

**Chemically Distinct Ligands Promote
Differential CB₁ 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

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@ncu.edu

smukhopadhyay@ncu.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; GDP β S, guanosine 5'-O-(3-thio)-diphosphate; GppNHp, guanylyl-imidodiphosphate; GTP γ S, guanosine 5'-O-(3-thio)-triphosphate; MAPK, mitogen-activated protein kinase; SDS, sodium dodecylsulfate.

ABSTRACT

In order to understand how structurally distinct ligands regulate CB₁ receptor interactions with Gi1, Gi2 and Gi3, we quantitated the G α i and β γ proteins that coimmunoprecipitate with the CB₁ 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 RG α i complexes, cannabinoid desacetylleonantradol for G α i1 and G α i2, and eicosanoid (R)-methanandamide for G α i3. Desacetylleonantradol maintained RG α i3 complexes and (R)-methanandamide maintained RG α i1 and RG α i2 complexes even in the presence of a non-hydrolyzable GTP analog. The biaryl pyrazole antagonist SR141716 maintained all three RG α i complexes. G β proteins, and to a certain extent G γ 2, exhibited the same association/dissociation pattern as the G α 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; desacetylleonantradol 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.

It has become generally accepted that different GPCRs¹ in a cell can couple selectively to different G α and G $\beta\gamma$ 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-HT₂ 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. GTP γ S 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 D₂ 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 CB₁ cannabinoid receptor. The CB₁ receptor is a GPCR found abundantly in brain and neuronal cells, and is coupled to the Gi/o family of G proteins to regulate effectors such as adenylyl cyclase and ion channels (see the review by the International Union of Pharmacology Cannabinoid Receptor Committee (Howlett *et al.*, 2002)). The CB₁ 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). CB₁ receptor-G α complexes readily exist in the absence of exogenously added agonist or inverse agonist ligands (Houston and Howlett, 1993; Howlett *et al.*, 1999; Mukhopadhyay *et al.*, 2000; Mukhopadhyay and Howlett, 2001).

We hypothesized that structurally distinct ligands would exhibit differential ability to regulate CB₁ receptor interactions. To test this hypothesis, we used a well-characterized neuronal model for CB₁ cannabinoid receptor-mediated signal transduction, the N18TG2 neuroblastoma cell, which endogenously expresses CB₁ receptors and all three subtypes of G_i (reviewed by (Mukhopadhyay *et al.*, 2002)). We quantitated the G_{αi} and βγ proteins that co-immunoprecipitate with the CB₁ 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-G_{αi} complexes and free receptors differentially depending upon the G_{αi} subtype. SR141716 maintained the receptor in a complex with all three G_{αi} subtypes. These results also provide evidence for the differential behavior of these ligands as agonists or inverse agonists depending upon the G_i 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 G_{αi1}, G_{αi2} or G_{αi3} 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

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

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

Membrane Preparation, Detergent Solubilization and Treatments: N18TG2 neuroblastoma cells were grown in 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 MgCl₂; 1 mM EDTA). After sedimentation at 1000 x g for 5 min at 4°C to remove unbroken cells and nuclei, the supernatant was collected and sedimented at 17,000 x 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 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 MgCl₂) 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 CB₁ 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 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: Following the incubation, the immunoprecipitation of the CB₁ 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 μ l aliquot of the ligand- or guanine nucleotide-treated CHAPS extract was incubated under constant rotation with sepharose bead-coupled anti-CB₁ antibody (20 μ l) for 6 h at 4°C. Thus, the addition of antibody-coupled matrix to the solubilized preparation resulted in a 20% dilution of the ligands or guanine nucleotides. The anti-CB₁ affinity matrix was then sedimented at 17,000 X g for 5 min and matrix was washed three times with 500 μ l 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 μ l Gly-Cl, pH 2.5 (100 mM), and the eluate was immediately neutralized with 450 μ l Tris-Cl, pH 8.0 (1.5 M). The protein from the neutralized eluate was precipitated by addition of 8 volumes of CHCl₃/CH₃OH/H₂O (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-CB₁(1-14) combined with the indicated anti-G α i antibody (1:1000) or G β (subunits 1-4) and G γ 2 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 CB₁ Receptor and Specific G α i proteins.

