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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, NC 27707

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Corresponding address

Somnath Mukhopadhyay, Ph.D. or Allyn C. Howlett, Ph.D.

Neuroscience of Drug Abuse Research Program

J. L. Chambers Biomedical/Biotechnology Research Institute

North Carolina Central University

700 George Street, Durham, NC 27707

Ph # (919) 530-7032 or 530-7762

FAX (919) 530-7760

ahowlett@nccu.edu

smukhopadhyay@nccu.edu

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ABBREVIATIONS: CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate; CHO, Chinese hamster ovary; DALN, desacetyllevonantradol; ECL, enhanced chemiluminescence; GPCR, G protein coupled receptor; GDPβS, guanosine 5'-O-(3-thio)-diphosphate; GppNHp, guanylyl-imidodiphosphate; GTPγS, guanosine 5'-O-(3-thio)-triphosphate; MAPK, mitogen-activated protein kinase; SDS, sodium dodecylsulfate.

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ABSTRACT

In order to understand how structurally distinct ligands regulate CB_1 receptor interactions with Gi1, Gi2 and Gi3, we quantitated the Gαi and βγ 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 WIN55212-2 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 non-hydrolyzable GTP analog. The biaryl pyrazole antagonist 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. WIN55212-2 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 in order to regulate individual G proteins.

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It has become generally accepted that different GPCRs[1] in a cell can couple selectively to different Gα and Gβγ subtypes (see Gudermann et al., for review (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 activation of different signal transduction pathways, was described in ternary complex equilibrium models of multiple activated receptor states coupling selectively to different G proteins (Clarke and Bond, 1998;Kenakin, 1995;Leff *et al.*, 1997). These models have been supported by observations of agonist-selective coupling of α_{1B}-adrenergic receptor mutants (Perez *et al.*, 1996) and 5-HT_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 CHO 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 cannabinoid 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 (see the review by the International Union of Pharmacology Cannabinoid Receptor Committee (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 (Meschler *et al.*, 2000; Pan *et al.*, 1998; 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).

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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 (reviewed by (Mukhopadhyay *et al.*, 2002)). We quantitated the Gαi and βγ proteins that co-immunoprecipitate with the CB_1 receptor from a CHAPS extract of N18TG2 cell membranes. We demonstrate here that the aminoalkylindole WIN55212-2, 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 upon the Gi subtype. A simplified working model is depicted in Fig. 1 as a basis for developing a platform for understanding the emerging data.

Experimental Procedures

Materials. The chemicals, including GTPγS, GDPβS, and GppNHp, were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. DALN was a gift from Pfizer, Inc. (Groton, CT). WIN 55212-2 and (R)-methanandamide were purchased from Calbiochem (CA) and Cayman Chemicals (Ann Arbor, MI), respectively. SR141716 and rabbit antisera against peptides selective for Gαi1, Gαi2 or Gαi3 were purchased from BIOMOL (Plymouth Meeting, PA). Urea was purchased from ICN (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 1-4 was purchased from Santa Cruz (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 (West Grove, PA). Rainbow molecular

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weight markers and ECL reagents were purchased from Amersham Life Sciences, Inc. (Arlington Heights, IL).

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)

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Membrane Preparation, Detergent Solubilization and Treatments: N18TG2 neuroblastoma cells were grown in DMEM media with 10% heat-inactivated calf serum and 1% Penicillin-streptomycin to 90% confluence. Cells were then harvested with PBS-EDTA, sedimented, and the cell pellet was homogenized in a glass homogenizer in ice-cold HME buffer (20 mM Na-HEPES, pH 8.0; 2 mM $MgCl_2$; 1 mM EDTA). After sedimentation at 1000 x g for 5 min at 4^{o}C to remove unbroken cells and nuclei, the supernatant was collected and sedimented at 17,000 x g for 20 min at 4^{o}C. The pellet (P2 membrane fraction) was resuspended in HME and the protein concentration was determined (Bradford, 1976). For solubilization, 5 mg membrane protein was sedimented at 17, 000 x g, resuspended in 500 µl solubilization buffer TM buffer (30 mM Tris-Cl, pH 7.4; 5 mM $MgCl_2$) containing 4 mg CHAPS and 20% glycerol according to the method described by Houston and Howlett (Houston and Howlett, 1993). CHAPS extracts were treated with the indicated CB_1 receptor ligands at varying concentrations (10 nM to1µM) in the presence or absence of 100 µM GTPγS, GppNHp or GDPβS in a final volume of 100 µl TM buffer for 20 min at 30^{o}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.

