Novel Insights into CB$_1$ Cannabinoid Receptor Signaling: A Key Interaction Identified between the Extracellular-3 Loop and Transmembrane Helix 2$^{3}$

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Received October 22, 2012; accepted February 19, 2013

ABSTRACT

Activation of the cannabinoid CB$_1$ receptor (CB$_1$) is modulated by aspartate residue D2.63$^{176}$ in transmembrane helix (TMH) 2. Interestingly, D2.63 does not affect the affinity for ligand binding at the CB$_1$ receptor. Studies in class A G protein-coupled receptors have suggested an ionic interaction between residues of TMH2 and 7. In this report, modeling studies identified residue K373 in the extracellular-3 (EC-3) loop in charged interactions with D2.63. We investigated this possibility by performing reciprocal mutations and biochemical studies. D2.63$^{176}$A, K373A, and the reciprocal mutant with the interacting residues juxtaposed D2.63176K-K373D were characterized using radioligand binding and guanosine 5'-3-[2-hydroxypropyl]triphosphate functional assays. None of the mutations resulted in a significant change in the binding affinity of N-(piperidiny-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride (SR141716A) or (−)3-cis-[2-hydroxy-4-(1,1-dimethyl-2-heptyl)phenyl]-trans-4-[3-hydroxyethyl-propyl] cyclohexan-1-ol (CP55,940). Modeling studies indicated that binding-site interactions and energies of interaction for CP55,940 were similar between wild-type and mutant receptors. However, the signaling of CP55,940, and (R)-(+)[2,3-dihydro-5-methyl-3-[4-morpholino][methyl]-pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl][1-naphthalenyl]-methanone mesylate (WIN55,212-2) was impaired at the D2.63$^{176}$A-K373A and the single-alanine mutants. In contrast, the reciprocal D2.63$^{176}$K-K373D mutant regained function for both CP55,940 and WIN55,212-2. Computational results indicate that the D2.63$^{176}$K-K373D ionic interaction strongly influences the conformation(s) of the EC-3 loop, providing a structure-based rationale for the importance of the EC-3 loop to signal transduction in CB$_1$. The putative ionic interaction results in the EC-3 loop pulling over the top (extracellular side) of the receptor; this EC-3 loop conformation may serve protective and mechanistic roles. These results suggest that the ionic interaction between D2.63$^{176}$ and K373 is important for CB$_1$ signal transduction.

Introduction

The cannabinoid CB$_1$ receptor (CB$_1$), a member of the class A rhodopsin-like family of G protein-coupled receptors (GPCRs) (see Fig. 1), is found primarily in the central nervous system (CNS) and is important in the regulation of neuronal activity. In addition, there is evidence that the CB$_1$ receptor is expressed in peripheral tissues (albeit to a lesser extent), including the adrenal gland, bone marrow, heart, lung, and prostate (Howlett et al., 2002). The CB$_1$ receptor, a G/o coupled GPCR binds five structurally diverse classes of ligands; these include the endocannabinoids (typified by anandamide and 2-arachidonoylglycerol), the classic and nonclassic cannabinoids (typified by δ-9-tetrahydrocannabinol and CP55,940 [(−)cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-[3-hydroxyethyl-propyl] cyclohexan-1-ol], respectively), the aminoaalkylindoles (typified by WIN55,212-2 [R]-(+)[2,3-dihydro-5-methyl-3-[4-morpholino][methyl]-pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl][1-naphthalenyl]-methanone mesylate), and the diarylpyprazole antagonists/inverse agonists (typified by SR141716A [N-(piperidiny-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride]) (see Fig. 2) (Picone et al., 2002).

Considering its fundamental role in the CNS, it is not surprising that the CB$_1$ receptor has been reported to mitigate numerous pathologies, including Alzheimer’s disease, cancer, obesity, and pain (Pertwee, 2009). Unfortunately, many attempts

ABBREVIATIONS: $E_{\text{max}}$, maximal binding; CB$_1$, cannabinoid CB$_1$ receptor; CL, confidence limit; CP55,940, (−)cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-[3-hydroxypropyl] cyclohexan-1-ol; EC, extracellular loop; $E_{\text{max}}$, maximal effective response; GPCRs, G protein-coupled receptors; GTP$\gamma$S, guanosine 5’-3-O-(thiophosphoryl)triphosphate; HEK, human embryonic kidney; IC loop, intracellular loop; $K_{\text{s}}$, equilibrium dissociation constant; $K_{i}$, inhibitory constant; SR141716A, N-(piperidiny-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride; TMH, transmembrane helices; WIN55,212-2,[R]-(+)[2,3-dihydro-5-methyl-3-[4-morpholino][methyl]-pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl][1-naphthalenyl]-methanone mesylate; WT, wild type.

This work was supported by the National Institutes of Health National Institute on Drug Abuse [Grants DA003934, DA021358, DA023204, and DA09158].

http://dx.doi.org/10.1124/jpet.112.201046.

