Structural Mimicry in Class A G Protein-coupled Receptor Rotamer Toggle Switches

THE IMPORTANCE OF THE F3.36(201)/W6.48(357) INTERACTION IN CANNABINOID CB1 RECEPTOR ACTIVATION*

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In this study, we tested the hypothesis that a CB1 TMH3-4/5-6 aromatic microdomain, which includes F3.25(190), F3.36(201), W5.43(280), and W6.48(357), is centrally involved in CB1 receptor activation, with the F3.36(201)/W6.48(357) interaction key to the maintenance of the CB1-inactive state. We have shown previously that when F3.36(201), W5.43(280), and W6.48(357) are individually mutated to alanine, a significant reduction in ligand binding affinity is observed in the presence of WIN 55,212-2 and SR141716A but not CP55,940 and anandamide. In the work presented here, we report a detailed functional analysis of the F3.36(201)A, F3.25(190)A, W5.43(280)A, and W6.48(357)A mutant receptors in stable cell lines created in HEK cells for agonist-stimulated guanosine 5'-3-(thio)triphosphate (GTPγS) binding and GIRK1/4 channel current effects in Xenopus oocytes where the mutant proteins were expressed transiently. The F3.36(201)A mutation showed statistically significant increases in ligand-independent stimulation of GTPγS binding versus wild type CB1, although basal levels for the W6.48(357)A mutant were not statistically different from wild type CB1, F3.36(201)A demonstrated a limited activation profile in the presence of multiple agonists. In contrast, enhanced agonist activation was produced by W6.48(357)A. These results suggest that a F3.36(201)/W6.48(357)-specific contact is an important constraint for the CB1-inactive state that may need to break during activation. Modeling studies suggest that the F3.36(201)/W6.48(357) contact can exist in the inactive state of CB1 and be broken in the activated state via a rotamer switch (F3.36(201) trans, W6.48(357) g+ → (F3.36(201) g+, W6.48(357) trans). The F3.36(201)/W6.48(357) interaction therefore may represent a “toggle switch” for activation of CB1.

The cannabinoid CB1 receptor belongs to the class A rhodopsin-like family of G protein-coupled receptors (GPCRs)1 (see helix net, Fig. 1) (2). The availability of high resolution crystal structures of rhodopsin (Rho) (3, 4) (Protein Data Bank accession code 1GZM) and the availability of biophysical data on the conformational changes that occur when rhodopsin and other class A receptors are activated (6–8) have greatly aided the study of structure-function relationships in class A GPCRs. Ballesteros et al. (9) have proposed that “structural mimicry” may occur in GPCRs such that different amino acids or alternate microdomains in class A receptors (e.g. the amine receptors) can support similar deviations from the regular a-helical structure seen in Rho, thereby resulting in similar tertiary structures. In the work presented here, we provide evidence that “structural (functional) mimicry” by alternate microdomains may also support the core function of signaling activation through transmembrane helix conformational change in the class A family.

There is a growing body of evidence in the literature that activation of GPCRs is accompanied by rigid domain motions and rotations of transmembrane helices (TMHs) 3 and 6 (6–8). At their intracellular ends, TMHs 3 and 6 in Rho are constrained by an E9.49(134)/R3.50(135)/E6.30(247) salt bridge that limits the relative mobility of the cytoplasmic ends of TMH3 and TMH6 in the inactive state (3) and acts like an “ionic lock” (10, 11). During activation, P6.50 of the highly conserved CWXP motif in TMH6 of GPCRs may act as a flexible hinge, permitting TMH6 to straighten upon activation, moving its intracellular end away from TMH3 and upwards toward the lipid bilayer (12).

Khorana and co-workers (13) have reported that even in the dark (inactive) state of Rho, only some strong constraints exist, whereas the majority of the molecule experiences conformational flexibility. Therefore, light activation of Rho does not require the breaking and forming of thousands of specific contacts within nanoseconds, rather only a few specific contacts restricting the inactive state, including indole side chain contacts of tryptophan residues, need to break on activation. These changes can then be transmitted through the entire membrane protein because of its dynamic plasticity. One of the tryptophan residues that Khorana and co-workers (13) have reported to be restricted is W6.48(265). In the dark (inactive) state of Rho, the β-ionone ring of 11-cis-retinal is close to W6.48(265) of the

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1 The abbreviations used are: GPCR, G protein-coupled receptor; CB, cannabinoid; TMH, transmembrane helix; GIRK, G protein-coupled inwardly rectifying potassium; Rho, rhodopsin; WT, wild type; GTPγS, guanosine 5’-3-(thio)triphosphate; CM, conformational memories.
CWXP motif on TMH F and helps constrain it in a \( \chi_1 = g^+ \) conformation (3). In the light-activated state, the \( \beta \)-ionone ring moves away from TMH F and toward TMH D where it resides close to A4.58(169) (14). This movement releases the constraint on W6.48(265), making it possible for W6.48(265) to undergo a conformational change. Lin and Sakmar (15) reported that perturbations in the environment of W6.48(265) of Rho occur during the conformational change concomitant with receptor activation. This suggests that the conformation of W6.48(265) when Rho is in its inactive/ground state (R; \( g^+ \)) changes during activation (i.e. W6.48(265) \( g^+ \rightarrow \) trans) (16).

In the class A cationic neurotransmitter receptors, a highly conserved cluster of aromatic amino acids is found on TMH6 that faces the binding site crevice bracketing W6.48 (F6.44, W6.48, F6.51, and F6.52) (16). Shi et al. (16) have proposed that an aromatic at 6.52 (F6.52) in the \( \beta_2 \)-adrenergic receptor may serve to constrain W6.48 in its inactive state (i.e. \( g^+ \chi_1 \)) and is part of a rotamer toggle switch (C6.47 trans/W6.48 g \( \leftarrow \) F6.52 g \( \rightarrow \) C6.47 g \( \leftarrow \) F6.52 trans) for activation of this receptor.