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Immunoprecipitation: Following the incubation, the immunoprecipitation of the CB_1 receptor and associated proteins from ligand- or guanine nucleotide-treated CHAPS extracts was performed 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 X g for 5 min and matrix was washed three times with 500 µl TBS-T buffer (20 mM Tris-Cl, pH 7.4; 140 mM NaCl; 0.1% Tween 20). Immunoprecipitated protein was eluted from the matrix with 50 µl Gly-Cl, pH 2.5 (100 mM), and the eluate was immediately neutralized with 450 µl Tris-Cl, pH 8.0 (1.5 M). The protein from the neutralized eluate was precipitated by addition of 8 volumes of $CHCl_3/CH_3OH/H_2O$ (1:4:3), dissolved in Laemmli's sample buffer containing 5 mM EDTA and heated at 65°C for 5 min. Samples were subjected to polyacrylamide gel electrophoresis (PAGE) 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 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 anti-Gαi antibody (1:1000) or Gβ (subunits 1-4) and 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 TBS-Tween 20, 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 National Institutes of Health Image Program (Scion Corp.) or using Alpha Innotech software. Data analysis and figures were produced using Graphpad Prism 3 (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, 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 co-immunoprecipitated 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 matix-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 exists 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 by 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 Gβγ. The co-immunoprecipitation method can provide a quantitative measure of the ability of ligands to modify the distribution of free versus complexed receptors and G proteins.

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Three structurally different CB_1 receptor agonists classes were tested to determine their effects on CB_1 receptor-Gαi (Gαi1 or Gαi2 or Gαi3) complexes in CHAPS-solubilized N18TG2 cell membranes. Representative Western immunoblots depicting the effects of ligands and the non-hydrolyzable 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 co-immunoprecipitated with the receptor in the same lane (Fig. 3A-C; Lanes 1, upper, middle and lower panels). 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 WIN55212-2 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). WIN55212-2 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 nM 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 about 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 WIN55212-2 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. 3A-C, lanes 5 for each Gαi subtype; Fig. 5A-C). 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

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GDP-GTPγS exchange appears to have gone to completion under the assay conditions utilized in the present study. In the absence of agonist ligands, this would represent spontaneous dissociation of GDP from 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 upon the ligand and the Gαi subtype. One sees little influence of WIN55212-2 on any of the three $G\alpha i_{GTP\gamma S}$ dissociated states, consistent with the relative non-selectivity 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).

In order 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 subtype to CB_1 receptor in immunoprecipitate (Fig. 5D.-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 ($G\alpha i_{GDP\beta S}$-βγ) that would have been able to associate with the CB_1 receptor.

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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. 5D.-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 would be predicted that if free Gαi proteins exist in solution under control conditions, then a greater population of Gαi proteins would 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% decline 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 Gαi_ were present in solution, one would predict that in the presence of high concentrations of GDPβS, SR141716 would stabilize a greater amount of co-immunoprecipitatable 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 Gαi2 (68% dissociated) and Gαi3 (69% dissociated), and a similar trend existed for Gαi1 (71% dissociated) (Fig. 6A). The GppNHp-induced dissociation of CB_1 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

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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 the subtypes of Gi protein (42% for Gi1, 46% for Gi2, 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-Gβγ complexes. The interaction of the CB_1 receptor with the Gβγ 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. Gγ2 did not show a pattern of dissociation from the CB_1 receptor. This may be due to 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. 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 Gβ and > 80% of the Gγ that was associated with the CB_1 receptor in CHAPS detergent (see Fig. 7). Under these conditions, the Gαi proteins were dissociated by 60% to 100% (see Fig. 5). Because the free $G\alpha i_{GTP\gamma S}$ is not likely to reassociate with Gβγ dimers to form heterotrimers, receptor-G protein complexes are not readily reestablished. In the presence of WIN55212, 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 would be 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-Gβ(γ) complex. This would be consistent with the receptor-G protein heterotrimer being stabilized by the

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occupancy of Gαi with GDPβS. SR141716 appeared to exert no influence on the CB_1 receptor-Gβγ interaction in the absence or presence of GDPβS. However, SR141716 served to counter the GTPγS-mediated dissociation of the CB_1 receptor-Gβγ complex.