CB₁ receptors solubilized from the membrane in CHAPS detergent exist in a state that is associated with various subtypes of the Gi protein family (G α i1, G α i2, G α i3) in the absence of exogenously added agonists (Fig. 2). It is particularly interesting to note that a significant fraction of the G α i proteins present in the CHAPS extract are co-immunoprecipitated with the CB₁ receptors (compare lane 1 (Load) with lane 2 (Immunoprecipitated)). Only a limited fraction of residual G α i proteins remained in the supernatant fraction (lane 3) or in any of the subsequent washes of the affinity matrix-bound CB₁ receptor-G protein complex. This indicates that the CB₁ receptor preferentially exists as a receptor-G protein complex in detergent solution under these experimental conditions. This association can be disrupted by incubation with pertussis toxin, demonstrating that the receptors and G proteins exist in a dynamic association/dissociation reaction mixture in detergent solution (Howlett *et al.*, 1999; Mukhopadhyay and Howlett, 2001). If these receptor-G protein complexes are functional, then they should be targets for functional interaction with CB₁ receptor ligands. Experimental conditions were chosen in which GTP and GDP are absent, so that association/dissociation reactions could proceed 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 α i_{GDP} with G $\beta\gamma$. 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.

Three structurally different CB₁ receptor agonists classes were tested to determine their effects on CB₁ receptor-G α i (G α i1 or G α i2 or G α i3) complexes in CHAPS-solubilized N18TG2 cell membranes. Representative Western immunoblots depicting the effects of ligands and the non-hydrolyzable GTP analog, GTP γ S, are shown in Fig. 3. The immunoblots depict the CB₁ receptor monomer found in cultured neuronal cells and the G α 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 CB₁ receptor band were calculated from multiple experiments, and the means and standard errors from multiple experiments are shown in Figs. 4 and 5. The aminoalkylindole ligand WIN55212-2 evoked partial dissociation of all three subtypes of G α i proteins from the receptor, reaching a maximum dissociation of only 50% of the control amount of receptor-G α i complexes (Figs. 3A and 4A). WIN55212-2 was relatively more potent in dissociating the receptor-G α i1 complex, achieving a maximal dissociation at 10 nM. In contrast, the dissociation of G α i2 and G α i3 from the receptor occurred between 10 nM and 100 nM. The cannabinoid ligand DALN (Figs. 3B and 4B) dissociated G α i1 and G α i2 from the CB₁ receptor-G α i complex in a dose-dependent manner. G α i2 was dissociated completely from the receptor at 1 μ M DALN (Fig. 4B). CB₁ receptor-G α i1 dissociation reached a maximum of about 50 % at 100 nM, with no further dissociation with increasing agonist concentrations. DALN had no effect on CB₁ receptor-G α i3 complexes. The eicosanoid (R)-methanandamide evoked dissociation of only CB₁ receptor-G α i3 (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 CB₁ receptor-G α i1 or G α i2 complexes.

Effect of guanine nucleotides on the CB₁ Receptor-G α i complex. Incubation of the CHAPS extract of N18TG2 membranes with the nonhydrolyzable GTP analog GTP γ S at 100 μ M resulted in 85% to 100 % dissociation of all three CB₁ receptor-G α i complexes (Fig. 3A-C, lanes 5 for each G α i subtype; Fig. 5A-C). Addition of GppNHp (100 μ M) also resulted in complete dissociation of all CB₁ receptor-G α i complexes (data not shown). The observation of complete receptor-G α i dissociation suggests that the

GDP-GTP γ S 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 GTP γ S, and dissociation of the heterotrimer to free receptor and G α i_{GTP γ S}. This process could have been facilitated by the absence of exogenous Na⁺ in the assay solutions.

The ability of GTP γ S to promote dissociation of the CB₁ receptor-G α i proteins was influenced differentially depending upon the ligand and the G α i subtype. One sees little influence of WIN55212-2 on any of the three G α i_{GTP γ S} dissociated states, consistent with the relative non-selectivity for any of the G α i subtype (Figs. 3A lanes 5-8 and 5A). DALN had no influence on the ability of GTP γ S to promote dissociation of the CB₁ receptor-G α i2 complex, and only limited influence on the CB₁ receptor-G α i1 complex (Figs. 3B lanes 5-8 and 5B). In similar experiments using an alternative GTP analog, GppNHp, dissociation of CB₁ receptor-G α i1 and G α i2 complexes was complete in the presence of DALN (data not shown). (R)-Methanandamide had little influence on the G α i_{GTP γ S} dissociated state for G α i3 (Figs. 3C lanes 5-8 and 5C). In contrast, the cannabinoid ligand DALN precluded the G α i3_{GTP γ S} dissociation, and partially attenuated the G α i1_{GTP γ S} dissociation (Figs. 3B and 5B). (R)-Methanandamide potently (10 nM) attenuated the G α i1_{GTP γ S} dissociation, and concentrations between 100 and 1000 nM attenuated the G α i2_{GTP γ S} dissociation (Figs. 3C and 5C).

