Allosteric Modulator ORG27569 Induces CB1 Cannabinoid Receptor High Affinity Agonist Binding State, Receptor Internalization, and $G_i$ Protein-independent ERK1/2 Kinase Activation*

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Background: ORG27569 is an allosteric modulator of CB1.

Results: Although ORG27569 inhibits G protein coupling, it induces CB1 high affinity agonist binding, receptor internalization, and downstream ERK phosphorylation.

Conclusion: The ORG27569-induced activation of ERK via CB1 is $G_i$ protein-independent.

Significance: This is the first case of CB1 allosteric ligand-biased signaling.

The cannabinoid receptor 1 (CB1), a member of the class A G protein-coupled receptor family, is expressed in brain tissue where agonist stimulation primarily activates the pertussis toxin-sensitive inhibitory G protein ($G_i$). Ligands such as CP55940 ((1R,3R,4R)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol) and $\Delta^2$-tetrahydrocannabinol are orthostERIC agonists for the receptor, bind the conventional binding pocket, and trigger $G_i$-mediated effects including inhibition of adenylate cyclase. ORG27569 (5-chloro-3-ethyl-1H-indole-2-carboxylic acid [2-(4-piperidin-1-yl-phenyl)ethyl]amide) has been identified as an allosteric modulator that displays positive cooperativity for CP55940 binding to CB1 yet acts as an antagonist of G protein coupling. To examine this apparent conundrum, we used the wild-type CB1 and two mutants, T210A and T210I (D’Antona, A. M., Ahn, K. H., and Kendall, D. A. (2006) Biochemistry 45, 5606–5617), which collectively cover a spectrum of receptor states from inactive to partially active to more fully constitutively active. Using these receptors, we demonstrated that ORG27569 induces a CB1 receptor state that is characterized by enhanced agonist affinity and decreased inverse agonist affinity consistent with an active conformation. Also consistent with this conformation, the impact of ORG27569 binding was most dramatic on the inactive T210A receptor and less pronounced on the already active T210I receptor. Although ORG27569 antagonized CP55940-induced guanosine 5'-3-O-(thio)triphosphate binding, which is indicative of G protein coupling inhibition in a concentration-dependent manner, the ORG27569-induced conformational change of the CB1 receptor led to cellular internalization and downstream activation of ERK signaling, providing the first case of allosteric ligand-biased signaling via CB1. ORG27569-induced ERK phosphorylation persisted even after pertussis toxin treatment to abrogate $G_i$ and occurs in HEK293 and neuronal cells.

Agonist-activated G protein-coupled receptors (GPCRs) undergo conformational changes that promote activation of the coupled guanine nucleotide-binding protein (G protein) and the subsequent stimulation of downstream effector proteins. Different agonists may promote distinct GPCR conformations, which lead to different activation states from partially active ($R^*$) to fully active ($R^{**}$). These different states of the receptor result in different signaling efficacies and may even promote different downstream signaling via distinct second messenger systems. Indeed, an increasing number of studies have demonstrated that some GPCRs can signal G protein-independently as well as G protein-dependently depending upon the agonist bound (1–3). This functional selectivity (also known as “biased agonism”) has been observed for some GPCRs that are involved in the regulation of cell proliferation and differentiation by stimulating mitogen-activated protein kinase (MAPK) cascades (4, 5). However, the molecular mechanisms underlying this differential signaling and its regulation by different ligands remain unclear. Nonetheless, utilization of pathway-biased ligands has great potential for the development of selective therapeutic agents.

The cannabinoid receptor 1 (CB1), a member of the class A GPCR family, is mainly expressed at high levels in mammalian cerebral cortex, hippocampus, and cerebellum where it primarily activates the pertussis toxin (PTX)-sensitive inhibitory G ($G_{i/o}$) protein (6). The consequences of its activation are modulation of adenylate cyclase activity and calcium and potassium channel conductance. In addition, CB1 may impact mitogen-activated protein kinases such as p42/p44 MAPK (ERK1/2), p38

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The abbreviations used are: GPCR, G protein-coupled receptor; CB, cannabinoid receptor; TM, transmembrane helix; ORG27569, 5-chloro-3-ethyl-1H-indole-2-carboxylic acid [2-(4-piperidin-1-yl-phenyl)ethyl]amide; CP55940, (1R,3R,4R)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; SR141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; GTPγS, guanosine 5’-3’-O-(thio)triphosphate; PTX, pertussis toxin; LAMP-1, lysosome-associated membrane protein 1; PCC, Pearson’s correlation coefficient.
MAPK, and c-Jun N-terminal kinase (JNK1/2); however, very little is known about this possibility (for a review, see Ref. 7). Like most GPCRs, the CB1 receptor is rapidly desensitized and lost from the cell surface upon prolonged exposure to agonist (8). This process appears to be regulated by a plethora of different proteins such as G protein-coupled receptor kinases, β-arrestins, and G protein receptor-associated sorting protein 1 (9–11). After endocytosis and localization to endosomes, GPCRs are either recycled to the plasma membrane or targeted to lysosomes for degradation (for a review, see Ref. 12). Interestingly, CB1 also displays relatively high basal activity and constitutive endocytosis with 85% of the receptor localized to the endosome in the absence of ligand (13, 14).

Although conventional agonists (“orthosteric ligands”) share overlapping binding sites with endogenous ligands, some compounds can bind to distinct sites on the receptor and regulate orthosteric ligand binding and/or its efficacy. These are termed allosteric modulators. A few compounds have been identified as allosteric modulators of the CB1 receptor including ORG27569, ORG29647, ORG27759, and PSNCBAM-1 (15, 16). As positive allosteric modulators, they increase the binding of the CB1 agonist [3H]CP55940 in membranes from cells expressing the CB1 receptor. Intriguingly, these studies suggested that these compounds antagonize CP55940-induced signaling by inhibiting [35S]GTPγS binding and reducing the E\text{max} value in the mouse vas deferens assays (15). This apparent conundrum, increased agonist binding with “insurmountable antagonism of receptor function” (15), opens pressing questions regarding their mechanism of action. Do the allosteric modulators promote a form of CB1 comparable with the active state? Does any downstream signaling occur? Is internalization a consequence? It is critical that we delineate these issues particularly in view of the potential therapeutic benefits of these modulators. These include the potential for a greater range of selectivity across receptor subtypes because allosteric binding sites are typically more divergent than orthosteric sites and because some allosteric modulators may be used alone or in combination with orthosteric ligands (17). The prospect for finely tuning CB1-mediated effects is especially compelling because CB1 is associated with appetite stimulation, pain reduction, anxiety and depression attenuation, and motor control (18–21).

