G Protein-coupled Receptors and Resistance to Inhibitors of Cholinesterase-8A (Ric-8A) Both Regulate the Regulator of G Protein Signaling 14 (RGS14)-Gαi1 Complex in Live Cells*5

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Background: Regulator of G protein signaling 14 (RGS14) is a G protein regulatory (GPR) protein that participates in unconventional G protein signaling independent of G protein-coupled receptors (GPCRs).

Results: RGS14 forms regulated complexes with GPCRs in live cells.

Conclusion: RGS14 integrates unconventional and conventional GPCR-dependent G protein signaling pathways.

Significance: GPR proteins appear to be at the nexus of divergent G protein signaling pathways.

Regulator of G protein Signaling 14 (RGS14) is a multifunctional scaffolding protein that integrates both conventional and unconventional G protein signaling pathways. Like other RGS (regulator of G protein signaling) proteins, RGS14 acts as a GTPase accelerating protein to terminate conventional Gαi/o signaling. However, unlike other RGS proteins, RGS14 also contains a G protein regulatory/GoLoco motif that specifically binds Gαi1,3-GDP in cells and in vitro. The non-receptor guanine nucleotide exchange factor Ric-8A can bind and act on the RGS14-Gαi1-GDP complex to play a role in unconventional G protein signaling independent of G protein-coupled receptors (GPCRs). Here we demonstrate that RGS14 forms a Gαi1-dependent complex with a Gβ-linked GPCR and that this complex is regulated by receptor agonist and Ric-8A (resistance to inhibitors of cholinesterase-8A). Using live cell bioluminescence resonance energy transfer, we show that RGS14 functionally associates with the α2A-adrenergic receptor (α2A-AR) in a Gαi1-dependent manner. This interaction is markedly disrupted after receptor stimulation by the specific agonist UK4304, suggesting complex dissociation or rearrangement. Agonist-mediated dissociation of the RGS14-α2A-AR complex occurs in the presence of Gαi/o, but not Gαi or Gαo. Unexpectedly, RGS14 does not dissociate from Gαi in the presence of stimulated α2A-AR, suggesting preservation of RGS14-Gαi1 complexes after receptor activation. However, Ric-8A facilitates dissociation of both the RGS14-Gαi1 complex and the Gαi1-dependent RGS14-α2A-AR complex after receptor activation.

Established models of G protein signaling suggest that heterotrimeric G proteins (Gaβγ subunits) are linked to specific G protein-coupled receptors (GPCRs),3 and that these receptors act as guanine nucleotide exchange factors (GEFs) toward the Gα subunit to promote nucleotide exchange and downstream signaling events (1, 2). The regulators of G protein signaling (RGS) proteins act as GTPase accelerating proteins on the activated Gαi/o subunit, catalyzing GTP hydrolysis to terminate G protein signaling (3–5).

Recent studies have explored novel unconventional G protein signaling pathways involved with cell division and synaptic signaling/plasticity that can operate independently of GPCRs (6–13). The hallmark of these unconventional G protein pathways are signaling complexes involving Gaα-GDP bound to proteins containing one or more G protein regulatory (GPR) motifs. Resistance to inhibitors of cholinesterase 8A (Ric-8A) is a cytosolic GEF that directly promotes nucleotide exchange on Gαi, Gαo, and Gαs subunits in unconventional G protein signaling (14). Ric-8A also recognizes, binds, and regulates the formation/dissociation of some GPR-Gαi1-G protein complexes, such as AGS3-Gαi1-GDP, LGN-Gαi1-GDP, and RGS14-Gαi1-GDP (15–17).