Discussion

Our present studies have examined the stability of CB_1 receptor complexes with three subtypes of Gi proteins in detergent solution in order 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 β_2-AR receptors in the absence of GTP or GTPγS (Brown and Schonbrunn, 1993; Lachance *et al.*, 1999; Law and Reisine, 1992; Law and Reisine, 1997). In the present investigation using CHAPS extracts from cultured N18TG2 neuronal cell membranes, and studies that we previously reported using rat brain membranes (Houston and Howlett, 1993; Houston and Howlett, 1998; Mukhopadhyay *et al.*, 2000), a significant fraction of the total Gαi was found to be associated with immunoprecipitable 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 about 20% in rat brain membranes, and 35% for WIN55212-2 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 (Meschler *et al.*, 2000; Pan *et al.*, 1998; 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 immunoprecipitable 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

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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}. WIN55212-2 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 which 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 discernable 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 (I)-supported inactive state (IR^oG_{GDP}), in the ternary complex equilibrium model (Fig. 1). This state was 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 through the MAPK or adenylyl cyclase pathways was blocked in the presence of SR141716 (Bouaboula *et al.*, 1997). 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 available to interact with agonists to induce the AR*G_ complex.

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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 (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 WIN55212-2 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 below 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 WIN55212-2, 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.

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. Interestingly, 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.

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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 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_ $\Rightarrow$ 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 (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 [^{35}S]GTPγS binding to purified Gαi (all subtypes) and Gαo proteins. Both Gi and Go proteins were activated to the maximum extent by HU210, and minimally by Δ^9-THC. WIN55212 and anandamide exhibited maximal or near-maximal activity for Gi, but only about 70% maximal activity for Go. An inhibition of [^{35}S]GTPγS binding by SR141716 was observed for both Gi and Go. Prather and colleagues (Prather *et al.*, 2000) demonstrated differences in the ED_{50} for G protein activation by WIN55212-2 utilizing [^{32}P] azidoanilido-GTP binding as the determinant of G protein activation. The ED_{50} for WIN55212-2 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 to ours because undifferentiated N18TG2 cells do not express an appreciable amount of Gαo, and those studies did not quantitate [^{32}P] azidoanilido-GTP incorporation into Gαi3.

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The studies of Glass and Northup (Glass and Northup, 1999) and Prather and colleagues (Prather *et al.*, 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 in order to achieve GDP-GTP exchange and release of G proteins from the receptor. Shim and Howlett (2004) have proposed a theoretical model whereby nonclassical cannabinoid compounds such as CP55940 can convert to low energy states within the binding pocket, providing a "steric trigger" for micro-conformational changes within the binding domain. Chemically distinct ligands may allow this transition to progress by multiple pathways due to their differential ability to provide the activation energy for micro-isomerization to unique conformations that can direct the activation of selected G protein subtypes (see (Kenakin and Onaran, 2002) for discussion). We previously determined 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 upon the type of Gα isoform expressed within cells and the relative abundance of G proteins in the environment coupled to receptors.

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Figure Legends

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 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 reentering 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 an inverse agonist-supported inactive state (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 the equilibrium box; however, evidence suggests that CB_1 agonists can compete (Meschler *et al.*, 2000), demonstrating the reversibility of this step.

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 50µg N18TG2

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membrane protein (400 µl) mixed with 100 µl TM buffer as control; 2, Immunoprecipitate: CHAPS extract from N18TG2 membranes (400 µl) mixed with 100 µl sepharose-anti CB_1 antibody affinity matix. Proteins eluted from the affinity matrix, neutralized and sedimented as described in the text; immunoprecipitated proteins were dissolved in 500 µl TBS-T; 3, Supernatant: CHAPS extract remaining after the affinity matix-bound protein was removed (approximately 475 µl); 4-6, Washings 1-3: Supernatants remaining after the affinity matix-bound protein was washed with 500 µl TBS-T as described in the text. For each of these fractions, a 25 µl aliquot was added to 25 µl 2X 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 (upper), Gαi2 (middle), or Gαi3 (lower) panels. Immunoreactive bands were visualized by ECL as described in the text.

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, 5), 10 nM (lanes 2, 6), 100 nM (lanes 3, 7) or 1 µM (lanes 4, 8) concentrations of the aminoalkylindole WIN55212-2 (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 co-stained for both CB_1 receptor and either Gαi1 (upper), Gαi2 (middle), or Gαi3 (lower) proteins. Immunoreactive bands were visualized by ECL. Results are shown from a single representative of at least 3 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 WIN55212 (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

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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 SEM 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.