In order to assess the possible spontaneous GDP release in the association/dissociation reaction, the CB₁ receptor-G α i complexes were incubated in the presence of a high concentration (100 μ M) of the GDP analog GDP β S. The addition of GDP β S to the detergent extract of N18TG2 membranes neither increased nor decreased the ratio of any of the G α i subtype to CB₁ receptor in immunoprecipitate (Fig. 5D.-F. bars 1 versus 2). If there existed any unoccupied G α i_ in the extract, it would have been predicted that GDP β S would bind, thereby promoting formation of additional heterotrimer (G α i_{GDP β S}- β γ) that would have been able to associate with the CB₁ receptor.

The failure of the GDP analog to promote a greater abundance of CB₁R-G α i complexes than in control extracts suggests that the CB₁ receptor-G α protein association was at its maximum as it existed in the CHAPS extract. The addition of GDP β S failed to alter the CB₁R-G α i 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 CB₁ receptor-G α heterotrimer complexes.

Inverse agonist influence on CB₁R-G α i complexes- SR141716 is a CB₁ receptor-selective competitive antagonist that has been shown to exhibit inverse agonist activity in signal transduction assays in recombinant cell models (Bouaboula *et al.*, 1997). It would be predicted that if free G α i proteins exist in solution under control conditions, then a greater population of G α i proteins would be found in a CB₁ receptor-G α i complex in the presence of SR141716. However, as shown in Fig. 6A, SR141716 exhibited little or no effect (< 10% decline in the amount of G α i associated with receptors) on the amount of receptor-G α i complex for any of the G α i subtypes. A similar finding was reported earlier for the CB₁ receptor associated with G α o in solubilized preparations from rat brain (Mukhopadhyay *et al.*, 2000). If a significantly greater population of unliganded G α i_ were present in solution, one would predict that in the presence of high concentrations of GDP β S, SR141716 would stabilize a greater amount of co-immunoprecipitable CB₁ receptor-Gi_{GDP β S} complexes. This was not the case for any of the G α i subtypes at any of the concentrations of SR141716 tested (Fig. 6B).

The GTP γ S-driven dissociation (85%-96% dissociated) was significantly attenuated in the presence of 1 μ M SR141716 for G α i2 (68% dissociated) and G α i3 (69% dissociated), and a similar trend existed for G α i1 (71% dissociated) (Fig. 6A). The GppNHp-induced dissociation of CB₁ receptor-G α i1 and G α 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 $G\alpha_{iGTP\gamma S}$ and complexed forms of $G\alpha_i$. A lower concentration (50 μM) of $GTP\gamma S$ produced only partial dissociation of the CB_1 receptor- $G\alpha_i$ complex compared with control for all the subtypes of Gi protein (42% for Gi_1 , 46% for Gi_2 , 40% for Gi_3). Various concentrations of SR141716 (10 nM to 1 μM) failed to influence the response to this lower concentration of $GTP\gamma S$.

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. $G\beta$ and $G\gamma$ 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 $G\alpha_i$ proteins, 40% to 70% of the $G\beta$ (isoforms 1-4) was dissociated. $G\gamma_2$ did not show a pattern of dissociation from the CB_1 receptor. This may be due to the profile of $G\gamma$ subtypes that are present in the N18TG2 cell membranes and associated with the $G\alpha_i$ proteins as a heterotrimer. This antibody does not recognize all $G\gamma$ subtypes that may potentially be present and/or associated with the CB_1 receptor. $G\gamma_2$ is only one of several $G\gamma$ subtypes that would be expected to be present in neuronal cells (Downes and Gautam, 1999).

