enhanced precoupled state. Analysis of the cellular activity of the L207A receptor showed elevated basal cyclic AMP accumulation relative to the wild type that is inhibited by SR141716A, consistent with receptor-mediated Gs precoupling. Using toxins to selectively abrogate Gs or Gi coupling, we found that CP55940 nonetheless induced only a Gi response suggesting a strong preference of this ligand-bound form for Gi in this system. Molecular dynamics simulations reveal that the single residue change of L207A impacts the association of TM3 and TM6 in the receptor by altering hydrophobic interactions involving L207, the salt bridge involving the Arg of the DRY motif, and the helical structure of TM6, consistent with events leading to activation. The structural alterations parallel those observed in models of a mutant CB₁ receptor T210I, with established constitutive activity (D'Antona, A.M., Ahn, K.H. and Kendall, D.A., 2006. Mutations of CB₁ T210 produce active and inactive receptor forms: correlations with ligand affinity, receptor stability, and cellular localization. Biochemistry, 45, 5606–5617).

Keywords

Cannabinoid; Cannabinoid receptor; CB₁; G-protein-coupled receptor; Ligand binding; Receptor activation

1. Introduction

The human cannabinoid receptor 1 (CB₁) is a member of the G-protein-coupled receptor (GPCR) superfamily and the family A rhodopsin-like receptors. The CB₁ receptor was originally isolated from a human brain stem cDNA library (Gerard et al., 1991) and is primarily found in the central nervous system. It selectively binds the major psychoactive constituent of *Cannabis sativa*, Δ^9 -tetrahydrocannabinol, and endogenous cannabinoids such as anandamide and 2-arachidonylglycerol (for a review, see Howlett et al., 2002). The cannabinoid receptor pathway is believed to be involved with synaptic transmission and the depression of neurotransmitter release in particular (Wilson and Nicoll, 2002). Like other GPCRs, the CB₁ receptor has seven α -helical transmembrane segments connected by three intracellular and three extracellular loops and is oriented with an extracellular amino terminus and an intracellular carboxyl terminus. Upon ligand binding and receptor activation, the intracellular face of the receptor transduces the signal to a heterotrimeric guanine nucleotide binding protein (G protein). Studies have shown that the CB₁ receptor is primarily coupled to pertussis toxin (PTX)-sensitive Gi/o type G proteins (Howlett and Fleming, 1984), which in turn interact with adenylate cyclase to inhibit its activity. However, in striatal neurons, the CB₁ receptor has also been shown to couple with Gs though to a lesser extent (Glass and Felder, 1997). Other events following CB₁ receptor activation can include increased conductance of K⁺ channels (Felder et al., 1995), decreased Ca²⁺ channel conductance (Mackie and Hille, 1992), as well as the release of arachidonic acid (Burstein et al., 1994).

The 2.2 Å crystal structure of rhodopsin (Okada et al., 2004) provides insight into the conformation of GPCRs and possible changes that may occur during receptor activation. However, the derived structure is of the resting state, and a detailed model of the activated state based on structural data does not exist. Studies focusing on the conformation changes of rhodopsin and the β_2 -adrenergic receptor during receptor activation have suggested a rearrangement of the transmembrane (TM) segments in which TM6 plays a primary role (Gether, 2000; Gouldson et al., 2004). In the resting state, the bend at a conserved CWXP motif within TM6 seems to be preserved through interactions with TM3. Located on TM3 is the highly conserved DRY motif in which the Arg 3.50 (Ballesteros and Weinstein, 1995) has 94% identity among all family A GPCRs. The Arg 3.50 is believed to form a salt bridge with Asp 3.49 on TM3 and Glu/Asp 6.30 on TM6 (Palczewski et al., 2000). Receptor activation is hypothesized to involve protonation of Asp 3.49, thereby disrupting the salt bridge and thus the stability of the inactive form of the receptor (Ballesteros et al., 2001; Scheer et al., 1996). These changes are accompanied by a straightening of TM6 that allows closer association with TM5.