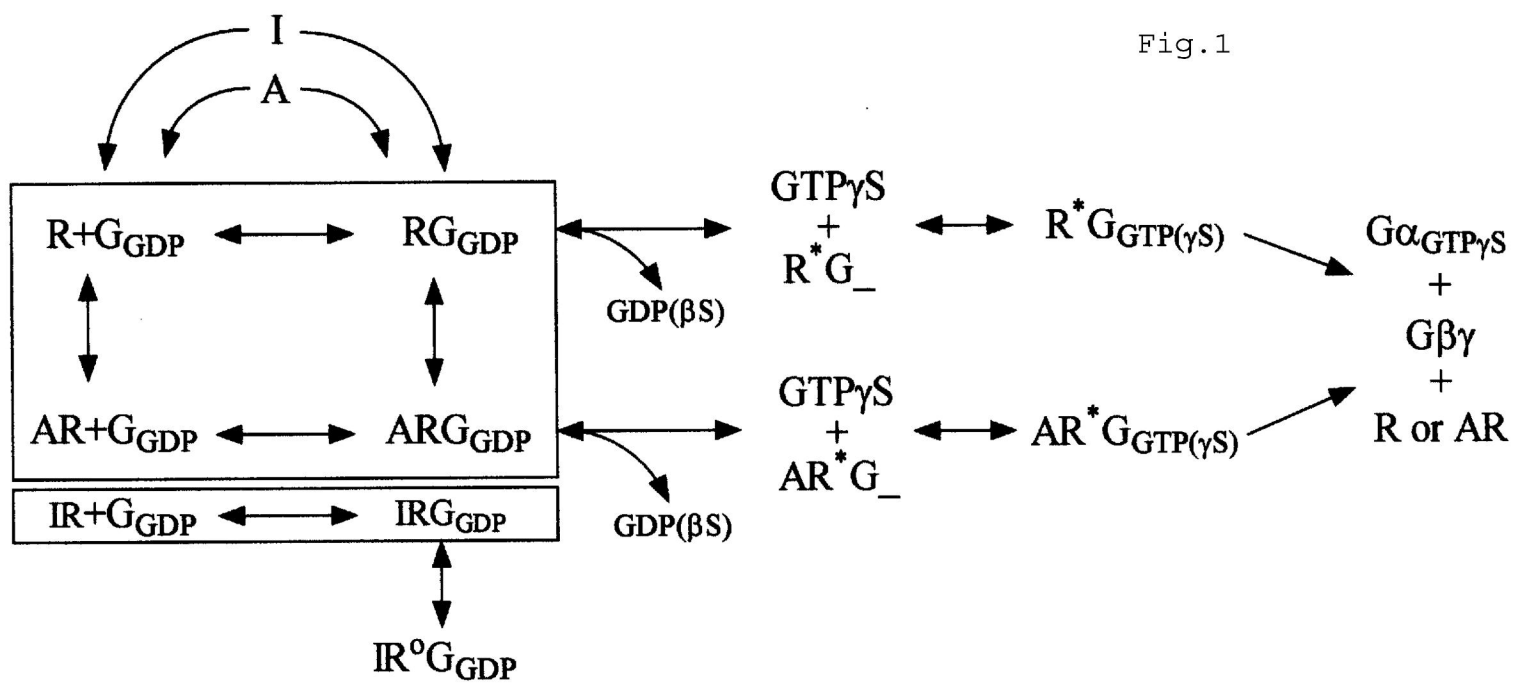
Fig. 5. Interactions of agonist-occupied CB_1 receptor and guanine nucleotide-occupied Gαi. CHAPS extracts from N18TG2 cell membranes were incubated with 100 μM GTPγS (A.-C.) or GDPβS (D.-F.) in the absence or presence of various concentrations of WIN55212-2 (A., D.), DALN (B., F.), or (R)-methanandamide (C., 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.-C. Data are the mean and SEM from n=3 independent experiments. A two-way ANOVA was used to determine contribution of variance. A significant difference from GTPγS alone is indicated by + at $p< 0.05$, and * at $p <0.001$. D.-F. Data are the mean and SD from n=2 (WIN55212-2) 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.

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 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 SD 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.

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 WIN55212-2, 100 nM DALN, 100 nM (R)-methanandamide, 1 μM SR141716, 100 μM GTPγS, 100 μM GDBβS, or combinations as indicated. Immunoprecipitation and Western blotting was performed with SDS-PAGE conditions modified to allow detection of low molecular weight proteins. Gβ and G γ proteins and the CB_1 receptor band densities were quantitated as described in the text and quantitated as a ratio compared to the CB_1 receptor band density. Data are mean and standard deviation 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.

