Chemically Distinct Ligands Promote Differential CB$_1$ Cannabinoid Receptor-Gi Protein Interactions

Somnath Mukhopadhyay and Allyn C. Howlett

Neuroscience of Drug Abuse Research Program, J. L. Chambers Biomedical/Biotechnology Research Institute, North Carolina Central University, Durham, North Carolina

Received August 4, 2004; accepted March 4, 2005

ABSTRACT

To understand how structurally distinct ligands regulate CB$_1$ receptor interactions with Gi1, Gi2, and Gi3, we quantified the Gai and G$_{i\beta}$ proteins that communoprecipitate with the CB$_1$ receptor from a detergent extract of N18TG2 membranes in the presence of ligands. A mixture of A, R, G$_{i\alpha}$ (or G$_i$), and ARG$_{i\alpha}$ complexes was observed in the presence of aminoalkylindole (R)-(+-)[2,3-dihydro-5-methyl-3-[4-morpholinylmethyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalemethane (WIN 55,212-2) for all three RG$_i$ complexes, cannabinoid desacetyllevonantradol for Gai1 and Gai2, and eicosanoid (R)-methanandamide for Gai3. Desacetyllevonantradol maintained RGai1 and RGai2 complexes and (R)-methanandamide maintained RGai1 and RGai2 complexes even in the presence of a nonhydrolyzable GTP analog. The biaryl pyrazole antagonist N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboximide hydrochloride (SR141716) maintained all three RGai complexes. Gj proteins, and to a certain extent Gj2, exhibited the same association/dissociation pattern as the G$_i$ proteins. A GDP analog had no influence on any of these association/dissociation reactions and failed to promote sequestration of G proteins. These results can be explained by invoking the existence of an inverse agonist-supported inactive state in the ternary complex equilibrium model. WIN 55,212-2 behaves as an agonist for all three Gi subtypes; SR141716 behaves as an inverse agonist for all three Gi subtypes; desacetyllevonantradol behaves as an agonist for Gi1 and Gi2, and an inverse agonist at Gi3; and (R)-methanandamide behaves as an inverse agonist at Gi1 and Gi2, and an agonist at Gi3. These ligand-selective G protein responses imply that multiple conformations of the receptor could be evoked by ligands to regulate individual G proteins.

It has become generally accepted that different GPCRs in a cell can couple selectively to different Ga and G$_{i\beta}$ subtypes (Gudermann et al., 1996). This selective coupling can occur even within the Gi/o subfamily (Cordeaux et al., 2001; Faivre et al., 2001; Yang et al., 2002). “Agonist trafficking”, which is the promotion by an agonist of receptor coupling to one G protein versus another leading to the activation of different signal transduction pathways, was described in ternary complex equilibrium models of multiple activated-receptor states coupling selectively to different G proteins (Kenakin, 1995; Leff et al., 1997; Clarke and Bond, 1998). These models have been supported by observations of agonist-selective coupling of $\alpha_{1H}$-adrenergic receptor mutants (Perez et al., 1996) and 5-hydroxytryptamine-2 receptors (Berg et al., 1998) to pertussis toxin-sensitive versus -insensitive G proteins to stimulate different phospholipase pathways. Agonist-selective signal transduction has been demonstrated for $\alpha_{2}$-adrenergic receptors coupled to Gs or Gi (Brink et al., 2000) and neurotransmitter receptors coupled to Gs, Gi, or Gq/11 (Skrzylska et al., 2003) in transfected Chinese hamster ovary cells. GTP-S binding to exogenous G proteins was shown to exhibit agonist selectivity for $\alpha_{2}$-adrenergic receptors activating Go versus Gi proteins in NIH3T3 cells (Yang and Lanier, 1999) and D$_2$ receptors activating Gi2 versus Go in Sf21 insect cells (Cordeaux et al., 2001).

Our studies herein examine the molecular mechanism for the agonist-receptor-G protein selectivity for the CB$_1$ canna-

