

Structural dynamics and energetics underlying allosteric inactivation of the cannabinoid receptor CB₁

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G protein-coupled receptors (GPCRs) are surprisingly flexible molecules that can do much more than simply turn on G proteins. Some even exhibit biased signaling, wherein the same receptor preferentially activates different G-protein or arrestin signaling pathways depending on the type of ligand bound. Why this behavior occurs is still unclear, but it can happen with both traditional ligands and ligands that bind allosterically outside the orthosteric receptor binding pocket. Here, we looked for structural mechanisms underlying these phenomena in the marijuana receptor CB₁. Our work focused on the allosteric ligand Org 27569, which has an unusual effect on CB₁—it simultaneously increases agonist binding, decreases G-protein activation, and induces biased signaling. Using classical pharmacological binding studies, we find that Org 27569 binds to a unique allosteric site on CB₁ and show that it can act alone (without need for agonist cobinding). Through mutagenesis studies, we find that the ability of Org 27569 to bind is related to how much receptor is in an active conformation that can couple with G protein. Using these data, we estimated the energy differences between the inactive and active states. Finally, site-directed fluorescence labeling studies show the CB₁ structure stabilized by Org 27569 is different and unique from that stabilized by antagonist or agonist. Specifically, transmembrane helix 6 (TM6) movements associated with G-protein activation are blocked, but at the same time, helix 8/TM7 movements are enhanced, suggesting a possible mechanism for the ability of Org 27569 to induce biased signaling.

GPCR | CB₁ | protein dynamics | allosteric | biased signaling

Classically, our understanding of G protein-coupled receptor (GPCR) signaling presumed that the receptor formed one unique, active receptor structure in response to agonist binding. We now know that this paradigm is too simple. A growing body of evidence shows that GPCRs can adopt different active conformations depending on the type of signal (ligand) bound, making it unlikely that only one GPCR structure is present at any given moment (1, 2). These different ligand-dependent conformations could explain why a wide range of activities can be observed for some GPCRs, such as coupling to multiple different G-protein types or signaling through non-G-protein signaling partners, such as the protein arrestin (3). This phenomenon—diverse ligands bound to the same receptor selectively eliciting different signaling pathways—is referred to as functional selectivity or biased signaling.

What are these different receptor conformations, and why might they result in biased signaling? One possibility is that they involve different orientations of transmembrane helix 5 (TM5) and TM6 in the cytoplasmic face. An outward movement of TM6 is critical for G-protein activation, because it exposes a hydrophobic binding site and enables formation of the ternary complex of receptor, ligand, and G protein (4–9). Newer evidence suggests that there is likely some plasticity in TM6 movement during activation, with differences in either the magnitude or probability of the movement explaining varying degrees of G-protein signaling (3, 10, 11).

Some types of biased signaling may also arise when TM7 and its attached helix 8 (H8) adopt different conformations in the cytoplasmic face, because movements in this region have been detected during receptor activation (12–14). However, H8/TM7

movement does not seem to be required for G-protein activation (15), and this region does not contact the G protein in the recent ternary complex structure (7). For these reasons, H8/TM7 movements may not be directly involved in G-protein binding but rather, may play a role in the binding of arrestin and/or kinase, thus triggering arrestin-centric signaling pathways (14, 16).

The mechanism(s) through which allosteric molecules alter GPCR structure is also an unresolved question and an area of increasing interest (17–19) for which novel approaches are being developed (20) because of the potential that these ligands offer for new treatment paradigms (21). Allosteric ligands for several GPCRs have now been identified, including ligands for the cannabinoid, muscarinic, and μ -opioid.

To gain more information about the structural changes accompanying both biased signaling and allosteric modulation of GPCRs, we have been studying the effects of an unusual allosteric ligand on CB₁, the marijuana GPCR. The use of this ligand, called Org 27569, provides a unique way to detect previously unidentified GPCR conformations for several reasons. First, because it binds allosterically, Org 27569 likely uses a different mechanism to act on CB₁. It also enables well-characterized radioactively labeled orthosteric CB₁ ligands to be used. Second, Org 27569 exhibits a number of unusual effects—it increases agonist binding to the receptor while simultaneously inhibiting G-protein activation (10, 22), and inducing biased signaling (23–25). Thus, it is hard to imagine how these different effects could occur unless the CB₁–Org 27569 bound state adopts a unique and different conformation. A cartoon representation of CB₁ and the putative Org 27569 binding site is shown in Fig. 1.

Recently, we reported that, although Org 27569 stabilizes CB₁ interactions with the agonist, it simultaneously blocks the TM6 movements required for G-protein activation discussed above

Significance

G protein-coupled receptors (GPCRs) are a major pharmaceutical target. Traditionally, they were thought to lead a binary existence, adopting either an off (inactive) or on (active) state. However, recent evidence suggests GPCRs can adopt multiple conformations and these might explain biased signaling—the phenomena where different drugs binding to the same orthosteric site on the receptor can cause activation of different signaling pathways, such as β -arrestin signaling. We show a previously unidentified structure is induced in the marijuana receptor CB₁ by an unusual allosteric ligand that blocks G-protein signaling but increases agonist binding and elicits biased signaling. We propose that a common structural state may exist for β -arrestin biased signaling, one that can also be attained by allosteric ligand binding.