Fig.1


I
A
R+G_GDP
RG_GDP
AR+G_GDP
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IR+G_GDP
IRG_GDP
IR°G_GDP
GDP(βS)
GDP(βS)
GTPγS
+
R*G_
R*G_GTP(γS)
Gα_GTPγS
+
Gβγ
+
R or AR
GTPγS
+
AR*G_
AR*G_GTP(γS)

Fig.2

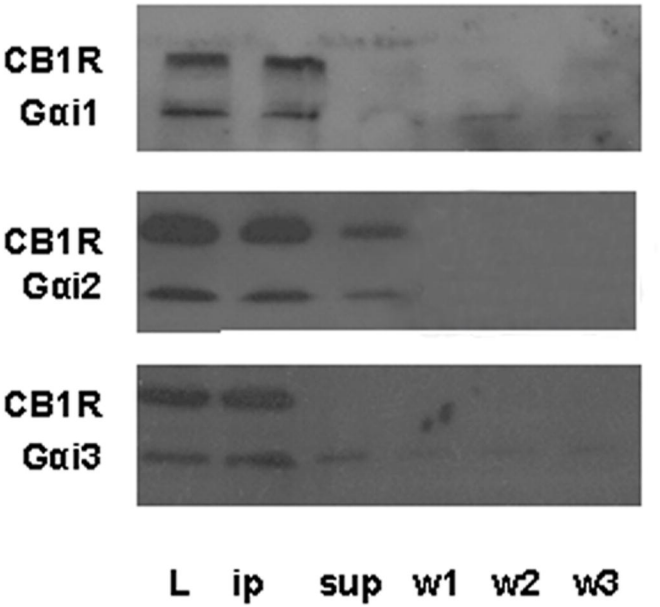


Fig. 3

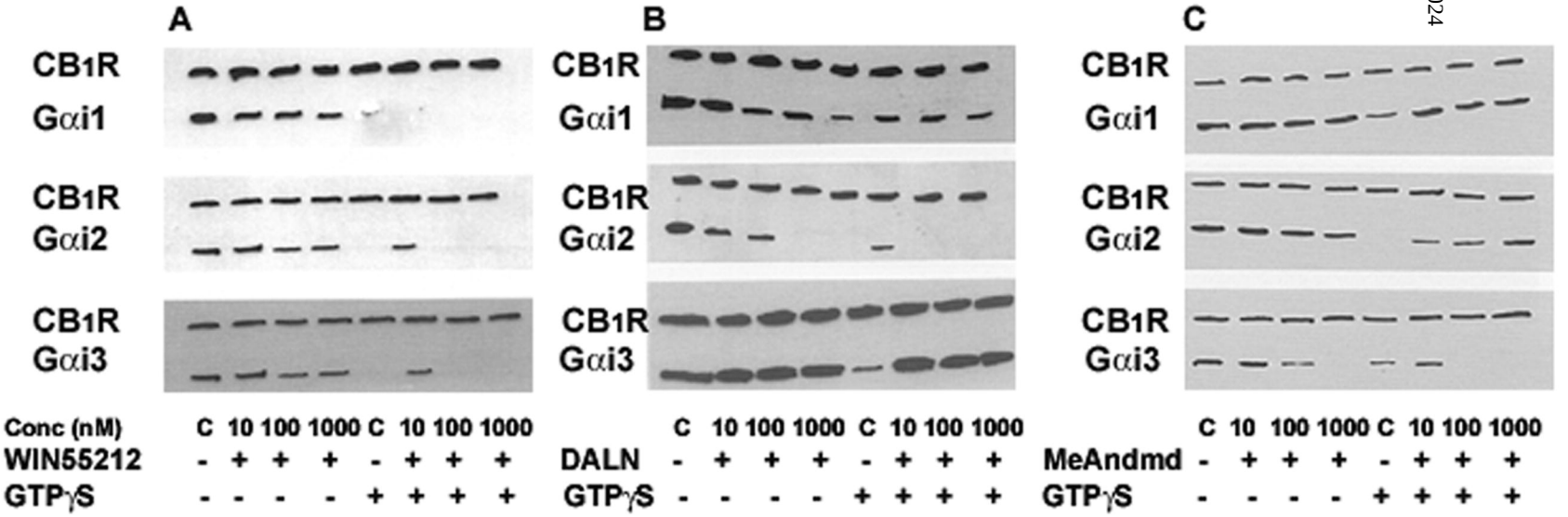
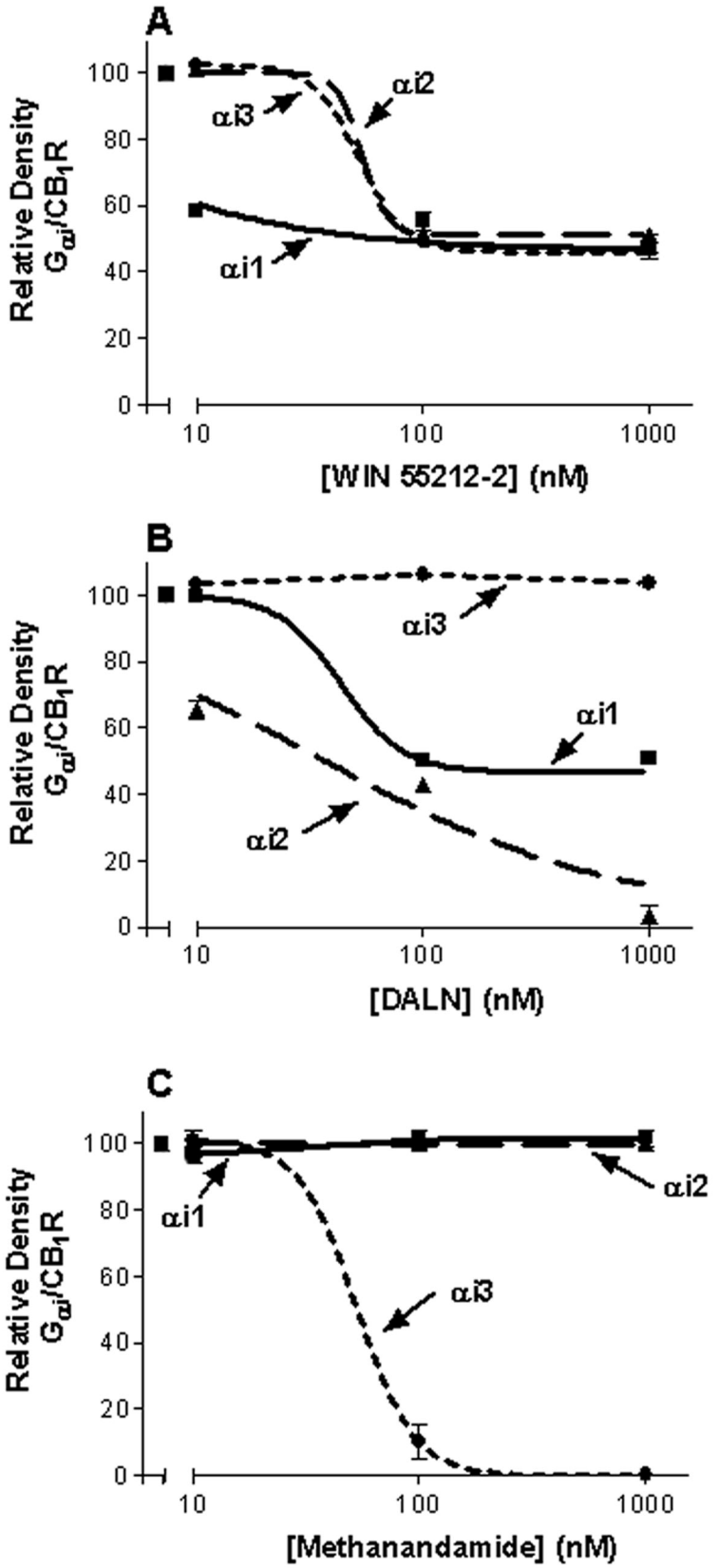