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http://jpet.aspetjournals.org/content/suppl/2013/02/20/jpet.112.201046.DC1

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at harnessing the therapeutic potential of the CB₁ receptor have failed due to unacceptable CNS-related side effects, such as euphoria, depression, and suicidal fixation (Christopoulou and Kiortsis, 2011). Clearly, a better understanding of the CB₁ receptor’s signal transduction mechanism(s) at a molecular level would be useful in realizing this receptor’s therapeutic potential.

Traditionally, the high degree of sequence homology of amino acid residues from transmembrane helices (TMHs) of different GPCRs has led to the identification of conserved residues, which have been shown to be crucial for receptor function using biochemical studies (Tao and Abood, 1998). In addition, charged interactions between amino acid residues from different TMH domains have been shown to be essential for either ligand binding or receptor function (Zhou et al., 1994; Sealfon et al., 1995; Xu et al., 1999). Residues from the extracellular (EC) loops demonstrate low sequence homology (Peeters et al., 2011b) and were initially thought to connect the TMH domains rather than to have a direct role in receptor functioning.

However, recent studies have demonstrated the critical role of the EC loops to ligand binding and receptor signaling. Mutation studies have demonstrated that the first EC loop (EC-1 loop) is important to the activation of the adenosine A₂B receptor (Peeters et al., 2011a). The second EC loop (EC-2 loop) has been shown to be important in ligand binding and activation at the V₁a vasopressin receptor (Connor et al., 2007), to be important to helix movement in rhodopsin (Ahuja et al., 2009), and to be involved in the binding of allosteric modulators at the M₂ acetylcholine receptor (Avlani et al., 2007). Less is known about the third EC loop (EC-3 loop); however, a key salt bridge between the EC-3 and EC-2 loops has been observed to influence ligand binding and receptor activation at the β₂-adrenergic receptor (Bokoch et al., 2010). The EC-1 and EC-2 loops of the CB₁ receptor (Murphy and Kendall, 2003; Ahn et al., 2009a; Bertalovitz et al., 2010) have been better characterized than its EC-3 loop. EC-3 loop modeling studies reported here suggest that the EC-3 loop residue K373 may form a functionally-important ionic interaction with a transmembrane residue, D2.63 (176). Our previous D2.63 (176) mutation studies have demonstrated that the negative charge of D2.63 (176) is critical for agonist efficacy but not ligand binding at the CB₁ receptor (Kapur et al., 2008). We hypothesized that this functional requirement (of a negatively charged residue at 2.63 (176)) may be due to this residue’s participation in an ionic interaction with K373 that is necessary for signal transduction. To test this hypothesis, three mutations that would disrupt this putative interaction, D2.63 (176)A, K373A, and D2.63 (176)A-K373A, and a charge-reversal mutation D2.63 (176)K-K373D that would restore the interaction were evaluated for their impact on ligand binding and agonist efficacy. The binding affinities for CP55,940 and SR141716A were not significantly affected by any of the mutations. However, the efficacy of CP55,940 and WIN55,212-2 was markedly reduced by the alanine-substitution mutations, while the charge-reversal mutation led to partial rescue of wild-type (WT) levels of efficacy. Computational results indicate that the D2.63 (176)-K373 ionic interaction strongly influences the conformation(s) of the EC-3 loop, providing a structure-based rationale for the importance of the EC-3 loop to signal transduction in CB₁. Specifically, the putative ionic interaction results in the EC-3 loop pulling over the top (EC side) of the receptor; this EC-3 loop conformation may serve protective and mechanistic roles.

Our results have for the first time identified an interaction between the residues from TMH2-EC3, suggesting the proximity of these two domains and their role in modulating CB₁ signal transduction.

**Materials and Methods**

**Materials**

[3H]CP55,940 (160-180 Ci/mmol) and [35S]GTPγS (guanosine 5′-3-O-(thio)triphosphate; 1250 Ci/nmol) were purchased from PerkinElmer (Boston, MA). WIN55,212-2, CP55,940, and SR141716A were obtained from Tocris (Ellisville, MI). The Pfu Turbo DNA polymerase for mutagenesis experiments was from Stratagene (La Jolla, CA). All
other reagents were obtained from Sigma-Aldrich (St. Louis, MO) or other standard sources. The CB$_1$ antibody was kindly provided by Ken Mackie (Indiana University).

**Amino Acid Numbering**

The numbering scheme suggested by Ballesteros and Weinstein (1995) was employed here. In this system, the most highly conserved residue in each TMH is assigned a locant of 0.50. This number is preceded by the TMH number and followed by the absolute sequence number in superscript. All other residues in a TMH are numbered relative to this residue. The sequence numbers used are human CB$_1$ sequence numbers unless otherwise noted (Bramblett et al., 1995).