$GTP\gamma S$ was able to dissociate 100% of the $G\beta$ and > 80% of the $G\gamma$ that was associated with the CB_1 receptor in CHAPS detergent (see Fig. 7). Under these conditions, the $G\alpha_i$ proteins were dissociated by 60% to 100% (see Fig. 5). Because the free $G\alpha_{iGTP\gamma S}$ 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 WIN55212, DALN or (R)-methanandamide, 40% to 70% of the control $G\beta$ and <10% of the control $G\gamma$ 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 $G\alpha_i$ subtypes. Similar to what was observed with $G\alpha_i$, $GDP\beta S$ alone did not alter the amount of $G\beta$ in association with the CB_1 receptor. However, $GDP\beta S$ could attenuate the agonist-promoted dissociation of the CB_1 receptor- $G\beta(\gamma)$ complex. This would be consistent with the receptor-G protein heterotrimer being stabilized by the

occupancy of $G\alpha_i$ with $GDP\beta\gamma$. SR141716 appeared to exert no influence on the CB_1 receptor- $G\beta\gamma$ interaction in the absence or presence of $GDP\beta\gamma$. However, SR141716 served to counter the $GTP\gamma S$ -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 G_i 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 $GTP\gamma S$ (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 $G\alpha_i$ 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 $GTP\gamma S$ 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 $GTP\gamma S$ 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 $GTP\gamma S$ 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 G_i subtypes, and promoted complete dissociation of the three $RG\alpha_i$ complexes in the presence of $GTP\gamma S$. This same behavior appeared in the presence of DALN for $G\alpha_i1$ and $G\alpha_i2$, and in the presence of the (R)-methanandamide for $G\alpha_i3$. The complete dissociation of G proteins from the CB_1 receptor evoked by DALN for G_i2 and by (R)-methanandamide for G_i3 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 $G\alpha$, would not be discernable from a functionally dissociated receptor.

Inverse agonist SR141716 maintained all three $RG\alpha_i$ complexes in the absence of GTP analogs, and exerted a very small effect on the $GTP\gamma S$ -promoted dissociation of G proteins from receptors. These results can be explained by invoking the existence of an inverse agonist (D)-supported inactive state (IR^0G_{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” G_i 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^0G_{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.

The conversion of the RG_{GDP} complex to a sustainable IR^0G_{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 $GTP\gamma S$ to drive forward the dissociation of Gi3, or Gi1 and Gi2, respectively. In previous studies (Houston and Howlett, 1998), $GTP\gamma S$ 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 $GTP\gamma S$ and Na^+ (Houston and Howlett, 1998). These findings are consistent with our current observation that in the presence of WIN55212-2, $GTP\gamma S$ was able to promote dissociation all three Gi subtypes from the CB_1 receptor, but that in the presence of DALN, $GTP\gamma S$ failed to dissociate Gi3.

An alternative mechanism might be that the inverse agonist-occupied receptors serve as guanine nucleotide exchange factors that act on $G\alpha_{iGTP\gamma S}$ to exchange GDP for $GTP\gamma S$. This mechanism is not likely because our studies indicated that $G\beta\gamma$ was dissociated from the CB_1 receptor, and there is a smaller probability that $G\alpha_{iGTP\gamma S}$ would be able to interact with the receptor in the absence of $G\beta\gamma$ (Clark *et al.*, 2001). Furthermore, the studies with $GDP\beta S$ 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 $GTP\gamma S$ -driven dissociation of $G\alpha_{iGTP\gamma S}$, suggesting that these ligands do not possess as great an inverse agonist efficacy to promote the isomerization to IR^0G_{GDP} as does DALN for Gi3 or (R)-methanandamide for Gi1 and Gi2.