Restriction of W6.48 by a TMH6 aromatic cluster is not possible in the cannabinoid receptors, as the CB1 receptor has leucines at 6.44, 6.51, and 6.52. Instead, the CB1 receptor contains a microdomain of aromatic residues that face into the ligand-binding pocket in the TMH3-4-5-6 region, including F3.25(190), F3.36(201), Y5.39(276), W5.43(280), and W6.48(357) (Fig. 2). In work reported here, we suggest that the F3.36(201)W6.48(357) interaction may act as a mimic of the 11-\( \text{cis} \)-retinal/W6.48 interaction in the Rho dark state and may serve as the "toggle switch" for CB1 activation, with F3.36(201) \( \chi_1 \) trans/W6.48(357) \( \chi_1 \) g \( \leftarrow \) representing the inactive (R) and F3.36(201) \( \chi_1 \) g \( \leftarrow \) W6.48(357) \( \chi_1 \) trans representing the active (R*) state of CB1. Modeling, mutation, and functional studies undertaken to test the importance of the TMH3-4-5-6 aromatic microdomain in ligand recognition and in the conformational changes that accompany activation of CB1 suggest that a F3.36(201)W6.48(357)-specific contact is an important constraint for the CB1 inactive state.
**EXPERIMENTAL PROCEDURES**

**Molecular Modeling**

**Amino Acid Numbering System**—In the discussion of receptor residues below, the amino acid numbering scheme proposed by Ballesteros and Weinstein (17) is used. In this numbering system, the most highly conserved residue in each transmembrane helix (TMH) is assigned a locant of 0.50. This number is preceded by the TMH number and followed by the sequence number. All other residues in a TMH are numbered relative to this residue. In this numbering system, for example, the most highly conserved residue in TMH2 of the mouse CB1 receptor is D2.50(164). The residue that immediately precedes it is A2.49(163). Fig. 1 serves as a reference for this numbering system in mouse CB1.

**Definition of Rotameric State of \( \psi_1 \)**—Different nomenclatures have been used to denote the rotameric state of the side chain torsion angles. The nomenclature employed here for the \( \psi_1 \) torsion angle is that described by Shi et al. (16). When the heavy atom at the \( \gamma \) position is opposite to the backbone nitrogen when viewed from the \( \beta \)-carbon to the \( \alpha \)-carbon, the \( \chi_1 \) is defined to be trans. When the heavy atom at the \( \gamma \) position is opposite to the backbone nitrogen when viewed from the \( \alpha \)-carbon to the \( \beta \)-carbon, the \( \chi_1 \) is defined to be gauche + (\( g^+ \)). When the heavy atom at the \( \gamma \) position is opposite to the \( \alpha \)-hydrogen when viewed from the \( \beta \)-carbon to the \( \alpha \)-carbon, the \( \chi_1 \) is defined to be gauche − (\( g^- \)). By using this nomenclature system, the side chain conformations discussed here are categorized into \( g^- (0° < \chi_1 < 120°) \), \( g^+ (120° < \chi_1 < 240°) \), or \( g^0 (240° < \chi_1 < 360°) \).

**Conformational Memories Studies of TMH6 and the W6.48A Mutant TMH6**—In order to explore the consequences of the W6.48A mutation upon TMH6, we used conformational memory (CM) method (18), a method that employs multiple Monte Carlo/simulated annealing random walks and the Amber* force field. Conformational memories has been shown to converge in a very practical number of steps and to be capable of overcoming energy barriers efficiently. By using CM, the conformational properties of a helix can be fully characterized by the free energy of each of the conformations that the helix can adopt, and this property includes not only the intrinsic conformation of each conformational state but also the probability that the helix will adopt each particular conformation relative to all other ones accessible in an equilibrated thermodynamic ensemble.

The calculation is performed in two phases. In the first phase, repeated runs of Monte Carlo/simulated annealing are carried out to map the entire conformational space of the helix. In the second phase, new Monte Carlo/simulated annealing runs are performed only in the populated regions identified in the first phase of the calculation.

**WT TMH6 Versus TMH6 W6.48A Mutant**—The CB1 TMH6 (from residue 6.30 to residue 6.57, DLRAKLTLVLLVIWCGPPLAIVMY) and the TMH6 W6.48A mutant (DLRAKLTLVLLVIWCGPPLAIVMY) were built using MacroModel (19). In the Rho 2.8-Å crystal structure (3), \( \chi_1 \) of W6.48 is \( g^- \). In order to be consistent with this result, W6.48 was chosen to be \( g^- \) in the WT model of TMH6 (as is found in Rho). Statistically, there are no significant differences between \( g^- \), \( g^+ \), and \( g^0 \) angles of the R* CB1, WT versus W6.48A R and R* helix families were evaluated in the two-sample independent \( t \) test computed using OriginPro version 7 (Origin Lab Corp.).

**Models of CB1, R and R* States**—In the present study, the literature on GPCR activation discussed above was used to generate an R* CB1, TMH bundle from a model of the inactive (R) CB1 receptor based on the 2.8-Å crystal structure of rhodopsin (3). The creation of these two forms of CB1 is described briefly below.

**Model of Inactive State (R) Form of CB1**—A model of the R form of CB1 was created using the 2.8-Å crystal structure of bovine Rh (3). First, the sequence of the mouse CB1 receptor (22) (see Fig. 1) was aligned with the sequence of bovine Rh using the same highly conserved residues as alignment points. We then used this alignment toinitialize a model of CB1 (22) to the R* TMH5 in CB1, i.e., using the observed proline in TMH5 of Rho. Therefore, the sequence of CB1, in the TMH5 region was aligned with that of Rho as described previously using its hydrophobicity profile (23). The mouse CB1, sequence (22) is 97.7% identical to the human CB1, sequence (2) overall and 100% identical within the transmembrane regions. The mouse sequence is one residue longer (473 residues) than the human sequence (472 residues) due to an additional residue in the N terminus.