Even with recent advances in X-ray structure determination of GPCRs, many questions remain unanswered regarding the interactions involved in stabilization of the resting state, the specific helical rearrangements in the progression through intermediate states, and the role of specific ligands in promoting these states and the activated form. Using an in situ reconstitution system, Glass and Northup (1999) showed that the efficacy of different cannabinoids for activating different CB₁-G protein complexes varies widely. These observations and others (Howlett, 2004) suggest that the cannabinoid receptor, like other GPCRs, may be induced to form ligand-specific activated forms. Evidence that distinct ligand-directed activated forms promote coupling to different G proteins comes from studies of several GPCRs. Agonists of the α_{1B} -adrenergic receptor (Perez et al., 1996) and the 5-hydroxytryptamine-2 receptor (Berg et al., 1998) differentially activate PTX-sensitive versus -insensitive G proteins. Furthermore, the α_2 -adrenergic receptor displays agonist-specific Go versus Gi coupling (Yang and Lanier, 1999). Studies of the CB₁ receptor using rat brain membrane preparations (Houston and Howlett, 1998) and CHAPS extracts from N18TG2 neuroblastoma cell membranes (Mukhopadhyay and Howlett, 2005) also showed that different agonists promote specific

receptor–Gai subtype complexes. The chemically distinct ligands tested are thought to provide different “microconformational” changes within the receptor binding pocket. It is these distinct changes within the protein that dictate specific G protein coupling and therefore signaling to different cellular pathways.

In this study, we have focused on the role of L207, proximal to the DRY motif of the CB₁ receptor, in the interconversion of the receptor resting and active states. Characterization of the mutant receptor L207A revealed enhanced agonist and diminished inverse agonist affinity relative to the wild type, a hallmark of constitutive activity as predicted by the extended Ternary Complex Model (Samama et al., 1993). Furthermore, the change in SR141716A affinity was GTPγS sensitive, as expected for a mutation-induced shift toward the active state. Interestingly, examination of the consequences of the mutation on cyclic AMP levels reveals a deviation in G protein subtype coupling for ligand-dependent versus -independent coupling.

2. Results

2.1. Rationale for the L207A mutation

In this study, we characterized the mutant CB₁ receptor L207A in which a Leu to Ala substitution was generated in the transmembrane region adjacent to the DRY sequence of TM3 (Fig. 1A). This location was particularly interesting because Leu 207 is predicted to be on the same face as the Arg 3.50 and because the Leu is conserved (Fig. 1B) among family A receptors (71%) and among all human GPCRs (62%). This Leu 3.43 is the most invariant residue of TM3 aside from the Arg of the DRY motif in this region of the helix (Horn et al., 2003). Moreover, Leu 207 is also found on the same face of TM3 as a constitutively active CB₁ mutant, T210I (D'Antona et al., 2006), which is only one turn away from Arg 3.50 (Fig. 1C).

2.2. The L207A receptor exhibits shifts in agonist and nverse agonist preferences

The L207A receptor expressed in HEK 293 cells displayed a marked change in its ligand-binding pattern as compared to the wild-type CB₁ receptor (Table 1). Using saturation binding analysis, we found $B_{\max}$ values of 1.4 ± 0.1 pmol/mg protein (L207A) and 1.5 ± 0.1 pmol/mg protein (wild type) and observed that the affinity for CP55940 increased approximately three-fold with the L207A receptor ($K_d = 1.7 \pm 0.1$ nM) relative to the wild type ($K_d = 4.7 \pm 0.8$ nM). Binding of two other structurally dissimilar agonists was tested using competition assays against [³H]CP55940. These were *R*-(+)-WIN55212-2, an aminoalkylindole, and *R*-(+)-methanandamide, a derivative of the endogenous agonist anandamide, which are thought to have different points of contact with the receptor (McAllister et al., 2003). Nonetheless, the L207A receptor also displayed an increased affinity (three- to six-fold) for these compounds. For *R*-(+)-WIN55212-2, the affinity for the L207A receptor was determined to have an inhibition constant of $K_i = 27.0 \pm 5.1$ nM while the K_i value for the wild-type receptor was found to be $K_i = 72.5 \pm 6.1$ nM; for *R*-(+)-methanandamide, the value was $K_i = 110 \pm 31$ nM for the L207A receptor, and for the wild type, $K_i = 630 \pm 1$ nM. Enhanced agonist binding within this range has been demonstrated for other constitutively active receptors (Ge et al., 2003; Scheer et al., 2000; Zuscik et al., 1998) and is predicted by the extended Ternary Complex Model for receptor intermediates that more closely resemble the fully active form than the resting state (Samama et al., 1993).