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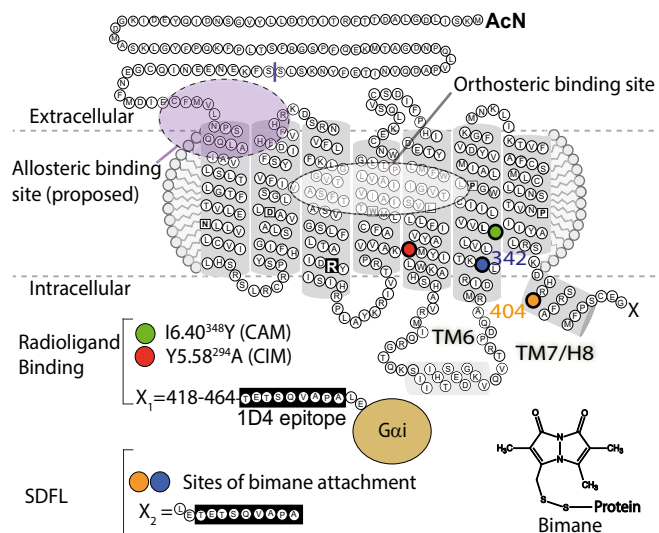


Fig. 1. A 2D cartoon model of CB₁ showing the approximate location of orthosteric and allosteric binding sites and the various mutations used. The traditional (orthosteric) ligand binding site is depicted as a dashed white oval, and the (proposed) allosteric ligand Org 27569 binding site is depicted as a dashed purple circle (50). Key point mutations in CB₁ include a CAM (I348Y^{6,40}; green) or CIM (Y294A^{5,58}; red). These mutations presumably cause their effect by altering interactions with a highly conserved Arg (R) in TM3 (black square). Radioligand binding studies used CB₁-G_{α_i}, a full-length human CB₁ receptor fused to the G-protein G_{α_i} (tan). SDFL studies used a minimal cysteine receptor (θ) with a truncated N terminus (Δ87) and a truncated C terminus (Δ417) to which the 1D4 epitope tag (black boxes) is attached to enable purification and unique reactive cysteines introduced at either TM6 (A342C^{6,34}; blue) or H8/TM7 (L404C^{8,50}; orange) to enable labeling with the fluorophore bimane (Inset). The different C-terminal modifications following residue 417 are indicated as X₁ where the sequence is either X₁ (for CB₁-G_{α_i}) or X₂ (for θ).

(10), thus explaining the negative effect of Org 27569 on G-protein signaling. These conclusions were based on site-directed fluorescence labeling (SDFL) studies of purified CB₁ that showed that, although Org 27569 induces CB₁ to adopt a high-affinity agonist binding conformation, it is not the high-affinity agonist binding conformation traditionally associated with the formation of the ternary complex with G protein (4).

Here, we set out to characterize the conformation and energetics underlying this unique Org 27569 trapped state and identify a mechanism for the unusual effects discussed above using a combination of classical pharmacology, mathematical modeling, and SDFL studies. One goal was to determine if Org 27569 could act on the receptor in the absence of an orthosteric ligand. Another goal was to explore the linkage between Org 27569 binding and TM6 movements in CB₁ by asking the question: because Org 27569 binding blocks TM6 movement, does impairing TM6 movement inhibit Org 27569 binding? We did this by creating and testing two different CB₁ mutants: one constitutively active mutant (CAM) and one constitutively inactive mutant (CIM). In these mutants, TM6 movement was either enhanced (CAM) or impaired (CIM). Radioligand binding studies were then performed on these mutants in the presence of Org 27569 to test the above hypothesis and assess the energetics underlying Org 27569 binding. [All radioligand binding and efficacy measurements, unless otherwise stated, were done in the absence of sodium, a well-known negative allosteric modulator of GPCR activity, to enhance basal activity and reduce allosteric variables. This fact could contribute to the relatively high R^*/R ratios that we observe for WT CB₁ along with the use of G-protein chimeras in our measurements (because G proteins can allosterically modulate receptor affinity).]

Finally, we used SDFL to probe the structural differences between active, inactive, and Org 27569-bound CB₁, with the goal of identifying other structural changes in the receptor that might explain the mechanism of allosteric modulation and biased signaling, specifically focusing on movements at TM6 as well as H8/TM7. Our results are intriguing—they show that Org 27569 binding stabilizes a different receptor conformation, one that may be related to its ability to induce biased signaling.