Under the present assay conditions, $G\beta\gamma$ was dissociated from the CB_1 receptor in parallel with $G\alpha_i$, 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 $G\beta$ (multiple isoforms) from the CB_1 receptors. In the presence of $GTP\gamma S$, agonists promoted the dissociation of a fraction of the $G\beta$ isoforms consistent with the $AR * G \rightleftharpoons AR + G\beta\gamma + G\alpha_i_{GTP\gamma S}$ forward reaction. Protein interaction studies by others have demonstrated that $G\beta\gamma$ can interact with both R and AR in the absence of $G\alpha$ in detergent solution and reconstituted lipid vesicles (Heithier *et al.*, 1992). In surface plasmon resonance studies of immobilized rhodopsin, $G\beta\gamma$ binding was transient, but was required to facilitate binding of $G\alpha$ (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]GTP γS binding to purified $G\alpha_i$ (all subtypes) and $G\alpha_o$ proteins. Both G_i and G_o 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 G_i , but only about 70% maximal activity for G_o . An inhibition of [^{35}S]GTP γS binding by SR141716 was observed for both G_i and G_o . 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 $G\alpha_i1$ and $G\alpha_o3$ to 3.7 μM for $G\alpha_o2$. It is not easy to compare their specific findings to ours because undifferentiated N18TG2 cells do not express an appreciable amount of $G\alpha_o$, and those studies did not quantitate [^{32}P] azidoanilido-GTP incorporation into $G\alpha_i3$.

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 G α subunit under conditions that restrict reversal of the reaction. The present investigation determined receptor-G α 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 CB₁ receptor juxtamembrane C-terminal fourth loop domain was responsible for coupling to G α_o and G α_i3 , but not to G α_i1 or G α_i2 (Mukhopadhyay *et al.*, 2000; Mukhopadhyay and Howlett, 2001). In contrast, the third intracellular loop was important for interaction with G α_i1 and G α_i2 (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 G α 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 CB₁ 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_o or AR*G_o complexes readily bind GTP, and dissociation of the heterotrimer allows G α _{GTP} and G $\beta\gamma$ proteins to interact with effectors (Waelbroeck, 1999). In an experimental situation in which GTP γ S is present in high concentrations, the transiently empty G protein (R*G_o or AR*G_o) is rapidly filled by GTP γ S. 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) GTP γ S/GDP exchange and the subsequent dissociation of G α _{GTP γ 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 CB₁ 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 CB₁ agonists can compete (Meschler *et al.*, 2000), demonstrating the reversibility of this step.

Fig. 2. Coimmunoprecipitation of CB₁ receptor-G α i 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 μ g N18TG2

membrane protein (400 μ l) mixed with 100 μ l TM buffer as control; 2, Immunoprecipitate: CHAPS extract from N18TG2 membranes (400 μ l) mixed with 100 μ l sepharose-anti CB₁ antibody affinity matrix. Proteins eluted from the affinity matrix, neutralized and sedimented as described in the text; immunoprecipitated proteins were dissolved in 500 μ l TBS-T; 3, Supernatant: CHAPS extract remaining after the affinity matrix-bound protein was removed (approximately 475 μ l); 4-6, Washings 1-3: Supernatants remaining after the affinity matrix-bound protein was washed with 500 μ l TBS-T as described in the text. For each of these fractions, a 25 μ l aliquot was added to 25 μ l 2X Laemmli sample buffer containing EDTA, and 35 μ l of this mixture was loaded on the lane. Western blot analysis was carried out by costaining with both anti-CB₁ receptor antibody and anti-G α : G α i1 (upper), G α i2 (middle), or G α i3 (lower) panels. Immunoreactive bands were visualized by ECL as described in the text.

Fig. 3. Western blot analysis of the effects of CB₁ receptor agonists and GTP γ S on G α i protein association with the CB₁ receptor. CHAPS detergent extracts of N18TG2 cell membranes were incubated for 20 min at 30°C with vehicle (lanes 1, 5), 10 nM (lanes 2, 6), 100 nM (lanes 3, 7) or 1 μ M (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 μ M GTP γ S. The CB₁ receptor and associated proteins were immunoprecipitated with affinity-purified anti-CB₁(1-14) and Western blot analysis was performed as described in the text. Each blot was co-stained for both CB₁ receptor and either G α i1 (upper), G α i2 (middle), or G α i3 (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 CB₁ receptor at 64 kDa, and the higher mobility band is the G α i protein at approximately 40 kDa.

Fig. 4. Agonist-evoked dissociation of G α i subtypes from the CB₁ 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 G α i band compared with the CB₁ receptor was calculated to normalize the data and correct for potential sample loading

differences, transfer or staining variability. For each experiment, the ratio of G α i/CB $_1$ band density for the control (vehicle) was defined as “100%”, and the ratios of G α i/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 G α i. CHAPS extracts from N18TG2 cell membranes were incubated with 100 μ M GTP γ S (A.-C.) or GDP β S (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 GTP γ S 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 GDP β S is indicated by #. ND: Not Determined.