For the CB₁ receptor, antagonists with inverse agonist activity are commercially available which permit further examination of the possibility of a shift of the mutant receptor population toward a constitutively active state. Saturation binding isotherms up to 30 nM [³H]SR141716A revealed a substantial loss in affinity for the L207A receptor (data not shown). Using a competitive binding assay with [³H]CP55940, displacement by SR141716A was observed and K_i determinations were feasible (Table 1). The data show a clear shift corresponding to a 35-

fold decrease in ligand affinity ($K_i = 260 \pm 61$ nM) relative to the wild-type receptor ($K_i = 7.5 \pm 1.6$ nM). A second biarylpyrazole inverse agonist, AM281, was also used in competition assays with [3 H] CP55940 (Table 1). The experiments with the L207A receptor revealed about a 19-fold loss in affinity for this ligand ($K_i = 590 \pm 110$ nM) versus the wild-type receptor ($K_i = 31.5 \pm 1.8$ nM). The overall pattern is striking when the ratios of binding constants for the mutant and wild-type CB₁ receptors are compared (Table 1). Furthermore, these ratios are remarkably similar to those of a T210I CB₁ receptor mutant (Table 1) with established constitutive activity (D'Antona et al., 2006). Collectively, the data suggest that the L207A receptor adopts a conformation approximating an active state.

2.3. Inhibition of G protein coupling reverses the loss in SR141716A affinity by the L207A receptor

To probe if the observed loss in binding affinity for inverse agonists exhibited by the L207A receptor was due to a change in the extent of G protein precoupling, we treated membrane preparations with nonhydrolyzable GTP γ S to shift the pool of receptors to the inactive state (Barker et al., 1994; Ehlert and Rathbun, 1990). Previously, the wild-type CB₁ receptor has been shown to exhibit some basal activity attributed to a low level of ligand-independent Gi precoupling; GTP γ S treatment resulted in an increased affinity for inverse agonist as expected with a shift toward the inactive form (Bouaboula et al., 1997). Consistent with this observation, the wild-type CB₁ receptor displayed a four-fold ($P < 0.05$) increase in SR141716A binding affinity in the presence of GTP γ S (Fig. 2A). This change was more pronounced with the L207A receptor, which exhibited a 10-fold increase in inverse agonist affinity and further indicates that this mutant adopts a more active ligand-independent form (Fig. 2B). However, the IC₅₀ value in the presence of GTP γ S for the L207A receptor remains about 23-fold higher than that for the wild-type receptor (Fig. 2C). This suggests that, even in the absence of G protein, the mutation sufficiently alters the allowable intramolecular interactions such that the inactive form cannot be completely adopted.

2.4. Elevated cyclic AMP accumulation levels of HEK 293 cells expressing the L207A receptor

To further explore the possibility that the L207A receptor is constitutively active, we examined the ligand-independent cyclic AMP levels. We found that expression of the L207A receptor resulted in basal and forskolin (FSK)-stimulated cyclic AMP levels significantly higher ($P < 0.05$) than when cells were transfected with the wild-type receptor (Fig. 3A) or with empty vector (data not shown). Basal levels of cyclic AMP for cells expressing the L207A receptor were 47 ± 7.9 pmol/ 10^6 cells compared with 8 ± 3.7 pmol/ 10^6 cells for wild-type receptor expressing cells. These differences became more apparent in the presence of FSK, with cells expressing the L207A receptor producing 635 ± 39 pmol/ 10^6 cells, a 4-fold increase over 170 ± 15 pmol/ 10^6 cells expressing the wild-type receptor. This value is significantly different from the wild type ($P < 0.05$), and multiple clones gave similar results. To verify that the observed cyclic AMP levels were CB₁-receptor-mediated, the wild-type- and L207A-expressing cells were treated with SR141716A (8 μ M) to inhibit ligand-independent signaling (Fig. 3A). As observed previously (Bouaboula et al., 1997), the wild-type-expressing cells exhibited a small increase in cyclic AMP accumulation following treatment, which is attributed to the reversal of a low level of basal, Gi-mediated, CB₁ constitutive activity. In contrast, treatment of cells expressing the L207A receptor with SR141716A resulted in a reduction of cyclic AMP accumulation consistent with substantial ligand-independent Gs signaling directed by the L207A receptor. The cyclic AMP levels following SR141716A treatment for the wild-type- and L207A-expressing cells are similar, as anticipated with inhibition of G protein precoupling.