**Mutagenesis and Cell Culture**

The D2.63$^{776}$A, K373A, D2.63$^{776}$A-K373A, and D2.63$^{776}$K-K373D mutants of the human CB$_1$ in the vector pcDNA3 were constructed using the QuikChange site-directed mutagenesis kit (Stratagene). The mutagenic oligonucleotides used were between 27 and 33 base pairs long. Restriction endonuclease digestion and DNA sequencing subsequently confirmed the presence of the mutation. Stably transfected human embryonic kidney (HEK)-293 cell lines were created by transfection with WT or mutant CB$_1$-pcDNA3 cDNA by the lipofectamine reagent (Invitrogen, Carlsbad, CA) and selected in growth medium containing geneticin (1 mg/ml), as previously described elsewhere (McAllister et al., 2003).

**Radioligand Binding and GTP$\gamma$S Binding Assay**

Protein membrane preparations harvested from stably transfected HEK293 cells were prepared and assayed as previously described elsewhere (Kapur et al., 2007). In brief, binding assays (saturation and competition binding assays) were initiated by the addition of 50 $\mu$g membrane protein to glass tubes pretreated with siliconizing fluid (Pierce, Rockford, IL; to reduce nonspecific binding) containing [3$\mathrm{H}$]SR141716A, and an appropriate volume of binding buffer A (50 mM Tris-Base, 1 mM EDTA, 3 mM MgCl$_2$, and 5 mg/ml bovine serum albumin, pH 7.4) to bring the final volume to 500 $\mu$l. Nonspecific binding was assessed in the presence of excess (1 $\mu$m) unlabeled SR141716A. Reactants were allowed to reach equilibrium (~1 hour). Subsequently, free and bound radioligand were separated by vacuum filtration through Whatman GF-C filters, and the radioactivity retained on the filters was quantified by a liquid scintillation counter.

The $K_d$ (equilibrium dissociation constant) and $B_{\text{max}}$ (maximal binding) values were determined by analyzing the saturation binding data by nonlinear regression and fitted to a one-site binding model using GraphPad Prism 4.0 software (GraphPad, San Diego, CA). The displacement log IC$_{50}$ values were determined by nonlinear regression and fitting the data to one-site competition and then were converted to $K_i$ (inhibitory constant) values using the Cheng and Prusoff method (Cheng and Prusoff, 1973) with the use of GraphPad Prism.

The GTP$\gamma$S assay was initiated by the addition of 20 $\mu$g of membrane protein into silanized glass tubes containing 0.1 nM [3$\mathrm{S}$]GTP$\gamma$S, 10 $\mu$g GDP in GTP$\gamma$S binding buffer (50 mM Tris-Base, 1 mM EDTA, 3 mM MgCl$_2$, and 0.2 mM EGTA, and 0.1% bovine serum albumin, pH 7.4). Nonspecific binding was assessed in the presence of 20 $\mu$g unlabeled GTP$\gamma$S. Free and bound radioligand were separated, and bound radioactivity was quantified as described previously. Nonlinear regression of log concentration values versus the percentage effect fitted to sigmoidal dose-response was used to obtain estimates of agonist concentrations that elicit half the maximal response ($EC_{50}$) and maximal response ($B_{\text{max}}$).

**Statistical Analyses**

Data are reported as the mean value of the replicates along with their 95% confidence limits (CL). The $K_d$ and log $EC_{50}$ values in the mutant and WT CB$_1$ receptors were compared using one-way analysis of variance with Bonferroni multiple comparison post tests. $P<.05$ was considered statistically significant.

**Molecular Modeling**

**Receptor Model Construction Protocol for Loop Calculations.**

*Wild-type CB$_1$ activated (R*) receptor model construction.* Using interactive computer graphics, extracellular (EC-1; R145–R150, IC-2 F221–T229, and IC-3 S303–M336) were manually added to our previously constructed TMH bundle model of the CB$_1$ R$_{3}$ (active state) receptor, with CP55,940 docked in its global minimum energy conformation (Kapur et al., 2007). The program Modeler was then used to refine loop structures (Sali and Blundell, 1993; Fiser et al., 2000). Because of their close spatial proximity, the conformations of all three EC loops were calculated together followed by calculation of the three IC loop conformations. Chosen loop conformations were those that produced a low value of the Modeler objective function. The loops were minimized in three stages (stages 1 to 3, as described later). Next, portions of the N and C termini were added, and conformations of each were refined in Modeler. The termini were minimized using stages 4 to 5 of the minimization protocol.