RGS14 is a functionally and structurally complex signaling protein that is most highly expressed in the brain but also present in spleen, thymus, and lymphocytes (18–21). Within brain,
RGS14 is predominately localized in the CA2 subregion of the hippocampus, where it is involved in spatial memory, learning, and synaptic plasticity (22). The unique structure of RGS14, which includes an RGS domain, two Ras/Rap binding domains, and a GPR (also known as GoLoco (23)) motif (20, 21) suggests that RGS14 functions in the brain through a variety of signaling mechanisms that may involve both G protein and MAP kinase signaling cascades (24). In addition to possessing GTPase accelerating protein activity toward activated Go, RGS14 also exhibits selective guanine nucleotide dissociation inhibitor activity toward Goi GDP subunits. Compelling evidence also indicates that RGS proteins directly interact with G protein signaling (AGS) proteins that are characterized by binding of its GPR motif (18, 19, 21, 25–27). In this regard RGS14 shares similarities with the family of Group II activators of G protein signaling (AGS) proteins that are characterized by free GPCRs (17) as well as certain GPCR signaling pathways (34, 35). The non-receptor GEF Ric-8A regulates the RGS14-Goi complex (17) as well as certain GPCR signaling pathways (34, 35). However, it remains unknown whether Ric-8A can modulate GPCR-G protein interactions, especially in the presence of a GPCR protein such as RGS14. Therefore, we also studied the effects of Ric-8A on RGS14-Goi-GPCR complex formation and whether RGS14 may be at the interface between conventional and unconventional G protein signaling pathways. Here we report the first evidence that the RGS14-Goi GDP complex is regulated in concert by both a Goi-linked GPCR and Ric-8A in live cells. We show that RGS14 forms a stable complex with Goi via its GPR motif and that this complex is proximal to GPCRs as evidenced by the presence of specific bioluminescence resonance energy transfer (BRET) signals between RGS14 and the α2a-adrenergic receptor (α2a-AR) in the presence of Goi. This RGS14-α2a-AR complex partially dissociates/rearranges after receptor agonist treatment and is further regulated by Ric-8A. Together, these findings illustrate that RGS14 functions together in both conventional and unconventional G protein signaling and that Ric-8A may recognize and act on GPCR-Goi-GPCR complexes to further regulate Goi signaling.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Antibodies**—The rat RGS14 cDNA used in this study (GenBank™ accession number U92279) was acquired as described (19). Rat RGS14 was used as a template in PCR reactions using TaKaRa Taq (Fisher) to generate Renilla luciferase (Luc) fusion protein constructs in the phRLucN2 vector graciously provided by Dr. Michel Bouvier (University of Montreal). The following oligonucleotides and restriction enzymes were used in the PCR amplification and subsequent digestion: RGS14 forward primer 5’-GCT CTC GAG GCC ACC ATG CCA GGG AAG CCC AAC CAC-3’, XhoI; reverse primer 5’-CGC GGT ACC TGG TGG AGG ATC GTC CTC AGA ACC ACC ACC CCC ACC-3’, KpnI.

The RGS14-Luc GPR mutant, in which invariant glutamine and arginine residues (Gln15 and Arg19) were both mutated to alanine, was generated by site-directed mutagenesis using a Strategene site-directed mutagenesis kit according to the manufacturer’s instructions and is referred to as RGS14(GPR-null). Oligonucleotide primers used to create RGS14-Q515A/R516A-Luc (RGS14(GPR-null)) are as follows: RGS14(GPR-null) forward primer 5’-GGG GCC CAT GAC GCC GCC GGA TCT CTT CTT GCC AAA GAA GAA TTC AGC GCC GCC GCC GTA ACT TTC TGG CAA GC-3’ and reverse primer 5’-CGC GGT ACC TGG TGG AGC CTC CTG AGA ACC TTC TGG CAA GC-3’. RGS14-Goi-null, in which invariant glutamic acid and asparagine (Glu152 and Asn153) residues were both mutated to alanine, was generated by site-directed mutagenesis using a Strategene kit and is referred to as RGS14(GRSnull). Oligonucleotide primers used to create RGS14-E92A/N93A-Luc (RGS14(RGS-null)) are as follows: RGS14(RGS-null) forward primer 5’-TTT CTT CGC AAA G-3’ and reverse primer 5’-TTT CTT GCC AAA GAA AAG CCC AAG CAC-3’. The RGS14-Luc RGS domain mutant, in which invariant glutamic acid and asparagine (Glu152 and Asn153) residues were both mutated to alanine, was generated by site-directed mutagenesis using a Strategene kit and is referred to as RGS14(GRS-null). Oligonucleotide primers used to create RGS14-E92A/N93A-Luc (RGS14(RGS-null)) are as follows: RGS14(RGS-null) forward primer 5’-GGG GCC CAT GAC GCC GCC GGA TCT CTT CTT GCC AAA GAA GAA TTC AGC GCC GCC GCC GTA ACT TTC TGG CAA GC-3’ and reverse primer 5’-CGC GGT ACC TGG TGG AGC CTC CTG AGA ACC TTC TGG CAA GC-3’.