Recent crystal structures of GPCRs have provided breakthroughs in our understanding of the molecular basis for receptor activation (22–24). These receptors are constrained in an inactive state by noncovalent interactions. Upon activation, some of those interactions are broken, and transmembrane helices undergo rearrangement. It is broadly accepted that most class A GPCRs are restrained in part in an inactive state by the salt bridge (“ionic lock”) between an arginine of the (D/E)RY motif in TM3 and a negatively charged aspartic acid or glutamic acid residue in TM6. In previous studies, we demonstrated that the single mutation of threonine at position 2103.46 (superscript denotes Ballesteros-Weinstein nomenclature (25)) of the human CB1 receptor, which is one turn above the arginine of the DRY motif in TM3, to isoleucine and alanine resulted in enhanced and reduced constitutively active receptor forms, respectively, relative to the wild-type receptor (26). Thus, the T210A, wild-type, and T210I CB1 receptors cover a spectrum of receptor states from inactive to partially active to more fully constitutively active and provide ideal reagents to characterize the conformational plasticity of CB1 and the outcomes of specific ligand interactions.

In this study, we elucidated the effect of ORG27569 on CB1 receptor activation by taking advantage of the previously identified inactive and constitutively active mutants, T210A and T210I CB1 receptors. We demonstrated that the allosteric modulator ORG27569 induces a CB1 receptor state that is characterized by enhanced agonist affinity and decreased inverse agonist affinity consistent with an active conformation. The impact of ORG27569 binding was most dramatic on the inactive T210A receptor and less pronounced on the already active T210I receptor. Although ORG27569 antagonized CP55940-induced [35S]GTPγS binding indicative of G protein coupling inhibition in a concentration-dependent manner, the ORG27569-induced conformational change of the CB1 receptor led to cellular internalization and downstream activation of ERK1/2 signaling, providing the first case of allosteric ligand-biased signaling via CB1.

**EXPERIMENTAL PROCEDURES**

**CB1 Expression and Membrane Preparation**—The plasmid DNA encoding mutant receptors and the corresponding C-terminally green fluorescent protein (GFP)-tagged receptors were prepared as described previously (26). HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 3.5 mg/ml glucose at 37 °C in 5% CO₂. For transient expression of the receptors, HEK293 cells were seeded at 800,000 cells/100-mm dish on the day prior to transfection. Transfection was carried out using the calcium phosphate precipitation method (27). 24 h post-transfection, membranes of transfected cells expressing the wild-type or mutant receptors were prepared as described previously (28). Hippocampal cultures were prepared as described previously (29). In brief, dissociated hippocampal neurons from embryonic day 18 rats were plated on 15-mm-diameter coverslips coated with poly-d-lysine (Sigma-Aldrich) and laminin (ATCC, Manassas, VA) in Neurobasal medium supplemented with B-27, 25 μM glutamate, 500 μM glutamine (Invitrogen), and 2-mercaptoethanol at a density of ~150/mm² and maintained at 37 °C in a humidified 5% CO₂ incubator. The cells were fed 5 days after plating and weekly thereafter with plating medium without glutamate and 2-mercaptoethanol.

**Radioligand Binding Assay**—Saturation binding assays were performed as described previously (30) with some modifications. Approximately 3–5 μg of membranes were incubated at 30 °C for 90 min in a total volume of 200 or 500 μl of TME buffer (25 mM Tris-HCl, 5 mM MgCl₂, and 1 mM EDTA, pH 7.4) containing 0.1% fatty acid-free bovine serum albumin using [3H]CP55940 (147.9 Ci/mmol; PerkinElmer Life Sciences) or [3H]SR141716A (43 Ci/mmol; PerkinElmer Life Sciences). The total assay volume and the amount of membrane samples were adjusted to avoid ligand depletion by keeping the bound ligand less than 10% of the total. At least nine radiolabeled ligand concentrations (typically ranging from 0.16 to 40.00 nM) were used to determine K_d values of the receptors. In competition binding
assays and assays used to determine the cooperativity between allosteric and orthosteric ligands, the cell membranes were incubated with a fixed tracer concentration typically at the $K_d$ of the receptor using at least nine concentrations of unlabeled ligand (ranging between 100 pm and 100 μm) as the displacing ligand. Nonspecific binding was determined in the presence of 1 μm unlabeled ligand. Reactions were terminated by adding 250 μl of TME buffer containing 5% BSA followed by filtration with a Brandel cell harvester through Whatman GF/C filter paper. Radioactivity was measured by liquid scintillation counting.

$\text{GTPyS Binding Assay}$—15 μg of membranes were incubated for 60 min at 30 °C in a total volume of 500 μL GTPyS binding assay buffer (50 mM Tris-HCl, pH 7.4, 3 mM MgCl2, 0.2 mM EGTA, and 100 mM NaCl) with unlabeled ligand (at least nine different concentrations were used ranging between 100 pm and 100 μm), 0.1 nM $[^{35}S]\text{GTPyS}$ (1250 Ci/mmol; PerkinElmer Life Sciences), 10 μM GDP, and 0.1% (w/v) BSA. The basal GTPyS binding was measured in the absence of ligand. Nonspecific binding was determined with 10 μM unlabeled GTPyS (Sigma). The reaction was terminated by rapid filtration through Whatman GF/C filters. The radioactivity trapped in the filters was determined by liquid scintillation counting.