**N terminus.** The first 89 residues of the N terminus were truncated, based on results from the Chin laboratory (Anderson et al., 2003) which showed that CP55,940 has WT binding affinity and efficacy at the N-terminal truncated CB$_1$, whereas the receptor has better cell surface expression than WT. X-ray crystal structures of class A GPCRs with lipid-derived endogenous ligands show that the N terminus occludes the binding pocket. In the crystal structure of rhodopsin (Li et al., 2004), the N terminus is positioned centrally, occluding the EC side of the bundle (i.e., the retinal plug). This general placement of the N termination is also observed in the crystal structure of the sphingosine 1-phosphate receptor (Hanson et al., 2012). Because CB$_1$ also has a lipid-derived endogenous ligand, a truncated N-terminal conformation (positioned centrally over the EC side of the receptor) was chosen.

**EC-2 loop.** One of the significant sequence divergences between rhodopsin and CB$_1$ is in the EC-2 loop region. This loop in CB$_1$ is shorter than in rhodopsin and is missing the conserved disulfide bridge between the cysteine in the EC-2 loop and C3.25 in TM3 of rhodopsin. Instead, there is a Cys at the extracellular end of TMH4 in CB$_1$ and a Cys near the middle of the EC-2 loop that experiments suggest may form a disulfide bridge (Fay et al., 2005). Consequently, the position of the EC-2 loop with respect to the binding site crevice in CB$_1$ around TMHs 3, 4, and 5 is likely to be quite different from that in rhodopsin. Therefore, this loop was modeled with an internal C257–C264 disulfide bridge based upon mutation results from the Farrens laboratory (Fay et al., 2005), which show that these two cysteines are required for high-level expression and receptor function.

To guide selection of an appropriate EC-2 loop conformation, we used mutation results from the Kendall laboratory (Ahn et al., 2009a; Bertalovitz et al., 2010), which demonstrate that mutation of EC-2 loop residue F268 to a tryptophan severely damages the binding affinity and efficacy of CP55,940 but has no significant effect on the binding affinity of SR141716A. Thus, an EC-2 loop conformation was chosen that placed F268 in close proximity to CP55,940. A F268W mutant bundle was constructed to verify that this mutation resulted in significant steric overlaps with CP55,940 in our model but not with SR141716A (Supplemental Fig. 1).

**EC-3 loop.** The EC loops were refined by use of Modeler in two stages. In the first stage, no harmonic distance constraints were used. This calculation was performed to examine the general conformational space of the EC-3 loop. The EC-3 loop conformation with the lowest objective function placed the EC-3 loop over the top of the receptor; in addition, the putative ionic interaction between D2.63$^{776}$

[3$\mathrm{S}$]GTP$\gamma$S, 10 $\mu$g GDP in GTP$\gamma$S binding buffer (50 mM Tris-Base, 1 mM EDTA, 3 mM MgCl$_2$, and 0.2 mM EGTA, and 0.1% bovine serum albumin, pH 7.4). Nonspecific binding was assessed in the presence of 20 $\mu$g unlabeled GTP$\gamma$S. Free and bound radioligand were separated, and bound radioactivity was quantified as described previously. Nonlinear regression of log concentration values versus the percentage effect fitted to sigmoidal dose-response was used to obtain estimates of agonist concentrations that elicit half the maximal response ($EC_{50}$) and maximal response ($B_{\text{max}}$).
and K373 had formed. In the second stage, a 3.0 kcal/mol harmonic distance constraint was placed between the EC-3 loop residue K373 and D2.63\(^{176}\). Specifically, the distance between the OD1 atom of D2.63\(^{176}\) and the NZ atom of K373 was constrained to 3.0 ± 2.0 Å. This second calculation was performed to obtain a focused conformational sampling of the EC-3 loop conformation with the lowest objective function (obtained in the first stage of the calculation).

**IC-3 loop.** The CB1 IC-3 loop is much longer than the corresponding sequence in rhodopsin. Nuclear magnetic resonance experiments have been performed on a peptide fragment composed of the CB1 sequence span from the IC end of TMH5 to the IC end of TMH6 in micelles (Uffers et al., 2002). This study suggested that part of the IC-3 loop is α-helical. This region occurs after the IC end of TMH5 (K5.64\(^{40}\)) and consists of a short α-helical segment from A201 to R307, followed by an elbow region (R307–I309) and an α-helical segment (Q310–S316) up to an II sequence (I317–I319) in IC-3. Based on these results, we replaced the initial Modeler-built IC-3 loop segment (Q310–I309) in IC-3 loops with this α-helix-α-helix region, and then the rest of IC-3 loop (I317–P332) was rebuilt and optimized using Modeler.

**C terminus.** A C-terminal fragment S414–G417, which contains a putative palmitoylation site at Cys\(^{415}\) (Fay et al., 2005), was added with this

Based on these results, we replaced the initial Modeler-built IC-3 loop segment (Q310–I309) in IC-3 loops with this α-helix-α-helix region, and then the rest of IC-3 loop (I317–P332) was rebuilt and optimized using Modeler.

