0026-895X/05/6706-2016–2024$20.00
MOLECULAR PHARMACOLOGY
Vol. 67, No. 6
3558/3032983
Mol Pharmacol 67:2016–2024, 2005
Printed in U.S.A.

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 Gαi and $\beta\gamma$ proteins that coimmunoprecipitate with the CB_1 receptor from a detergent extract of N18TG2 membranes in the presence of ligands. A mixture of A, R, G_{GDP} (or G_), and ARG_{GDP} (or ARG_) 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-naphthalenylmethanone (WIN 55,212-2) for all three RGαi complexes, cannabinoid desacetyllevonantradol for Gαi1 and Gαi2, and eicosanoid (*R*)-methanandamide for Gαi3. Desacetyllevonantradol maintained RGαi3 complexes and (*R*)-methanandamide maintained RGαi1 and RGαi2 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-1*H*-pyrazole-3-carboximide hydrochloride (SR141716) maintained all three RGαi complexes. Gβ proteins, and to a certain extent Gγ2, exhibited the same association/dissociation pattern as the Gα 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 Gα and G$\beta\gamma$ 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 α_{1B}-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 α_2-adrenergic receptors coupled to Gs or Gi (Brink et al., 2000) and neurotensin receptors coupled to Gs, Gi, or Gq/11 (Skrzydelski et al., 2003) in transfected Chinese hamster ovary cells. GTPγS binding to exogenous G proteins was shown to exhibit agonist selectivity for α_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-

This work was supported by National Institute on Drug Abuse grants R01-DA03690, R01-DA06312, K05-DA00182, and U24-DA12385.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.104.003558.

ABBREVIATIONS: GPCR, G protein-coupled receptor; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate; DALN, desacetyllevonantradol; ECL, enhanced chemiluminescence; GDPβS, guanosine 5′-*O*-(3-thio)-diphosphate; GppNHp, guanylyl-imidodiphosphate; TBS, Tris-buffered saline; TBST, Tris-buffered saline/Tween 20; GTPγS, 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-naphthalenylmethanone; SR141716, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride; HU-210, (−)-7-OH-Δ-6-tetrahydrocannabinol-dimethylheptyl; CP55940, 5-(1,1-dimethylheptyl)-2-(5-hydroxy-2-(3-hydroxypropyl)cyclohexyl)phenol; HME, sodium-HEPES/$MgCl_2$/EDTA; TM, Tris-Cl/$MgCl_2$; ANOVA, analysis of variance; IR^oG_{GDP}, inverse agonist-supported inactive state.

binoid receptor. The CB_1 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 adenylyl cyclase and ion channels (Howlett et al., 2002). The CB_1 receptor exhibits properties of agonist-independent receptor-G protein precoupling and constitutive activity in both recombinant (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). CB_1 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 CB_1 receptor interactions. To test this hypothesis, we used a well-characterized neuronal model for CB_1 cannabinoid receptor-mediated signal transduction, the N18TG2 neuroblastoma cell, which endogenously expresses CB_1 receptors and all three subtypes of Gi (Mukhopadhyay et al., 2002). We quantified the Gαi and $\beta\gamma$ proteins that coimmunoprecipitate with the CB_1 receptor from a CHAPS extract of N18TG2 cell membranes. We demonstrate 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 Gi 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 antisera against peptides selective for Gαi1, Gαi2, or Gαi3 were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Urea was purchased from Valeant 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).

CB_1 Receptor Antibody and Affinity Matrix Preparation. Rabbit polyclonal antibodies were raised against the N-terminal 14 amino acids of the CB_1 receptor as described previously (Howlett et al., 1998; Mukhopadhyay and Howlett, 2001). Anti-CB_1(1–14) was affinity-purified using a peptide comprising the N-terminal 14 amino acid residues of the rat CB_1 receptor as the affinity ligand attached to agarose matrix using the SulfoLink Immobilization procedure (Pierce, Rockford, IL). An affinity resin for the rat CB_1 cannabinoid receptor was prepared by coupling affinity-purified anti-CB_1(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