$Ligand and GTPyS Binding Data Analysis$—All ligand binding assays and GTPyS binding assays were carried out in duplicate. Data are presented as the mean ± S.E. value or the mean with the corresponding 95% confidence limits from at least three independent experiments. The $K_d$ and $B_{max}$ values were calculated by nonlinear regression using GraphPad Prism Software (GraphPad Software Inc., San Diego, CA). To evaluate the interaction between the radioligands and the allosteric modulator, the following allosteric ternary complex model (31) was applied as described by Price et al. (15).

$$Y = \frac{[A]/([A] + (K_a(1 + [B]/K_b)/(1 + \alpha[B]/K_b)))}{(1 + \alpha[B]/K_b)} \tag{Eq. 1}$$

where $Y$ denotes the fractional specific binding and $[A]$ and $[B]$ denote the concentration of orthosteric and allosteric modulator, respectively. $K_a$ and $K_b$ are the equilibrium dissociation constant for orthosteric ligand and allosteric modulator, respectively. $\alpha$ is the antilogarithm of the cooperativity factor. When $\alpha = 1.0$, the modulator does not alter orthosteric ligand binding. If $\alpha$ is less than 1.0, the modulator reduces ligand binding (negative allosteric modulation). If $\alpha$ is greater than 1.0, the modulator increases ligand binding (positive allosteric modulation). For competition binding assays, $IC_{50}$ values were determined by nonlinear regression. $K_i$ values were then calculated using the Cheng-Prusoff equation (32) based on $K_d$ values obtained from saturation binding analyses. The binding constants including $K_p$, $K_m$, and $K_i$ values for the wild-type and mutant receptors were compared using analysis of variance followed by Bonferroni’s post hoc test for significance. $p$ values of <0.05 were considered to be statistically significant.

$Confocal Microscopy$—HEK293 cells expressing CB1 receptors C-terminally fused to GFP were seeded onto 35-mm glass-bottomed dishes (MatTek Corp., Ashland, MA) precoated with poly-d-lysine. Cells were treated with different ligands for various lengths of time as indicated in the figures and then washed three times with PBS followed by fixation with 4% paraformaldehyde for 10 min at room temperature. For co-localization studies, the cells were permeabilized by 0.1% Triton X-100 in DME containing 5% normal goat serum, pH 7.6. After incubating with blocking solution (5% normal goat serum in DME) for 30 min at room temperature, the cells were incubated with the lysosome-associated membrane protein 1 (LAMP-1) (H4A3) antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) diluted 1:200 in DME containing 5% normal goat serum. After washing with PBS, cells were incubated with Cy3-labeled donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:200 for 30 min at room temperature. Cells were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and visualized using a Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzler, Germany). Images were collected from at least three independently transfected cell dishes and processed for presentation in figures using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA). Quantification of co-localization was performed using ImageJ software (National Institutes of Health, Bethesda, MD) with the JACoP plugin (33) as described previously (13, 33). For the analysis, images of ~10–15 cells were taken. Images for the receptor-GFP (green) and LAMP-1 (red) were processed in parallel, background-subtracted, and converted into eight-bit format for analysis. The extent of co-localization was quantified using intensity correlation analysis. Pearson’s correlation coefficient (PCC) ($r$), which provides an estimate of the goodness of co-localization, was determined ($-1$ = a negative correlation, 0 = no correlation, and +1 = a positive correlation). PCC ($r$) is presented as the mean ± S.E.

$Immunoblotting Studies$—Cells expressing CB1 receptors were exposed to the different compounds for the times indicated. For toxin treatment to abrogate Gi coupling effects, PTX was added to the medium at 5 ng/ml (Calbiochem). Following an 18-h incubation in the presence of toxin, cells were washed twice with PBS and treated with compounds (34). Cells were washed with ice-cold PBS, and cell lysates were obtained by harvesting the cells with ice-cold lysis buffer (150 mM NaCl, 1% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 7.5 containing 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin as protease inhibitors) (Sigma). Samples were subjected to SDS-polyacrylamide gel electrophoresis in 10% gels, and proteins were transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking with blocking buffer (Fisher Scientific), the membrane was incubated for 1 h with the corresponding antibody (1:3000 phospho-p44/42 and p44/42 MAPK antibodies; 1:3000 phospho-SAPK/JNK and SAPK/JNK antibodies; Cell Signaling Technology, Danvers, MA). The membranes were washed with PBS, and then they were incubated with anti-rabbit peroxidase-conjugated secondary antibody (1:6000; Cell Signaling Technology) for 1 h at room temperature. The specific immunoreactive proteins were detected using the SuperSignal West Femto Chemiluminescent Substrate System (Thermo Fisher Scientific, Rockford, IL) follow-
ing the manufacturer’s protocol. Immunoblots were quantified with the ImageJ program.

RESULTS

**ORG27569 Impacts Ligand Binding Consistent with Induction of High Affinity Conformation for Agonist**—ORG27569, a CB1 receptor allosteric modulator, increases the binding of the CB1 agonist \([3H]CP55940\) but decreases the binding of the inverse agonist \([3H]SR141716A\) to CB1 in mouse brain membranes (15). It is generally accepted that a GPCR in its active state displays enhanced agonist affinity but decreased inverse agonist affinity, whereas the inactive conformation of the receptor exhibits the converse properties, preferring inverse agonist affinity, whereas the inactive conformation of the receptor exhibits the converse properties, preferring inverse agonist binding to agonist binding. Thus, we hypothesize that the ORG27569-induced changes in agonist and inverse agonist binding affinities may be due to the transition between inactive and active conformations of the receptor. To evaluate this possibility, we assessed the effect of the ORG compound on \([3H]CP55940\) binding to the CB1 wild-type receptor and two mutant receptors, T210A and T210I, that we previously demonstrated were more fully inactive and constitutively active, respectively, than the wild-type receptor (26).