Results

Allosteric Ligand Org 27569 Simultaneously Enhances Agonist Binding While Inhibiting CB₁ Signaling, and It Can Inactivate Receptor Signaling by Itself in the Absence of Agonist. As shown in Fig. 2A and noted previously (22), the allosteric ligand Org 27569 can enhance binding of the agonist CP 55940 to CB₁ in membranes but does not stimulate antagonist SR141716A binding. Additional analysis suggests that this inhibition of antagonist binding is not competitive but rather, occurs through an allosteric mechanism (Fig. S1). To test if Org 27569 can bind and act on CB₁ by itself in the absence of other ligands, in Fig. 2B, we measured its effect on CB₁ constitutive activity (that is, the activation of G proteins by CB₁ in the absence of agonist).

Similar to the antagonist SR141716A, Org 27569 lowers basal G-protein activation in membranes expressing a CB₁-G_{α_i} fusion protein (measured as GTPγS binding), although it does so with less potency (EC₅₀ of 31 ± 5 μM vs. 7.0 ± 0.8 nM). Interestingly, the ability of Org 27569 to inhibit CB₁ activity is enhanced if agonist CP 55940 is present (EC₅₀ = 1.9 ± 0.3 μM) (Fig. 2B), which would be expected for an allosteric ligand with positive cooperativity with respect to agonist binding. Sodium, a well-known allosteric antagonist for many GPCRs, including CB₁ (26, 27), also further enhances this observed potency (Fig. S2).

Org 27569 Affinity for CB₁ Is Different for CB₁ Mutants with Altered TM6 Movements and Thus, Altered $R \rightleftharpoons R^*$ Equilibrium. We hypothesized that Org 27569 may act by shifting the equilibrium between active (R^*) and inactive (R) CB₁ receptors, because it can suppress the basal activity of CB₁ by itself (Fig. 2B). To test this hypothesis, we measured the effect of Org 27569 on CAMs and CIMs. These

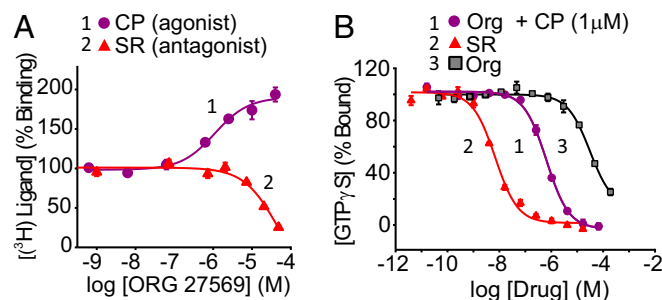


Fig. 2. The allosteric ligand Org 27569 enhances agonist binding while simultaneously inactivating CB₁, and it can also inactivate receptor signaling, even in the absence of agonist. (A) Specific equilibrium binding of either radioactive agonist (0.4 nM ³H CP 55940; purple circles) or antagonist (0.2 nM ³H SR141716A; red triangles) was determined in the presence of increasing concentrations of the allosteric ligand Org 27569. The K_d^{Org} values are 1.23 ± 0.32 μM with respect to agonist and 32 ± 18 μM with respect to antagonist when fit to the allosteric ternary complex model. Org 27569 increases binding of the agonist, whereas it has little effect on the antagonist binding until much higher concentrations are used. (B) Dose-response curves measuring the inhibition of GTPγS binding to WT CB₁-G_{α_i} by the following compounds: antagonist (SR141716A; red triangles), the allosteric inverse agonist Org 27569 (gray squares), and Org 27569 in the presence of 1 μM agonist CP 55940 (purple circles). The EC₅₀ values are 7.0 ± 0.8 nM for SR141716A, 31 ± 5 μM for Org 27569, and 1.9 ± 0.3 μM for CP 55940/Org 27569. Each symbol represents the mean ± SEM for at least two independent experiments performed in duplicate.

mutants were created by introducing mutations in the receptor that stabilize or destabilize the TM6 movement that accompanies active-state formation. The mutants targeted a key residue involved in stabilizing TM6 in the active-state structure: the arginine (R) found in the highly conserved Asp-Arg-Tyr (DRY) motif in TM3 (R3.50). To create the active mutant (CAM), we introduced a tyrosine at I348^{6,40} to interact with R214^{3,50} and stabilize TM6 in an outward, active-state conformation (28–30). The inactive receptor (CIM) was created by mutating the highly conserved Y294^{5,58} in TM5 to an alanine (A), thus removing its stabilizing interactions with R214^{3,50} (31–34).

The CB₁ CAM and CIM receptors exhibited the anticipated reduction or enhancement in basal G-protein activation (Fig. S3). Importantly, their traditional (orthosteric) ligand binding pocket was not significantly perturbed, because they did not show large alterations in their agonist binding affinities [$K_d = 5.6 \pm 0.9$ nM for WT CB₁-G α_i , 7.7 ± 1.6 nM for the CIM (Y294A^{5,58})-G α_i , and 3.9 ± 0.3 nM for the CAM (I348Y^{6,40})-G α_i]. Their expression was also largely unaffected, which was confirmed by homologous radioligand binding and comparative Western blot analysis (Fig. S3).