ABBREVIATIONS: GPCR, G protein-coupled receptor; CAPS, 3-[cyclohexylaminio]-1-propanesulfonic acid; CHAPS, 3-[[3-cholamidopropyl]dimethylammonio]propanesulfonate; DALN, desacetyllevonantradol; ECL, enhanced chemiluminescence; GDPjS, guanosine 5’-O-(3-thio)-diphosphate; GppNHp, guanyl-imidodiphosphate; TBS, Tris-buffered saline; TBST, Tris-buffered saline/Tween 20; GTPyS, guanosine 5’-O-(3-thio)-triphosphate; WIN 55,212-2, (R)-(+-)[2,3-dihydro-5-methyl-3-[4-morpholinylmethyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalemethane; SR141716, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboximide hydrochloride; HU-210, (--)7-OH-1,6-tetralohncannabinol-dimethylheptyl; CP55940, 5-(1,1-dimethylheptyl)-2-(5-hydroxy-2-(3-hydroxypropyl)cyclohexyl)phenol; HME, sodium-HEPES/MgCl$_2$/EDTA; TM, Tris-Ci/MgCl$_2$/ANoVA, analysis of variance; IRI$_G^{\Delta G_{i\alpha}}$, inverse agonist-supported inactive state.
binoid receptor. The CB₁ receptor is a GPCR found abundantly in brain and neuronal cells and is coupled to the Gi/o family of G proteins to regulate effectors such as adenyllyl cyclase and ion channels (Howlett et al., 2002). The CB₁ receptor exhibits properties of agonist-independent receptor-G protein precoupling and constitutive activity in both recombiant (Bouaboula et al., 1997; Pan et al., 1998; Vasquez and Lewis, 1999) and native cell models (Pan et al., 1998; Meschler et al., 2000; Sim-Selley et al., 2001). CB₁ receptor-Gα complexes readily exist in the absence of exogenously added agonist or inverse agonist ligands (Houston and Howlett, 1993; Howlett et al., 1999; Mukhopadhyay et al., 2000; Mukhopadhyay and Howlett, 2001).

We hypothesized that structurally distinct ligands would exhibit differential ability to regulate CB₁ receptor interactions. To test this hypothesis, we used a well-characterized neuronal model for CB₁ cannabinoid receptor-mediated signal transduction, the N18TG2 neuroblastoma cell, which endogenously expresses CB₁ receptors and all three subtypes of Gi (Mukhopadhyay et al., 2002). We quantified the Gαi and βγ proteins that coimmunoprecipitate with the CB₁ receptor from a CHAPS extract of N18TG2 cell membranes. We demonstrated here that the aminoalkylindole WIN 55,212-2, the cannabinoid DALN, and the eicosanoid (R)-methanandamide promote a mixture of receptor-Gαi complexes and free receptors differentially depending upon the Gαi subtype. SR141716 maintained the receptor in a complex with all three Gαi subtypes. These results also provide evidence for the differential behavior of these ligands as agonists or inverse agonists, depending on the Gαi subtype. A simplified working model is depicted in Fig. 1 as a basis for developing a platform for understanding the emerging data.

**Materials and Methods**

**Materials.** The chemicals, including GTPγS, GDPβS, and GppNHp, were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. DALN was a gift from Pfizer, Inc. (New York, NY). WIN 55,212-2 and (R)-methanandamide were purchased from Calbiochem (San Diego, CA) and Cayman Chemical (Ann Arbor, MI), respectively. SR141716 and rabbit antiserum against peptides selective for Gαi1, Gαi2, or Gαi3 were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Urea was purchased from Vances Pharmaceuticals (Costa Mesa, CA). SDS, acrylamide, bisacrylamide, ammonium persulfate, and polyvinylidene difluoride membranes were obtained from Bio-Rad (Hercules, CA). Antibody against an epitope common to Gβ subtypes 1 to 4 was purchased from Santa Cruz Biochemicals (Santa Cruz, CA). The Gγ2 antibody was a gift from N. Gautam (Washington University, St. Louis, MO). Anti-rabbit and anti-mouse IgG-horseradish peroxidase was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Rainbow molecular weight markers and ECL reagents were purchased from Amersham Biosciences (Piscataway, NJ).

**CB₁ Receptor Antibody and Affinity Matrix Preparation.** Rabbit polyclonal antibodies were raised against the N-terminal 14 amino acids of the CB₁ receptor as described previously (Howlett et al., 1998; Mukhopadhyay and Howlett, 2001). Anti-CB₁(1–14) was affinity-purified using a peptide comprising the N-terminal 14 amino acid residues of the rat CB₁ receptor as the affinity ligand attached to agarose matrix using the SulfoLink Immobilization procedure (Pierce, Rockford, IL). An affinity resin for the rat CB₁ cannabinoid receptor was prepared by coupling affinity-purified anti-CB₁(1–14) to Affi-Prep-Hz matrix (Bio-Rad) according to the manufacturer’s instructions. This method binds periodate-oxidized carbohydrate moieties on the antibody heavy chain to hydrazide-activated methacrylate matrix (O’Shannessy and Hoffman, 1987).