**Assessment of Pairwise Interaction and Total Energies**

Interaction energies between CP55,940 and the WT, K373A, D2.63\(^{176}\)A, D2.63\(^{176}\)A-K373A, or D2.63\(^{176}\)K-K373D receptors were calculated using Macromodel (Schrodinger). After defining the atoms of CP55,940 as one group (group 1) and the atoms corresponding to a residue that lines the binding site in the final ligand/CB1 R* complex as another group (group 2), Macromodel was used to output the pairwise interaction energy (coulombic and van der Waals) for a given pair of atoms. The pairs corresponding to group 1 (ligand) and group 2 (residue of interest) were then summed to yield the interaction energy between the ligand and the receptor.

**Results**

The binding of \(^{3}H\)SR141716A to WT and mutant receptors stably expressed in HEK 293 cells was measured to generate an estimate of the \(K_d\) and \(B_{\text{max}}\) values. Similar cell surface expression of WT and mutant cell lines was verified by immunofluorescence staining (unpublished data).

**Radioligand Binding Assays**

Saturation binding analysis of \(^{3}H\)SR141716A at the D2.63\(^{176}\)A, K373A, D2.63\(^{176}\)A-K373A, and D2.63\(^{176}\)K-K373D mutations displayed \(K_d\) (CL) values of 4.2 (0.1–9.8) nM, 1.7 (0.2–3.5) nM, 4.4 (0.1–9.1) nM, and 3.5 (0.1–24) nM, respectively (see Table 1). The \(K_d\) for the WT CB1 receptor was 2.2 (0.4–3.9) nM. The \(K_d\) values for the mutants versus WT were not statistically significantly different. Similarly, the \(B_{\text{max}}\) values for each cell line demonstrated that expression levels of these receptors between the different cell lines were comparable. The cell lines D2.63\(^{176}\)A, K373A, and D2.63\(^{176}\)A-K373A, and D2.63\(^{176}\)K-K373D respective \(B_{\text{max}}\) (CL) values were 2.3 (1.0–3.5) pmol/mg, 1.8 (0.1–3.7) pmol/mg, 2.7 (1.7–3.7) pmol/mg, and 0.7 (0.1–2.4) pmol/mg. The WT CB1 cell line displayed a \(B_{\text{max}}\) of 2.4 (1.9–2.9) pmol/mg.

**Competitive Binding Assays**

We investigated the binding affinity of the bicyclic cannabinoïd agonist CP55,940 to displace \(^{3}H\)SR141716A bound to the WT and mutant hCB1 receptors. The \(K_i\) values between WT and mutant receptors overlapped and were not statistically significantly different (see Fig. 3; Table 2). The \(K_i\) (CL) values for WT, D2.63\(^{176}\)A, K373A, D2.63\(^{176}\)A-K373A, and D2.63\(^{176}\)K-K373D.
D2.63176K-K373D were 17 (5.3–53) nM, 4.9 (0.6–43) nM, 17 (3.3–55) nM, 5.1 (0.63–42) nM, and 15 (3.0–69) nM, respectively.

**Agonist Stimulated GTPγS Binding**

We used [35S]GTPγS binding to measure the stimulation of WT and mutant cannabinoid receptors upon stimulation with different classes of cannabinoid ligands (see Fig. 4; Table 3). The EC50 and Emax values were generated for WT and mutant receptor activation in the presence of CP55,940 and WIN55,212-2. The EC50 values of CP55,940 and WIN55,212-2 at WT CB1 were 12.6 nM and 36.7 nM, respectively. The D2.63176A mutation statistically significantly increased EC50 values for CP55,940 and WIN55,212-2 to 67 nM (5.3-fold) and 231 nM (6.3-fold), respectively, and the maximum agonist responsive-ness was lower. The K373A mutation resulted in similar effects on the EC50 and Emax values. The K373A mutant generated a statistically significant increase in EC50 values from WT for CP55,940 and WIN55,212-2 to 39.8 nM (5.6-fold) and 156 nM (7.5-fold), respectively.

However, when the ionic interaction between D2.63176 and K373 was disrupted by double-alanine substitutions, the receptor activity was severely reduced. The D2.63176A-K373A mutant resulted in dramatic shifts of either or both the EC50 and Emax values and for CP55,940 and WIN55,212-2 to 39.8 nM (Emax = 29%) and 561 nM (Emax = 59%), respectively. The largest shift observed was from WIN55,212-2, 15.3-fold above the WT value. In contrast, the charge-reversal mutant D2.63176K-K373D displayed an EC50 and Emax for WIN55,212-2 of 126 nM and 79%, respectively. Likewise, the D2.63176K-K373D mutant EC50 and Emax values for CP55,940 were 38 nM and 82%, respectively.