Org 27569 Favors a Receptor with an Inactive Conformation and Disfavors an Active Species. Although the WT, CAM, and CIM CB₁ receptors showed similar expression levels and agonist binding properties, their behavior in response to Org 27569 was dramatically different. Org 27569 no longer enhanced agonist binding for the active (CAM) receptor, whereas its potency (ability to enhance agonist binding) was increased ~10-fold for the inactive (CIM) receptor ($K_d^{\text{Org}} = 1.23 \pm 0.32$ μM vs. 127 ± 86 nM). Remarkably, Org 27569 potency was so enhanced for the CIM that, at higher concentrations, it seemed to reduce agonist binding (Fig. 3A, dashed red line). Presumably, this behavior occurs because high concentrations of Org 27569 slows radiolabeled agonist dissociation so much that equilibrium was not achieved during the experiment (18). The ability of Org 27569 to inhibit CB₁ function (agonist-stimulated GTP γ S binding) also showed a similar trend, exhibiting the following rank order of potency: CIM > WT > CAM. This result again suggests that Org 27569 favors a receptor with an inactive TM6 conformation and disfavors an active species (Fig. 3B).

Differences in Org 27569 Affinity for the CB₁ Active and Inactive Mutants Can Be Used to Assess Differences in Free Energy Between the R and R* States. To assess the relative affinity differences of Org 27569 for the WT, CIM, and CAM receptors, we first fit the ligand binding dose–response data (Fig. 3A) to a simple allosteric ternary complex model, which is described in *SI Experimental Procedures*. The results show that Org 27569 affinity is greatest for the CIM and least for the CAM, although the lack of enhanced agonist binding for the latter yielded a poor fit (CIM $K_d^{\text{Org}} = 127 \pm 86$ nM, WT $K_d^{\text{Org}} = 1.23 \pm 0.32$ μM , and CAM $K_d^{\text{Org}} = 17 \pm 72$ μM).

We next carried out more detailed modeling to assess the affinity of Org 27569 for each receptor and other parameters of the system. In brief, this process involved using global analysis to simultaneously fit both sets of data (Fig. 3A and B) to an extended allosteric ternary complex model (*SI Experimental Procedures* and Fig. S4). The goal was to find shared parameters that best described both the receptor ligand binding data and the active receptor population (the latter was estimated by assuming that bound GTP γ S reflects the amount of active receptor because of the 1:1 receptor:G-protein stoichiometry in the CB₁-G α_i fusion proteins). Interestingly, only two parameters show striking differences between the WT and mutant receptors in this analysis—the affinities for the allosteric ligand (K_d^{Org}) and L , the ratio of active to inactive receptor forms ($[R^*]/[R]$) (Fig. S4 and Table S1).

Together, these results suggest that Org 27569 favors an inactive receptor species, because they show the K_d for Org 27569