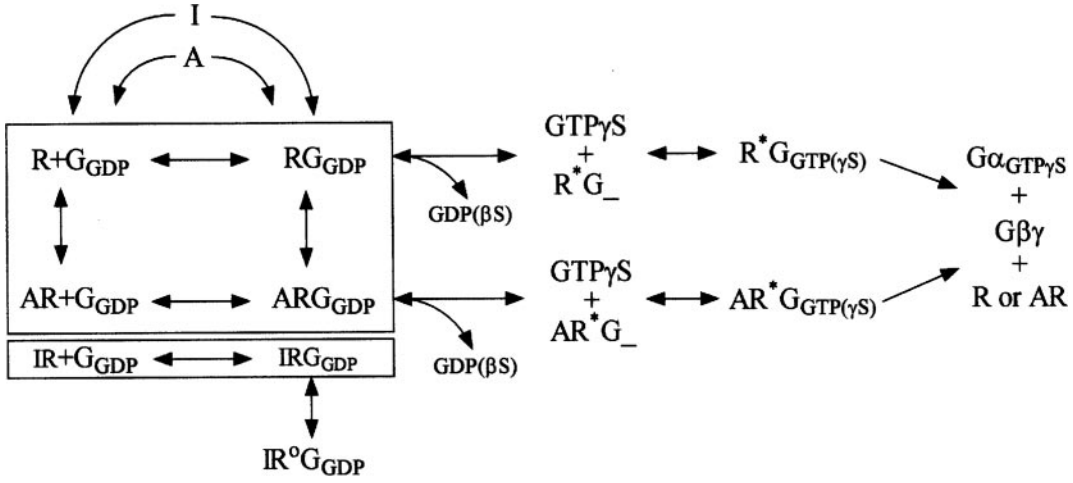

Fig. 1. Equilibrium ternary complex model of ligand-receptor-G protein regulated by agonists or inverse agonists. In the ternary complex model of agonist action (Leff et al., 1997), the receptor is denoted as R in the ground state and R* in the state that activates the G protein, and the heterotrimer bearing GDP is denoted as G_{GDP}. An agonist (A) can bind either to R or to RG_{GDP} complexes, creating an equilibrium with the "ternary complex" ARG_{GDP}. Consistent with evidence that CB_1 receptors exhibit constitutive activity (Bouaboula et al., 1997), a probability exists that a fraction of the RG_{GDP} complexes become spontaneously activated in the absence of agonist. Isomerization of the RG_{GDP} complex or ARG_{GDP} ternary complex to an active state will lead to dissociation of GDP. In an intact cell in which GTP is abundant, the resulting R*G_ or AR*G_ complexes readily bind GTP, and dissociation of the heterotrimer allows $G\alpha_{GTP}$ and G$\beta\gamma$ proteins to interact with effectors (Waelbroeck, 1999). In an experimental situation in which GTPγS is present in high concentrations, the transiently empty G protein (R*G_ or AR*G_) is rapidly filled by GTPγS. Steps that would exclude the complex from re-entering the equilibrium (inside the box) would include 1) GDP dissociation in the absence of added exogenous GDP or a GDP analog, and 2) GTPγS/GDP exchange and the subsequent dissociation of $G\alpha_{GTP\gamma S}$. Addition of the inverse agonist ligand (I) to precoupled RG_{GDP} induces IR^oG_{GDP}, originally proposed by Bouaboula and colleagues to describe a mechanism for the CB_1 receptor to "sequester" Gi proteins, thereby explaining their data that basal signal transduction was blocked in the presence of SR141716 (Bouaboula et al., 1997). The working model has depicted this complex as existing outside of the equilibrium box; however, evidence suggests that CB_1 agonists can compete (Meschler et al., 2000), demonstrating the reversibility of this step.

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 $MgCl_2$, and 1 mM EDTA). After sedimentation at 1000*g* for 5 min at 4°C to remove unbroken cells and nuclei, the supernatant was collected and sedimented at 17,000*g* 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 17,000*g*, resuspended in 500 μl of solubilization TM buffer (30 mM Tris-Cl, pH 7.4, and 5 mM $MgCl_2$) 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 CB_1 receptor ligands at varying concentrations (10 nM to 1 μM) in the presence or absence of 100 μM GTPγS, GppNHp, 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 CB_1 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-CB_1 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-CB_1 affinity matrix was then sedimented at 17,000*g* 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 protein was 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 $CHCl_3/CH_3OH/H_2O$ (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-CB_1(1–14) combined with the indicated antibodies to Gαi (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 CB_1 Receptor and Specific Gαi Proteins. CB_1 receptors solubilized from the membrane in CHAPS detergent exist in a state that is associated with various subtypes of the Gi protein family (Gαi1, Gαi2, and Gαi3) in the absence of exogenously added agonists (Fig. 2). It is particularly interesting to note that a significant fraction of the Gαi proteins present in the CHAPS extract are coimmunoprecipitated with the CB_1 receptors [compare lane 1 (Load) with lane 2 (Immunoprecipitated)]. Only a limited fraction of residual Gαi proteins remained in the supernatant fraction (lane 3) or in any of the subsequent washes of the affinity matrix-bound CB_1 receptor-G protein complex. This indicates that the CB_1 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 CB_1 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 $G\alpha i_{GDP}$ with G$\beta\gamma$. 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.

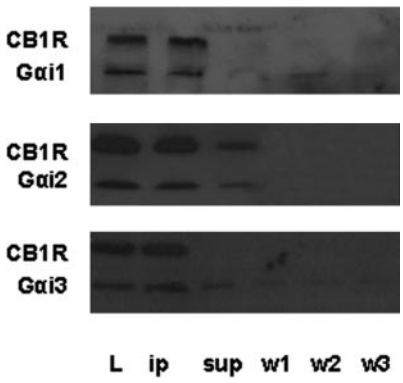

Fig. 2. Coimmunoprecipitation of CB_1 receptor-Gαi complexes from CHAPS-solubilized N18TG2 cell membranes. CHAPS extracts were prepared and the immunoprecipitation procedure was followed with care to preserve equivalent volumes at each step. Lanes were as follows: 1, load: CHAPS extract from 50 μg of N18TG2 membrane protein (400 μl) mixed with 100 μl of TM buffer as control; 2, immunoprecipitate: CHAPS extract from N18TG2 membranes (400 μl) mixed with 100 μl of Sepharose-anti-CB_1 antibody affinity matrix. Proteins were eluted from the affinity matrix, neutralized, and sedimented as described in the text; immunoprecipitated proteins were dissolved in 500 μl of TBST; 3, supernatant: CHAPS extract remaining after the affinity matrix-bound protein was removed (approximately 475 μl); 4 to 6, washings 1 to 3: supernatants remaining after the affinity matrix-bound protein was washed with 500 μl of TBST as described in the text. For each of these fractions, a 25-μl aliquot was added to 25 μl of 2× Laemmli sample buffer containing EDTA, and 35 μl of this mixture was loaded on the lane. Western blot analysis was carried out by costaining with both anti-CB_1 receptor antibody and anti-Gα: Gαi1 (top), Gαi2 (middle), or Gαi3 (bottom). Immunoreactive bands were visualized by ECL as described in the text.

Three structurally different CB_1 receptor agonist classes were tested to determine their effects on CB_1 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 CB_1 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 CB_1 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 CB_1 receptor-Gαi complex in a dose-dependent manner. Gαi2 was dissociated completely from the receptor at 1 μM DALN (Fig. 4B). CB_1 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 CB_1 receptor-Gαi3 complexes. The eicosanoid (*R*)-methanandamide evoked dissociation of only CB_1 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 CB_1 receptor-Gαi1 or Gαi2 complexes.