**Experimental Procedures**

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Anti-sera used include anti-Gα₁₁ (Millipore and Santa Cruz Biotechnologies, Inc.), anti-Gα₂ (Abcam), anti-Gα₁₃ and anti-Gαq (gifts from Dr. Thomas Gettys at Pennington Biomedical Research Center, Baton Rouge, LA), anti-FLAG (Sigma), anti-Ric-8A (provided by Dr. Gregory Tall, University of Rochester School of Medicine and Dentistry), anti-Gαq (Santa Cruz Biotechnologies, Inc.), anti-Gαq (Santa Cruz Biotechnologies, Inc.), Alexa 546 goat anti-rabbit secondary IgG (Invitrogen), Alexa 633 goat anti-mouse secondary IgG (Invitrogen), peroxidase-conjugated goat anti-mouse IgG (Rockland Immunochemicals, Inc.), and peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad).

Cell Culture and Transfection—HEK293 cells were maintained in Dulbecco’s minimal essential medium (without phenol red) containing 10% fetal bovine serum (5% after transfection), 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were incubated at 37 °C with 5% CO₂ in a humidified environment. Transfections were performed using previously described protocols with polyethyleneimine (PolySciences, Inc.) (32). For immunofluorescence, cells were seeded onto glass coverslips before transfection.

BRET—BRET experiments were performed as previously described (31, 32). Briefly, HEK293 cells were transiently transfected with BRET donor and acceptor plasmids using polyethyleneimine. Forty-eight hours after transfection, the culture medium was removed, and cells were washed once with PBS and harvested with Tyrode’s solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.37 mM Na₂HPO₄, 24 mM NaHCO₃, 10 mM HEPES, and 0.1% glucose (w/v), pH 7.4). Each group of cells was distributed into gray 96-well OptiPlates (PerkinElmer Life Sciences) in triplicate, with each well containing 1 × 10⁶ cells. The acceptor (YFP/Venus-tagged) protein expression levels were evaluated by measuring total fluorescence using the TriStar LB 941 plate reader (Berthold Technologies) with excitation and emission filters at 485 and 535 nm, respectively. Data were analyzed using the MikroWin 2000 program. After fluorescence measurement, coelenterazine H (Nanolight Technologies; 5 μM final concentration) was added and luminescence-detected in the 480 and 530 ± 20 nm windows for donor (Luc) and acceptor (YFP/Venus), respectively, by the TriStar LB 941 plate reader. BRET signals were determined by calculating the ratio of the light intensity emitted by the YFP/Venus divided by the light intensity emitted by Luc. Net BRET values were corrected by subtracting the background BRET signal detected from the expression of the donor fusion protein (Luc) alone. Agonists used were UK14304 (Sigma) and isoproterenol (Sigma). Immunoblots were performed as described previously (39).

Immunofluorescence and Confocal Imaging—Transfected HEK293 cells were treated with either vehicle or 10 μM UK14304 diluted in serum-free DMEM for 5 min at 37 °C. Cells were then fixed at room temperature for 15 min in buffer containing 3.7% paraformaldehyde diluted in PBS. Cells were washed in PBS and incubated for 8 min with 0.4% Triton X-100 diluted in PBS. Cells were then blocked for 1 h at room temperature in PBS containing 10% goat serum and 3% bovine serum albumin. Next, cells were incubated in this same buffer with a 1:1000 dilution of rabbit anti-FLAG and/or mouse anti-Gα₁₁ antibodies at room temperature for 2 h. Cells were washed with PBS (3 ×) and incubated with 1:300 dilutions of Alexa 546 goat anti-rabbit and/or Alexa 633 goat anti-mouse secondary antibodies at room temperature for 1 h. Cells were washed with PBS again (3 ×) and mounted with ProLong Gold Antifade Reagent (Invitrogen). Confocal images were taken using a 63 × oil immersion objective from a LSM510 laser scanning microscope with AxioObserver Stand (Zeiss). Images were processed using the ZEN 2009 Light Edition software and Adobe Photoshop 7.0 (Adobe Systems).

RESULTS

RGS14 Interacts Selectively with Gα₁₁ through Its GPR Motif—RGS14 has two distinct Gα-binding domains. The RGS domain binds active Gα₁₀ subunits (18, 19, 21), whereas the GPR motif binds inactive Gα₁₁ and Gα₁₃ (19, 26, 27, 40). That RGS14 is recruited from the cytosol to the plasma membrane and colocalizes with wild-type Gα₁₁ (Fig. 1A, supplemental Fig. S1, and Refs. 17 and 27) suggests that RGS14 forms a stable complex with Gα₁₁ at the plasma membrane, which we sought to quantitatively measure using BRET. We therefore measured the strength and selectivity of a BRET signal between RGS14-Luc and various YFP-tagged Gα subunits (36, 41–43) (Fig. 1B). Of note, the YFP tag was inserted into the loop joining the αβ and αC helices of each Gα (36, 41, 43), preserving nucleotide binding and hydrolysis properties similar to the wild-type protein (36). Transfection of HEK cells with increasing amounts of Gα-YFP plasmid and a fixed amount (5 ng) of RGS14-Luc plasmid showed a robust, saturable BRET signal in the presence of Gα₁₁-YFP, whereas no BRET signal was observed between RGS14-Luc paired with either Gα₁₀-YFP or Gα₁₃-YFP (Fig. 1B). This BRET signal saturation is indicative of a specific interaction between RGS14 and Gα₁₁ (44).