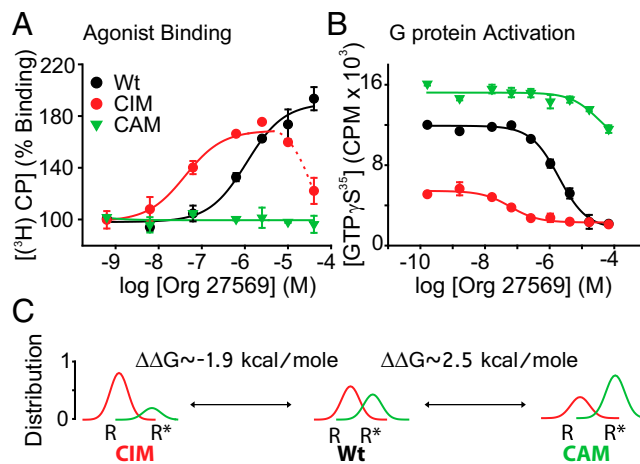


Fig. 3. The affinity for the allosteric ligand Org 27569 is affected by activating and inactivating mutations that alter the $R=R^*$ equilibrium, and these values can be used to assess energetic differences between receptor states. (A) Org 27569 affects agonist [^3H]CP 55940 binding differently for WT and activating (CAM) and inactivating (CIM) CB₁-G α_i fusion proteins. Org 27569 does not enhance agonist binding for the CAM-G α_i (green) compared with the WT-G α_i (black). In contrast, the CIM-G α_i (red) shows such enhanced potency that nonequilibrium conditions appear to occur (agonist binding appears inhibited) at higher concentrations (dashed red line). (B) Org 27569 affects agonist-stimulated G-protein activation differently for WT, CAM, and CIM CB₁-G α_i fusion proteins, which is indicated by its effect on GTP γ S binding in the presence of 1 μM agonist (CP 55940). (C) Simultaneously fitting the data in A and B using global analysis and an extended allosteric ternary complex model (Fig. S4 and Table S1) suggests significant changes in the relative K_d^{Org} values (CIM $K_d^{\text{Org}} = 58 \pm 6$ nM vs. WT $K_d^{\text{Org}} = 1.29 \pm 0.1$ μM vs. CAM $K_d^{\text{Org}} = 77 \pm 6$ μM). Using these values, the $\Delta\Delta G$ values (free energy difference of Org 27569 binding for the CAM and CIM vs. WT) are ~ 2.5 kcal/mol greater for the CAM and ~ 1.9 kcal/mol less for the CIM. These values are consistent with the loss or gain of a hydrogen bond interaction between a Tyr and the Arg at 3.50 thought to stabilize or destabilize the active receptor state. The graphs suggest the relative distribution of receptor in each state and were determined from the isomerization constant L (the ratio of active R^* to inactive R states) (Table S1) determined from the above analysis. Note the greater amount of R^* for the CAM and smaller amount of R^* for the CIM.

increases for the active (CAM) receptor and decreases for the inactive (CIM) receptor (CIM $K_d^{\text{Org}} = 58 \pm 6$ nM, WT $K_d^{\text{Org}} = 1.29 \pm 0.1$ μM , and CAM $K_d^{\text{Org}} = 77 \pm 6$ μM). The resulting isomerization constants (L values) derived for each receptor indicate that the CAM produces more receptor in the active R^* form (compared with WT) and that the CIM produces less (for the CIM, $L = 0.11 \pm 0.01$; for the WT, $L = 0.72 \pm 0.08$; and for the CAM, $L = 2.0 \pm 0.1$).

Finally, as described in *SI Experimental Procedures*, we estimated the difference in free energy between the CAM and CIM receptors relative to WT using their difference in the free energy for Org 27569 binding as a proxy. For the inactive (CIM) receptor, this analysis yielded $\Delta\Delta G = -1.9 \pm 1.2$ kcal/mol, and for the active (CAM) receptor, $\Delta\Delta G = 2.5 \pm 0.8$ kcal/mol. These results and the relative distribution of inactive R to active R^* (calculated from L , the isomerization constant) are shown in Fig. 3C and Table S1.

SDFL Studies Confirm That the Binding of Org 27569 to CB₁ Creates a Unique State: One That Blocks Agonist-Induced TM6 Movement but Enhances Conformational Changes at H8/TM7. Although informative, the above experiments and analysis contain several assumptions and are based on agonist binding and the ability to activate G protein. However, they cannot discern if more than one type of G protein-inactive state (such as R , R' , and R'') is present. Thus, we next used SDFL to determine if Org 27569 inactivates CB₁ by trapping a unique, inactive receptor conformation or instead, acts

by stabilizing the same inactive conformation trapped by traditional CB₁ antagonists, like SR141716A.

Previously, using SDFL, we discovered that Org 27569 blocks TM6 movement (10). However, in that work, we could not unequivocally determine if the Org 27569 bound structure was the same or different from the antagonist bound form. Here, to see if Org 27569 causes changes in other parts of the receptor, we used SDFL to look for movements at H8, the membrane-associated amphipathic helix located directly above TM7 in GPCRs. H8 has been proposed to undergo conformational changes that potentially play a role in biased signaling for orthosteric ligands (2, 14, 16, 35). Because the allosteric ligand Org 27569 is reported to induce arrestin signaling (23–25), we hypothesized that it might also induce changes in this region of the receptor.

We reconfirmed our previous observation (10) that agonists cause fluorescence changes in a bimane probe on TM6 (at site 342) and that these changes are blocked by both the antagonist SR141716A and the allosteric ligand Org 27568 (Fig. 4 *A* and *B*). We next tested the effect of these ligands on CB₁ with a bimane probe attached on H8 (at site 404). To our initial surprise, the results were very different—the agonist caused an ~20% decrease in bimane fluorescence for the probe at H8 (Fig. 4*D*). Moreover, adding Org 27569 did not block this change but rather, causes an ~20% additional decrease in fluorescence. Both of these effects are in stark contrast to the lack of changes observed in the presence of antagonist SR141716A (Fig. 4*E*).