Initial helix ends for mouse CB1, were chosen in analogy with those of Rho (3). With the exception of TMH1, these helix ends were found to be within one turn of the helix ends originally calculated by us and reported in 1995 (23). Two changes dictated by the CB1 sequence were made in the helix ends. The shortness of the E1 loop region in CB1, which is not present in Rho, (as is found in Rho). The break in helicity caused by the GNW sequence motif on the extracellular end of TMH4 necessitated that TMH4 end at 4.62 instead of 4.66 (as is found in Rho). Changes to the general Rho structure that were necessitated by sequence divergences included the absence of helix-kinking proline residues in TMH1 and TMH5, the lack of a GG motif in TMH2, as well as the presence of extra flexibility in TMH6 (24). Our recent conformational memories study of TMH6 in CB1, revealed that TMH6 in CB1, has high flexibility due to the small size of residue 6.49 (a glycine) immediately preceding Pro 6.50. The conformer selected from our CM results for inclusion in the CB1, R bundle (Pro kink angle = 53.1°) was chosen so that R3.50(215) and D6.30(339) could form a salt bridge at the intracellular ends of TMH3 and -6 in the CB1, TMH bundle. A flexible residue has been shown to be an important stabilizer of the inactive state of the \( \beta \)-adrenergic receptor, the 5HT-2a receptor (10), and to be present in Rho (3). Because of the extreme flexibility of TMH6 in CB1, we have proposed that an additional TMH3–6 salt bridge, K3.28(193)–D6.58(367), stabilizes the inactive state on the extracellular side of the TMH bundle (25).

**Model of Active (R*) Form of CB1**—Based upon experimental results for rhodopsin and the \( \beta \)-adrenergic receptor (16, 15, 26), the R* (active) CB1 bundle was created from the inactive (R) model of CB1, by substituting a less kinked TMH6 (21.8° kink angle) from our CM results (25) for which the R3.50(215) and D6.30(339) salt bridge would be broken due to the movement of the intracellular end of TMH6 away from

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2 M. Mezei, personal communication.
that of TMH3 and out into lipid (10). Rotations of both TMH3 and TMH6 were also central to the creation of the R* model. The details of these rotations are presented elsewhere (24).

Preparation of Helices—Each helix of the model was capped as the acetamide at its N terminus and as the N-methyl amide at its C terminus. Ionizable residues in the first turn of either end of the helix were neutralized, as were any lipid facing charged residues. Ionizable residues were considered charged if they appeared anywhere else in the helix.

Energy Minimization, Unoccupied Cell States—The energy of the CB1 or CB2, R* TMH bundle complex was minimized using the AMBER* united atom force field in Macromodel 6.5 (Schrodinger Inc., Portland, OR). A distance-dependent dielectric, 8.0-Å extended nonpolar dielectric, was employed. The CB1 Ro C B1 R* TMH bundle complex was minimized using the AMBER* united atom force field in Macromodel 6.5 (Schrodinger Inc., Portland, OR). A distance-dependent dielectric, 8.0-Å extended nonpolar dielectric, was employed.

Assessment of Aromatic Stacking Interactions—Residues were designated here as participating in an aromatic stacking interaction if subject rings had centroid to centroid distances (d) between 4.5 and 7.0 Å. These interactions were further classified as "tilted t" arrangements if the force constant on the helix backbone atoms was reduced by a factor of 225 kJ/mol was used on the helix backbone atoms in order to obtain the helix backbone atoms were reduced to 50 kJ/mol in order to allow the helix backbone to adjust. Stages one and two were repeated with the number of CG stages in stage two incremented from 100 to 500 steps until a gradient of 0.001 kJ/(mol Å) was reached. This same protocol was followed for the W6.48(357)A 3.2 (1.9–4.5) 45° (22–92) 0.3 (0.1–0.5) 23° (23–45) that of TMH3 and out into lipid (10). Rotations of both TMH3 and TMH6 were also central to the creation of the R* model. The details of these rotations are presented elsewhere (24).

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Toggle Switch Activation of CB₁ Receptor

We have recently published mutation and modeling studies of the CB₁ TMDH3-4-5-6 aromatic microdomain in which binding sites for the inverse agonist SR141716A and the CB₁ agonists WIN 55212-2 and anandamide were identified (1).

**Functional Analysis of Mutant Receptors**

We have shown previously that the aromatic residues F3.36(201), W5.43(280), and W6.48(357) form specific interaction sites in CB₁ for aminoalkylindole agonist (WIN 55,212-2) and diaryl pyrazole inverse agonist/antagonist (SR141716A) ligands but not for endogenous (anandamide) and bicyclic cannabinoid (CP55,940) agonists (1). When the aromatic residues were individually mutated to alanine, a significant reduction in ligand binding affinity was only observed in the presence of WIN 55,212-2 and SR141716A but not CP55,940 and anandamide. The study presented here is a detailed functional analysis of these mutant receptors using two different cellular backgrounds. The mutants were studied in stable cell lines created in HEK cells or in oocytes where the mutant proteins were expressed transiently. The mutations were analyzed in two ways. Direct receptor-G protein stimulation in HEK cells was assessed using [35S]GTPγS binding, and G protein stimulation downstream of the receptor was evaluated by measuring enhancement of GIRK channel activity in oocytes.

**RESULTS**

The TMDH3-4-5-6 Aromatic Microdomain at the CB₁ Receptor: Comparison of R and R* State Models in the Absence of Ligand

CB₁ R State—Fig. 2A illustrates key features of the CB₁ TMD bundle model in the inactive (R) state in the TMDH3-4-5-6 region. One of the significant features of this model is a salt bridge between K3.28(193) and D6.58(367) (N-O distance = 2.6 Å; N-H-O angle = 159°) (51). This salt bridge is made possible by the profound flexibility in TMH6 due to the presence of G6.49 in the CWXP motif of TMH6 (25). The TMDH3-4-5-6 region of the R bundle in the absence of ligand is characterized by a W6.48(357)/F3.25(201)/F3.36(201) aromatic cluster in which W6.48(357) stacks with F3.36(201) (d = 5.3 Å, α = 90°), whereas F3.36(201) stacks with W5.43(280) (d = 5.6 Å, α = 40°). W5.43(280) also has an off-set parallel stack with Y5.39(276) (d = 5.9 Å, α = 0°), whereas Y5.39(276) stacks with W4.64(256) (d = 6.5 Å, α = 90°) (see “Experimental Procedures” for definitions of d and α).