Fig. 6. Effect of SR141716 and guanine nucleotides on the CB $_1$ receptor-G α i interaction. A. CHAPS-solubilized extracts of N18TG2 cell membranes were incubated in the presence of 1 μ M SR141716, 100 μ M GTP γ S or both as indicated. B. CHAPS-solubilized extracts of N18TG2 cell membranes were incubated in the presence of 100 μ M GDP β S in the absence or presence of 10 nM, 100 nM or 1 μ M SR141716. Immunoprecipitation and Western blotting for individual G α i 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 G α i 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 GTP γ S and GTP γ S plus SR141716 are indicated by a connecting bracket.

Fig. 7. Effect of ligands and guanine nucleotides on the CB₁ receptor interaction with G β (A.) and G γ (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 μ M SR141716, 100 μ M GTP γ S, 100 μ M GDB β S, or combinations as indicated. Immunoprecipitation and Western blotting was performed with SDS-PAGE conditions modified to allow detection of low molecular weight proteins. G β and G γ proteins and the CB₁ receptor band densities were quantitated as described in the text and quantitated as a ratio compared to the CB₁ 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

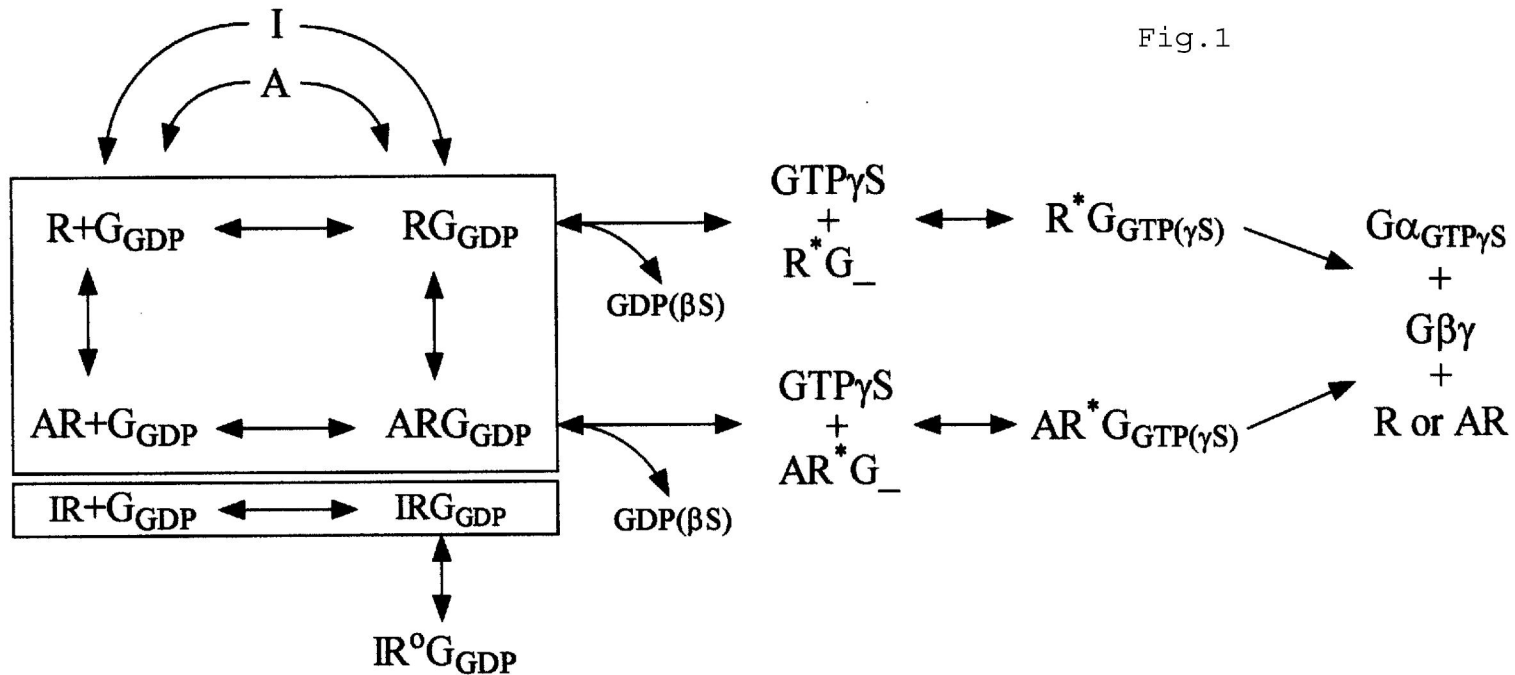
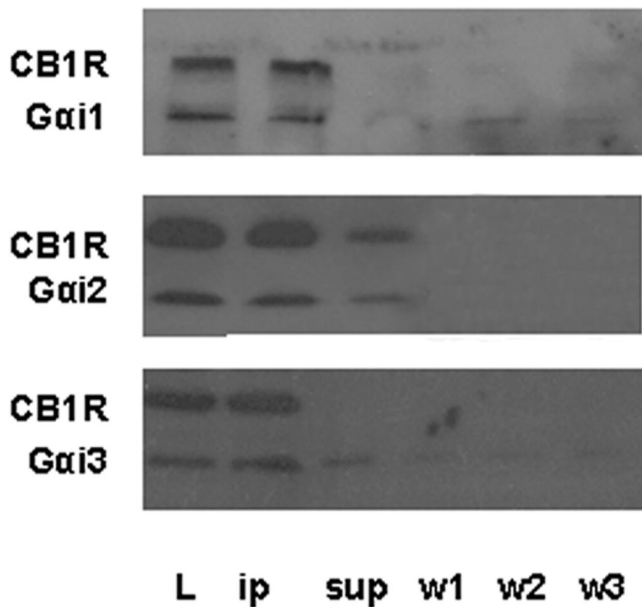