### Initial ORG27569 Binding Experiments

Initial ORG27569 binding experiments were performed using \([3H]CP55940\) as the tracer to determine the extent of cooperativity between the allosteric modulator and the binding of the orthosteric ligand. Fig. 2 shows the equilibrium dissociation constant \((K_B)\) values for the allosteric modulator and the cooperativity factor \((\alpha)\), which quantifies the magnitude of the allosteric effect on orthosteric ligand binding. The constitutively inactive T210A receptor \((K_B = 644.2 \text{ nM})\) shows a 3-fold increased \(K_B\) value for ORG27569 with respect to the wild-type receptor \((K_B = 217.3 \text{ nM})\). Because the T210A receptor has a 3.6-fold lower affinity for CP55940 than the wild-type receptor \((K_d\) values of 2.15 ± 0.48 and 7.82 ± 1.30 nM for the wild-type and T210A receptors, respectively), it displays a slightly better cooperativity for ORG27569. In contrast, the presence of ORG27569 did not affect \([3H]CP55940\) binding for the highly constitutively active T210I receptor (Fig. 2A). These data suggest that ORG27569 has a varying effect on agonist binding depending on the extent of activation of the receptor. The T210A receptor showed the greatest enhancement in CP55940 binding; the wild-type receptor showed a slightly lesser increase compared with the T210A receptor, whereas the T210I receptor showed no change in CP55940 binding with increasing concentration of ORG27569 (Fig. 2B).

To further investigate the effect of ORG27569 on the affinity of the receptors for agonist and inverse agonist, saturation binding experiments were performed for \([3H]CP55940\) and \([3H]SR141716A\) in the absence and presence of ORG27569. As shown previously (26), the T210I receptor had the greatest affinity for CP55940 among the three receptors with a \(K_d\) of 0.31 ± 0.12 nM followed by the wild-type and T210A receptors.
**Allosteric Modulator ORG27569-induced CB1 Biased Signaling**

**Table 1**

|                      | Vehicle alone | ORG27569 |
|----------------------|---------------|----------|
|                      | $K_d$ (nM)   | $K_d$ (receptor:WT) | $B_{max}$ | $K_d$ (nM) | $K_d$ (receptor:WT) | $B_{max}$ |
| CB1 WT               | 2.15 ± 0.48  | 3072 ± 256 | 0.29 ± 0.06 | 3798 ± 127 |
| CB1 T210I            | 0.31 ± 0.12a | 3353 ± 266 | 0.42 ± 0.14 | 3658 ± 327 |
| CB1 T210A            | 7.82 ± 1.30b | 2603 ± 285 | 0.30 ± 0.05 | 3843 ± 166 |

* Statistical significance difference from wild type ($p < 0.05$) using an analysis of variance followed by Bonferroni’s post hoc test for significance.

**Table 2**

|                      | Vehicle alone | ORG27569 |
|----------------------|---------------|----------|
|                      | $K_d$ (nM)   | $K_d$ (receptor:WT) | $B_{max}$ | $K_d$ (nM) | $K_d$ (receptor:WT) | $B_{max}$ |
| CB1 WT               | 1.93 ± 0.48  | 4278 ± 217 | 15.68 ± 5.85 | 4731 ± 704 |
| CB1 T210I            | 17.75 ± 3.67a | 3915 ± 880 | 22.60 ± 5.67b | 5191 ± 491b |
| CB1 T210A            | 0.93 ± 0.27m | 4470 ± 316 | 13.92 ± 5.23 | 5313 ± 724 |

* Statistical significance difference from wild type ($p < 0.05$) using an analysis of variance followed by Bonferroni’s post hoc test for significance.

* No apparent saturable binding of $[^3H]$SR141716A was observed up to 80 nM. Instead, $K_d$ and $B_{max}$ values for SR141716A were determined from homologous competition binding using 22 nM $[^3H]$SR141716A as a tracer and a wide range of cold ligand concentrations.

with $K_d$ values of 2.15 ± 0.48 and 7.82 ± 1.30 nM, respectively (Table 1). In the presence of 10 µM ORG27569, however, the three receptors exhibited similar $K_d$ values for CP55940 (0.29 ± 0.06, 0.42 ± 0.14, and 0.30 ± 0.05 nM for the wild-type, T210I, and T210A receptors, respectively) that correspond to the high affinity observed with the T210I receptor. All three receptors showed negligible $B_{max}$ changes in $[^3H]$CP55940 binding with ORG27569.

Table 2 shows the saturation binding parameters for $[^3H]$SR141716A for the wild-type, T210I, and T210A receptors. Here, the rank order of the affinity of the receptors for the inverse agonist is the reverse relative to CP55940. The wild-type receptor ($K_d = 1.93 ± 0.48$ nM) has a 9-fold higher affinity for $[^3H]$SR141716A than the T210I receptor ($K_d = 17.75 ± 3.67$ nM) but a 2-fold lower affinity than the T210A receptor ($K_d = 0.93 ± 0.27$ nM). In the presence of 10 µM ORG27569, the wild-type and T210A receptors exhibited 8- and 13-fold $K_d$ shifts to weaker affinity, respectively, from the $K_d$ values in the absence of ORG27569. For the T210I mutant receptor, we observed no apparent saturable binding of $[^3H]$SR141716A up to 80 nM concentration of the ligand. Due to the lack of extremely concentrated stock of the radiolabeled ligand, we could not perform a saturation binding assay at the higher concentration needed for statistical reliability. Instead, homologous competition binding assays were performed to estimate $K_d$ and $B_{max}$ values (36, 37). The T210I receptor exhibited only a marginal increase in $K_d$ value with 10 µM ORG27569 (17.75 ± 3.67 and 22.60 ± 5.67 nM in the absence and presence of ORG27569, respectively). Interestingly, all three receptors exhibited similar $K_d$ values in the presence of ORG27569 as shown for $[^3H]$CP55940 only in the direction of weaker affinity for the inverse agonist. All three receptors showed negligible $B_{max}$ changes in $[^3H]$SR141716A binding with ORG27569. Taken together, the addition of ORG27569 resulted in an enhanced affinity for agonist but a decreased affinity for inverse agonist comparable with that of the receptor in its active conformation.