Effect of Guanine Nucleotides on the CB_1 Receptor-Gαi 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 CB_1 receptor-Gαi complexes (Fig. 3, A–C, lane 5 for each Gαi subtype; Fig. 5, A–C). The addition of GppNHp (100 μM) also resulted in complete dissociation of all CB_1 receptor-Gαi complexes (data not shown). The observation of complete receptor-Gαi dissociation suggests that the GDP-GTPγS exchange seems to have gone to completion under the assay conditions used in the present study. In the absence of agonist ligands, this would represent spontaneous dissociation of GDP from the receptor-G_{GDP} complex, perhaps as a result of the spontaneous isomerization to the activated state, exchange of GDP for GTPγS, and dissociation of the heterotrimer to free receptor and $G\alpha i_{GTP\gamma S}$. This process could have been facilitated by the absence of exogenous Na^+ in the assay solutions.

The ability of GTPγS to promote dissociation of the CB_1 receptor-Gαi proteins was influenced differentially depending on the ligand and the Gαi subtype. One sees little influence of WIN 55,212-2 on any of the three $G\alpha i_{GTP\gamma S}$ dissociated states, consistent with the relative nonselectivity for any of the Gαi subtype (Figs. 3A, lanes 5–8, and 5A). DALN had no influence on the ability of GTPγS to promote dissociation of the CB_1 receptor-Gαi2 complex and only limited influence on the CB_1 receptor-Gαi1 complex (Figs. 3B, lanes 5–8, and 5B). In similar experiments using an alternative GTP analog, GppNHp, dissociation of CB_1 receptor-Gαi1 and Gαi2 complexes was complete in the presence of DALN (data not shown). (*R*)-Methanandamide had little influence on the $G\alpha i_{GTP\gamma S}$-dissociated state for Gαi3 (Figs. 3C, lanes 5–8, and 5C). In contrast, the cannabinoid ligand DALN precluded the $G\alpha i3_{GTP\gamma S}$ dissociation and partially attenuated the $G\alpha i1_{GTP\gamma S}$ dissociation (Figs. 3B and 5B). (*R*)-Methanandamide potently (10 nM) attenuated the $G\alpha i1_{GTP\gamma S}$ dissociation, and concentrations between 100 and 1000 nM attenuated the $G\alpha i2_{GTP\gamma S}$ dissociation (Figs. 3C and 5C).

To assess the possible spontaneous GDP release in the association/dissociation reaction, the CB_1 receptor-Gαi complexes were incubated in the presence of a high concentration (100 μM) of the GDP 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 CB_1 receptor in the immunoprecipitate (Fig. 5, D–F, bars 1 versus 2). If there existed any unoccupied Gαi_ in the extract, it would have been predicted that GDPβS would bind, thereby promoting formation of additional heterotrimer ($G\alpha i_{GDP\beta S}$-$\beta\gamma$) that would have been able to associate with

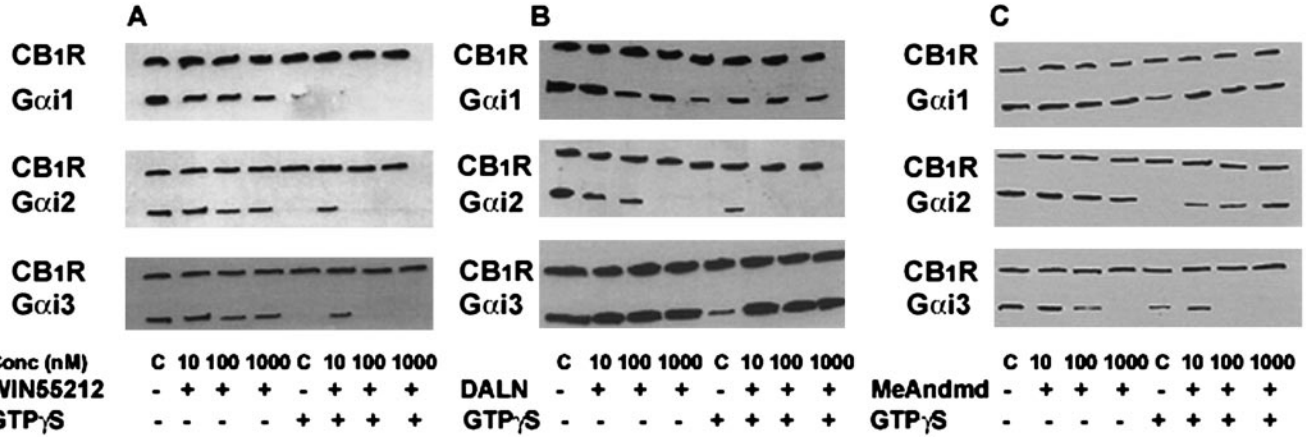

Fig. 3. Western blot analysis of the effects of CB_1 receptor agonists and GTPγS on Gαi protein association with the CB_1 receptor. CHAPS detergent extracts of N18TG2 cell membranes were incubated for 20 min at 30°C with vehicle (lanes 1 and 5), 10 nM (lanes 2 and 6), 100 nM (lanes 3 and 7), or 1 μM (lanes 4 and 8) concentrations of the aminoalkylindole 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 CB_1 receptor and associated proteins were immunoprecipitated with affinity-purified anti-CB_1(1–14), and Western blot analysis was performed as described in the text. Each blot was costained for both CB_1 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 CB_1 receptor at 64 kDa, and the higher mobility band is the Gαi protein at approximately 40 kDa.