Fig.2



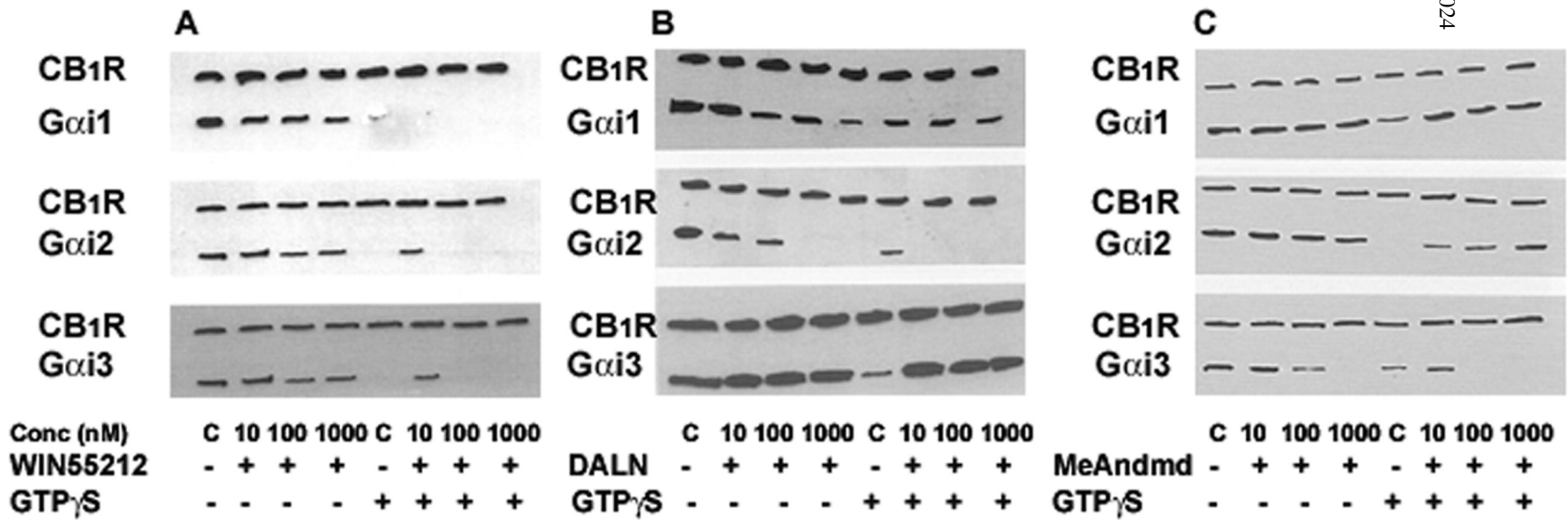


Fig.4