Cells were separately treated with the agonist CP55940 (1 μ M) in the presence of FSK to determine if a ligand-dependent response could also be detected by cyclic AMP level changes (Fig. 3A). For cells expressing L207A, this resulted in about 60% inhibition of the FSK-

stimulated cyclic AMP accumulation, and this relative level of inhibition is comparable to the response of cells expressing the wild-type receptor. Moreover, as shown in Fig. 3B, the L207A-expressing cells exhibit a dose-dependent response to CP55940, with a statistically significant ($P < 0.05$) shift in the EC_{50} (2.6 ± 0.42 nM) compared to the wild type (5.2 ± 0.28 nM) which parallels the increase in binding affinity observed with this ligand (Table 1).

2.5. Gs and Gi coupling activities of the L207A receptor

The L207A mutation was further characterized using bacterial toxins to abrogate Gi or Gs signaling. The cells were incubated with PTX which renders Gi unable to interact with the receptor (Fig. 3C). However, the elevation of cAMP in the L207A-expressing cells, relative to the wild type, is clearly retained with PTX treatment alone and with the addition of FSK. With the subsequent addition of CP55940 (1 μ M), no change in cyclic AMP levels relative to PTX treatment in the absence of ligand, for either the L207A- or wild-type-expressing, FSK-treated cells, was observed. When the data are normalized relative to FSK-stimulated cAMP levels in the absence of CP55940, the similar response to agonist for cells expressing the wild-type and mutant receptors is evident (Fig. 3C, inset). In separate experiments, cells were treated with cholera toxin (CTX), which decreases the intrinsic GTPase activity of Gs, persistently activating it (Cassel and Selinger, 1977) and attenuating the ability of the receptor to activate Gs. The addition of CP55940 (1 μ M) to the CTX-treated cells produced a decrease in cAMP accumulation (Fig. 3D). The levels are 60% and 43% of the levels in the absence of ligand for the L207A- and wild-type-expressing cells, respectively (Fig. 3D, inset). Taken together, the results of PTX and CTX treatment are consistent with the notion that CP55940 binding promotes the Gi coupled form of the receptor even for L207A that exhibits a ligand-independent preference for Gs.

2.6. Molecular dynamics simulations of the wild-type and constitutive mutant receptors

The above analyses indicate that the amino acid change in the mutant receptor perturbed interactions that normally stabilize the resting state. To reveal the key alterations, we examined the interactions among the transmembrane helices from 1 ns simulations of the wild-type, L207A, and T210I receptors, using the microdomain approach (Luo et al., 1994). The forces and torques active during molecular dynamics simulations of the mutant receptor were calculated and compared to those observed for the wild type, providing an unbiased strategy for probing the structural changes induced by the single-site mutation. Analysis of the L207A receptor simulation illustrates that this single amino acid substitution in TM3 impacts an entire set of conformational transitions involving domains within TM2, TM4, and TM6 proximal to the cytoplasm. Closer examination of the averaged structures calculated over the last 100 ps of the simulations reveals changes that can be ascribed to specific residues. For example, in the wild-type receptor, Leu 3.43 (207) is located in a hydrophobic pocket formed by Tyr 5.60 (296), Leu 6.37 (345), and Leu 6.41 (349) as illustrated in Fig. 4A. The mutant L207A receptor model shows that this arrangement is altered with Leu 6.37 (345), moving away from the hydrophobic cluster (Fig. 4B). Interestingly, the model suggests that Arg 3.50 (214) of the DRY motif and Asp 6.30 (338), which Ballesteros et al. (2001) and Greasley et al. (2002) have suggested to form an ionic interaction to stabilize the inactive state of a GPCR, are shifted in the L207A receptor jeopardizing this interaction. In addition, a loss of helical structure in TM6 is observed. Since a T210I mutant receptor also involves an amino acid substitution in the same region as that in the L207A receptor (Fig. 1C) and exhibits similar shifts in binding affinity for agonists and inverse agonists relative to the wild-type receptor (Table 1) and other features of constitutive activity (D'Antona et al., 2006), we carried out molecular dynamic simulations on this receptor in parallel with the L207A receptor. The derived model reveals comparable structural changes to those observed for the L207 receptor, though not quite as large. The greatest RMSD changes were for the cytoplasmic ends of TM1, TM6, and TM7. Nonetheless, a similar increase in the distance between the Arg 3.50 and the Asp 6.30 is observed (Fig. 4C),

making salt bridge formation unfeasible. As seen for the L207A receptor, the unwinding of the regular helical structure of TM6 in the T210I mutant is also strikingly evident.