**ORG27569 Inhibits Basal and Agonist-induced G Protein Coupling in Concentration-dependent Manner**—It was shown previously that ORG27569 reduces the efficacy of CP55940 in a reporter-gene assay and the efficacy of WIN55212-2 for inhibition of electrically evoked contractions in a mouse vas deferens preparation (15). To investigate the effect of ORG27569 in CB1-mediated G protein coupling, $[^35S]$GTPγS binding assays were performed in the presence of ORG27569. This assay monitors the level of G protein activation by determining the extent of binding of the nonhydrolyzable analog to Gα subunits. First, we evaluated the impact of the allosteric modulator on agonist CP55940-induced $[^35S]$GTPγS binding in the presence of various concentrations of ORG27569. We observed a progressive decrease in the $E_{max}$ values with an increasing concentration of ORG27569 for the CB1 wild-type receptor (Fig. 3A). The addition of 0.1 and 0.3 µM ORG27569 resulted in a 52.4 and 67.4% reduction in $E_{max}$ values ($E_{max} = 70.5$ and 63.1 fmol/mg), respectively, compared with that of wild type in the absence of ORG27569. Treatment with ORG27569 at 1 µM showed a complete inhibition of CP55940-stimulated $[^35S]$GTPγS binding. Interestingly, treatment with 10 µM ORG27569 further inhibited GTPγS binding to a point that is below the basal level (in the absence of agonist) observed.

The basal G protein coupling activity level of the T201A, wild-type, and T210I receptors is consistent with its inactive, partially active, and more fully constitutively active states (Fig. 3B). ORG27569 in the absence of CP55940 also antagonized the basal level of $[^35S]$GTPγS binding to the wild-type and T210I receptors in a concentration-dependent manner. Treatment with 10 µM ORG27569 resulted in very low levels of $[^35S]$GTPγS binding to the receptors comparable with the effect of 10 µM inverse agonist SR141716A, which produces the
Allosteric Modulator ORG27569-induced CB1 Biased Signaling

ORG27569 Promotes Internalization of CB1 Receptor—Following prolonged activation, GPCRs typically become desensitized to further agonist binding and subsequently become internalized in the cell. To investigate the apparent conundrum that ORG27569 induces high affinity conformation for agonist yet inhibits G protein coupling, we evaluated the cellular response of CB1 with ORG27569 treatment using confocal microscopy of cells expressing GFP-tagged CB1 receptors. Initially, we examined the internalization of the CB1 wild-type-GFP, T210I-GFP, and T210A-GFP receptors at various time points following treatment with 10 μM ORG27569. To evaluate the extent of internalization of the receptors from the cell surface, co-localization of receptors with LAMP-1, the marker for the late endosome/lysosome, was assessed at various time points. Consistent with previous findings (13, 14, 28, 38), the wild-type receptor localized mainly to intracellular vesicles in HEK293 cells in the absence of ligand consistent with its partial constitutive activity (Fig. 4A, top). This localization pattern has been demonstrated in many cell types including endogenously CB1-expressing and stably expressing cells (39–42). The T210I receptor exhibited almost exclusively intracellular localization (Fig. 4A, bottom) as shown previously (26). Because both receptors were internalized in the absence of ORG27569 as anticipated, a 3-h treatment with 10 μM ORG27569 showed no difference, and both wild-type and T210I receptors were found in intracellular compartments (Fig. 4B). The results are comparable at earlier time points (Fig. 4E). Because the majority of both receptors were constitutively internalized from the cell surface, it is impossible to accurately assess the effect of ORG27569 treatment for these two receptors. Consequently, we took advantage of the inactive T210A receptor, which is mainly expressed at the cell surface (Fig. 4C) in the absence of ligand and thus provides a window in which the extent of receptor internalization can be monitored. Interestingly, ORG27569 treatment produced substantial T210A receptor internalization after a 3-h treatment compared with the receptors with vehicle (0.03% DMSO) treatment only (Fig. 4, C and D).

Using the T210A receptor, we additionally examined how ORG27569 affects CP55940-induced CB1 receptor internalization because ORG27569 displays an antagonizing effect on CP55940-induced G protein activation. Our data show that the CB1 T210A receptor underwent pronounced internalization upon treatment with CP55940 by 40 min (Fig. 5, A and C). Interestingly, co-treatment with CP55940 and ORG27569 resulted in robust acceleration in the internalization process of the CB1 T210A receptor (Fig. 5, B and C). High levels of receptor internalization were observed at 10 min and plateaued by 20 min (Fig. 5C). This indicates that although ORG27569 antagonizes G protein coupling activity the mutant receptor, like the wild-type receptor, is internalized after the production of an activated receptor state.

FIGURE 3. Effect of ORG27569 on stimulation of [35S]GTPγS binding to HEK293 cell membranes expressing CB1 wild-type, T210I, or T210A receptors. Dose-response curves for CP55940-induced [35S]GTPγS binding in membrane preparations of HEK293 cells expressing the wild-type receptor in the absence (C) or presence of 0.1, 0.3, or 1 μM ORG27569 are shown (A). The basal level of [35S]GTPγS binding was measured for the wild-type, T210I, and T210A receptors (B). Inhibition of [35S]GTPγS binding by SR141716A and ORG27569 was tested at the indicated concentrations for each receptor. Data are presented as specific binding of GTPγS to the membranes. Nonspecific binding was determined in the presence of 10 μM unlabeled GTPγS. Each data point represents the mean ± S.E. (error bars) of at least three independent experiments performed in duplicate. The solid line indicates the level of non CB1-mediated GTPγS binding obtained from [35S]GTPγS binding to the mock-transfected membrane sample.