**Membrane Preparation, Detergent Solubilization, and Treatments.** N18TG2 neuroblastoma cells were grown in Dulbecco’s...
modified Eagle’s medium with 10% heat-inactivated calf serum and 1% penicillin-streptomycin to 90% confluence. Cells were then harvested with phosphate-buffered saline/EDTA, sedimented, and the cell pellet was homogenized in a glass homogenizer in ice-cold HME buffer (20 mM sodium-HEPES, pH 8.0, 2 mM MgCl₂, and 1 mM EDTA). After sedimentation at 1000g for 5 min at 4°C to remove unbroken cells and nuclei, the supernatant was collected and sedimented at 17,000g for 20 min at 4°C. The pellet (P2 membrane fraction) was resuspended in HME, and the protein concentration was determined (Bradford, 1976). For solubilization, 5 mg of membrane protein was sedimented at 17,000g, resuspended in 500 μL of solubilization TM buffer (30 mM Tris-Cl, pH 7.4, and 5 mM MgCl₂) containing 4 mg of CHAPS and 20% glycerol according to the method described by Houston and Howlett (1993). CHAPS extracts were treated with the indicated CB₁ receptor ligands at varying concentrations (10 nM to 1 μM) in the presence or absence of 100 μM GTPγS, Gpp(NH)₇, or GDPβS in a final volume of 100 μL of TM buffer for 20 min at 30°C. Control samples were treated with the vehicle for the ligands (TM buffer) under identical conditions. The ligands and guanine nucleotides were present throughout the immunoprecipitation procedure.

Immunoprecipitation. After the incubation, the immunoprecipitation of the CB₁ receptor and associated proteins from ligand- or guanine nucleotide-treated CHAPS extracts was performed by following the method used in this laboratory previously (Mukhopadhyay et al., 2000; Mukhopadhyay and Howlett, 2001). A 100-μL aliquot of the ligand- or guanine nucleotide-treated CHAPS extract was incubated under constant rotation with Sepharose bead-coupled anti-CB₁ antibody (20 μL) for 6 h at 4°C. Thus, the addition of antibody-coupled matrix to the solubilized preparation resulted in a 20% dilution of the ligands or guanine nucleotides. The anti-CB₁ affinity matrix was then sedimented at 17,000g for 5 min, and matrix was washed three times with 500 μL of TBST buffer (20 mM Tris-Cl, pH 7.4, 140 mM NaCl, and 0.1% Tween 20). Immunoprecipitated proteins were eluted from the matrix with 50 μL of glycine-chloride, pH 2.5 (100 mM), and the eluate was immediately neutralized with 450 μL of Tris-Cl, pH 8.0 (1.5 M). The protein from the neutralized eluate was precipitated by the addition of 8 volumes of CHCl₃/CH₃OH/H₂O (1:4:3), dissolved in Laemmli sample buffer containing 5 mM EDTA, and heated at 65°C for 5 min. Samples were subjected to polyacrylamide gel electrophoresis on 10% polyacrylamide/0.1% SDS/6 M urea gels.

Western Immunoblot Analysis. Electrophoretic transfer of proteins from the gel to polyvinylidene difluoride membranes was carried out in 10 mM CAPS buffer with 0.01% SDS, pH 11, for 16 h (0–4°C) at 20 V using a Bio-Rad Trans-Blot Cell equipped with a cooling coil. Blots were rinsed with TBS buffer and were incubated with blocking buffer (5% nonfat dry milk plus 5% normal goat serum in TBS) at room temperature for 1 h to eliminate nonspecific binding. Blots were then incubated with affinity-purified anti-CB₁(1–14) combined with the indicated antibodies to Goi (1:1000), Gβ (subtypes 1–4), or Gγ2 in blocking buffer for 90 min at room temperature, followed by washing three times with TBS containing 0.1% Tween 20. Control experiments were performed using separate incubations with individual antibodies, and the results were the same as experiments stained with combined antibodies. Blots were incubated with horseradish peroxidase-coupled anti-rabbit and anti-mouse IgG sequentially for 1 h at room temperature, followed by one rinse with TBS, seven rinses with TBST, and four rinses with water. Immunoreactive bands were detected by ECL reaction and exposure of Hyperfilm. Densitometric scanning was analyzed using a modified version (version 1.59) of the Scion Image software (Scion Corporation, Frederick, MD) or using Alpha Innotech software (Alpha Innotech, San Leandro, CA). Data analysis and figures were produced using Prism 3 (GraphPad Software Inc., San Diego, CA).