To further show BRET signal selectivity for RGS14-Luc interactions with Gα₁₁-YFP, we performed a competition assay in cells co-expressing untagged Gα subunits (Fig. 1C) to determine which Gα subunits could displace Gα₁₁-YFP from RGS14-Luc and disrupt the BRET signal. As expected, the previously reported RGS14 binding partners Gα₁₂ and Gα₁₃ each disrupted the RGS14/Gα₁₁ BRET signal, indicative of competition with Gα₁₁-YFP for RGS14 binding. By contrast, Gα₁₂, Gα₁₃, and Gα₁₅ did not disrupt Gα₁₁-YFP binding to RGS14. This selectivity for Gα₁₁ and Gα₁₃ binding is entirely consistent with earlier reports showing RGS14 binding to only Gα₁₃ and Gα₁₅ but not other Gα through its GPR motif, further validating our BRET system (18, 19, 21, 26, 27, 40).

Findings in Fig. 1 suggested that the BRET signal we observed between RGS14 and Gα₁₁ occurs via the GPR motif. To test this hypothesis, we constructed mutants of RGS14-Luc that rendered it insensitive to binding Gα₁₁-YFP through either the RGS domain (RGS14-E92A/N93A-Luc; RGS-null) (18), the GPR motif (RGS14-Q515A/R516A-Luc; GPR-null) (25, 45), or both (RGS14-E92A/N93A/Q515A/R516A-Luc; RGS/GPR-null) (Fig. 2, A and B). The BRET signal between wild-type RGS14 (WT) and Gα₁₁ was comparable with that between RGS14(RGS-null) and Gα₁₁, suggesting that the majority of the observed BRET signal was not due to the RGS domain interacting with Gα₁₁. However, the BRET signal between RGS14(GPR-
null) and Gαi1 was ~5-fold lower than that of the RGS14-WT/Gαi1 pair. This indicates that the observed BRET signal between RGS14 and Gαi1 is primarily due to the GPR motif. As an additional approach, we generated Gαi1-YFP mutants that were insensitive to binding either the RGS domain (Gαi1-G183S-YFP; RGSi) (46), the GPR motif (Gαi1-N149I-YFP; GPRi) (47, 48), or both (Gαi1-G183S/N149I-YFP; RGSi/GPRi) (Fig. 2 C). Consistent with findings in Fig. 2B, the BRET signal between RGS14 and Gαi1-GPRi was substantially (~8-fold) lower than that generated by RGS14 paired with either wild-type Gαi1 (WT) or Gαi1-RGSi. Taken together, these findings are entirely consistent with the idea that the majority of the BRET signals observed between RGS14 and Gαi1 are due to the interaction between the RGS14 GPR motif and Gαi1.

RGS14 Forms a Complex with the Gαi/o-dependent Manner—The GPR proteins AGS3 and AGS4 form Gαi-dependent complexes with GPCRs that are regulated by receptor activation (31, 32). Therefore, we sought to investigate whether the RGS14/Gαi1 complex can also be regulated by GPCRs in cells. Subcellular localization data showed that although RGS14 remained predominately cytosolic in the presence of co-expressed Gαi/o, it was recruited to the plasma membrane in the presence of both overexpressed Gαi/o and Gαi1 in the absence of agonist (Fig. 3, left panel). Although RGS14 and Gαi1 remained at the plasma membrane, the Gαi/o internalized in the presence of agonist UK14304 (Fig. 3, right panel).