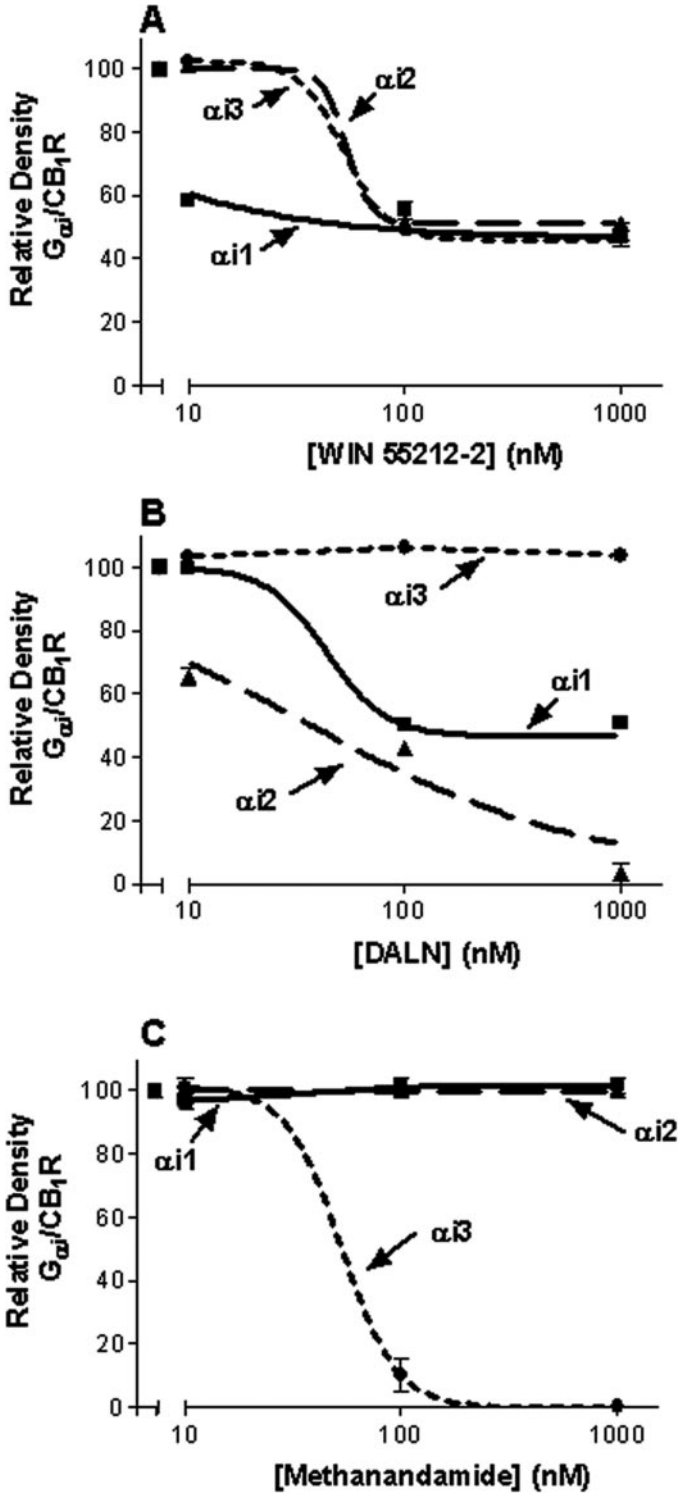

Fig. 4. Agonist-evoked dissociation of Gαi subtypes from the CB_1 receptor by WIN 55,212-2 (A), DALN (B), and (*R*)-methanandamide (C). Western blots were performed as described in Fig. 3, and the densities of the bands on the film were quantified. For each lane, the ratio of the density of the Gαi band compared with the CB_1 receptor was calculated to normalize the data and correct for potential sample loading differences, transfer, or staining variability. For each experiment, the ratio of Gαi/CB_1 band density for the control (vehicle) was defined as 100%, and the ratios of Gαi/CB_1 band densities for the experimental samples were expressed in relation to the control (% Control). The mean and S.E.M. values from three separate experiments were determined. Where error bars are not showing, the bars were smaller than the symbol. Data points were plotted, and spline or straight lines were drawn to connect the points for ease of visualization.

the CB_1 receptor. The failure of the GDP analog to promote a greater abundance of CB_1R-Gαi complexes than in control extracts suggests that the CB_1 receptor-Gα protein association was at its maximum as it existed in the CHAPS extract. The addition of GDPβS failed to alter the CB_1R-Gαi 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 CB_1 receptor-Gα heterotrimer complexes.

Inverse Agonist Influence on CB_1R-Gαi Complexes. SR141716 is a CB_1 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 found in a CB_1 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 associated with receptors) on the amount of receptor-Gαi complex for any of the Gαi subtypes. A similar finding was reported earlier for the CB_1 receptor associated with Gαo in solubilized preparations from rat brain (Mukhopadhyay et al., 2000). If a significantly greater population of unliganded G$\alpha i_$ were present in solution, one would predict that in the presence of high concentrations of GDPβS, SR141716 would stabilize a greater amount of coimmunoprecipitatable CB_1 receptor-$Gi_{GDP\beta S}$ 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 G$\alpha i2$ (68% dissociated) and G$\alpha i3$ (69% dissociated), and a similar trend existed for G$\alpha i1$ (71% dissociated) (Fig. 6A). The GppNHp-induced dissociation of CB_1 receptor-G$\alpha i1$ and G$\alpha i2$ 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 $G\alpha i_{GTP\gamma S}$ and complexed forms of Gαi. A lower concentration (50 μM) of GTPγS produced only partial dissociation of the CB_1 receptor-Gαi complex compared with control for all of the subtypes of Gi protein (42% for Gi1, 46% for Gi2, and 40% for Gi3). 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 CB_1 Receptor-G$\beta\gamma$ Complexes. The interaction of the CB_1 receptor with the G$\beta\gamma$ dimer was examined in Fig. 7. Gβ and Gγ proteins were both detected in the protein complex immunoprecipitated by the CB_1 antibody. Upon incubation with agonist ligands at concentrations that promoted dissociation of those selective Gαi proteins, 40 to 70% of the Gβ (isoforms 1–4) was dissociated. G$\gamma 2$ did not show a pattern of dissociation from the CB_1 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 CB_1 receptor. G$\gamma 2$ 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 Gβ and >80% of