**Modeling Studies**

**Modeler Results: EC Loop Conformations in the WT CB1, R* and the D2.63176A, K373A, D2.63176A-K373A, and D2.63176K-K373D Mutants.** As described in Materials and Methods, low-energy WT and mutant loop conformations were added to our previous CB1 R* bundle (Kapur et al., 2007). Consistent with the experimental results, the WT model includes an ionic interaction between D2.63176 and EC-3 loop residue K373 (see Fig. 5). This ionic interaction causes the EC-3 loop to pull across the top (EC side) of the receptor. Clearly, this specific ionic interaction cannot form in the D2.63176A, K373A, or the D2.63176A-K373A mutant. By not forming this ionic interaction, the EC-3 loops of the mutant receptors experience greater conformational freedom. As illustrated in Fig. 5, the modeled loop conformations of D2.63176A, K373A, and D2.63176A-K373A position the EC-3 loop away from the center and are more directly above TMHs 6 and 7. It is noteworthy that these three mutants have very similar EC-3 loop conformations and that these conformations are fundamentally different from the WT EC-3 loop conformation.

Unlike the D2.63176A, K373A, or the D2.63176A-K373A mutant, the D2.63176K-K373D swap mutant can form the putative ionic interaction. In agreement with the experimental results, the model of this mutant includes an ionic interaction between D2.63176 and EC-3 loop residue K373 (see Fig. 6). This ionic interaction causes the EC-3 loop to pull across the top (EC side) of the receptor. As observed in Fig. 6, the WT and the D2.63176K-K373D EC loops have a remarkable degree of conformational similarity in their EC loops. The formation of

**TABLE 1** Radioligand binding properties of wild-type and mutant cell lines

<table>
<thead>
<tr>
<th>Radioligand</th>
<th>Cell Line</th>
<th>Kd (nM)</th>
<th>95% CL</th>
<th>Emax (pmol/ mg)</th>
<th>95% CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]SR141716A</td>
<td>WT hCB1</td>
<td>2.2</td>
<td>(0.4–3.9)</td>
<td>2.4</td>
<td>(1.9–2.9)</td>
</tr>
<tr>
<td>D2.63176A</td>
<td>4.2</td>
<td>(0.1–9.8)</td>
<td>2.3</td>
<td>(1.0–3.5)</td>
<td></td>
</tr>
<tr>
<td>K373A</td>
<td>1.7</td>
<td>(0.2–3.5)</td>
<td>1.8</td>
<td>(0.1–3.7)</td>
<td></td>
</tr>
<tr>
<td>D2.63176A-K373A</td>
<td>4.4</td>
<td>(0.1–9.1)</td>
<td>2.7</td>
<td>(1.7–3.7)</td>
<td></td>
</tr>
<tr>
<td>D2.63176K-K373D</td>
<td>3.5</td>
<td>(0.1–24)</td>
<td>0.7</td>
<td>(0.1–2.4)</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 2** The effects of amino acid mutations of recombinant hCB1 receptors on the displacement of [3H]SR141716A by CP55,940

<table>
<thead>
<tr>
<th>Radioligand</th>
<th>CP 55,940 (Kd) + 95% CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>17 nM (5.3–53)</td>
</tr>
<tr>
<td>D2.63176A</td>
<td>4.9 nM (0.6–43)</td>
</tr>
<tr>
<td>K373A</td>
<td>17 nM (3.3–85)</td>
</tr>
<tr>
<td>D2.63176A-K373A</td>
<td>5.1 nM (0.6–42)</td>
</tr>
<tr>
<td>D2.63176K-K373D</td>
<td>15 nM (3.0–69)</td>
</tr>
</tbody>
</table>
the putative ionic interaction (despite having switched the residues at 2.63176 and 373) explains how the D2.63176K-K373D swap mutant is capable of promoting an EC-3 loop conformation that is very similar to the WT EC-3 loop conformation.

In addition, the negatively charged K373D may be able to form ionic interactions with K370 and K7.32376 (see Fig. 1). These interactions may reduce the frequency of the D2.63176K-K373D ionic interaction. Energetically favorable interactions with K370 and K7.32376 are highly unlikely for K373 (in WT), as its positive charge would be repelled by the positive charge on K370 and K7.32376.

**CP55,940/Receptor Pairwise and Total Interaction Energies.** The results of the saturation and competitive binding assays demonstrate that all mutations do not significantly affect the binding affinity of the ligands studied. Therefore, to test whether our models agreed with these results, pairwise and total interaction energies were calculated for the WT and mutant models (the total interaction energies are listed in Table 4; the complete pairwise interaction energies are listed in Supplemental Tables 1–5). Only five residues contribute at least 5% of the total interaction energy between CP55,940 and each of the models (Supplemental Tables 1–5). Strikingly, these five important residues are the same in the WT and mutant models (Q1.32116, F2.57170, K3.28192, S7.39383, and L7.43387). This consistency (in which residues contribute at least 5% of the total interaction energy) qualitatively suggests that CP55,940 binds WT and mutant receptors similarly. Quantitatively, Table 4 shows that none of the mutations resulted in significant change in the total interaction energy between CP55,940 and the receptor. These results indicate that our computational models are consistent with the results of the binding assays.