To further examine the regulatory effects of GPCRs on RGS14-Gαi1 complexes, we analyzed the BRET signals between RGS14-Luc and Venus-tagged α2A-AR or α2C-AR (Fig. 4). As expected, little to no detectable BRET signal was observed between RGS14 and the Gs-linked β2-AR in the absence or presence of both Gαi1 and the receptor agonist isoproterenol.
Very low specific BRET signals were observed between RGS14 and α2A-AR both in the absence and presence of receptor agonist UK14304 (Fig. 4B, filled circles and open circles, respectively). However, a 3-fold increase in BRET signal was observed between α2A-AR and RGS14 in the presence of co-expressed Gαi1 (Fig. 4B, filled triangles). This signal was reduced by ~50% in the presence of UK14304 (Fig. 4B, open triangles). This agonist-induced reduction in BRET correlates with the lack of co-localization between RGS14 and the α2A-AR after agonist stimulation (Fig. 3, right panel). Furthermore, ago-
Specific BRET signals were observed between RGS14 and the $\alpha_{2A}$-AR. Measurements were taken after treatment with either vehicle or isoproterenol (100 nM) for 5 min. A schematic representing the BRET principle used in all experiments of Fig. 4, which includes BRET measured between RGS14-Luc and a GPCR-Venus (Ven) in the presence or absence of untagged $G_o$, is shown within the graph. The very low BRET signals observed in the presence of all four $G_o$ subunits used in samples with transfected $G_{i/o}$ family members (Fig. 4C). BRET signals measured between RGS14-Luc and $\alpha_{2A}$-AR-Venus under each condition is shown. Data are expressed as the mean of four separate experiments with triplicate determinations. Bottom panel, shown is a representative immunoblot for $G_o$ subunits used. Data are expressed as the mean of three separate experiments with triplicate determinations. Right panel, shown is a representative immunoblot of the different $G_o$ subunits used. Data are expressed as the mean of two separate experiments with triplicate determinations. BRET signals measured between RGS14-Luc and $\alpha_{2A}$-AR-Venus pair are shown for HEK cells transfected with 5 ng of RGS14-Luc, 25 ng of $\alpha_{2A}$-AR-Venus, and 750 ng of $G_{i1}$ plasmids. Measurements were taken after treatment with vehicle or 10 nM UK14304 (10 µM) for 5 min. A schematic representing the BRET principle used in all experiments of Fig. 4, which includes BRET measured between RGS14-Luc and a GPCR-Venus (Ven) in the presence or absence of untagged $G_o$, is shown within the graph. Measurements were taken after treatment with either vehicle or $\alpha_{2A}$-AR agonist UK14304 (10 µM) for 5 min. A schematic representing the BRET principle used in all experiments of Fig. 4, which includes BRET measured between RGS14-Luc and a GPCR-Venus (Ven) in the presence or absence of untagged $G_o$, is shown within the graph. The very low BRET signals observed in the presence of all four $G_o$ subunits used in samples with transfected $G_{i/o}$ family members (Fig. 4C). BRET signals measured between RGS14-Luc and $\alpha_{2A}$-AR-Venus under each condition is shown. Data are expressed as the mean of four separate experiments with triplicate determinations. Bottom panel, shown is a representative immunoblot for $G_o$ subunits used. Data are expressed as the mean of three separate experiments with triplicate determinations. Right panel, shown is a representative immunoblot of the different $G_o$ subunits used. Data are expressed as the mean of two separate experiments with triplicate determinations. BRET signals measured between RGS14-Luc and $\alpha_{2A}$-AR-Venus pair are shown for HEK cells transfected with 5 ng of RGS14-Luc, 25 ng of $\alpha_{2A}$-AR-Venus, and 750 ng of $G_{i1}$ plasmids. Measurements were taken after treatment with vehicle or 10 nM UK14304 (10 µM) for 5 min. A schematic representing the BRET principle used in all experiments of Fig. 4, which includes BRET measured between RGS14-Luc and a GPCR-Venus (Ven) in the presence or absence of untagged $G_o$, is shown within the graph. Measurements were taken after treatment with either vehicle or $\alpha_{2A}$-AR agonist UK14304 (10 µM) for 5 min. To determine which domains of RGS14 are important for associating with the $\alpha_{2A}$-AR, we performed BRET experiments using the RGS14 constructs with mutations in the RGS domain and GPR motif as described in Fig. 2B (Fig. 4D). BRET signals observed between either RGS14-WT or RGS14-(RGS-null) and the $\alpha_{2A}$-AR in the presence of co-expressed $G_{i1}$ were compa-
rable, with similar reductions in response to receptor agonist UK14304. This suggests that the RGS domain of RGS14 is not required for the formation of the Gαi1-dependent complex with the α2A-AR. In contrast, the BRET signals observed between the α2A-AR and RGS14(GPR-null) in the presence of Gαi1 were reduced by ~50% in the absence of agonist compared with RGS14-WT, indicating that the GPR motif is critical to forming a complex with the α2A-AR in the presence of Gαi1. Together, these results indicate that RGS14 forms a complex with the α2A-AR in the presence of a Gαi1 protein and that the GPR motif is critical to promoting the formation of this complex (see supplemental Fig. S2A).