Results

Ligand-Mediated Redistribution of the CB₁ Receptor and Specific Goi Proteins. CB₁ receptors solubilized from the membrane in CHAPS detergent exist in a state that is associated with various subtypes of the Gi protein family (Goi1, Goi2, and Goi3) in the absence of exogenously added agonists (Fig. 2). It is particularly interesting to note that a significant fraction of the Goi proteins present in the CHAPS extract are coimmunoprecipitated with the CB₁ receptors (compare lane 1 (Load) with lane 2 (Immunoprecipitated)). Only a limited fraction of residual Goi proteins remained in the supernatant fraction (lane 3) or in any of the subsequent washes of the affinity-matrix-bound CB₁ receptor-G protein complex. This indicates that the CB₁ receptor preferentially exists as a receptor-G protein complex in detergent solution under these experimental conditions. This association can be disrupted by incubation with pertussis toxin, demonstrating that the receptors and G proteins exist in a dynamic association/dissociation reaction mixture in detergent solution (Howlett et al., 1999; Mukhopadhyay and Howlett, 2001). If these receptor-G protein complexes are functional, then they should be targets for functional interaction with CB₁ receptor ligands. Experimental conditions were chosen in which GTP and GDP are absent so that association/dissociation reactions could proceed in which free agonist, receptor, and G protein could coexist with ternary complexes. In the absence of GTP, the G protein cycle would not be able to continue through GTPase-dependent hydrolysis and reassociation of GaγGDP with Gβγ. The coimmunoprecipitation method can provide a quantitative measure of the ability of ligands to modify the distribution of free versus complexed receptors and G proteins.
Three structurally different CB₁ receptor agonist classes were tested to determine their effects on CB₁ receptor-Gαi (Gαi1, Gαi2, or Gαi3) complexes in CHAPS-solubilized N18TG2 cell membranes. Representative Western immunoblots depicting the effects of ligands and the nonhydrolyzable GTP analog, GTPγS, are shown in Fig. 3. The immunoblots depict the CB₁ receptor monomer found in cultured neuronal cells and the Gα subunits coimmunoprecipitated with the receptor in the same lane (Fig. 3, A–C; each lane 1, top, middle, and bottom). The ratio of the densities of the G protein band compared with the CB₁ receptor band were calculated from multiple experiments, and the means and standard errors from multiple experiments are shown in Figs. 4 and 5. The aminoalkylindole ligand WIN 55,212-2 evoked partial dissociation of all three subtypes of Gαi proteins from the receptor, reaching a maximum dissociation of only 50% of the control amount of receptor-Gαi complexes (Figs. 3A and 4A). WIN 55,212-2 was relatively more potent in dissociating the receptor-Gαi1 complex, achieving a maximal dissociation at 10 nM. In contrast, the dissociation of Gαi2 and Gαi3 from the receptor occurred between 10 and 100 nM. The cannabinoid ligand DALN (Figs. 3B and 4B) dissociated Gαi1 and Gαi2 from the CB₁ receptor-Gαi complex in a dose-dependent manner. Gαi2 was dissociated completely from the receptor at 1 μM DALN (Fig. 4B). CB₁ receptor-Gαi1 dissociation reached a maximum of approximately 50% at 100 nM, with no further dissociation with increasing agonist concentrations. DALN had no effect on CB₁ receptor-Gαi3 complexes. The eicosanoid (R)-methanandamide evoked dissociation of only CB₁ receptor-Gαi3 (Figs. 3C and 4C), and this disruption was nearly complete at 100 nM. Unlike WIN 55,212-2 or DALN, (R)-methanandamide failed to produce any dissociation of CB₁ receptor-Gαi1 or Gαi2 complexes.

**Effect of Guanine Nucleotides on the CB₁ Receptor-Gai Complex.** Incubation of the CHAPS extract of N18TG2 membranes with the nonhydrolyzable GTP analog GTPγS at 100 μM resulted in 85 to 100% dissociation of all three CB₁ receptor-Gαi complexes (Figs. 3A, lanes 5 and 7 for each Gαi subtype; Fig. 5A). The addition of GDPγS (100 μM) had no influence on the ability of GTPγS to promote dissociation of the CB₁ receptor-Gαi complex and only limited influence on the CB₁ receptor-Gαi1 complex (Figs. 3B, lanes 5 and 7). In similar experiments using an alternative GTP analog, GppNHp, dissociation of CB₁ receptor-Gαi1 and Gαi2 complexes was complete in the presence of DALN (data not shown). (R)-Methanandamide had little influence on the Gαi3GTPγS-dissociated state for Gαi3 (Figs. 3C, lanes 5–8, and 5C). In contrast, the cannabinoid ligand DALN precluded the Gαi3GTPγS dissociation and partially attenuated the Gαi1GTPγS dissociation (Figs. 3B and 5B). (R)-Methanandamide potently (10 nM) attenuated the Gαi1GTPγS dissociation, and concentrations between 100 and 1000 nM attenuated the Gαi2GTPγS dissociation (Figs. 3C and 5C).