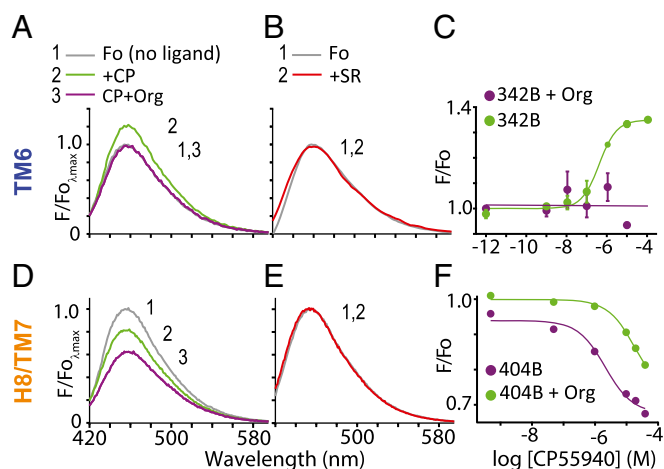


Fig. 4. SDFL studies indicate that the allosteric ligand Org 27569 captures a unique receptor conformation: one that lacks TM6 movement but has enhanced conformational changes around H8/TM7. A bimane fluorescent probe on TM6 (at site 342) or H8 (site 404) detects different conformational changes in CB₁, on agonist and allosteric ligand binding. The order of ligand addition is indicated by the number and compound. (*A*) For the probe at TM6, agonist (CP 55940; 10 μ M; green) causes an ~25% increase in bimane fluorescence compared with the apo state (Fo; gray). This change is reversed by the subsequent addition of allosteric ligand Org 27569 (20 μ M; purple). (*B*) Antagonist (SR141716A; 10 μ M; red) causes no change. (*C*) Dose-response plots in the presence or absence of Org 27569 (5 μ M) confirm the results shown in *A* and our previous studies (10). (*D*) For the bimane probe at H8, agonist (CP 55940; 20 μ M; 30 min incubation, green) causes an ~20% decrease in fluorescence intensity compared with the apo state (Fo; gray). Addition of Org 27569 (20 μ M, 15 min incubation, purple) to the CP 55940-treated sample does not block the fluorescence change but rather, causes an additional 20% decrease in intensity. Each spectrum is normalized to the apo state (gray). (*E*) In contrast, antagonist (SR141716A; 10 μ M; red) causes no fluorescence change. (*F*) Dose-response plots of the experiments in *D*. Both the potency and magnitude of this agonist-induced decrease in bimane fluorescence for the probe at H8 are enhanced in the presence of Org 27569 (10 μ M).

Importantly, the fluorescence changes at both sites are reproducible and occur in a dose-dependent fashion (Fig. 4 *C* and *F*). As discussed below, these results show that Org 27569 binding produces a distinct receptor conformation that is significantly different from the other ligand-bound receptor states.

Discussion

Our goal was to gain new structural insights into the mechanism and energetics underlying allosteric modulation of GPCR activation, attenuation, and biased signaling. We focused on studying how an allosteric ligand for CB₁ (Org 27569) can induce positive cooperativity for agonist binding but simultaneously, reduce signaling by the agonist bound receptor and how these effects might be related to the reported biased signaling that this compound can induce in CB₁ (23–25).

Initial evidence that Org 27569 and the antagonist SR141716A trap different receptor conformations is seen in the radioligand binding studies (Fig. 2). They show that Org 27569 does not need agonist to bind CB₁ and inhibit R* formation (Fig. 2*B*), although cobinding with agonist does enhance Org 27569 potency. In contrast, Org 27569 impairs antagonist SR141716A binding (Fig. 2*A*), presumably through an allosteric mechanism (Fig. S1).

Additional evidence that Org 27569 traps a unique receptor conformation is clear in the functional studies of two different CB₁ mutants (Fig. 3). In these mutants, TM6 movement was altered to produce either an active (CAM) receptor (mutant I348Y^{6,40}) or an inactive (CIM) receptor (mutant Y294A^{5,58}) using substitutions well-established to impart these properties in other GPCRs (28, 31–33, 36). Importantly, these mutations are outside the orthosteric ligand binding pocket and cause no significant change in receptor expression levels or agonist affinity (Fig. S3). Thus, dose-response experiments between these mutants could be directly compared to assess the effect of Org 27569.

These experiments show that the ability of Org 27569 to act on CB₁ is directly linked to the TM6 movement that is associated with G-protein activation. When TM6 movements are impaired (as in the CIM), Org 27569 potency is greatly enhanced. When TM6 movements are enhanced (as in the CAM), Org 27569 potency is greatly reduced in regards to both stimulation of agonist binding and inhibition of G-protein activation (Fig. 3).

Our additional analysis of these data using a simplified version of the allosteric ternary two-state model (37) revealed several insights (Fig. S4 and Schemes S1–S4). First, the results suggest that some level of conformational selection might also be involved in Org 27569 binding, because Org 27569 affinity is inversely proportional to the amount of active R* CB₁ present. Together, these data provide additional evidence that Org 27569 prefers an inactive receptor state that lacks TM6 movement. Second, the $\Delta\Delta G$ for Org 27569 binding to these mutants relative to WT (estimated from the differences in K_d for Org 27569) shows an ~2.5 kcal/mol increase for the CAM and ~1.9 kcal/mol decrease for the CIM.