The RGS14-Gαi1 Complex Remains Intact after α2A-AR Stimulation—Because we observed Ric-8A regulation of RGS14-Gαi1 complexes in vitro (17), we sought to quantitatively measure Ric-8A-mediated dissociation of RGS14-Gαi1 complexes in live cells using BRET (Fig. 6A). As expected (17), Ric-8A protein levels induced a decrease in BRET between RGS14-Luc and Gαi1-YFP (Fig. 6C). Ric-8A-induced reductions in RGS14/Gαi1 BRET were inhibited by pertussis toxin (+PTX) (Fig. 6C), which blocks Ric-8A binding and GEF activity toward Gαi subunits (49). Expression of Ric-8A also induces an increase in Gαi1-YFP protein expression levels (Fig. 6B), which is consistent with recent evidence showing that Ric-8A is important for the functional expression and stability of Gαi subunits (50). Interestingly, the effect of Ric-8A on Gαi1-YFP expression levels was not blocked by pertussis toxin pretreatment, suggesting that the effect of Ric-8A on Gαi expression is independent of its GEF activity.

We next studied the effects of Ric-8A on RGS14-Gαi1 complexes in the presence of the α2A-AR (Fig. 7A). In the absence of Ric-8A, RGS14-Gαi1 complexes remained intact after receptor stimulation as before (see Fig. 5A). In the absence of receptor agonist, Ric-8A promoted a decrease in the RGS14/Gαi1 BRET signal. In the presence of agonist, Ric-8A induced an even greater decrease in the BRET signal (Fig. 7A). These findings suggest that Ric-8A can recognize and act on RGS14-Gαi1 complexes in the presence of GPCRs and even more so in the presence of activated receptors.

Ric-8A Potentiates Dissociation of the RGS14-Gαi1 Complex Caused by Receptor Agonist—Because Ric-8A induced dissociation of Gαi1 from RGS14 in the presence of the α2A-AR, we next investigated the effect of Ric-8A on the RGS14-α2A-AR complex in the presence of Gαi1 (Fig. 7B). Ric-8A had little
effect on the RGS14-AR complex in the presence of co-expressed Goi in the absence of agonist. However, BRET signals between RGS14 and the AR in the presence of Goi and receptor agonist were further reduced by ~25% in the presence of Ric-8A (red lines) compared with the absence of Ric-8A (black lines) (Fig. 7B). These findings suggest that Ric-8A acts to facilitate dissociation of RGS14 from activated α2A-AR in the presence of Goi (see supplemental Fig. S2C).

**DISCUSSION**

RGS14 is unusual among RGS protein family members in that it possesses two distinct Go binding domains; that is, an
RGS domain that accelerates GTP hydrolysis on activated Gαi/o subunits (18, 19, 21) and a GPR motif that forms a tight complex with inactive Gαi/o subunits (17, 19, 25–27). RGS14 also belongs to a second family of signaling proteins, the Group II AGS proteins, which are characterized by the presence of one or more GPR motifs that mediate newly appreciated “ unconventional” G protein signaling events (28, 29). Recent studies of AGS3 and AGS4 demonstrate that these GPR-domain-containing proteins interact with Gαi/o to form complexes with Gαi/o–linked GPCRs in cells (31, 32). Our results with RGS14 support those findings but also highlight some important differences that will be discussed. Overall, our findings indicate the following: 1) RGS14 selectively interacts with Gαi1/3 in live cells through its GPR motif, 2) RGS14 forms a Gαi1/3–dependent complex with the Gαi/o–linked α2AR in live cells, 3) RGS14 dissociates from the α2AR after agonist treatment but remains bound to Gαi1/3, 4) Ric-8A potentiates agonist-stimulated dissociation of the RGS14–α2AR–G protein complex, and 5) Ric-8A induces dissociation of Gαq and αqαAR from RGS14, having a greater effect in the presence of stimulated α2AR. Taken together, these findings suggest that RGS14 integrates both unconventional Ric-8A/G protein signaling and conventional GPCR/G protein signaling. A summary and interpretation of these findings is shown in Fig. 8.

RGS14 Selectively Interacts with Inactive Gαi1/3 in Live Cells through Its GPR Motif—Our BRET analysis and confocal imaging indicate that the interaction of RGS14 with inactive Gαi1/3 occurs at the plasma membrane of live cells (Fig. 1 and supplemental Fig S1). Consistent with previous studies (18, 19, 26, 27, 40), the capacity of both Gαi1 and Gαq (but not Gαo2, Gαo, or Gαoq) to disrupt the BRET between RGS14–Luc and Gαi1–YFP indicates that the observed BRET signal is specific for interactions between RGS14 and Gαi1/3 (Fig. 1C).