To assess the possible spontaneous GDP release in the association/dissociation reaction, the CB₁ receptor-Gαi complexes were incubated in the presence of a high concentration (100 μM) of the GTP analog GDPβS. The addition of GDPβS to the detergent extract of N18TG2 membranes neither increased nor decreased the ratio of any of the Gαi subtypes to CB₁ receptor in the immunoprecipitate (Fig. 5, D–F, bars 1 versus 2). If there existed any unoccupied Gαi1 in the extract, it would have been predicted that GDPβS would bind, thereby promoting formation of additional heterotrimer (Gαi1GDPβSβ3γ3) that would have been able to associate with

---

**Fig. 3.** Western blot analysis of the effects of CB₁ receptor agonists and GTPγS on Gαi protein association with the CB₁ receptor. CHAPS detergent extracts of N18TG2 cell membranes were incubated for 20 min at 30°C with vehicle (lanes 1 and 5), 10 nM (lanes 2 and 6), 100 nM (lanes 3 and 7), or 1 μM (lanes 4 and 8) concentrations of the aminoalkylindole ligand WIN 55,212-2 (A), cannabinoid DALN (B), or (R)-methanandamide (C) in the absence (lanes 1–4) or presence (lanes 5–8) of 100 μM GTPγS. The CB₁ receptor and associated proteins were immunoprecipitated with affinity-purified anti-CB₁(1–14), and Western blot analysis was performed as described in the text. Each blot was contained for both CB₁ receptor and either Gαi1 (top), Gαi2 (middle), or Gαi3 (bottom) proteins. Immunoreactive bands were visualized by ECL. Results are shown from a single representative of at least three experiments. The lower mobility band is the CB₁ receptor at 64 kDa, and the higher mobility band is the Gαi protein at approximately 40 kDa.
the CB₁ receptor. The failure of the GDP analog to promote a greater abundance of CB₁-R-Gi complex than in control extracts suggests that the CB₁ receptor-Gα protein association was at its maximum as it existed in the CHAPS extract. The addition of GDPβS failed to alter the CB₁-R-Gi complex when incubated with cannabinoid receptor ligands (Fig. 5, D–F, bars 3 versus 4). This observation would support predictions from the ternary complex model that the GDP analog should not promote dissociation of the agonist-bound CB₁ receptor-Gα heterotrimer complexes.

**Inverse Agonist Influence on CB₁,R-Gi Complexes.** SR141716 is a CB₁ receptor-selective competitive antagonist that has been shown to exhibit inverse agonist activity in signal transduction assays in recombinant cell models (Bouaboula et al., 1997). It may be predicted that if free Gαi proteins exist in solution under control conditions, then a greater population of Gαi proteins could be present in a CB₁ receptor-Gαi complex in the presence of SR141716. However, as shown in Fig. 6A, SR141716 exhibited little or no effect (<10% decrease in the amount of Gαi bound to receptors) on the amount of receptor-Gαi complex for any of the Gαi subtypes. A similar finding was reported earlier for the CB₁ receptor associated with Gαo in solubilized preparations from rat brain (Mukhopadhyay et al., 2000). If a significantly greater population of unliganded Gαi were present in solution, one would predict that in the presence of high concentrations of GDPβS, SR141716 would stabilize a greater amount of communoprecipitable CB₁ receptor-GiGDPβS complexes. This was not the case for any of the Gαi subtypes at any of the concentrations of SR141716 tested (Fig. 6B).

The GTPγS-driven dissociation (85–96% dissociated) was significantly attenuated in the presence of 1 μM SR141716 for Gαi2 (68% dissociated) and Gαi3 (69% dissociated), and a similar trend existed for Gαi1 (71% dissociated) (Fig. 6A). The GppNHP-induced dissociation of CB₁ receptor-Gαi1 and Gαi2 complexes was also partially reversed (50%) by SR141716 (data not shown). This effect of SR141716 was not robust, indicating that the presence of this ligand on the receptor exerts a modest influence on the distribution between free GαiGTPγS and complexed forms of Gαi. A lower concentration (50 μM) of GTPγS produced only partial dissociation of the CB₁ receptor-Gαi complex compared with control for all of the subtypes of Gαi protein (42% for Gαi1, 46% for Gαi2, and 40% for Gαi3). Various concentrations of SR141716 (10 nM to 1 μM) failed to influence the response to this lower concentration of GTPγS.

**Agonist and Guanine Nucleotide Effects on CB₁, Receptor-Gβγ Complexes.** The interaction of the CB₁ receptor with the Gβγ dimer was examined in Fig. 7. Gβγ and Gγ proteins were both detected in the protein complex communoprecipitated by the CB₁ antibody. Upon incubation with agonist ligands at concentrations that promoted dissociation of those selective Gαi proteins, 40 to 70% of the Gβγ isoforms (1–4) was dissociated. Gγ2 did not show a pattern of dissociation from the CB₁ receptor. This may be caused by the profile of Gγ subtypes that are present in the N18TG2 cell membranes and associated with the Gαi proteins as a heterotrimer. This antibody does not recognize all Gγ subtypes that may potentially be present and/or associated with the CB₁ receptor. Gγ2 is only one of several Gγ subtypes that would be expected to be present in neuronal cells (Downes and Gautam, 1999).