Residue 3.25 (190) does not stack with the other aromatic residues in the TMDH3-4-5-6 region but still appears to be an important residue. In the R bundle, F3.25(190) is directed directly extraacellular to K3.28 and is close enough to K3.28 to be able to form a cation-π interaction with K3.28 (193) (NZ-centroid distance, r = 3.1 Å, CE-centroid distance, r’ = 4.9 Å; see “Experimental Procedures” for definitions of distances). In the R state, the χ₁ for K3.28 (193) and for F3.25(190) is trans.

CB₁ R* State—Fig. 2B illustrates key features of the CB₁ TMD bundle model in the active (R*) state in the TMDH3-4-5-6 region. The conformational changes that occur upon receptor activation result in rotations of TMH3 and -6 as well as a change in the conformation of TMH6 (by modulation of its proline kink angle) (6–8, 10). In our models, both W6.48(357) and F3.36(201) undergo a change in their χ₁ values from R to R*, χ₁ in W6.48(357) changes from g+ to trans and χ₁ of F3.36(201) changes from trans to g+ (see “Experimental Procedures” for definition of χ₁). In the R* TMH bundle, the K3.28(193) and D6.58(367) salt bridge is broken (N-O distance = 16.8 Å) because TMH3 and -6 rotate (counterclockwise from extracellular view) during the R to R* transition (i.e. activation) (26, 32). K3.28(193) has rotated away from D6.58(367) toward TMH2/TMH7, and D6.58(367) has rotated toward the TMH5–6 interface and is raised higher above the ligand-binding pocket due to the moderation of the TMH6 proline kink angle. The W6.48(357)/F3.36(201)/W5.43(280)/Y5.39(276)/W4.64(256) aromatic cluster present in the inactive state in the absence of ligand also undergoes rearrangement, with F3.36(201) no longer part of this cluster. In the TMDH3-4-5-6 region of R* in the absence of ligand, W6.48(357) and W5.43(280) form an off-set parallel aromatic stacking interaction with each other (d = 4.9 Å, α = 30°). W5.43(280) also stacks with Y5.39 (d = 6.6 Å, α = 60°), whereas Y5.39 stacks with W4.64 (d = 5.7 Å, α = 90°). This series of aromatic stacking interactions results in a large aromatic stack in R* comprised of W6.48(357)/W5.43(280)/Y5.39(276)/W4.64(256). F3.36(201) (χ₁ = g+) is not located near another TMDH3-4-5-6 aromatic residue in the R* bundle, instead F3.36(201) is bounded by V3.40(205), V3.32(197), and L6.44(353).

As stated above, in the R* state, the rotation of TMH3 upon activation causes K3.28(193) to point toward TMH2/TMH7, and because F3.25(190) is one turn above K3.28(193), it also now faces the TMH2/TMH7 region. The χ₁ for both K3.28(193) and F3.25(190) is g+ in the R* bundle.
activity of GIRK1/4 was evaluated between F3.36A(201) and WT CB1 (Fig. 8 and Table IV). Although the amount of receptor expressed in oocytes used for the GIRK assay cannot be determined, the $B_{\text{max}}$ values for the WT and F3.36(201)A cell lines used for the GTPyS assay were not statistically different from one another (WT CB1 $B_{\text{max}} = 4.4$ (3.5–5.3) pmol/mg; F3.36(201)A $B_{\text{max}} = 5.2$ (3.6–6.9) pmol/mg; Table I). Thus, the 73% increase (relative to WT) in the level of GTPyS binding seen in the HEK cells stably transfected with F3.36A(201) cannot be attributed to an overexpression of mutant receptor protein (Table I). These combined data strongly suggested the F3.36A(201) mutant receptor had even greater constitutive activity than WT CB1. To further support this hypothesis, the properties of the mutant receptor were evaluated using the CB$_{1}$-selective inverse agonist SR141716A. An inverse agonist should reduce the basal activity of the constitutively activated mutant receptor because the inverse agonist will force the receptor to adopt an inactive conformation; this was the case (Figs. 3E and 7). Furthermore, compared with WT CB1, the inverse agonist response of SR141716A at F3.36A(201) was significantly increased (Fig. 3E). No inverse agonism was produced at WT CB1 except at the highest (5 M) concentration (2% $^{a}$, n = 3) (Fig. 3E). However, inhibition of GTPyS binding occurred with F3.36A(201)A in the presence of nanomolar concentrations of SR141716A with EC$_{50}$ and $E_{\text{max}}$ values of 0.6 (0.1–3.2) nM and −24 (−30 to −18) %, respectively.

Modeling studies also suggest that the F3.36A(201)A mutant should be constitutively active, if it is assumed that changes in
The substantial reductions in potency, in two cell systems, may be due in part to the decreased affinity of WIN 55,212-2 at W5.43(280).A. The affinity of WIN 55,212-2 at W5.43(280)A was reduced 16.8-fold in HEK cells, whereas the affinity of CP55,940 and anandamide was unaffected (Table I) (1). Because the affinities of CP55,940 and anandamide were unaffected at this mutation, the functional responses produced by these ligands were tested at W5.43(280)A to determine whether the effects observed in the presence of WIN 55,212-2 were primarily a result of a reduction in affinity at the mutant receptor. As shown in Fig. 4, C and D, there was a substantial reduction in the potency of both anandamide and CP55,940 at W5.43(280)A. It is clear in Table I, however, that the $B_{max}$ of WIN 55,212-2 at W5.43(280).A mutant was statistically lower than that of WT CB1. Lower receptor expression levels can produce a right-shift in plots of response versus log[ligand] compared with a system in which a greater amount of receptor protein is expressed, despite the fact that the ligand has equal affinity in both systems. Thus, the reduced expression level of the W5.43(280)A mutant compared with WT CB1, may explain the rightward shifts in Fig. 4, C and D, seen for anandamide and CP55940 (40). There were no significant changes in agonist-independent activation of W5.43(280)A in either cell system tested (Figs. 7 and 8 and Table IV).