the Gγ that was associated with the CB_1 receptor in CHAPS detergent (Fig. 7). Under these conditions, the Gαi proteins were dissociated by 60 to 100% (Fig. 5). Because the free $G\alpha i_{GTP\gamma S}$ is not likely to reassociate with G$\beta\gamma$ 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 CB_1 receptor. This is consistent with heterotrimer dissociation if one considers the mixed responses that were observed with selective agonists and Gαi subtypes. Similar to what was observed with Gαi, GDPβS alone did not alter the amount of Gβ in association with the CB_1 receptor. However, GDPβS could attenuate the agonist-promoted dissociation of the CB_1 receptor-G$\beta(\gamma)$ complex. This is consistent with the receptor-G protein heterotrimer being stabilized by the occupancy of Gαi with GDPβS. SR141716 seemed to exert no influence on the CB_1 receptor-G$\beta\gamma$ interaction in the absence or presence of GDPβS. However, SR141716 served to counter the GTPγS-mediated dissociation of the CB_1 receptor-G$\beta\gamma$ complex.

Discussion

Our present studies have examined the stability of CB_1 receptor complexes with three subtypes of Gi proteins in detergent solution to gain insight regarding the role that

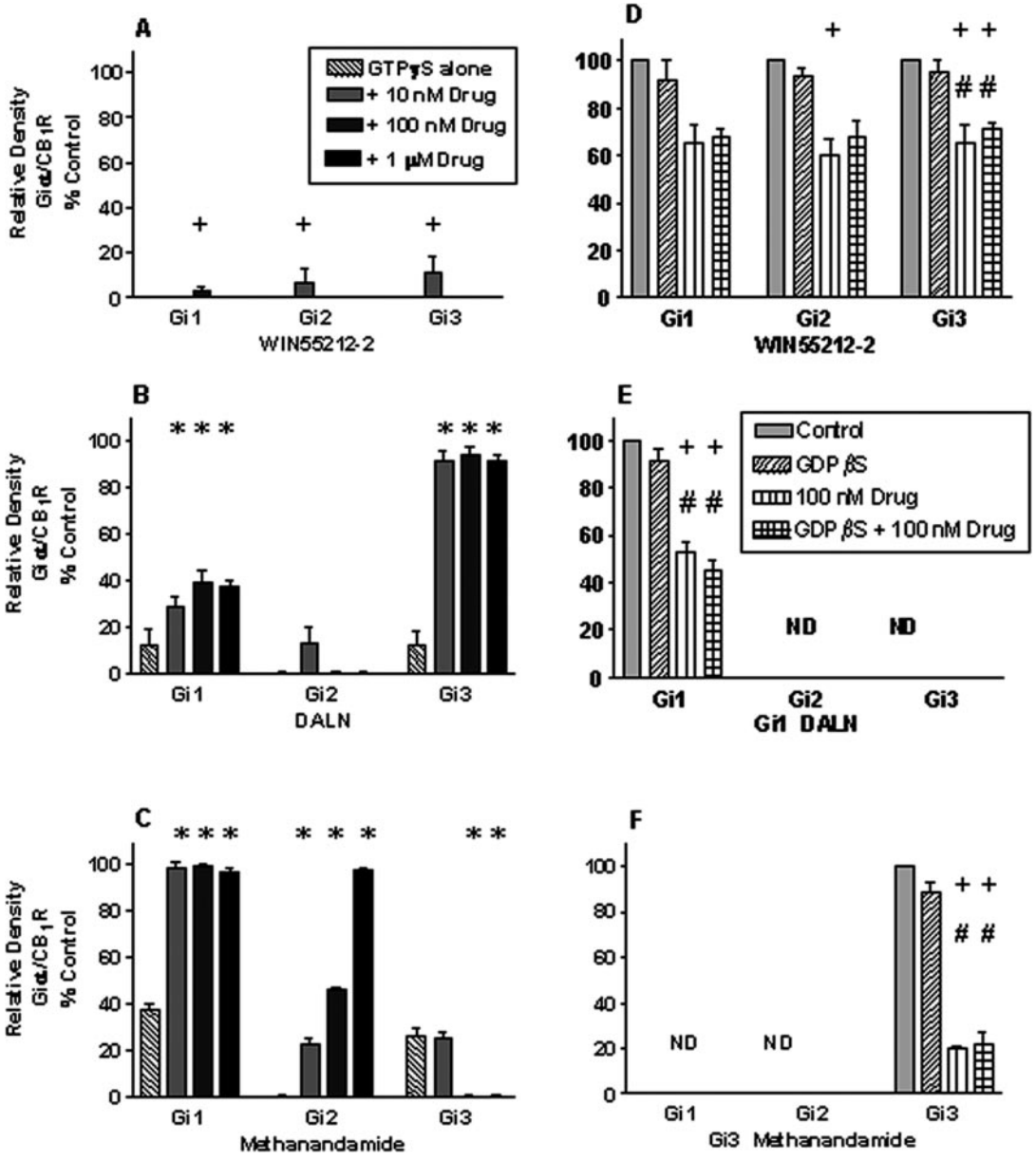

Fig. 5. Interactions of agonist-occupied CB_1 receptor and guanine nucleotide-occupied Gαi. CHAPS extracts from N18TG2 cell membranes were incubated with 100 μM GTPγS (A–C) or GDPβS (D–F) in the absence or presence of various concentrations of WIN 55,212-2 (A and D), DALN (B and F), or (*R*)-methanandamide (C and F) as indicated. Immunoprecipitation, Western blotting, and data analyses were carried out as described in the text and in legends to Figs. 3 and 4. A to C, data are the mean and S.E.M. from $n = 3$ independent experiments. A two-way ANOVA was used to determine the contribution of variance. A significant difference from GTPγS alone is indicated by +, $p < 0.05$, and by *, $p < 0.001$. D to F, data are the mean and S.D. from $n = 2$ (WIN 55,212-2) or $n = 3$ (DALN or (*R*)-methanandamide) independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. A significant difference ($p < 0.05$) from control is indicated by + and from GDPβS is indicated by #. ND, not determined.