ORG27569 Increases ERK1/2 Phosphorylation but Not JNK/SANK Phosphorylation—The rapid cellular internalization resulting from ORG27569 treatment inspired us to investigate its effect on downstream signaling. Our data indicate that ORG27569 induces subcellular internalization of the receptor, suggesting that the receptor interacts with endocytic scaffold proteins such as arrestin, which may also serve as a key component of the signal transduction pathway (2, 43–45). Previous studies suggest that upon agonist treatment the CB1 receptor activates the MAPK signaling pathway in a variety of cell types in a G protein-dependent manner (46–50). To characterize the role of the allosteric modulator ORG27569 in cellular signaling, we investigated the activation of MAPK family members including JNK and ERK1/2 (Fig. 6). The CB1 wild-type receptor displayed a CP55940-stimulated JNK phosphorylation with a
Allosteric Modulator ORG27569-induced CB1 Biased Signaling

FIGURE 4. Cellular internalization of CB1 receptors upon treatment with allosteric modulator ORG27569. HEK293 cells expressing the CB1 wild type-GFP (A and B, top) and T210I-GFP receptors (A and B, bottom) were incubated with vehicle alone (0.03% DMSO) or 10 μM ORG27569 for 3 h as indicated (A). HEK293 cells expressing the CB1 T210A-GFP receptor were incubated with vehicle alone (0.03% DMSO) (C) or 10 μM ORG27569 (D) for 0, 3, and 4 h as indicated. After incubation, cells were washed and fixed as described under “Experimental Procedures.” Localization of GFP-tagged wild-type, T210I, and T210A receptors (green; left) and the late endosome/lysosome marker LAMP-1 (red; middle) and an overlay of the fluorescence images (yellow; right) are shown. Images are representative of at least three independent transfections. Scale bar, 15 μm (white bar; see A). The extent of co-localization quantified using the intensity correlation analysis is shown (E). Twelve images of each condition were analyzed (n = 12). The PCC (r), which provides an estimate of the goodness of co-localization, was calculated at each time point. PCC (r) is presented as the mean ± S.E. (error bars).

peak at 5 min. Western blot analysis of each sample lysate was performed with phospho- and total JNK antibodies for comparison (Fig. 6, A–C, upper panel, phospho-JNK, and lower panel, total JNK). The two bands correspond to phosphorylated p46 (JNK1) and p54 (JNK2), the predominant isoforms of JNK. The CP55940-induced increase in JNK phosphorylation was also observed for the two mutant receptors, T210I and T210A. This effect was blocked by 10 μM SR141716A, indicating that the JNK phosphorylation is CB1-mediated (data not shown). However, in agreement with the [35S]GTPγS binding data, ORG27569 abrogated the JNK phosphorylation induced by CP55940 for all three receptors (Fig. 6, A–C). It is important to note that both phosphorylated forms of JNK responded similarly to the treatment, whereas the total JNK remained constant for all three receptors regardless of treatment, indicating that the impact of ORG27569 was not a result of expression level differences. These data suggest that JNK phosphorylation is only G protein-dependent via CB1 consistent with a previous report by Rueda and colleagues (51).

Next, we investigated the signaling pathway involving ERK1/2 because ERK1/2 phosphorylation has been demonstrated to occur both G protein-dependently and G protein-independently by the μ-opioid receptor (52), angiotensin AT1A receptor (53), and parathyroid hormone receptor (45). Consistent with previously reported data in mouse neuroblastoma cells (54), CP55940 (0.2 μM) induced a time-dependent stimulation of ERK1/2 with a maximum phosphorylation at 5 min (Fig. 6D). The two bands correspond to the 42- (p42: ERK2) and 44 (p44: ERK1)-kDa isoforms, and both were increased by CP55940. Interestingly, ORG27569 (10 μM) alone caused a significantly higher increase in transient ERK1/2 phosphorylation compared with the stimulation by CP55940 alone in cells expressing the CB1 wild-type receptor. The phosphorylation peaked at 5 min and diminished after 10 min. When the cells were co-treated with ORG27569 and CP55940, a peak at 5 min was also observed that was somewhat diminished relative to ORG27569 alone, perhaps reflecting some competition between G protein-mediated and G protein-independent pathways. As a control, ORG27569 treatment showed no effect on ERK1/2 phosphorylation in untransfected HEK293 cells, suggesting that the ORG27569-induced ERK1/2 phosphorylation is CB1-mediated.

CP55940, ORG27569, and both CP55940 and ORG27569 treatments increased ERK1/2 phosphorylation levels by 5.0-, 10-, and 8-fold, respectively, over the basal level at 5 min, whereas expression of phosphorylated plus unphosphorylated ERK1/2 remained constant. ORG27569 treatment and co-treatment with ORG27569 and CP55940 also increased ERK1/2 phosphorylation for the cells expressing the T210I and T210A receptors relative to the basal level of each receptor. CP55940, ORG27569, and both CP55940 and ORG27569 treatment of the cells expressing the T210I increased phosphorylation levels by 10-, 15-, and 15-fold, respectively, whereas the same treatments for the cells expressing the T210A increased phosphorylation levels by 7-, 10-, and 10-fold, respectively, over each basal level at its 5-min peak. It is important to note that the basal level of ERK1/2 phosphorylation for each receptor is different and consistent with their activity profiles; the T210A receptor is inactive, the wild-type receptor is slightly active, and the T210I receptor is highly constitutively active. Thus, the fold change over the basal level does not allow us to directly compare the effect of ORG27569 on ERK1/2 phosphorylation among the receptors. In each case, total ERK1/2 levels were constant (Fig. 6, D–F).
To ensure that the effect of ORG27569 on ERK1/2 signaling is not limited to HEK293 cells, we tested ORG27569-induced JNK and ERK1/2 phosphorylation with hippocampal neurons endogenously expressing CB1. Consistent with the phosphorylation pattern of HEK293 cells, CP55940 treatment increased phosphorylation of JNK and ERK1/2 1.8- and 1.5-fold, respectively, relative to basal levels (Fig. 7). In contrast, ORG27569 treatment inhibited the level of JNK phosphorylation, whereas it increased ERK1/2 phosphorylation by 2.2-fold. Collectively, these data indicate that ORG27569 is a positive allosteric agonist that enhances ERK1/2 signaling in neuronal cells as it does in HEK293 cells.

ORG27569-induced ERK1/2 Phosphorylation Is Gi Protein-independent—To further evaluate the involvement of Gi protein in ORG27569-induced ERK1/2 phosphorylation, we treated the cells expressing CB1 with the G\textsubscript{i/o} inhibitor PTX. PTX attenuated the CP55940-induced JNK phosphorylation (Fig. 8A), suggesting that CP55940 activates JNK signaling via a PTX-sensitive G\textsubscript{i/o}-coupled pathway. Strikingly, PTX treatment completely abolished CP55940-induced ERK1/2 phosphorylation (Fig. 8B). However, in marked contrast, ORG27569-induced ERK1/2 phosphorylation was not affected by PTX treatment (Fig. 8B). Both JNK and ERK1/2 phosphorylation remained unaffected by cholera toxin (data not shown), suggesting that G\textsubscript{i} is not involved. Taken together, these results indicate that the activation of the ERK1/2 signaling pathway induced by ORG27569 is G\textsubscript{i}-independent.