Fig.4


A
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$G_{\alpha i}/CB_1R$
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80
60
40
20
0
αi3
αi2
αi1
10
100
1000
[WIN 55212-2] (nM)
B
Relative Density
$G_{\alpha i}/CB_1R$
100
80
60
40
20
0
αi3
αi1
αi2
10
100
1000
[DALN] (nM)
C
Relative Density
$G_{\alpha i}/CB_1R$
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80
60
40
20
0
αi1
αi2
αi3
10
100
1000
[Methanandamide] (nM)

Fig.5

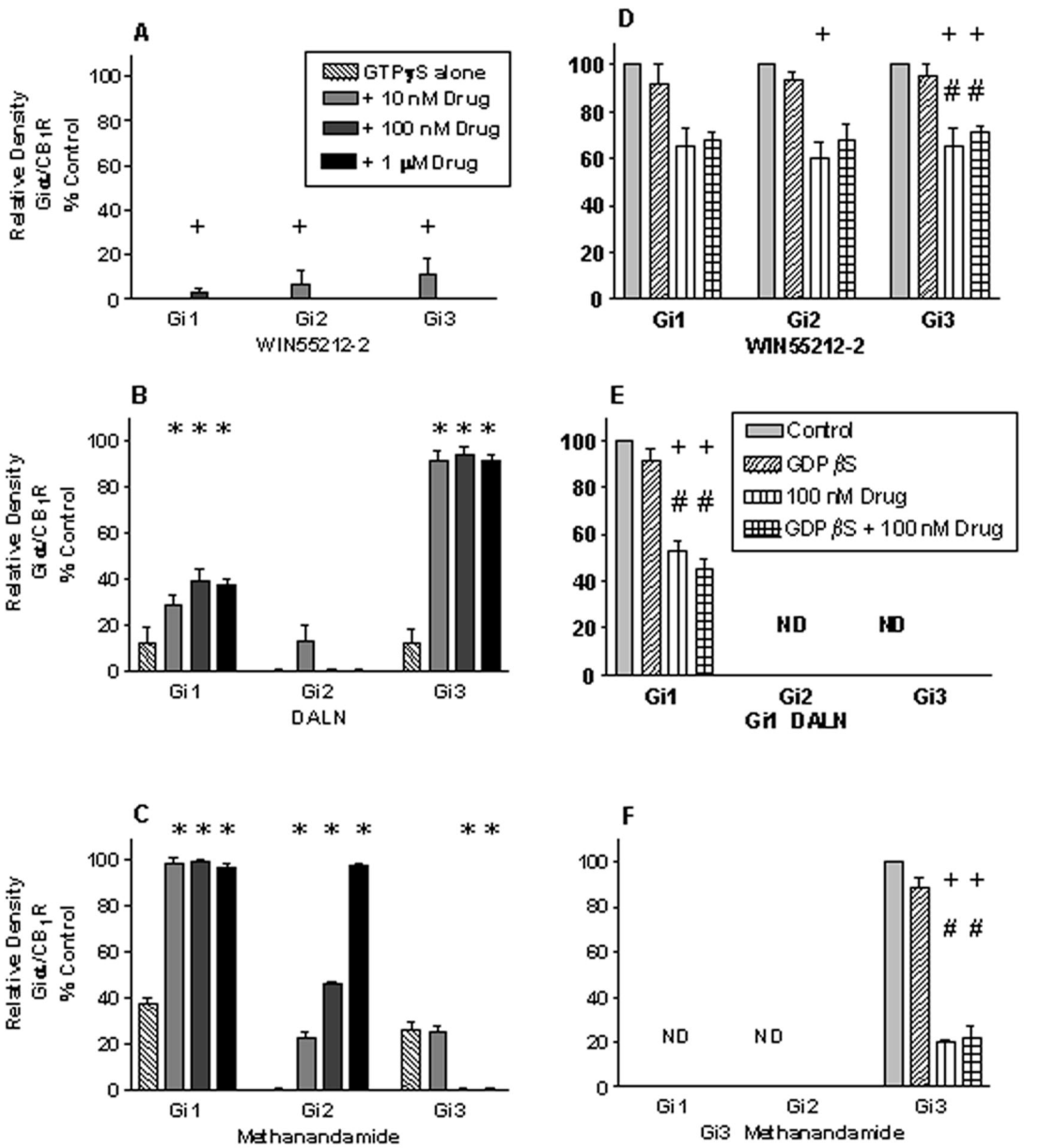

A
GTPγS alone
+ 10 nM Drug
+ 100 nM Drug
+ 1 μM Drug
Relative Density
Giα/CB1R
% Control
Gi1
Gi2
Gi3
WIN55212-2
D
Gi1
Gi2
Gi3
WIN55212-2
B
DALN
C
Methanandamide
E
Control
GDP βS
100 nM Drug
GDP βS + 100 nM Drug
ND
Gi1 DALN
F
Gi3 Methanandamide