W6.48(357)—The EC$_{50}$ values reported in Tables II and III show that the potency of WIN 55,212-2 at the W6.48(357)A mutant was reduced 12- and 1.8-fold when this receptor was studied using GTP$^\gamma$S binding or by measuring enhancement of GIRK channel activity, respectively (Fig. 5, A and B, and Tables II and III). However, the loss of potency observed with GIRK channel activity was not significant. The effects on potency may be due to the decreased affinity of WIN 55,212-2 at W6.48(357)A. The affinity of WIN 55,212-2 at W6.48(357)A was reduced 3.8-fold in HEK cells, whereas the affinity of CP55,940 and anandamide was unaffected (Table I) (1). Because the affinity of CP55,940 and anandamide was unaffected at this mutation, the functional responses produced by these ligands were tested at W6.48(357). As shown in Fig. 5, C and D, and Tables II and III, there was no significant reduction in the potency of either CP55,940 or anandamide at W6.48(357). These data suggest the loss in potency observed for WIN 55,212-2 stimulation of GTP$^\gamma$S binding at W6.48(357)A was the result of the selective loss of affinity for WIN 55,212-2 at this mutant receptor. It should be noted that a comparison of the shifts in potency for WIN 55,212-2 at both W6.48(357)A and W5.43(280)A demonstrate the GTP$^\gamma$S assay is more sensitive to this observable effect compared with studying GIRK channel activity (Figs. 4, A and B and Fig. 5, A and B, and Tables II and III).

Most interestingly, the maximum stimulation of GTP$^\gamma$S activity produced by WIN 55,212-2 at W6.48(357)A was significantly lower than that of W5.43(280)A. This is consistent with the decreased affinity of WIN 55,212-2 at W6.48(357)A compared with WT CB1, and suggests that the reduced efficacy of WIN 55,212-2 at W6.48(357)A may be due to decreased efficacy at this site. Therefore, the reduced efficacy of WIN 55,212-2 at W6.48(357)A compared with WT CB1, may explain the rightward shifts in Fig. 5, C and D, and Tables II and III (40). There were no significant changes in agonist-independent activation of W5.43(280)A in either cell system tested (Figs. 7 and 8 and Table IV).

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cantly enhanced compared with WT CB₁ (Fig. 5A and Table II). To determine whether this effect was specific for aminoalkylindoles, the bicyclic cannabinoid CP55,940 was also tested at W6.48(357)A. As observed with WIN 55,212-2, CP55,940 also produced enhanced stimulation of GTP\textsubscript{S} activity at W6.48(357)A compared with WT CB₁ (Fig. 5D). The enhanced activity of WIN 55,212-2 at W6.48(357)A was not observed when GIRK channel activity was measured (Fig. 5B).

There were no significant changes in agonist-independent activation of W6.48(357)A in either cell system tested (Figs. 7 and 8 and Table IV). Consistent with unchanged constitutive activity, the response to the inverse agonist at W6.48(357)A was not different from the response of WT CB₁, to the inverse agonist (Fig. 5E). Modeling studies also suggest that the W6.48(357)A mutant should not be constitutively active, if it is assumed that changes in the TMH3-4-5-6 aromatic cluster influence the state preference for the receptor. The W6.48A mutation will affect the TMH3-4-5-6 aromatic cluster in both the R and R* states. In the R state, the W6.48A mutation will result in the loss of one aromatic stacking interaction, i.e. the W6.48(357)/F3.36(201)/W5.43(280)/Y5.39(276)/W4.64(256) cluster in R will become a F3.36(201)/W5.43(280)/Y5.39(276)/W4.64(256) cluster in the mutant. In the R* state, the W6.48(357)/W5.43(280)/Y5.39(276)/W4.64(256) cluster present in the R* state will become a W5.43(280)/Y5.39(276)/W4.64(256) cluster in the mutant. Because aromatic stacking in both the R and R* states will be equally impacted, it is reasonable to expect that the W6.48A mutant will not produce a change in basal levels for the W6.48A mutant relative to WT CB₁, and this is what was seen experimentally.

F3.25(190)A—We previously reported that F3.25(190) is not part of the binding site of WIN 55,212-2, SR141716A, or CP55,940 but is a part of the anandamide-binding pocket (1). When F3.25(190) was mutated to an alanine, a 6-fold reduction in binding affinity was observed in the presence of anandamide, but no changes were observed in the presence of WIN 55,212-2, SR141716A, and CP55,940 (Table I). When GIRK channel activity was measured there was a significant reduction in both the potency and efficacy of anandamide at F3.25(190)A but no change in the presence of WIN 55,212-2 (Fig. 6, A and B, and Table III).

This result correlates with the binding data and receptor modeling. The effect seen with anandamide is likely to be related in part to the decreased affinity of anandamide for the F3.25(190)A mutant, as F3.25(190) is part of the anandamide-binding site (1). Anandamide stimulation of GTP\textsubscript{S} turnover, in HEK cells transfected with F3.25(190)A, could not be measured because the partial agonist nature of the endogenous ligand results in a nonsignificant amount of GTP\textsubscript{S} stimulation (41). Therefore, the findings with anandamide and the GIRK channel activity could not be compared with GTP\textsubscript{S} stimulation because anandamide did not produce a significant functional response in HEK cells. There was a 6.2-fold decrease...
in the potency of WIN 55,212-2 when receptor activation was assessed by measuring GTP$^\gamma$S activity.

These agonists do not exhibit affinity changes from WT in the F3.25(190)A mutant. However, the effects observed on potency and/or efficacy reported here for WIN 55212-2 at the F3.25(190)A mutant may be related to the general role F3.25 plays in positioning K3.28 in the TMH bundle. As is clear from Fig. 2, F3.25(190) is just above K3.28 in the inactive state of CB1 and has a cation-$\pi$ interaction ($r = 5\, \text{Å}$) with this residue. Gallivan and Dougherty (29) have reported that 14.5% of all cation-$\pi$ interactions found in the “Protein Data Bank Select” list of Sander and co-workers (42, 43) represent such Lys-Phe interactions. Our modeling studies suggest that these residues move in concert with each other, as they both have trans$_i^4$ in the R state and g$_i^1$ in the R$^*$ state. F3.25(190) may act as a chaperone of K3.28 to position it in the correct location in R$^*$ for ligand interaction and to shield it from the extracellular milieu in the R state. Because residue positions in TMH3 R$^*$ may be changed in the absence of aromaticity at position 3.25, it is possible that ligands that do not bind to F3.25(190) can have their potency and/or efficacy affected by the F3.25(190)A mutation.