To clarify which RGS14 domains are involved in the RGS14–Gαi1 interaction, we measured the BRET signal between mutant forms of RGS14–Luc and Gαi1–YFP that specifically blocked RGS and/or GPR motif functions (Fig. 2). These studies show that the majority of the observed RGS14–Gαi1 interaction is conferred by the GPR motif of RGS14 interacting with Gαi1. The fact that the BRET signal was never completely abolished in the presence of the RGS14 and Gαi1 double mutants that ablate Gαi binding to both the GPR and RGS domains (Fig. 2, B and C) is consistent with the existence of a third G protein binding site on RGS14, as has been postulated (51).

RGS14 Selectively Interacts with the α2AR Receptor in a Gαi/o–dependent Manner—Because RGS14 interacts with Gαi/o family members, we examined whether RGS14 can be regulated by a Gαi/o-linked GPCR, specifically the α2AR. RGS14, Gαi1, and the α2AR co-localized at the plasma membrane when all three proteins were expressed together in cells (Fig. 3, left panel), consistent with the possibility that a ternary protein complex forms at the plasma membrane. After treatment with the α2AR agonist UK14304, RGS14 and Gαi1 remained at the plasma membrane, whereas the α2AR partially internalized (Fig. 3, right panel), suggesting that the ternary complex dissociates. This hypothesis was supported in our BRET experiments. Co-expression of Gαi1 resulted in an approximate 3-fold increase in RGS14–α2AR–G protein BRET compared with RGS14 and α2AR alone (Fig. 4B). The Gαi1–dependent RGS14–α2AR–G protein BRET signal was reduced ~50% after receptor activation by agonist, and this agonist effect was blocked by pertussis toxin pretreatment (Fig. 4B, right panel). This implies that functional coupling of the α2AR to Gαi1 disrupts the RGS14–α2AR–G protein complex. It is possible that the interacting sites between GPCR–Gαi/o are different between the inactive and active states, the latter being sensitive to PTX. This is suggested by previous work on the phenomenon of guanine nucleotide-sensitive agonist binding to GPCRs and more recent work demonstrating preformed complexes of GPCRs and G proteins (52, 53).

As expected, RGS14 interaction with the α2AR is dependent on the presence of Gαi/o, as Gαo2 and Gαo failed to elicit a robust RGS14–α2AR–G protein BRET signal. Somewhat unexpectedly, RGS14–α2AR association is promoted indiscriminately by the presence of any Gαi/o family member (Gαi1, Gαi2, Gαi3, and Gαo) (Fig. 4C). This is surprising given that the RGS14–α2AR–G protein interaction was highly dependent on the GPR motif (Fig. 4D), which only interacts with Gαi1 and Gαi3 in the absence of receptor. One possible explanation may be that RGS14 recognizes a receptor if the receptor is bound to any Gαi/o protein, reflecting the promiscuity of RGS14 GTPase accelerating protein activity toward activated Gαi/o subunits. In this regard, RGS14 is similar to RGS2. In the absence of receptor, RGS2 acts specifically on Gαq (54). However, RGS2 is capable of interacting with Gαi/o in the presence of a Gαi/o–linked GPCR (55), albeit with 30-fold lower affinity than for Gαq (56). We note that
RGS14 complexes with receptor are dependent on both the G protein and the receptor because the Gs-linked β2-AR failed to interact with RGS14 in the presence of Ga11 (Fig. 4A).

The GPR motif interaction with Ga11 is important in promoting formation of the RGS14α2a-AR complex (Fig. 4D). The RGS14/α2a-AR BRET signal was greatly reduced in the presence of RGS14(GPR-null) compared with RGS14-WT, indicating that Ga11 has a reduced capacity to bring RGS14 and the α2a-AR in close proximity when it cannot bind the GPR motif. Even when Ga11 could no longer bind either the GRS domain or GPR motif, there was still a slight BRET signal between RGS14(RGS/GPR-null) and the α2a-AR. Several possibilities exist to explain these results; 1) there may be another (unde-}