3. Discussion

GPCR activation encompasses structural changes that involve a concerted rearrangement of the seven-transmembrane helical bundle and exposure of G protein binding sites in a conformation appropriate for activation. In the absence of ligand, these changes are normally inhibited by noncovalent associations (McAllister et al., 2004), in part, contributed by residues in the region of the DRY motif on TM3 (Ballesteros et al., 2001). Perturbation of these constraints by a naturally occurring mutation in the human lutropin/choriogonadotropin hormone receptor, where the conserved Leu at position 3.43 (the location of L207 of the CB₁ receptor) is changed to an Arg, leads to male precocious puberty in children possessing that mutant allele (Latronico et al., 1998). The corresponding mutant receptor *in vitro* was found to be constitutively active (Min et al., 1998). However, studies with other receptors suggest that the extent to which particular residues in this region are involved in controlling interconversion of receptor resting and active states varies among individual GPCRs. In this study, we have examined the consequence of a mutation at L207 (3.43) of the cannabinoid receptor, two turns away from R3.50 of the DRY motif. Investigation of the ligand binding profiles of the L207A receptor revealed a striking pattern of enhanced agonist affinity and diminished inverse agonist affinity (Table 1). This pharmacological profile is a trademark of a constitutively active mutant (CAM) receptor (e.g. α_{2B} -adrenoceptor mutants; Ge et al., 2003) described by the extended Ternary Complex Model (Samama et al., 1993).

Molecular dynamics of the wild-type CB₁ receptor reveals the identification of residues that participate in interactions with L207 and likely play a role in the stabilization of the resting state. As shown in Fig. 4A, a hydrophobic pocket formed by Tyr 296 of TM5 and Leu 345 and Leu 349 of TM6 ideally accommodates the large hydrophobic leucine side chain. Such interactions involving hydrophobic residues on adjacent surfaces of TM3 and TM6 have been proposed to stabilize the inactive state of the C5a receptor (Baranski et al., 1999). Replacement of L207 in the CB₁ receptor with the smaller alanine alters this contact, and Leu 6.37 (345) is no longer positioned to participate in the interaction (Fig. 4B). No ligand binding was detected with L207R transformed cells (data not shown), suggesting the possibility that exchange with the more obtrusive, charged residue may have perturbed the structure so substantially that assembly was not possible. Modeling of the L207A receptor also reveals an increase in the distance between Arg 3.50 (214) and Asp 6.30 (338) and the loss of a regular helical structure at the intracellular end of TM6 relative to that in the wild-type receptor. The modeling of the constitutively active mutant receptor T210I revealed a parallel set of changes, further emphasizing the involvement of this face of TM3 in maintaining the resting state form of the CB₁ receptor (Fig. 4C). Based on work with other receptors, it has been proposed that ionic interactions involving Asp/Glu 3.49, Arg 3.50, and Glu/Asp 6.30 are critical for maintaining the cytoplasmic ends of TM3 and TM6 immobilized in the inactive state (Ballesteros et al., 2001; Shapiro et al., 2002). The distance between Arg 3.50 (214) and Asp 6.30 (338) in L207A and T210I makes this ionic lock not feasible.

For promiscuous receptors, mutations that influence G protein association can impact Gi and Gs coupling differentially. For example, mutation of different residues in the α_{2A} -adrenergic receptor resulted in a spectrum of phenotypes ranging from impaired Gs coupling and normal Gi coupling to normal Gs and Gi coupling but diminished Gi activation (Wade et al., 1999). Intriguingly, the L207A mutation on TM3 and a mutation involving inversion of a leucine and alanine in intracellular loop 3 (Abadji et al., 1999) of CB₁ result in receptors with constitutive activity that favors Gs coupling based on the SR141716A-reversible, elevated levels of cAMP in the absence of ligand. However, coupling of wild-type CB₁ to Gs seems to be cell-system-

dependent (Glass and Felder, 1997) and adenylyl-cyclase-isoform-dependent (1, 3, 5, 6, and 8 are inhibited via CB₁ while 2, 4, and 7 are stimulated) (Rhee et al., 1998). In HEK 293 cells, in the absence or presence of PTX to inhibit Gi coupling, no Gs response is detected unless the D₂ dopamine receptor is coexpressed with the CB₁ receptor to sequester Gi (Jarrahian et al., 2004). With the L207A mutant, we also examined the agonist-dependent changes in cAMP; we observed a shift in EC₅₀ corresponding to the shift in CP55940 binding affinity, establishing a link between affinity and efficacy for this mutant. Interestingly, CP55940 induces an inhibition of cyclic AMP accumulation via this mutant, indicating that, if sufficient Gi is available, CP55940 induces a CB₁ conformation preferable for its coupling.