There were no significant changes in agonist-independent activation of F3.25(190)A in either cell system tested (Fig. 7 and Fig. 8 and Table IV). F3.25(190) is not part of the TMH3-4-5-6 aromatic cluster that characterizes the R and R$^*$ states of CB$_1$ (see Fig. 2). Therefore, the mutation of this aromatic residue to a nonaromatic residue would not be expected to affect basal levels.

**Toggle Switch Residues**

Fig. 9 illustrates the relationship between F3.36(201) and W6.48(357) in the R and R$^*$ states of CB$_1$. In the context of the inactive state model (see Fig. 9, left), F3.36(201) ($\chi_1 = \text{trans}$) is located opposite W6.48(357) ($\chi_1 = \text{g}^+$) and has an aromatic stacking interaction with W6.48(357) that would prevent the $\chi_1$ of W6.48(357) from changing from $\text{g}^+$ to trans, thus stabilizing TMH6 in its inactive conformation. In the active state of CB$_1$, F3.36(201) and W6.48(357) change conformations ($\chi_1 = \text{trans}/W6.48(357)/\chi_1 = \text{g}^+) \rightarrow F3.36(201)/W6.48(357)/\chi_1 = \text{g}^+/W6.48(357)/\chi_1 = \text{trans}$) in order to rotate past each other and $\chi_1$ and W6.48(357) are located too far apart in R$^*$ to interact with each other (see Fig. 9, right).

**Conformational Memories Study of WT TMH6 Versus W6.48A TMH6—TMH6 in the class A GPCRs contains the highly conserved sequence motif, CWXP. This proline containing region of TMH6 is thought to act as a flexible hinge during GPCR activation (44). Proline residues are known to perturb the structure of helices by introducing a kink between the segments preceding (pre-proline helix) and following the proline residue (post-proline helix). The distortion of the helical structure results from the avoided steric clash between the ring of the proline at position $i$ and the backbone carbonyl at position $i - 4$, as well as the elimination of helix backbone...
hydrogen bonds for the carbonyls at positions (i − 3) and (i − 4). Both the departure from the ideal helical pattern and the reduction in H-bond stabilization contribute to the observed

flexibility of a proline-containing α-helix. Table V lists the results of ProKink analyses of WT TMH6 CM output and the W6.48A TMH6 CM output. This analysis yields values not only

FIG. 6. Comparison of WT (■) CB₁ and F3.25(190)A (▲) receptor activation. Anandamide (A) and WIN 55,212-2 (B) were used for concentration-response analysis in oocytes co-expressing GIRK1/4 and CB₁ (WT or mutant) cRNAs. The amount of RNA injected and the recording protocol are described under the “Experimental Procedures.” Each data point in the concentration-response curve is the mean ± S.E. of 4–15 determinations made from at least two batches of oocytes. C, WIN 55,212-2 was used for concentration-response analysis of [35S]GTPγS binding in HER cell membranes expressing wild type or mutant receptor protein. Each data point shown is the mean ± S.E. of at least three independent experiments performed in triplicate.

FIG. 7. Agonist-independent activation of WT CB₁ and mutant receptors was assessed by measuring basal turnover of [35S]GTPγS binding. (SR) indicates reduction of basal [35S]GTPγS binding in the presence of 1 µM SR141716A. Data shown are the mean ± S.E. of at least three experiments performed in triplicate. The * indicates statistically significant differences from wild type (p < 0.05).

FIG. 8. Agonist-independent activity of GIRK1/4 channels was assessed by measuring Iₐ, in oocytes expressing WT or mutant CB₁ receptors. A large number of oocytes (n = 20–46) were analyzed for each condition from at least two batches of oocytes. The amount of RNA injected and the recording protocol are described under the “Experimental Procedures.” The * indicates statistically significant differences from wild type (*, p < 0.05, one-way analysis of variance).
for the bend angle of this proline-containing helix but also the wobble angles and face shifts for these helices. The bend angle is the angle between the two parts when the helix is kinked along its axis. The wobble angle is the angle that defines the orientation of the post-proline helix in three-dimensional space, with respect to the pre-proline helix. The face shift measures the distortion that causes a twisting of the helix “face” in such a way that amino acids that used to be on the same side (face) of the helix are shifted and are on different sides of the helix as a result of the bend.

The results of conformational memories calculations on WT CB₁ and the W6.48A mutant are summarized in Table V. In each case, Δ-cluster identified two major clusters of conformers. The first cluster contained TMH6s with large bend angles, whereas the second cluster contained TMH6s with straighter helices (i.e. smaller bend angles). Because TMH6 is more kinked in the inactive state (R) and straightens in the activated state (R*), these two clusters are labeled R and R* in Table V (12). The ProKink program (20) was used to analyze each helix within each cluster in terms of bend angle, wobble angle, and face shift and to compute averages and standard deviations for each cluster.

For WT CB₁, cluster 1 contained 40 members with an average proline bend (kink) angle of 75.9 ± 1.0°, whereas cluster 2 contained 51 members with average bend angle of 33.7 ± 1.1°. The TMH6 used in our CB₁ R model was selected from the more bent cluster, cluster 1, whereas the TMH6 used in our CB₁ R* model was selected from the straighter cluster, Cluster 2 (see “Experimental Procedures”). For the TMH6 W6.48A mutant, cluster 1 contained 19 members with an average proline bend (kink) angle of 74.0 ± 1.0°, whereas cluster 2 contained 72 members with an average proline bend angle of 36.8 ± 1.2°.

At the 0.01 level, the difference of population means for the kink, wobble, and face shift angles for the W6.48A mutant reported in Table V were not significantly different from the corresponding measures in WT CB₁, except for the R* wobble angle. Here the W6.48A R* wobble angle (−105.6 ± 3.4°) was found to be significantly different from the WT R* wobble angle (−120.5 ± 4.6°) at the 0.01 level. Fig. 10 illustrates the steric consequence of this 15° difference in wobble angle. In the R to R* transition, the salt bridge between R3.50 and D6.30 is thought to be broken via a conformational change in TMH6 mediated by the flexible hinge region (CWXP motif) of TMH6. Fig. 10 shows that D6.30 in the W6.48A mutant is capable of pulling further away from the intracellular end of TMH3 and R3.50 than is D6.30 in WT TMH6.