GTPγS was able to dissociate 100% of the Gβγ and >80% of
the Gγ that was associated with the CB₁ receptor in CHAPS detergent (Fig. 7). Under these conditions, the Goi proteins were dissociated by 60 to 100% (Fig. 5). Because the free GoiGTPγS is not likely to reassociate with Gβγ dimers to form heterotrimers, receptor-G protein complexes are not readily reestablished. In the presence of WIN 55,212-2, DALN, or (R)-methanandamide, 40 to 70% of the control Gβ and <10% of the control Gγ was dissociated from the CB₁ receptor. This is consistent with heterotrimer dissociation if one considers the mixed responses that were observed with selective agonists and Goi subtypes. Similar to what was observed with Goi, GDPβS alone did not alter the amount of Gβ in association with the CB₁ receptor. However, GDPβS could attenuate the agonist-promoted dissociation of the CB₁ receptor-Gβγ complex. This is consistent with the receptor-G protein heterotrimer being stabilized by the occupancy of Goi with GDPβS. SR141716 seemed to exert no influence on the CB₁ receptor-Gβγ interaction in the absence or presence of GDPβS. However, SR141716 served to counter the GTPγS-mediated dissociation of the CB₁ receptor-Gβγ complex.

## Discussion

Our present studies have examined the stability of CB₁ receptor complexes with three subtypes of Gi proteins in detergent solution to gain insight regarding the role that...
agonists and inverse agonists play in the ternary complex equilibrium and G protein activation cycle models. Stable ternary ARG complexes in detergent solution were promoted by agonists for somatostatin, δ-opioid, and β₂-adrenergic receptors in the absence of GTP or GTPγS (Law and Reisine, 1992, 1997; Brown and Schonbrunn, 1993; Lachance et al., 1999). In the present investigation using CHAPS extracts from cultured N18TG2 neuronal cell membranes, and studies that we reported previously using rat brain membranes (Houston and Howlett, 1993, 1998; Mukhopadhyay et al., 2000), a significant fraction of the total Gαi was found to be associated with immunoprecipitatable CB₁ cannabinoid receptor in the absence of exogenous agonists. The fraction of receptors having high affinity for agonists (believed to be the fraction of receptors in RG complexes) was approximately 20% in rat brain membranes and 35% for WIN 55,212-2 and 50% for DALN in CHAPS extracts (Houston and Howlett, 1998). Constitutive activity is readily observed in recombinant cell systems (Bouaboula et al., 1997; Pan et al., 1998; Vasquez and Lewis, 1999) and native cell systems under favorable experimental conditions (Pan et al., 1998; Meschler et al., 2000; Sim-Selley et al., 2001). Thus, a facile GαGDP association is likely to occur in vivo. The model in Fig. 1 can be used to conceptualize the data regarding alterations in the equilibrium between G proteins bound to immunoprecipitatable receptors (RG_GTP or RG_) and free CB₁ receptors.

As depicted in the model, the demonstration that GTPγS alone promoted dissociation of the G proteins from the CB₁ receptor indicates that the RG_GDP complexes can become spontaneously activated in the absence of agonist, permitting GDP release and a transiently empty R^G_ state. Once GTPγS binds, the GαGTPγS dissociates and can no longer participate in the association/dissociation reaction (Fig. 1). The model depicts the ability of agonists to facilitate this association/dissociation reaction, leading to mixtures in the absence of GTP or GTPγS comprising equal amounts of the receptor in an ARG_GDP complex and in the dissociated state as AR plus G αGDP. WIN 55,212-2 promoted development of this mixture for all three Gαi subtypes and promoted complete dissociation of the three RGαi complexes in the presence of GTPγS. This same behavior appeared in the presence of DALN for Gαi1 and Gαi2 and in the presence of the (R)-methanandamide for Gαi3. The complete dissociation of G proteins from the CB₁ receptor evoked by DALN for Gαi2 and by (R)-methanandamide for Gαi3 suggests that an isomerization to AR^G may have been induced. AR^G_ would exist as a very transient complex in intact cells that possess an abundance of GTP to fill the guanine nucleotide binding site. Under the present experimental conditions, with no GTP present to promote Gαi_GTP dissociation, the AR^G complex may be susceptible to protein denaturation, as has been observed for conformationally relaxed constitutively active mutants of GPCR (Gether et al., 1997). In our experimental model, a denatured receptor that is unable to bind Go would not be discernible from a functionally dissociated receptor.