The extended Ternary Complex Model (Samama et al., 1993) of receptor activation and the expansion of this model (Caramellini and Leff, 1998) provide useful models for considering the multiple active states possible for promiscuous receptors. In its simplest form, the receptor can exist in an inactive state (R) and two different active states (R* and R**) distinguished by G protein preferences (e.g. R*Gs and R**Gi). CB₁ may be poised such that the mutation we have generated readily destabilizes the resting state to produce R*, and, in the absence of Gi and/or an agonist able to induce R**, coupling with Gs occurs. However, with all equilibria linked, an agonist that enriches one receptor state will do so at the expense of others. Consequently, in the presence of CP55940, R** forms and is stabilized by Gi, provided that availability is not limiting. The free energy change associated with inter-conversions between the different states R, R*, and R** must be small, and the prominence of one state over another is readily influenced by receptor mutation, availability of different G proteins, and the preferences of ligands. Understanding the receptor features which distinguish resting and active states selective for different effectors remains a key area for study to understand the mechanisms of signal transduction and for the development of therapeutics which impact specific cellular pathways.

4. Experimental procedures

4.1. Mutagenesis and expression of wild-type and L207A receptors

A pcDNA3.1 expression vector coding the human CB₁ receptor (Chin et al., 1998) was used for site-directed mutagenesis with QuikChange (Stratagene, La Jolla, CA). The mutation was verified by DNA sequencing using the Prism Automated Sequencing System (Applied Biosciences, Forest City, CA). For most studies, HEK 293 cells (ATCC, Manassas, VA) were maintained and transiently transfected with wild-type or mutant receptor expression vectors as previously described (Murphy and Kendall, 2003). For cyclic AMP analysis, stable transformants were selected following transfection using 1.2 mg/ml G418 sulfate (EMD Biosciences, La Jolla, CA). After 2 weeks, single clones were maintained in 0.6 mg/ml G418. Screening of single clones was performed using RT-PCR and binding analysis as previously described (Chin et al., 1998).

4.2. Amino acid numbering system

Residue L207 is identified in the text by the natural linear sequence number of the CB₁ receptor. In order to make comparisons with the GPCR superfamily, some key residues are noted by the amino acid numbering scheme developed by Ballesteros and Weinstein (1995) and the natural linear sequence number. An amino acid is identified by the transmembrane helix number in which it is located and then by reference number which relates it to the most conserved residue in that helix, which is assigned a locant value of 0.50. The reference number for the other residues either ascend or descend to the C- or N-terminus, respectively. For example, L3.43 would be located on TM3 and is located amino terminal to the most conserved residue of TM3 by seven residues. In some instances, CB₁ residues are identified both by the Ballesteros and Weinstein scheme and by the number in the natural sequence of CB₁, given in parenthesis.

4.3. Membrane preparations and ligand binding assays

Membrane preparations of transfected HEK 293 cells were generated as previously described (Abadji et al., 1999). Briefly, cells were washed twice with, and suspended in, phosphate-buffered saline (PBS) + 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Cells were then fractionated using nitrogen cavitation at 750 psi using a Parr Cell Disruption Bomb. The cell fractions were centrifuged at $500 \times g$ for 10 min at 4 °C. The supernatant was spun at $100,000 \times g$ to pellet membrane vesicles, which were then resuspended in TME buffer (25 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 1 mM EDTA) and 7% sucrose (w/v). The membrane preparations used for R-(+)-methanandamide binding were treated with freshly made phenylmethanesulfonyl fluoride (Sigma-Aldrich, St. Louis, MO) prior to pelleting (Lin et al., 1998). Total protein concentration was determined using the method of Bradford (1976). Binding assays were performed as previously described (Murphy and Kendall, 2003). In saturation binding assays, 39 µg of total protein was incubated with [³H]CP55940 (PerkinElmer, Wellesley, MA) or [³H]SR141716A (GE Health-care, Piscataway, NJ) for 90 min at 30 °C. Competition binding experiments used 39 µg total protein with 4 nM [³H]CP55940 and a range of unlabeled displacing ligand. GTPγS treatment was carried out by incubating the membranes with 100 µM GTPγS (Sigma-Aldrich, St. Louis, MO) during the 90 min binding incubation at 30°C. Nonspecific binding was determined with 1 µM unlabeled ligand, and all reactions were performed in duplicate. Reactions were terminated with 250 µl cold TME + 5% bovine serum albumin (BSA). Bound ligand was separated from free by filtration with a Brandell cell harvester through Whatman GF/C filter paper washed with cold TME and analyzed as previously described (Chin et al., 1999).