**DISCUSSION**

Mutation of the toggle switch residues, F3.36(201) and W6.48(357), yielded perhaps the most interesting results of all aromatic microdomain mutations reported here. Mutation of F3.36(201) resulted in elevated basal signaling (increased constitutive activity) and reduced ligand efficacies. Mutation of W6.48(357) resulted in unaltered basal signaling but greatly enhanced ligand efficacies. These results are discussed below.

**F3.36(201)A Mutation—**F3.36(201) is revealed here to be a key residue both for ligand binding and for CB₁ activation. One of the significant results in the work reported here is that although WT CB₁ and the F3.36(201)A mutant have statistically equivalent protein expression levels in HEK293 cells (see Table I), an F3.36(201)A mutation results in a statistically significant higher level of ligand-independent activation of CB₁ (i.e. higher basal levels, increased constitutive activity) as assessed by [³⁵S]GTPγS binding. In addition to the demonstration of elevated basal levels, another way to test for constitutive activity in a mutant receptor is to examine the effects on basal signaling produced by an inverse agonist. If the receptor is constitutively active, then the inverse agonist should be able to reduce basal levels (i.e. reduce [³⁵S]GTPγS binding). We have shown here by assessing basal turnover of [³⁵S]GTPγS binding, that the CB₁ inverse agonist, SR141716A reduces basal signaling in the F3.36(201)A mutant, and this effect is statistically significant (p < 0.05) (see Fig. 7 and Table IV). Another traditional expectation of constitutively active receptors is that the affinities of agonists should increase, and the affinities of inverse agonists should decrease due to the shift in the receptor population toward R*. The majority of GPCR mutations that have led to constitutive activity have been mutations in the intracellular half-of the TMH bundle, away from the ligand-binding pocket. Very typically, these have been mutations to TMH6 (45). However, the mutation that produced an increase in constitutive activity here, F3.36(201)A, involves a key residue in the ligand-binding pocket. For this reason, measured ligand affinities for agonists, for example, would be the result of two opposing factors as follows: an increase in affinity due to the higher percentage R* in the F3.36(201)A mutant, but a
constitutive activity here. It is not appropriate to use a shift in ligand affinities as a test for actions and/or in the binding pocket itself. For this reason, it is binding site) due to a change in the ligand-binding site inter-
relative to R3.50(215) in the W6.48A mutant (yellow).

The W6.48(357)A and F3.36(201)A mutants (see Table V). Statistically, these two clusters differed only in their wobble angles (see Table V). The view is through the plane of the proline kink. Here it is clear that the wobble angle difference between the two groups results in a change in the three-dimensional placement of the intracellular ends of these two clusters. Right, a single helix from the WT R* cluster (bend angle = 33.8° and wobble angle = −120.9°) and a single helix from the W6.48A R* cluster (bend angle = 35.5° and wobble angle = −105.1°) are shown here superimposed at their extracellular ends and positioned inside the R* bundle. These helices have similar bend angles but differ by 15.8° in their wobble angles. The receptor state symbolized in the figure is the activated (R*) state. The clockworkwise (from extracellular perspective) rotations of TMH3 and -6 and the straightening of TMH6 upon activation result in the loss of the R3.50(215)/D6.30(339) salt bridge as R3.50(215) and D6.30(339) move away from each other, opening a space between TMH3 and -6 at their intracellular ends. It is clear here that the change in wobble angle between the WT TMH6 (green) and W6.48A mutant TMH6 (yellow) results in a greater displacement of D6.30(339) relative to R3.50(215) in the W6.48A mutant (yellow). decrease in affinity (some likely more than others depending on binding site) due to a change in the ligand-binding site interactions and/or in the binding pocket itself. For this reason, it is not appropriate to use a shift in ligand affinities as a test for constitutive activity here.

The W6.48(357)/F3.36(201) Toggle Switch in WT CB₁—In recently published Monte Carlo/stochastic dynamics calculations on the inactive state of CB₁, we found that one of the most persistent aromatic stacking interactions in the inactive state of CB₁ is the F3.36(201)/W6.48(357) interaction (46). Models of the CB₁ inactive (R) and active (R*) TMH bundles illustrated here in Fig. 9 show that in the inactive state residues W6.48(357) and F3.36(201) (X₁ = trans) are engaged in a direct aromatic stacking interaction. In this interaction, F3.36(201) appears to serve the function of confining W6.48(357) to the g+ X₁ rotamer state and restricting movement of W6.48. In Rho, the β-ionone ring of 11-cis-retinal appears to serve the same function as F3.36(201) in CB₁ (3, 14) (Fig. 11), i.e. it sterically confines W6.48(265) to the g+ X₁ rotamer state and restricts the movement of W6.48. Khorana and co-workers (13) have recently reported one of the specific contacts that must break in Rho for activation involves W6.48(265).

The conformational changes that occur upon receptor activation result in rotations of TMH3 and -6, as well as a change in the conformation of TMH6 (by moderation of its proline kink angle) (6–8,12). In the light-activated state of Rho, the β-ionone ring moves away from TMH F and toward TMH D where it resides close to A4.58(169) (14). This movement releases the constraint on W6.48, making it possible for W6.48(265) to undergo a conformational change. For the CB₁ receptor, clockworkwise (from the extracellular side) rotations of TMH3 and -6 concomitant with activation (26, 32) would move F3.36(201) and W6.48(357) past each other, with W6.48(357) moving toward the viewer in Fig. 11 and F3.36(201) moving away from the viewer (also see Fig. 9). Our modeling studies of the CB₁ TMH bundle suggest that these rotations cannot take place without a rotamer change for both W6.48(357) and F3.36(201) due to steric clashing. In our models, activation is accompanied by a X₁ change in W6.48(357) from g+ → trans and a X₁ change in F3.36(201) from trans → g+ (see above and Fig. 9). The W6.48(357)/F3.36(201) interaction may act as the toggle switch for CB₁ activation, with W6.48(357) X₁ g+→F3.36(201) X₁ trans representing the inactive and W6.48(357) X₁ trans/F3.36(201) X₁ g+ representing the active state of CB₁ (see Fig. 9). If this is true, then mutation of F3.36(201) to a smaller residue, a residue that is no longer a steric block to conformational change in W6.48, would be expected to increase ligand-independent activation of CB₁, and this is what is seen here experimentally.