Coincidentally, these energy values are consistent with a loss or gain of a hydrogen bond between tyrosine and the arginine at position 3.50—interactions previously proposed to either stabilize (CAM) or destabilize (CIM) the active receptor state because of their effect on TM6 movements (28, 31–33, 36). We note, however, that these interpretations are only suggestive. The activation pathway likely results in the formation/breaking of many other interactions, resulting from not only movements of TM6 but also, rearrangements in TM5 and TM7 and alteration of water-mediated interhelical interactions. Thus, the effect of these mutations could be because of altering one or more steps on this activation pathway and not simply the breaking or making of one hydrogen bond. Interestingly, although not directly comparable, these energy differences are of a similar magnitude as the activation energy barrier for TM6 movement previously measured for the corresponding CAM mutation in rhodopsin (38).

Another important caveat regarding our pharmacological studies—although they provide a good first approximation for assessing the receptor behavior with regards to agonist binding and G-protein activation, our modified allosteric ternary complex model cannot discern if more than one type of G protein-inactive state (such as R , R' , and R'') is present. Thus, it cannot discriminate a truly inactive (R) species from one that seems inactive in the above measurements but exhibits biased arrestin signaling (because of a different inactive receptor conformation, R').

To address this issue, we next turned to SDFL studies to look for possible differences in inactive-state conformations that the above pharmacological analysis could not identify.

SDFL Studies Confirm That Org 27569 Traps the Receptor in a Different Inactive Conformation than the Traditional Orthosteric Antagonist SR141716A. The SDFL results directly rule out the possibility that Org 27569 acts by simply shifting the equilibrium toward the traditional, inactive conformation. They show that Org 27569 binding produces a unique, inactive-like receptor state, which is different from the antagonist bound, inactive state. Moreover, the SDFL data show something interesting—agonist induces fluorescence changes for a probe on either TM6 or H8/TM7, whereas the antagonist does not. In contrast, the allosteric ligand enhances the fluorescence changes for the probe at H8/TM7, whereas it blocks agonist-induced changes at TM6 (Fig. 4) (10).

These observations yield two important new insights. First, they show that Org 27569 affects movements at H8/TM7 in a different way than it affects movements at TM6. Second, they show that H8/TM7 can move independently of TM6. The latter observation is especially intriguing, because we find that agonist-induced changes at TM6 occur more slowly (10) than they do at H8/TM7 (Fig. S5). This observation may suggest that H8/TM7 movements occur before TM6 on receptor activation. Interestingly, analysis of molecular dynamics simulations of the β_2 -adrenoreceptor suggests that H8/TM7 and TM6 can move independently and that two inactive receptor states with differential H8/TM7 movements can occur (2).

In Fig. 5, we highlight the differences between the effects caused by binding of an antagonist, the allosteric ligand Org 27569, or an agonist on the structure of the CB₁ cytoplasmic face in cartoon form. The model is consistent with our SDFL results from the bimane probe on TM6 (site 342) (Fig. 5, blue circle) and H8 (site 404) (Fig. 5, orange circle) as well as previous biophysical and crystallographic studies of other GPCRs (10, 39). The model shows no changes in the antagonist bound state (R) (Fig. 5, red). In contrast, the Org 27569 bound intermediate state (R') (Fig. 5, purple) exhibits enhanced changes around the H8/TM7 area but restricted TM6 movement. Finally, the active agonist bound state (R^*) (Fig. 5, green) exhibits changes both at TM6 and around H8/TM7.

As discussed previously (40, 41), these different types of receptor conformations can also be conceptualized in terms of free energy landscapes. As shown in Fig. 5B, the different ligand-stabilized conformational states shown in Fig. 5A can be thought to correspond to differences in the energy profiles as the receptor progresses from a fully inactive (antagonist bound) toward a more active receptor. Note that the final ternary complex state (agonist–receptor–G protein) is not depicted.

Altering the Conformational Equilibrium at TM6 and H8/TM7 May Be a General Mechanism Involved in β -Arrestin Biased Signaling Induced by both Orthosteric and Allosteric Ligands. A current hypothesis proposes that biased ligands produce their differential response because they favor conformational changes in one region of the receptor over another. Both NMR studies of the β_2 -adrenoreceptor and fluorescent studies in the V₂ vasopressin receptor suggest that TM7 movements are more predominant for β -arrestin-biased ligands, whereas TM6 movements are more predominant for G protein-mediated signaling (14, 16). Likewise, a crystallographic