4.4. Cyclic AMP accumulation determination

HEK 293 cells stably expressing either wild-type or L207A receptors, and verified to have equivalent ligand binding properties as in the transient expression system, were used for cyclic AMP level determinations. Cells were harvested at 80% confluency by first lifting the cells from 100 mm dishes with warm PBS containing 0.5 mM EDTA. Cells were then counted and resuspended in DME containing 2.5% fatty-acid-free BSA, 20 mM HEPES, pH 7.4, 0.2 mM RO 20-1724 and incubated for 10 min at 37 °C. Aliquots of 1×10^6 cells were incubated with 1 µM FSK and the appropriate cannabinoid ligand in a final reaction volume of 250 µl for 10 min at 37 °C. The reaction was terminated by the addition of HCl and freezing. Cells were then thawed and neutralized with 2 M HEPES, pH 7.5, pelleted and the supernatant removed. Cyclic AMP accumulation was determined using a [³H]cyclic AMP assay system (GE Healthcare, Piscataway, NJ). To delineate G_i versus G_s coupling effects, toxins were added to growth media at 5 ng/ml for PTX or 2 µg/ml for CTX (Calbiochem, La Jolla, CA). Following an 18-hour incubation in the presence of toxin, cells were washed twice with PBS and evaluated for cyclic AMP levels as described (Abadji et al., 1999).

4.5. CB₁ modeling and molecular dynamics simulation

Generating a wild-type CB₁ receptor model was accomplished with the Modeler program. The method utilizes rhodopsin as the template to generate the topological arrangement of the seven-transmembrane helices of the CB₁ receptor. The loops and N- and C-termini were positioned in a favored minimal energy state with the GROMACS program. The Insight program was then used to construct the L207A and T210I mutants from the wild-type receptor model. To mimic the cell environment, a biphasic simulation cell of water/decane/water was employed essentially as described previously (Rolz and Mierke, 2001). This places the loops and N- and C-termini in water above and below the transmembrane domains which are in a decane layer. Molecular dynamics simulations were carried out for 1 ns at 300 K using the GROMACS program. A final structure of each receptor was obtained by averaging the structures over the last 100 ps of the trajectories. The 7-transmembrane helices were each divided into 15

microdomains for the analysis of specific domain motions within each helix and the interaction among these domains was calculated by the method of Weinstein and coworkers (Luo et al., 1994). The forces and torques generated among these domains were compared to those of the wild-type receptor and traced back to the interaction producing the force/torque, allowing us to identify those due to the mutation.

4.6. Data analysis

Results were analyzed with Prism Graphpad Software (San Diego, CA) as previously described (Chin et al., 1999). K_i values were determined using the Cheng–Prusoff equation (Cheng and Prusoff, 1973). Comparisons between wild-type and mutant receptors were made using unpaired *t* tests of logged values. *P* values < 0.05 were defined as statistically significant.

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Fig. 1. Location of the L207A mutation in the CB₁ receptor characterized in this study. (A) Helical-net representation of the human cannabinoid receptor 1. Gray-shaded circles represent residues of the proposed transmembrane helices. White circles are the connecting extracellular (E1–E3) and intracellular (I1–I3) loops and the membrane proximal residues of the N- and C-termini. Black circles indicate the most conserved residue, among family A GPCRs, in each helix (Baldwin et al., 1997). The arrow indicates the location of the mutation made in this study. (B) Carboxyl terminal portions of TM3 from the CB₁ and CB₂ receptor sequences were aligned using the conserved arginine of the DRY sequence (italics) with the family A GPCR consensus sequence and the consensus sequence of human GPCRs (Horn et al., 2003). (C)

Longitudinal and cross-sectional views of the C-terminal region of TM3 of the CB₁ receptor depicting the locations of Leu 207 and Thr 210 relative to Arg 214 of the DRY motif.