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 β_2-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 CB_1 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 RG_{GDP} 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 CB_1 receptors.

As depicted in the model, the demonstration that GTPγS alone promoted dissociation of the G proteins from the CB_1 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 $G\alpha_{GTP\gamma S}$ 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_{GDP}. WIN 55,212-2 promoted development of this mixture for all three Gi 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 CB_1 receptor evoked by DALN for Gi2 and by (*R*)-methanandamide for Gi3 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 $G\alpha_{GTP}$ dissociation, the AR*G complex may be susceptible to protein denaturation, as has been observed for conformationally relaxed constitutively active mutants of GPCRs (Gether et al., 1997). In our experimental model, a denatured receptor that is unable to bind Gα 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^oG_{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 CB_1 receptor to "sequester" Gi 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 CB_1 receptors in an IR^oG_{GDP} complex would reduce the fraction of RG_{GDP} complex that could spontaneously convert to R*G_ or become

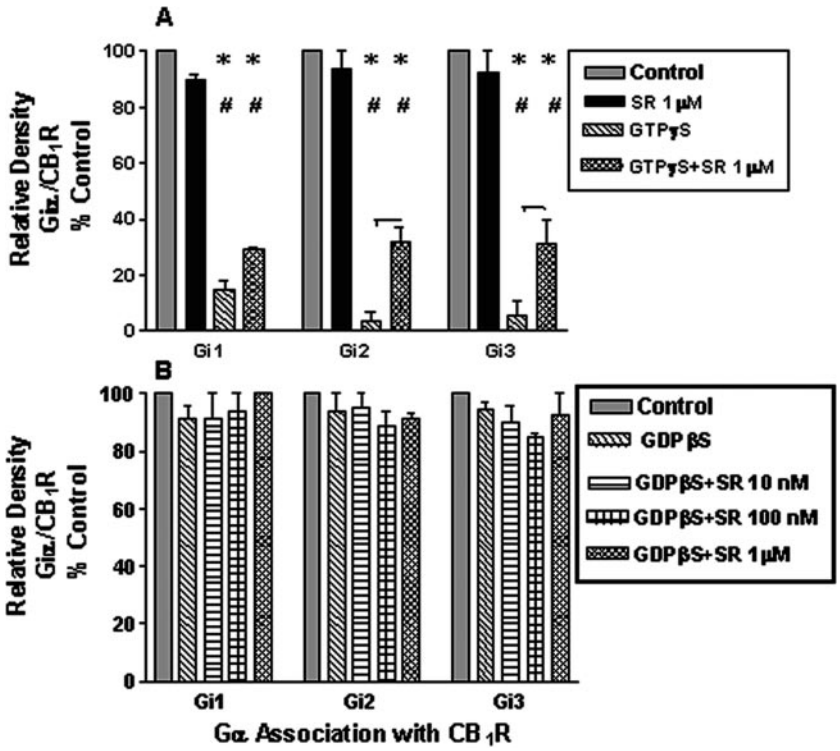

Fig. 6. Effect of SR141716 and guanine nucleotides on the CB_1 receptor-Gαi interaction. A, CHAPS-solubilized extracts of N18TG2 cell membranes were incubated in the presence of 1 μM SR141716, 100 μM GTPγS, or both as indicated. B, CHAPS-solubilized extracts of N18TG2 cell membranes were incubated in 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 CB_1 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 CB_1 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.

available to interact with agonists to induce the AR*G_ complex.

The conversion of the RG_{GDP} complex to a sustainable IR^oG_{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 CB_1 receptor, but that in the presence of DALN, GTPγS failed to dissociate Gi3.

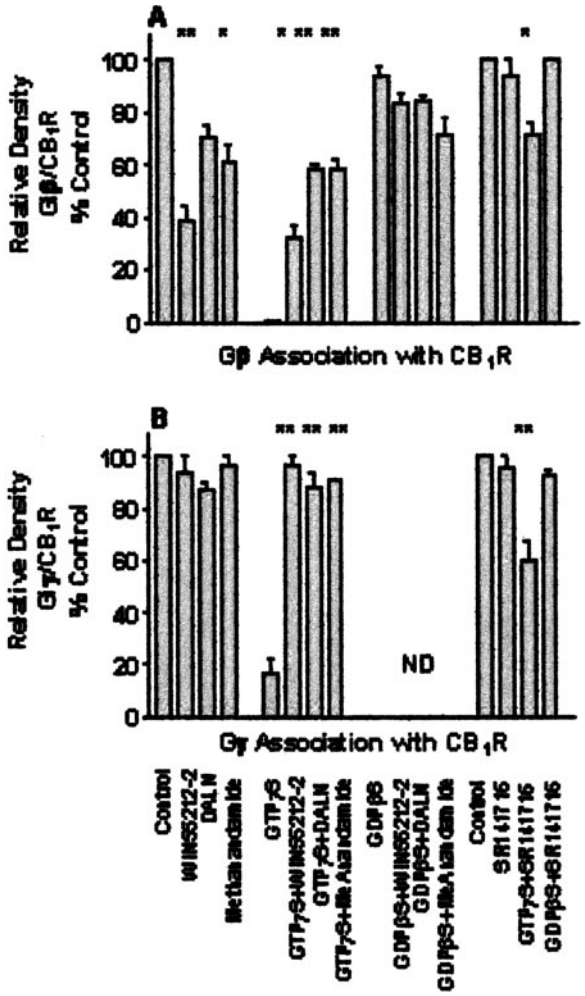

Fig. 7. Effect of ligands and guanine nucleotides on the CB_1 receptor interaction with Gβ (A) and Gγ (B). CHAPS-solubilized extracts of N18TG2 membranes were incubated in the absence or presence of 100 nM WIN 55,212-2, 100 nM DALN, 100 nM (*R*)-methanandamide, 1 μM SR141716, 100 μM GTPγS, 100 μM GDPβS, or combinations as indicated. Immunoprecipitation and Western blotting was performed with SDS-polyacrylamide gel electrophoresis conditions modified to allow for the detection of low-molecular-weight proteins. Gβ and Gγ proteins and the CB_1 receptor band densities were quantified as described in the text and quantified as a ratio compared with the CB_1 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.