ric-8A Is a Key Regulator of the GPCR-Ga11 Complex—Although RGS14 dissociated from the α2a-AR after agonist treatment in the presence of co-expressed Ga11 (Fig. 4), it remained in complex with Ga11 via the GPR motif (Fig. 5). This finding is unexpected and differs from previous observations that show AGS3 and AGS4 dissociating from Ga11 after receptor activation (Fig. 5A and Refs. 31 and 32). Our result suggests that RGS14 and Ga11 remain bound after receptor activation. This result is reminiscent of other findings showing that, in contrast to established models of G protein signaling (1), Gβγ may not necessarily always dissociate from Ga. In some cases Gβγ may rearrange relative to GaαGTP after receptor activation (53), although in others Gβγ does appear to dissociate (Refs. 57–59 and references therein). Irrespective of the mechanism involved, our findings represent a novel mechanism of action for GPCR-Gαi/RGS complexes, where the active conformation of the α2a-AR favors release of an RGS14-Ga11 complex that may then be able to function as a signaling complex on its own or with other binding partners (such as potential MAP kinase signaling partners (24)). This complex may be regulated and function independently of the GPCR.

Ric-8A Is a Key Regulator of the GPCR-Ga11 Complex—Although Ric-8A has been shown to influence GPCR signaling (34, 35, 60), little is known mechanistically about if or how Ric-8A may directly interact with and regulate GPCR-G protein complexes. We recently demonstrated that Ric-8A induces dissociation of RGS14 from Ga11 in vitro (17). In this study we sought to quantitatively measure the dissociative effects of Ric-8A on RGS14-Ga11 complexes in live cells using BRET (Fig. 6). Pertussis toxin blocked Ric-8A-mediated dissociation of the RGS14-Ga11 complex (Fig. 6, C and D), consistent with recent reports showing that pertussis toxin inhibits Ric-8A GEF activity on Ga11, and that Ric-8A binds to Ga11 at a region overlapping with the pertussis toxin binding site (17, 49). In the absence of pertussis toxin, Ric-8A facilitated RGS14-Ga11 complex dissociation (Fig. 6, C and D). Ric-8A also induced dissociation of the RGS14-Ga11 complex in the presence of the α2a-AR, even in the absence of α2a-AR stimulation (Fig. 7A). This may be explained by Ric-8A effects on Ga11 expression levels. Because Ric-8A overexpression also induced an increase in Ga11 expression (Fig. 6B), it may be that there is an overabundance of Ga11 that is free to bind RGS14. The number of RGS14-Ga11 complexes may, therefore, outnumber the number of α2a-ARs, resulting in free RGS14-Ga11 complexes on which Ric-8A may act in the absence of receptor activation.

Ric-8A did not induce dissociation of the RGS14α2a-AR complex in the absence of receptor stimulation (Fig. 7B). This is in contrast to its effects on the RGS14-Ga11 complex in the presence of unstimulated receptor. It is possible that Ric-8A facilitates dissociation of RGS14-Ga11 complexes that are not associated with receptors, accounting for the decrease in RGS14-Ga11 BRET seen in the presence of unstimulated receptor (Fig. 7A). In a cellular signaling context, Ric-8A may function similarly to the Arr4 protein in yeast that serves a feed-forward facilitating role in pheromone receptor-G protein signaling mating responses (61). Consistent with this idea is that Ric-8A potentiates taste-receptor signaling by a potential feed-forward mechanism (34).

Taken together, these studies show that RGS14 can associate with a GPCR-Ga11 complex in a regulated fashion and that Ric-8A is a regulatory partner in this process. Although Ric-8A potentiated dissociation of RGS14-Ga11 complexes from the α2a-AR in both the absence and presence of receptor stimulation, it had no effect on dissociating the RGS14α2a-AR complex itself in the absence of stimulation. We postulate that two pools of RGS14-Ga11 complexes may exist (Fig. 8). One subset resides at membranes (plasma and others?) in the absence of a GPCR, and the other directly complexes to a cell surface receptor. Ric-8A acts differently on the RGS14-Ga11 complex depending on whether or not the complex is coupled to a GPCR. In the absence of a GPCR (Fig. 8, bottom), Ric-8A can recognize and induce dissociation of the RGS14-Ga11 complex. When the RGS14-Ga11 complex is associated with a GPCR (Fig. 8, top), Ric-8A may not affect RGS14-Ga11 complexes unless the receptor is activated. In this case Ric-8A induces dissociation of Ga11 from RGS14 and subsequently RGS14 from receptor.

Our findings demonstrate that RGS14 functions in a unique mechanism to integrate both conventional GPCR-G protein signaling and unconventional GPCR-independent G protein signaling. These results highlight newly appreciated roles of GPR proteins at the interface of G protein signaling pathways, making them significant targets in the study of non-canonical G protein regulation and function.

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