**Discussion**

In our present study, we have used computational methods together with model-guided mutagenesis to evaluate the functional importance of a putative intramolecular ionic interaction within the CB1 receptor. Our previous mutation studies demonstrated the importance of a negative charge at residue 2.63176, possibly indicating its involvement in an essential ionic interaction (Kapur et al., 2008). Our previous modeling studies indicated that the EC-3 loop residue K373 might be the ionic partner to D2.63176. To test this hypothesis, four substitution mutant CB1 receptors were constructed—D2.63176A, K373A, D2.63176A-K373A, and D2.63176K-K373D—to evaluate the effect of removing the putative ionic interaction. The charge-reversal mutant was designed to determine whether switching the positions of the ionic partners could rescue WT levels of function. Finally, computational methods were also used to explore how the putative ionic interaction influences receptor structure.

**Table 3**

Concentration-effect data for agonist stimulation of [35S]GTPγS binding of WT and mutant receptors stably expressed in HEK293 cells.

<table>
<thead>
<tr>
<th>Drug</th>
<th>EC50 (95% CI)</th>
<th>Emax/Top (95% CI)</th>
<th>Mutant/WT EC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT CP55,940</td>
<td>12.6 nM (3.6–43.7)</td>
<td>127% (109–145)</td>
<td>NA</td>
</tr>
<tr>
<td>WIN55,212-2</td>
<td>36.7 nM (12.4–108.3)</td>
<td>95% (87–116)</td>
<td>NA</td>
</tr>
<tr>
<td>D2.63176A CP55,940</td>
<td>67 nM (17–259)*</td>
<td>59% (48–71)*</td>
<td>5.3*</td>
</tr>
<tr>
<td>WIN55,212-2</td>
<td>231 nM (27–1912)*</td>
<td>89% (60–118)</td>
<td>6.3*</td>
</tr>
<tr>
<td>K373A CP55,940</td>
<td>70 nM (5.6–870)*</td>
<td>70% (44–100)</td>
<td>5.6*</td>
</tr>
<tr>
<td>WIN55,212-2</td>
<td>274 nM (61–1230)*</td>
<td>83% (53–112)</td>
<td>7.5*</td>
</tr>
<tr>
<td>D2.63176A-K373A CP55,940</td>
<td>38.8 nM (10–153)*</td>
<td>29% (23–35)*</td>
<td>3.2*</td>
</tr>
<tr>
<td>WIN55,212-2</td>
<td>561 nM (60–5193)*</td>
<td>59% (38–81)*</td>
<td>15.3*</td>
</tr>
<tr>
<td>D2.63K-K373D CP55,940</td>
<td>38 nM (6.9–209)</td>
<td>82% (73–93)</td>
<td>3</td>
</tr>
<tr>
<td>WIN55,212-2</td>
<td>126 nM (26–607)</td>
<td>79% (63–95)</td>
<td>3.4</td>
</tr>
</tbody>
</table>

*P < 0.05.
Ligand binding affinity was not significantly affected by any of the mutations performed here. These results are consistent with our prior characterization of D2.63176, which was shown to be crucial for signal transduction but did not participate in high affinity agonist binding (Kapur et al., 2008). In addition, the binding affinity data reported here are consistent with the predictions made from our WT and mutant models. Specifically, CP55,940 was found to have a similar total interaction energy in the WT and mutant receptor models; this is not surprising, as neither D2.63176 nor the EC-3 loop are part of the predicted CP55,940 binding pocket. Indeed, reports of EC loop mutations that only affect agonist efficacy (and not ligand binding) are well documented in the GPCR literature.

Residues in EC-2 loop of the M3 muscarinic receptor could be mutated without affecting ligand binding; however, a significant reduction in agonist efficacy was observed (Scarselli et al., 2007). Similar results have been observed in the EC-1 loop of the adenosine A2A receptor (Peeters et al., 2011a). Analogously, our results suggest that the ionic interaction between D2.63176 and K373 is not important for CB1 WT function. The $E_{\text{max}}$ values for either CP55,940 or SR141716A. These results suggest that the mutations reported here did not cause the receptor to fold incorrectly or fail to express at the cell surface.

In contrast to the binding affinity results, the D2.63176A, K373A, D2.63176A-K373A or D2.63176K-K373D mutations caused a significant change in CP55,940's $E_{\text{max}}$ compared with WT. However, the $E_{\text{max}}$s of the D2.63176A, K373A, or the D2.63176K-K373D mutants when compared with the D2.63176A-K373A mutant were not significantly different. This is consistent with our hypothesis that it is the ionic interaction between the charged residues D2.63176 and K373 (and not the residues independently) that is important to agonist efficacy. If D2.63176 and K373 were independently important to function, one would expect that the $E_{\text{max}}$ of the double-alanine mutant would be higher than either of the single-alanine mutants.