Inverse agonist SR141716 maintained all three RGαi complexes in the absence of GTP analogs and exerted a very small effect on the GTPγS-promoted dissociation of G proteins from receptors. These results can be explained by invoking the existence of an inverse agonist-supported inactive state (IR^G_GDP) in the ternary complex equilibrium model (Fig. 1). This state was originally proposed by Bouaboula and colleagues (1997) to describe a mechanism for the CB₁ receptor to “sequester” G proteins, thereby explaining their data that basal signal transduction through the mitogen-activated protein kinase or adenylyl cyclase pathways was blocked in the presence of SR141716. We propose that inverse agonist sequestration of G proteins with CB₁ receptors in an IR^G_GDP complex would reduce the fraction of RG_GDP complex that could spontaneously convert to R^G_ or become

![Fig. 6. Effect of SR141716 and guanine nucleotides on the CB₁ receptor-Gαi interaction. A, CHAPS-solubilized extracts of N18TG2 cell membranes were incubated in the presence of 1 μM SR141716, 100 μM GTPγS, or both as indicated. B, CHAPS-solubilized extracts of N18TG2 cell membranes were incubated in the presence of 100 μM GDPβS in the absence or presence of 10 nM, 100 nM, or 1 μM SR141716. Immunoprecipitation and Western blotting for individual Gαi proteins and the CB₁ receptor were performed as described in the text and in previous figure legends. Band densities were determined and reported as a ratio of Gαi density to the CB₁ receptor density. Data are mean and S.D. from n = 2 experiments. Data were analyzed by two-way ANOVA and a Bonferroni post hoc test. Significant differences (p < 0.05) from control are indicated by * and from SR141716 alone are indicated by #. Significant differences (p < 0.05) between GTPγS and GTPγS plus SR141716 are indicated by a connecting bracket.](https://www.molpharm.org/content/95/1/2022.full.pdf)
available to interact with agonists to induce the AR*G_ complex.

The conversion of the RG_GDP complex to a sustainable IR*G_GDP complex by inverse agonist SR141716 was mimicked by DALN for Gi3 and by (R)-methanandamide for Gi1 and Gi2. The property of these ligands to behave as inverse agonists for these G protein subtypes was manifest as the failure of these RG_GDP complexes to participate in the reversible dissociation to R + G_GDP. This would explain the ability of DALN or (R)-methanandamide to preclude the ability of GTPγS to drive forward the dissociation of Gi3, or Gi1 and Gi2, respectively. In previous studies (Houston and Howlett, 1998), GTPγS converted the majority of the high-affinity WIN 55,212-2 binding sites (ARG_GDP or AR*G_) to the low-affinity state (AR). In contrast, the fraction of receptors remaining in the high-affinity state for DALN was never reduced less than 25% even in the presence of GTPγS and Na^+. (Houston and Howlett, 1998). These findings are consistent with our current observation that in the presence of WIN 55,212-2, GTPγS was able to promote dissociation all three Gi subtypes from the CB₁ receptor, but that in the presence of DALN, GTPγS failed to dissociate Gi3.

An alternative mechanism might be that the inverse agonist-occupied receptors serve as guanine nucleotide-exchange factors that act on Ga_i_GTP-S to exchange GDP for GTPγS. This mechanism is not likely, because our studies indicated that Gβγ was dissociated from the CB₁ receptor, and there is a smaller probability that Ga_i_GTP-S would be able to interact with the receptor in the absence of Gβγ (Clark et al., 2001). Furthermore, the studies with GDPβS failed to support the notion that SR141716 could increase the population of receptor-G protein complexes by filling the guanine nucleotide-binding site of unoccupied G proteins in the presence of an excess of the GDP analog. It is interesting that the effects of SR141716 on all three Gi subtypes, and DALN on Gi1, were only partially disruptive of the GTPγS-driven dissociation of Ga_i_GTP-S, suggesting that these ligands do not possess as great an inverse agonist efficacy to promote the isomerization to IR*G_GDP, as does DALN for Gi3 or (R)-methanandamide for Gi1 and Gi2.

Under the present assay conditions, Gβγ was dissociated from the CB₁ receptor in parallel with Ga_i, supporting the notion that the heterotrimer dissociation allows the release of both components of the heterotrimer from the receptor. Agonists, but not SR141716, could facilitate dissociation of a fraction of the population of Gβ (multiple isoforms) from the CB₁ receptors. In the presence of GTPγS, agonists promoted the dissociation of a fraction of the Gβ isoforms consistent with the AR*G_ → AR + Gβγ + Ga_i_GTP-S forward reaction. Protein-interaction studies by others have demonstrated that Gβγ can interact with both R and AR in the absence of Ga in detergent solution and reconstituted lipid vesicles (Heitner et al., 1992). In surface-plasmon resonance studies of immobilized rhodopsin, Gβγ binding was transient but was required to facilitate binding of Ga (Clark et al., 2001).