Whereas the F3.36(201)A mutation leads to a high degree of constitutive activity as is indicated in Fig. 7, this mutation also results in the reduced efficacy for WIN and CP. This reduction cannot be explained simply by reduced ligand affinity, as the affinity of CP is not affected by this mutation, but its efficacy is affected. It is possible that because the WT CB₁ receptor itself exhibits a high level of constitutive activity (33–35) and because the F3.36(201)A mutant exhibits an increase in constitutive activity relative to WT CB₁, the population of F3.36(201)A mutant receptors is already shifted heavily toward R*. This should not interfere with agonist binding, as agonists have higher affinity for the R* state. However, this would leave fewer receptors for ligands to activate, resulting in a reduced Eₘₐₓ. This explanation is consistent with that of Milligan (45) in a recent review concerning constitutive activity of GPCRs. Milligan (45) noted that “a number of GPCRs do seem to have significant levels of constitutive activity when expressed in cell lines; in some cases, ligand-induced stimulation of activity is relatively small compared with the signal in the absence of ligand” (45). Alternatively, the profound effect on ligand-dependent activation seen here for the F3.36(201)A mutation may indicate that aromaticity at residue 3.36 is cen-

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**Table V**

Results of conformational memories study of WT versus W6.48(357)A TMH6

<table>
<thead>
<tr>
<th>No. members</th>
<th>Bend angle</th>
<th>Wobble angle</th>
<th>Face shift</th>
</tr>
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<td></td>
<td>degrees ± S.E.</td>
<td>degrees ± S.E.</td>
<td>degrees ± S.E.</td>
</tr>
<tr>
<td>WT</td>
<td>R CLS 1</td>
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<td>75.9 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>R* CLS 2</td>
<td>51</td>
<td>33.7 ± 1.1</td>
</tr>
<tr>
<td>W6.48(357)A</td>
<td>R CLS 1</td>
<td>19</td>
<td>74.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>R* CLS 2</td>
<td>72</td>
<td>36.8 ± 1.2</td>
</tr>
</tbody>
</table>

a CLS, cluster.
b Statistically significant difference from WT at the p = 0.01 level.
reciprocal to WT CB1 (see Fig. 7). Our CB1 modeling studies have resulted in a statistically significant change in basal levels relative to the CB1 agonist-induced activation process and, consequently, that mutation to a nonaromatic residue impairs the function of this mutant CB1.

W6.48(357)A Mutation—The W6.48(357)A mutation did not result in a statistically significant change in basal levels relative to WT CB1 (see Fig. 7). Our CB1 modeling studies have shown that when W6.48(357) is mutated to alanine, helix packing allows F3.36(201) to interact with both A6.48 and L6.51. This interaction would serve the same function as F3.36(201) serves in WT CB1, i.e. effectively helping to stabilize TMH6 in its inactive state conformation. Therefore, these results suggest that basal levels should not change between WT CB1 and the W6.48(357)A mutant. Looking at this from another perspective, consideration of the changes in the aromatic microdomain as a result of this mutation also suggests that aromatic stacking in both the R and R* states will be equally impacted by the W6.48(357)A mutation (see “Results”), it is reasonable then to expect that the W6.48(357)A mutant will not produce a change in basal levels relative to WT CB1, and this is what was seen experimentally.

Although the W6.48(357)A mutation had no effect on basal levels, it had noticeable effects on ligand-induced activation, such that the efficacy of every ligand tested was enhanced. One of the steps that has been proposed to occur during GPCR activation is the breaking of an ionic lock (salt bridge) between R3.50(215) in TMH3, resulting in a wider “blossomed” intracellular end of CB1 in the TMH6 W6.48(357)A mutant. Such a wider opening may permit better coupling between the G protein and the W6.48(357)A mutant upon ligand activation, thus resulting in increased $E_{\text{max}}$. Values.

This phenomenon of dramatic enhancement of $E_{\text{max}}$ values upon mutation of residue W6.48(357) has been seen previously in the CCK-B gastrin receptor (49). Blaker and co-workers (49) found that whereas a W6.48(346)A mutation did not affect basal inositol phosphate production, this mutation affected the functional activity of PD-135,158 (from 20 ± 1% for WT CCK-B to 43 ± 5% in the W6.48(346)A mutant). An enhancement of $E_{\text{max}}$ has also been seen previously for mutations at other loci. For example, in the 6-opioid receptor, Beftor and co-workers (50) found that a Y308F mutation in TMH7 resulted in a marked increase in the efficacy of the potent agonist BW373U86. Consistent with the interpretation of the CB1 W6.48(357)A mutation discussed above, these authors (50) attributed this increase to a change in the receptor active state conformation, such that the receptor can interact with heterotrimeric G proteins more effectively.

Conclusions—Modeling, mutation, and functional studies undertaken to test the importance of the TMH3-4-5-6 aromatic microdomain in ligand recognition and in the conformational changes that accompany activation of CB1 suggest that a F3.36(201)/W6.48(357)-specific contact is an important constraint for the CB1-inactive state that may need to break during activation. Modeling studies suggest that the F3.36(201)/W6.48(357) contact can exist in the inactive state of CB1, and be broken in the activated state via a 1α rotamer switch (F3.36(201) trans, W6.48(357) g+) → (F3.36(201) g+, W6.48(357) trans). The F3.36(201)/W6.48(357) interaction, therefore, may represent a toggle switch for activation of CB1.

REFERENCES

21. Deleted in proof
Structural Mimicry in Class A G Protein-coupled Receptor Rotamer Toggle Switches: THE IMPORTANCE OF THE F3.36(201)/W6.48(357) INTERACTION IN CANNABINOID CB1 RECEPTOR ACTIVATION

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