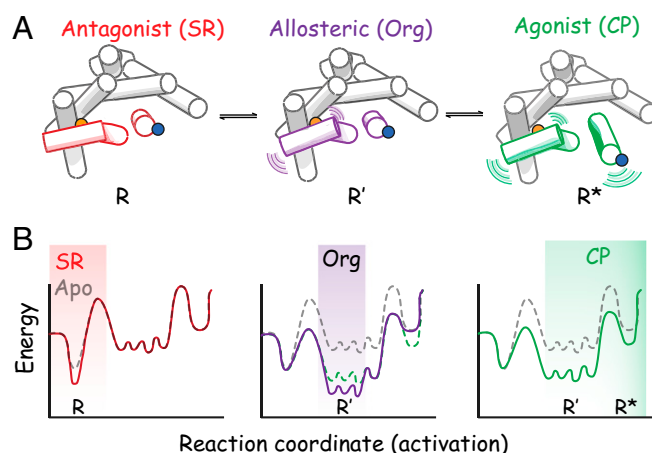


Fig. 5. Cartoon model proposing the structural differences in CB₁ trapped by antagonist, the allosteric ligand Org 27569, and agonist along with their corresponding energy landscape diagrams. The sites probed in the SDFL studies are indicated on TM6 (site 342; blue circles) and H8 (site 404; orange circles). (A) Cartoon models depicting dynamic snapshots of the cytoplasmic face of CB₁ in response to antagonist, allosteric ligand, or agonist. The antagonist bound state (R ; red) shows no significant changes in either TM6 or H8/TM7. In contrast, the Org 27569 bound state (R' ; purple) exhibits enhanced changes around the H8/TM7 area but restricted TM6 movement. The active, agonist bound state (R^* ; green) exhibits both enhanced movements of TM6 and changes around the H8/TM7 area. (B) Representative free energy landscapes for the different ligand-stabilized conformational states in A depicting differences in the receptor energy profile as it progresses from fully inactive (antagonist bound) toward a more active receptor.

model of the arrestin biasing ligand ergotamine bound to the LSD receptor (5-hydroxytryptamine receptor type 2B) also shows active-like rearrangements of TM7 (42).

Similarly, our evidence that Org 27569 traps CB₁ in a G protein-inactive intermediate state (R') could explain why Org 27569 acts as classical antagonist in regards to G-protein activation, while at the same time, eliciting varying degrees of MAPK signaling (23, 43), presumably by acting as an arrestin-biased allosteric agonist (23, 24). The reduced TM6 movement would inhibit productive G-protein coupling, whereas the enhanced H8/TM7 movements could change the accessibility of the C-terminal tail, enabling the receptor to preferentially engage with β -arrestin. Together, these combined effects on TM6 and H8/TM7 movements could skew the system toward more biased signaling and β -arrestin activation.

We also speculate that β -arrestin signaling, caused by the low-affinity “hanging arrestin” binding described by Lefkowitz and coworkers (44) in the recent EM structures of β -arrestin bound receptor, may be precipitated by changes in H8/TM7 but not TM6 movements. Consistent with this idea, we note that Org 27569 blocks agonist-induced CB₁ internalization (45) (presumably because high-affinity β -arrestin binding cannot occur when TM6 movements are blocked) but that biased signaling still persists.

How might this process occur? Arrestin recruitment and MAPK signaling by the G-protein inactive receptor could be triggered by interactions of an arrestin signaling complex with a distinct receptor phosphorylation pattern that may occur for the Org 27569 bound receptor, analogous to the effect of carvedilol on the β_2 -adrenoreceptor (46). Such a distinct phosphorylation pattern could be instigated by GRK5 (or GRK6), which has recently been shown to phosphorylate inverse agonist bound β_2 -adrenoreceptor and even rhodopsin (47).

Finally, we note that alterations of the conformational equilibrium at TM6 and H8/TM7 regions of the receptor may exist as a general mechanism used in the biased signaling of GPCRs and occur for biased allosteric ligands as well as biased orthosteric

ligands (14, 16). Thus, the approach that we describe here, especially the use of SDFL to look for independent ligand-induced conformational changes at TM6 vs. H8/TM7, could be generally useful for discovering new biased ligands for GPCRs.

Experimental Procedures

Buffers, Cloning, Mutagenesis, Transfection, and Purification of CB₁ Mutants.

Site-directed mutants were constructed in the appropriate CB₁ gene constructs: shCB₁-G α_i , shCB₁, or θ (10, 48). The mutant- θ is a nonreactive, minimal cysteine construct that enables fluorescence labeling for SDFL studies and has N- and C-terminal deletions to facilitate purification (10). Transfection was carried out transiently in COS-1 cells (10, 38). Mutants used for SDFL studies were grown in the presence of 100 nM SR141716A. Immunoaffinity purification and bimane labeling of the CB₁ mutants were carried out as described (10, 48). *SI Experimental Procedures* has more details.

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Radioligand Binding and Modeling of Binding Data. Competitive inhibition binding and agonist-stimulated GTP γ S incorporation for CB₁ mutants in membranes were carried out as described (48, 49). Experiments were done at least two times in either quadruplicate or duplicate. All binding experiments were done in the absence of sodium, unless otherwise indicated. Modeling was performed using an extension of methods used in our previous work (10, 48) as described in *SI Experimental Procedures*.

Fluorescence Measurements. Experiments were performed using a Photon Technology International fluorescence spectrometer, and fluorescence spectra were buffer-subtracted and corrected for dilution as previously described (10).

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