Fig.6

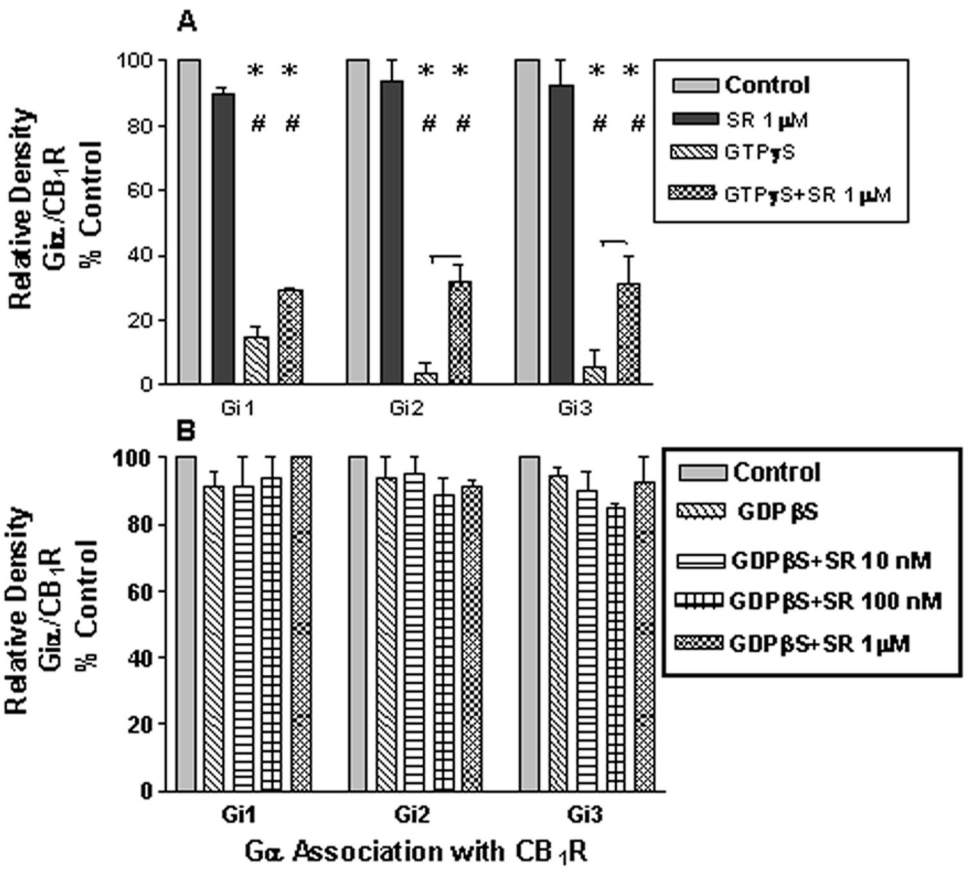
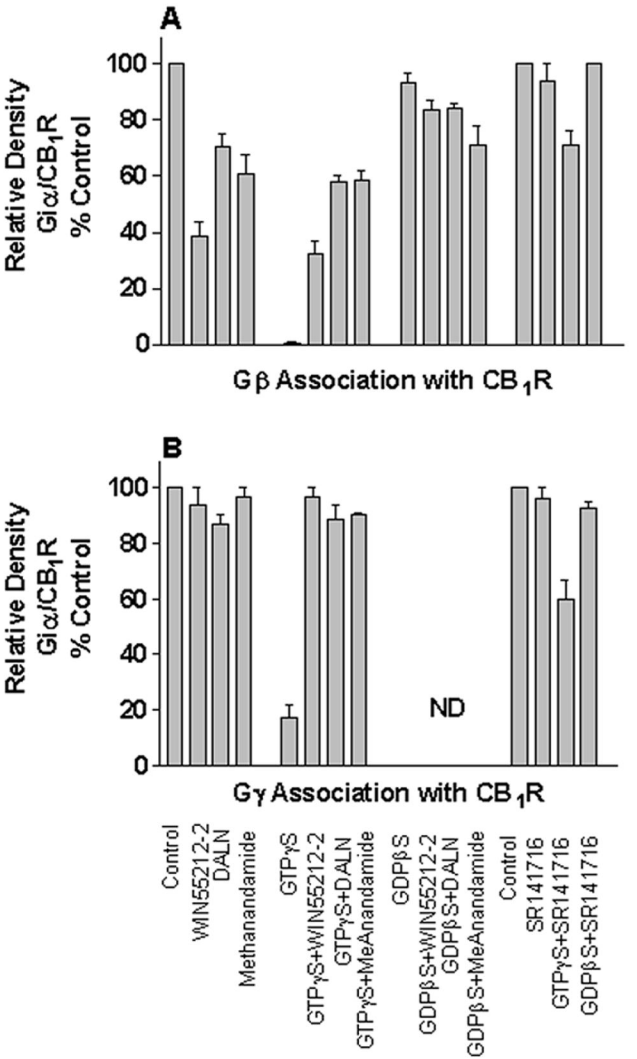

A
Relative Density
Giα/CB$_1$R
% Control
100
80
60
40
20
0
* * * *
# # # #
Gi1
Gi2
Gi3
Control
SR 1 μM
GTPγS
GTPγS+SR 1 μM
B
Relative Density
Giα/CB$_1$R
% Control
100
80
60
40
20
0
Gi1
Gi2
Gi3
Control
GDPβS
GDPβS+SR 10 nM
GDPβS+SR 100 nM
GDPβS+SR 1μM
Gα Association with CB$_1$R

Fig.7


A
Relative Density
Giα/CB1R
% Control
100
80
60
40
20
0
Gβ Association with CB1R
B
Relative Density
Giα/CB1R
% Control
100
80
60
40
20
0
ND
Gγ Association with CB1R
Control
WIN55212-2
DALN
Methanandamide
GTPγS
GTPγS+WIN55212-2
GTPγS+DALN
GTPγS+MeAnandamide
GDPβS
GDPβS+WIN55212-2
GDPβS+DALN
GDPβS+MeAnandamide
Control
SR141716
GTPγS+SR141716
GDPβS+SR141716