An alternative mechanism might be that the inverse agonist-occupied receptors serve as guanine nucleotide-exchange factors that act on $G\alpha i_{GTP\gamma S}$ to exchange GDP for GTPγS. This mechanism is not likely, because our studies indicated that Gβγ was dissociated from the CB_1 receptor, and there is a smaller probability that $G\alpha i_{GTP\gamma S}$ 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 $G\alpha i_{GTP\gamma S}$, suggesting that these ligands do not possess as great an inverse agonist efficacy to promote the isomerization to IR^oG_{GDP} as does DALN for Gi3 or (*R*)-methanandamide for Gi1 and Gi2.

Under the present assay conditions, Gβγ was dissociated from the CB_1 receptor in parallel with Gαi, 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 CB_1 receptors. In the presence of GTPγS, agonists promoted the dissociation of a fraction of the Gβ isoforms consistent with the AR*G_ → AR + Gβγ + $G\alpha i_{GTP\gamma S}$ forward reaction. Protein-interaction studies by others have demonstrated that Gβγ can interact with both R and AR in the absence of Gα in detergent solution and reconstituted lipid vesicles (Heithier et al., 1992). In surface-plasmon resonance studies of immobilized rhodopsin, Gβγ binding was transient but was required to facilitate binding of Gα (Clark et al., 2001).

Our studies can be compared with other investigations of CB_1 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 CB_1 receptors in Sf9 cell membranes to activate guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding to purified Gαi (all subtypes) and Gαo proteins. Both Gi and Go proteins were activated to the maximum extent by HU-210 and minimally by Δ^9-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′-*O*-(3-[^{35}S]thio)triphosphate binding by SR141716 was observed for both Gi and Go. Prather and colleagues (2000) demonstrated differences in the ED_{50} value for G protein activation by WIN 55,212-2 using [^{32}P]azidoanilido-GTP binding as the determinant of G protein activation. The ED_{50} value for WIN 55,212-2 to activate various G protein subtypes in rat cerebellum membranes ranged from 100 nM for Gαi1 and Gαo3 to 3.7 μM for Gαo2. It is not easy to compare their specific findings with ours because undifferentiated N18TG2 cells do not express an appreciable

amount of Gαo, and those studies did not quantify [^{32}P]azidoanilido-GTP incorporation into Gαi3.

The studies of Glass and Northup (1999) and Prather and colleagues (2000) both determined the exchange of a GTP analog for GDP on the Gα subunit under conditions that restrict reversal of the reaction. The present investigation determined receptor-Gα 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 microisomerization to unique conformations that can direct the activation of selected G protein subtypes (Kenakin and Onaran, 2002). We determined previously that the CB_1 receptor juxtamembrane C-terminal fourth loop domain was responsible for coupling to Gαo and Gαi3 but not to Gαi1 or Gαi2 (Mukhopadhyay et al., 2000; Mukhopadhyay and Howlett, 2001). In contrast, the third intracellular loop was important for interaction with Gαi1 and Gαi2 (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 induce alterations predominantly 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 Gα isoform expressed within cells and the relative abundance of G proteins in the environment coupled to receptors.

References

Berg KA, Maayani S, Goldfarb J, Scaramellini C, Leff P, and Clarke WP (1998) Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus. *Mol Pharmacol* **54:**94–104.

Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M, Barth F, Calandra B, Pecceu F, Lupker J, et al. (1997) A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. *J Biol Chem* **272:**22330–22339.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.

Brink CB, Wade SM, and Neubig RR (2000) Agonist-directed trafficking of porcine α_{2A}-adrenergic receptor signaling in Chinese hamster ovary cells: L-isoproterenol selectively activates G_s. *J Pharmacol Exp Ther* **294:**539–547.

Brown PJ and Schonbrunn A (1993) Affinity purification of a somatostatin receptor-G-protein complex demonstrates specificity in receptor-G-protein coupling. *J Biol Chem* **268:**6668–6676.

Clark WA, Jian X, Chen L, and Northup JK (2001) Independent and synergistic interaction of retinal G-protein subunits with bovine rhodopsin measured by surface plasmon resonance. *Biochem J* **358:**389–397.

Clarke WP and Bond RA (1998) The elusive nature of intrinsic efficacy. *Trends Pharmacol Sci* **19:**270–276.

Cordeaux Y, Nickolls SA, Flood LA, Graber SG, and Strange PG (2001) Agonist regulation of D_2 dopamine receptor/G protein interaction. Evidence for agonist selection of G protein subtype. *J Biol Chem* **276:**28667–28675.

Downes GB and Gautam N (1999) The G protein subunit gene families. *Genomics* **62:**544–552.

Faivre S, Regnauld K, Bruyneel E, Nguyen QD, Mareel M, Emami S, and Gespach C (2001) Suppression of cellular invasion by activated G-protein subunits Gαo, Gαi1, Gαi2 and Gαi3 and sequestration of Gβγ. *Mol Pharmacol* **60:**363–372.

Gether U, Ballesteros JA, Seifert R, Sanders-Bush E, Weinstein H, and Kobilka BK (1997) Structural instability of a constitutively active G protein-coupled receptor. Agonist-independent activation due to conformational flexibility. *J Biol Chem* **272:**2587–2590.

Glass M and Northup JK (1999) Agonist selective regulation of g proteins by cannabinoid CB_1 and CB_2 Receptors. *Mol Pharmacol* **56:**1362–1369.

Gudermann T, Kalkbrenner F, and Schultz G (1996) Diversity and selectivity of receptor-G protein interaction. *Annu Rev Pharmacol Toxicol* **36:**429–459.