We previously reported that presence of a negatively charged residue at position 2.63176 is crucial for receptor function (Kapur et al., 2008). In this study, we demonstrate that an ionic interaction between D2.63176 and K373 (not simply the negative charge on D2.63176 per se) is required for CB1 WT function. The $E_{\text{max}}$ values for either CP55,940 or SR141716A.
WIN55,212-2 at the double-alanine mutant showed a significant decrease in function. This result suggests that the interaction between D2.63 and K373 is important for signaling at CB₁. This result is also reinforced by results for the charge-reversal mutant D2.63K-K373D, as both CP55,940 and WIN55,212-2 showed a restoration of function compared with the double-alanine mutation. Therefore, the ability to switch the residues at 2.63 and 373 (dramatically flipping the polarity on both residues) and preserve near WT levels of efficacy strongly supports the existence of a functionally required ionic interaction between D2.63 and K373.

The single-alanine mutants showed increased E_{max} values relative to the double-alanine mutant (E_{max} = 59% at D2.63A and E_{max} = 29% for CP55,940; E_{max} = 39% at D2.63A and E_{max} = 59% for WIN55,212-2) that are larger than what might be expected for disruption of the same ionic interaction as seen in the double-alanine mutant. Our models suggest that residues near the putative ionic interaction may help rescue function in these single-alanine mutants. There are two additional lysines (K370 and K7.32) that are in close proximity to K373. These lysines may be able to form an ionic interaction with D2.63, thus partially rescuing function at the K373A mutant. Likewise, there is a negatively charged aspartate (D184) and two hydrophilic residues (H181 and D185) on the EC-1 loop that are in close proximity to D2.63. These residues may be able to form an ionic interaction (or a simple hydrogen bond in the case of H181 and S185) that enables the partial rescue of function at the D2.63A mutant. In the double mutant D2.63A-K373A, no such rescue would be possible because the polar residues at each site (D2.63 or K373) have been replaced with a nonpolar residue (Ala).

### Table 4

Total interaction energy of CP55,940 at the WT and mutant CB₁ R⁺ models.

<table>
<thead>
<tr>
<th>Model</th>
<th>Total Interaction Energies (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coulombic</td>
</tr>
<tr>
<td>CB₁ WT</td>
<td>-22.12</td>
</tr>
<tr>
<td>D2.63⁶⁷⁶A</td>
<td>-23.34</td>
</tr>
<tr>
<td>K373A</td>
<td>-23.34</td>
</tr>
<tr>
<td>D2.63⁶⁷⁶A-K373A</td>
<td>-23.61</td>
</tr>
<tr>
<td>D2.63⁶⁷⁶K-K373D</td>
<td>-24.72</td>
</tr>
</tbody>
</table>

EC-2 loop and TMH5. Motion of EC-2 may allow the EC end of the TMH6-EC-3-TMH7 segment to pivot toward the center of the protein and conversely allow the IC end of TMH6 to rotate outward (Ahuja et al., 2009). In some class A GPCRs, such as chemokine receptor 4, a specific interaction between the EC-3 loop and N terminus (disulfide bridge) acts as a “microswitch” that is crucial to the chemokine receptor 4 signaling (Rana and Baranski, 2010).

The computational results reported here illustrate how the ionic interaction between D2.63 and K373 causes the EC-3 loop to pull across the top (EC side) of the receptor. Notably, this EC-3 loop conformation is preserved in the charge-reversal mutant D2.63K-K373D. As described in Results, a strikingly different EC-3 loop conformation is observed in the three alanine-substitution mutants. These results suggest that the putative ionic interaction strongly influences the conformation of the EC-3 loop. This promoted EC-3 loop conformation could serve two important structural roles. First, this EC-3 loop conformation may contribute to forming a protected, closed EC surface, as has been reported in the crystal structures of rhodopsin (Li et al., 2004) and the sphingosine 1-phosphate receptor (Hanson et al., 2012). Second, this ionic interaction creates a noncovalent "tether" between the EC ends of TMHs 2, 6, and 7, allowing conformational changes that occur on one side of the receptor to be transmitted to the other side of the receptor. Thus, the alanine-substitution mutants are less capable of transmitting conformational changes throughout the receptor, and efficacy is consequently impaired. In conclusion, we have identified the EC-3 loop conformation that is mechanistically important in the signaling cascade in CB₁.

### Acknowledgments

The authors thank Dr. Linda Console-Bram for comments on an earlier version of this manuscript.

### Authorship Contributions

**Participated in research design:** Marcu, Abood, Shore, Makriyannis, Reggio, Kapur.

**Conducted experiments:** Marcu, Trznadel, Kapur, Shore.

**Performed data analysis:** Marcu, Kapur, Shore.

**Wrote or contributed to the writing of the manuscript:** Abood, Reggio, Shore, Marcu.

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