Our studies can be compared with other investigations of CB₁ receptor activation of G proteins that have detected differences in agonist efficacy to produce a response. Glass and Northup (1999) examined differential agonist activation of G proteins by measuring the ability of recombinant CB₁ receptors in Sf9 cell membranes to activate guanosine 5’S-O-(3-[35S]thio)triphosphate binding to purified Ga_i (all subtypes) and Ga_o proteins. Both Gi and Go proteins were activated to the maximum extent by HU-210 and minimally by Δ²-tetrahydrocannabinol. WIN 55,212-2 and anandamide exhibited maximal or near-maximal activity for Gi but only approximately 70% maximal activity for Go. An inhibition of guanosine 5’S-O-(3-[35S]thio)triphosphate binding by SR141716 was observed for both Gi and Go. Prather and colleagues (2000) demonstrated differences in the ED₅₀ value for G protein activation by WIN 55,212-2 using [32P]lazidocainilido-GTP binding as the determinant of G protein activation. The ED₅₀ value for WIN 55,212-2 to activate various G protein subtypes in rat cerebellum membranes ranged from 100 nM for Ga_i and Ga_o3 to 3.7 μM for Ga_o2. It is not easy to compare their specific findings with ours because undifferentiated N18TG2 cells do not express an appreciable

Fig. 7. Effect of ligands and guanine nucleotides on the CB₁ receptor interaction with Gi3 (A) and Gγ (B). CHAPS-solubilized extracts of N18TG2 membranes were incubated in the absence or presence of 100 nM WIN 55,212-2, 100 nM DALN, 100 nM (R)-methanandamide, 1 μM SR141716, 100 μM GTPγS, 100 μM GDPβS, or combinations as indicated. Immunoprecipitation and Western blotting was performed with SDS-polyacylamide gel electrophoresis conditions modified to allow for the detection of low-molecular-weight proteins. Gβγ and Gγ proteins and the CB₁ receptor band densities were quantified as described in the text and quantified as a ratio compared with the CB₁ receptor band density. Data are mean and S.D. of n = 2 experiments. Each group of data were analyzed by one-way ANOVA and a Bonferroni post hoc test. Significant differences in control versus drug for each group are indicated as *, p < 0.05, and **, p < 0.01. ND, not determined.
amount of Gao, and those studies did not quantify $[^{32}P]a$-zidanoanito-GTP incorporation into Ga3. The studies of Glass and Northup (1999) and Prather and colleagues (2000) both determined the exchange of a GTP analog for GDP on the Go subunit under conditions that restrict reversal of the reaction. The present investigation determined receptor-Ga interaction, with the dissociation of the ternary complex as the measure of G protein activation. It has been proposed that the stability of the ternary complex can be determined by the dissociation rate of the interacting G proteins (Waelbroeck, 1999). It is likely that the agonist-receptor-G protein complex requires a sequence of transitions that must overcome a series of energy barriers to achieve release of G proteins from the receptor and GDP-GTP exchange. Shim and Howlett (2004) have proposed a theoretical model whereby nonclassic cannabinoid compounds such as CP55940 can convert to low-energy states within the binding pocket, providing a "steric trigger" for microconformational changes within the binding domain. Chemically distinct ligands may allow this transition to progress by multiple pathways because of their differential ability to provide the activation energy for microsomerization to unique conformations that can direct the activation of selected G protein subtypes (Kenakin and Onaran, 2002). We determined previously that the CB1 receptor juxtamembrane C-terminal fourth loop domain was responsible for coupling to Go and Ga3 but not to Go1 or Goi2 (Mukhopadhyay et al., 2000; Mukhopadhyay and Howlett, 2001). In contrast, the third intracellular loop was important for coupling with Go1 and Goi2 (Mukhopadhyay and Howlett, 2001). This implies that certain agonists could induce a conformational change that is limited to the third intracellular loop, whereas others could alterate predominately in the juxtamembrane C-terminal fourth loop. Clear clinical implications can be made from these studies in the demonstration that pharmacological selectivity can be determined regarding ligand-directed responses depending on the type of Go isoform expressed within cells and the relative abundance of G proteins in the environment coupled to receptors.

References

Address correspondence to: Dr. Somnath Mukhopadhyay, Neuroscience of Development, Research Foundation, the National Institute on Drug Abuse, Research Institute, North Carolina Central University, 700 George Street, Durham, NC 27707. E-mail: smukhopadhay@ncsu.edu