Heithier H, Frohlich M, Dees C, Baumann M, Haring M, Gierschik P, Schiltz E, Vaz WL, Hekman M, and Helmreich EJ (1992) Subunit interactions of GTP-binding proteins. *Eur J Biochem* **204:**1169–1181.

Houston DB and Howlett AC (1993) Solubilization of the Cannabinoid receptor from rat brain and its functional interaction with guanine nucleotide-binding proteins. *Mol Pharmacol* **43:**17–22.

Houston DB and Howlett AC (1998) Differential receptor-G-protein coupling evoked by dissimilar cannabinoid receptor agonists. *Cell Signal* **10:**667–674.

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, et al. (2002) International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* **54:**161–202.

Howlett AC, Mukhopadhyay S, Shim JY, and Welsh WJ (1999) Signal transduction of eicosanoid CB1 receptor ligands. *Life Sci* **65:**617–625.

Howlett AC, Song C, Berglund BA, Wilken GH, and Pigg JJ (1998) Characterization of CB1 cannabinoid receptors using receptor peptide fragments and site-directed antibodies. *Mol Pharmacol* **53:**504–510.

Kenakin T (1995) Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. *Trends Pharmacol Sci* **16:**232–238.

Kenakin T and Onaran O (2002) The ligand paradox between affinity and efficacy: can you be there and not make a difference? *Trends Pharmacol Sci* **23:**275–280.

Lachance M, Ethier N, Wolbring G, Schnetkamp PP, and Hebert TE (1999) Stable association of G proteins with beta 2AR is independent of the state of receptor activation. *Cell Signal* **11:**523–533.

Law SF and Reisine T (1992) Agonist binding to rat brain somatostatin receptors alters the interaction of the receptors with guanine nucleotide-binding regulatory proteins. *Mol Pharmacol* **42:**398–402.

Law SF and Reisine T (1997) Changes in the association of G protein subunits with the cloned mouse δ opioid receptor on agonist stimulation. *J Pharmacol Exp Ther* **281:**1476–1486.

Leff P, Scaramellini C, Law C, and McKechnie K (1997) A three-state receptor model of agonist action. *Trends Pharmacol Sci* **18:**355–362.

Meschler JP, Kraichely DM, Wilken GH, and Howlett AC (2000) Inverse agonist properties of *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide HCl (SR141716A) and 1-(2-chlorophenyl)-4-cyano-5-(4-methoxyphenyl)-1H-pyrazole-3-carboxylic acid phenylamide (CP-272871) for the CB_1 cannabinoid receptor. *Biochem Pharmacol* **60:**1315–1323.

Mukhopadhyay S and Howlett AC (2001) CB1 receptor-G protein association. Subtype selectivity is determined by distinct intracellular domains. *Eur J Biochem* **268:**499–505.

Mukhopadhyay S, McIntosh HH, Houston DB, and Howlett AC (2000) The CB_1 cannabinoid receptor juxtamembrane C-terminal peptide confers activation to specific G proteins in brain. *Mol Pharmacol* **57:**162–170.

Mukhopadhyay S, Shim JY, Assi AA, Norford D, and Howlett AC (2002) CB_1 cannabinoid receptor-G protein association: a possible mechanism for differential signaling. *Chem Phys Lipids* **121:**91–109.

O'Shannessy DJ and Hoffman WL (1987) Site-directed immobilization of glycoproteins on hydrazide-containing solid supports. *Biotechnol Appl Biochem* **9:**488–496.

Pan X, Ikeda SR, and Lewis DL (1998) SR 141716A acts as an inverse agonist to increase neuronal voltage-dependent Ca^{2+} currents by reversal of tonic CB1 cannabinoid receptor activity. *Mol Pharmacol* **54:**1064–1072.

Perez DM, Hwa J, Gaivin R, Mathur M, Brown F, and Graham RM (1996) Constitutive activation of a single effector pathway: evidence for multiple activation states of a G protein-coupled receptor. *Mol Pharmacol* **49:**112–122.

Prather PL, Martin NA, Breivogel CS, and Childers SR (2000) Activation of cannabinoid receptors in rat brain by WIN 55212–2 produces coupling to multiple G protein α-subunits with different potencies. *Mol Pharmacol* **57:**1000–1010.

Sim-Selley LJ, Brunk LK, and Selley DE (2001) Inhibitory effects of SR141716A on G-protein activation in rat brain. *Eur J Pharmacol* **414:**135–143.

Skrzydelski D, Lhiaubet AM, Lebeau A, Forgez P, Yamada M, Hermans E, Rostene W, and Pelaprat D (2003) Differential involvement of intracellular domains of the rat NTS1 neurotensin receptor in coupling to G proteins: a molecular basis for agonist-directed trafficking of receptor stimulus. *Mol Pharmacol* **64:**421–429.

Vasquez C and Lewis DL (1999) The CB1 cannabinoid receptor can sequester G-proteins, making them unavailable to couple to other receptors. *J Neurosci* **19:**9271–9280.

Waelbroeck I (1999) Kinetics versus equilibrium: the importance of GTP in GPCR activation. *Trends Pharmacol Sci* **20:**477–481.

Yang J, Wu J, Jiang H, Mortensen R, Austin S, Manning DR, Woulfe D, and Brass LF (2002) Signaling through Gi family members in platelets. Redundancy and specificity in the regulation of adenylyl cyclase and other effectors. *J Biol Chem* **277:**46035–46042.

Yang Q and Lanier SM (1999) Influence of G protein type on agonist efficacy. *Mol Pharmacol* **56:**651–656.

Address correspondence to: Dr. Somnath Mukhopadhyay, Neuroscience of Drug Abuse Research Program, J. L. Chambers Biomedical/Biotechnology Research Institute, North Carolina Central University, 700 George Street, Durham, NC 27707. E-mail: smukhopadhyay@nccu.edu