DISCUSSION

An important finding of this study is that the allosteric modulator ORG27569 induces ligand-biased signaling (also termed...
functional selectivity) via the cannabinoid CB1 receptor by promoting a conformational change comparable with the active state adopted by the highly constitutive T210I receptor. Using HEK293 membrane preparations expressing CB1 receptors, we demonstrated that ORG27569 substantially enhances CP55940 binding affinity but decreases the binding affinity of the inverse agonist SR141716A, suggesting the involvement of ORG27569 in promoting a conformational change in the CB1 receptor toward a high affinity state for agonist. However, [35S]GTP binding data demonstrated that ORG27569 antagonizes the basal activity of the receptor as well as the stimulation of [35S]GTP binding by CP55940. Despite this inhibition of G protein coupling, ORG27569 treatment of HEK293 cells expressing the CB1 receptor showed receptor internalization as it does following agonist treatment (9, 55). Interestingly, ORG27569 was found to increase the phosphorylation of the ERK1/2 signaling pathway, whereas it failed to increase the phosphorylation of JNK. This is consistent with the finding of others (43, 56, 57) that JNK phosphorylation is G protein-mediated, whereas ERK1/2 phosphorylation can be G protein-dependent or -independent. Our data indicate that ORG27569 does indeed induce a conformation of the receptor that leads to biological activity but one that signals in a G-protein independent manner. Intriguingly, the angiotensin II receptor has been shown to be activated by a biased agonist that facilitates G protein-independent enhancement in ERK1/2 activation (53, 58).

Although a few small molecule allosteric modulators (Organon and Prosidion compounds) for the CB1 receptor were reported previously by Price et al. (15) and Horswill et al. (16), very little is known about the allosteric binding site on the receptor and the molecular basis underlying the conformational change caused by the binding of the allosteric modulator. In this study, we used three CB1 receptors (T210A, wild type, and T210I) to provide a spectrum of receptor activity that was ideal to study the influence of ORG27569. As shown in Table 1, the ORG27569-induced affinity change of the T210A, wild-type, and T210I receptors for CP55940 was progressively more extensive for the most active to the inactive T210A receptor. Although the $K_d$ values for CP55940 are different for these receptors depending on their extent of constitutive activity,
very similar \( K_d \) values were obtained for CP55940 in the presence of ORG27569. This suggests that the allosteric compound readily promotes a conformational change comparable with a high affinity agonist binding conformation that is similar for the three receptors including the originally inactive T210A receptor. However, additional work is needed to determine whether the high affinity states adopted by these receptors are truly the same, whether they have a similar activation mechanism, and whether they induce comparable second messenger signaling cascades.

One of the fundamental questions that will aid understanding the molecular basis of ORG27569 binding is whether or not it can bind to the receptor in the absence of the orthosteric agonist and induce a conformational change. Due to the lack of radiolabeled ORG27569, it is challenging to measure an accurate binding affinity for this compound to the CB1 receptor. Fig. 2 shows an equilibrium dissociation constant (\( K_d \)) for ORG27569 and the cooperativity between the orthosteric and allosteric binding sites using \([^{3}H]CP55940\) as tracer. This, however, does not directly reflect ORG27569 binding to the receptor per se. To assess whether or not ORG27569 alone can bind to the receptor, we investigated the effect of ORG27569 on \([^{35}S]GTP\gamma S\) binding, cellular localization of the receptor, and downstream signaling in the absence of orthosteric agonist. The antagonizing effect of ORG27569 on \([^{35}S]GTP\gamma S\) binding leading to inhibition of basal activity for the wild-type and T210I receptors clearly suggests that it binds to aporeceptors, which have constitutive activity. For the fully inactive T210A receptor, which displays no basal \([^{35}S]GTP\gamma S\) binding, we assessed the effect of ORG27569 on cellular localization of the receptor. Although the T210A receptor internalized with 10 \( \mu M \) ORG27569 treatment at a rate approximately 4 times slower than that with treatment of CP55940 (0.5 \( \mu M \)), these data also suggest that ORG27569 bound the apo-T210A receptor. The wild-type and T210I receptors could not be used to evaluate the internalization due to their constitutive endocytosis.

The \( E_{\text{max}} \) values determined from the \([^{35}S]GTP\gamma S\) binding for the wild-type receptor decreased with increasing ORG27569 concentration, suggesting that when the allosteric compound is present in sufficiently high concentrations G protein coupling is precluded. A few different explanations could account for the loss in coupling. First, we cannot rule out the possibility that the allosteric compound-induced conformation preferentially binds other G\( \alpha \) subtypes such as G\(_{q}\) or G\(_{q,15}\), which have a substantially lower rate of basal guanine nucleotide exchange compared with that of G\(_{q,11}\) and could go undetected in the \([^{35}S]GTP\gamma S\) binding assay system. However, in view of the work of Ross and co-workers (15) that demonstrates no G protein coupling using different assays, this seems unlikely. In

**FIGURE 7.** CP55940- and ORG27569-promoted MAPK signaling in hippocampal neuronal cells. Rat hippocampal neurons endogenously expressing CB1 were exposed to CP55940 (0.5 \( \mu M \)) or ORG27569 (10 \( \mu M \)) for 5 min. Cell lysates were resolved by SDS-PAGE and JNK (A) and ERK1/2 (B) phosphorylation was detected as described in Fig. 5. Representative blots of phosphorylated (p-JNK and p-ERK1/2) and total (JNK and ERK1/2) MAPK signals are depicted for each receptor. Graphs show the quantified JNK and ERK1/2 phosphorylation levels deduced from four independent experiments. Statistical significance of the differences was assessed using one-way analysis of variance and Bonferroni’s post-hoc test: *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \).