Fig. 2. Effect of GTP γ S treatment on SR141716A binding. Dose-dependent displacement of [3 H] CP55940 binding to the wild-type (A) or L207A (B) receptors by SR141716A in the absence (Δ) or presence ($\blacktriangle$) of 100 μ M GTP γ S. Nonspecific binding was determined using 1 μ M unlabeled CP55940. Experiments were performed with membrane preparations from HEK 293 cells transfected with wild-type or mutant receptors. Data represent the mean $\pm$ SEM of three independent experiments performed in duplicate. (C) The corresponding IC_{50} values of the dose-dependent displacement of [3 H]CP55940 by SR141716A for the wild-type and L207A receptors in the presence or absence of GTP γ S. The asterisk (*) represents values for binding

by the receptors in the presence of GTP γ S that are statistically different ($P < 0.05$) from those in the absence of GTP γ S.

Fig. 3.

Cyclic AMP accumulation measurements from HEK 293 cells expressing wild-type or L207A receptors. (A) Basal and FSK-stimulated cyclic AMP levels were measured for the mutant and the wild-type receptor expressing cells. FSK-stimulated levels were also measured in the presence of 1 μ M CP55940 or 8 μ M SR141716A as indicated. (B) Dose-response curves showing inhibition of FSK-stimulated cAMP levels as a function of increasing CP55940 concentrations for the wild-type (□) and L207A (▲) receptor expressing cells. Values are expressed as a percent of FSK-stimulated cyclic AMP levels (set at 100%) for each clone. (C) Cells expressing wild-type or L207A receptors were exposed for 12–16 h to PTX. FSK-stimulated cAMP levels were measured in the presence or absence of 1 μ M CP55940. The values of cAMP accumulation in cells treated with PTX alone, treated with PTX and FSK, and treated with PTX, FSK, and CP55940 (CP) in pmol/10⁶ cells are shown. The values expressed

as a percent of FSK-stimulated cyclic AMP levels (set at 100%) for each individual cell line are also given (inset). (D) Cells expressing wild-type or L207A receptors were exposed for 12–16 h to CTX. Cyclic AMP levels were measured in the presence or absence of 1 μ M CP55940 as indicated. The measured values of cAMP accumulation in cells untreated with CTX, treated with CTX alone, and treated with CTX and CP55940 (CP) in pmol/ 10^6 cells are shown. The values are also expressed as a percent of CTX-stimulated cyclic AMP levels (set at 100%) for each individual cell line (inset). Data represent the mean $\pm$ SEM of three or more independent experiments performed in duplicate.

Fig. 4.

Structural models of the wild-type CB₁ and mutant receptors. Models were derived from molecular dynamics simulations, as described in Experimental procedures, using a rhodopsin template to establish the topological arrangement of the seven transmembrane helices of CB₁. Residues are identified by their numerical sequence in CB₁. For clarity, only relevant TM helices are shown. TM6 is red, TM3 is blue, and others are gray. In each frame, R3.50 (214) and D6.30 (338) are represented for reference purposes. Views are of the wild-type CB₁ (A), L207A (B) and T210I (C) receptors. Each frame displays the model at slightly different angles.

Table 1Binding properties of wild-type and L207A CB₁ receptors ^a

Ligand	Receptor		Ratio of binding constants	
	wild type (nM)	L207A (nM)	WT:L207A	WT:T210I
CP55940	4.7 ± 0.8	1.7 ± 0.1 *	3:1	3:1
R-(+)-WIN55212-2	72.5 ± 6.1	27.0 ± 5.1 *	3:1	3:1
R-(+)-methanandamide	630 ± 1	110 ± 31 *	6:1	3:1
AM281	31.5 ± 1.8	590 ± 110 *	1:19	1:19
SR141716A	7.5 ± 1.6	260 ± 61 *	1:35	1:27

^aK_d values (nM) were determined for [³H]CP55940 binding. For all other ligands tested, K_i values (nM) were determined by competition binding against 4 nM [³H]CP55940. The wild-type receptor K_i values and the ratio of binding constants with T210I (D'Antona et al., 2006) are shown for comparison. Values in bold represent the ratio of binding constants for wild-type to mutant receptors. Values represent the mean ± SEM of three or more independent experiments performed in duplicate.

The asterisk (*) represents values for binding by the L207A receptor that are statistically different (P < 0.05) from those for the wild-type receptor.