**FIGURE 8.** Pertussis toxin sensitivity of JNK and ERK1/2 phosphorylation by ORG27569. HEK293 cells expressing CB1 receptors were incubated with vehicle or 5 ng/ml PTX for 18 h and treated with CP55940 (0.5 \( \mu M \)) or ORG27569 (10 \( \mu M \)) for 5 and 15 min as indicated. Equal amounts of cell lysate were resolved by SDS-PAGE, and JNK (A) and ERK1/2 (B) phosphorylation was detected as described in Fig. 5. Representative blots of phosphorylated (p-JNK and p-ERK1/2) and total (JNK and ERK1/2) MAPK signals are depicted for each receptor. Graphs show the quantified JNK and ERK1/2 phosphorylation levels deduced from four independent experiments. Data are expressed as the -fold increase above the basal level of phosphorylation. Data represent the mean ± S.E. (error bars) of at least three independent experiments. Statistical significance of the differences was assessed using one-way analysis of variance and Bonferroni’s post-hoc test: **, \( p < 0.01 \); and ***, \( p < 0.001 \).
Allosteric Modulator ORG27569-induced CB1 Biased Signaling

addition, our unpublished data showed that ERK1/2 phosphorylation induced by ORG27569 was cholera toxin-insensitive, suggesting that Gαi is not involved. Second, ORG27569 may induce an active receptor conformation that has a higher affinity for binding signaling molecules other than G proteins (such as β-arrestins). Third, ORG27569 might function through the intracellular region of the receptor directly or indirectly. If ORG27569 binds to the intracellular region of the receptor, then it may block the interaction sites with G protein, which are formed by the intracellular loops and C terminus in most class A GPCRs. Indirectly, ORG27569 binding could affect the structure of the intracellular region of the receptor. Dowal et al. (59) demonstrated that an allosteric modulator modulates proteinase-activated receptor 1 activity through helix 8, which has been found in most class A receptors including CB1 (13). This finding is intriguing because it provides an example in which the allosteric modulator plays a role in selective G protein coupling through the intracellular domain and results in differential downstream signaling. Fourth, it is also possible that ORG27569 binding promotes a different oligomerization state of this receptor. Przybyla and Watts (60) demonstrated that receptor activation affects the formation of CB1 and dopamine D2L receptor heterodimers. It can be speculated that a different receptor conformation induced by ORG27569 might confer different Gα coupling on the partnering receptor in the dimeric form, resulting in differential signaling via different downstream effectors.

It is noted that 1 μM ORG27569 inhibited CP55940-stimulated GTPγS binding but not the basal level of GTPγS binding. The basal GTPγS binding was reduced at a higher concentration (10 μM ORG27569) to a level comparable with inverse agonist SR141716A treatment. We cannot rule out the possibility that other effects of ORG27569 might take place. However, partial inhibition of basal GTPγS binding by intermediate ORG27569 concentrations suggests that the inhibition is receptor-mediated. Moreover, receptor-mediated effects were observed in several different assays at this concentration. For example, we demonstrated that the receptor undergoes robust endocytosis at a 10 μM concentration of ORG27569. Also, the presence of 10 μM ORG27569 shifted the binding affinity of CP55940 and SR141716A for the receptor. Additionally, the levels of ERK1/2 and JNK phosphorylation for the sample from untransfected cells remained unchanged upon treatment with 10 μM ORG27569 (Fig. 6, A and D). Taken together, these data suggest that 10 μM ORG27569 displays its distinct effects directly through CB1.

Numerous studies support that GPCRs can adopt different conformations to produce different degrees of functional activity depending on the agonist bound (61, 62). More recently, it has become evident that some allosteric modulators can also activate the receptor for signal transduction on their own (termed “allosteric agonist”) (63, 64). In addition to modulating the binding and efficacy of an orthosteric ligand, these allosteric agonists were shown to directly affect receptor activation, cellular localization, or down-regulation of various GPCRs including the M1 muscarinic acetylcholine receptor, mGluR7, and adenosine A1 receptor (65–68). Here, we show that ORG27569 can activate CB1 and promote downstream effects in the absence of the orthosteric agonist.

Tschammer et al. (69) have identified a single residue in TM6 as a key determinant of ligand-biased signaling in the dopamine D2L receptor. Similarly, Gregory et al. (70) identified critical residues in the orthosteric and allosteric binding regions of the M2 muscarinic acetylcholine receptor, mutation of which caused differential efficacy of agonists consistent with a role of the allosteric modulator in differential signaling. These data demonstrate that the binding of different agonists promotes different active conformations of the receptor that are pathway-specific. Further studies are required to reveal the detailed molecular mechanisms involved in ligand-biased signaling for CB1. As recent studies indicate that β-arrestins can serve as a scaffold for G protein-independent signaling (53, 71), β-arrestin is a candidate that needs to be explored here and could have significant therapeutic consequences. Another possible mechanism may involve a factor associated with neutral sphingomyelinase. It has been shown that CB1-mediated release of ceramide through sphingomyelin hydrolysis activates ERK signaling, and this activation may be Gαi-independent (72–74).

Because allosteric modulators bind a site remote from the orthosteric ligand binding site and regulate its binding affinity, their selectivity can be of enormous advantage. For example, although CP55940 can bind both CB1 and CB2 receptors with the same affinity, our unpublished data show that ORG27569 only regulates CP55940 binding to CB1 but not to CB2. This suggests that ORG27569 binds a site with higher sequence divergence (e.g. loops and/or termini) than the highly conserved orthosteric ligand binding pocket within the TMs. Thus, ORG27569 provides an example of subtype selectivity. Moreover, as we demonstrated in this study, allosteric modulators as well as orthosteric ligands can have the ability to alter the conformational equilibrium of the receptor, resulting in discrete signaling output through different effectors. This functional selectivity of an allosteric modulator can be used to either attenuate an endogenous ligand-induced response or promote a very different response from that of the endogenous ligand. It is clear that allosteric modulation of CB1 provides an avenue for specific regulation of receptor function that may provide valuable strategies for impacting disease.

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