Receptor-regulated Interaction of Activator of G-protein Signaling-4 and Goαi

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Activator of G-protein signaling-4 (AGS4), via its three G-protein regulatory motifs, is well positioned to modulate G-protein signal processing by virtue of its ability to bind Goαi-GDP subunits free of Gβγ. Apart from initial observations on the biochemical activity of the G-protein regulatory motifs of AGS4, very little is known about the nature of the AGS4-G-protein interaction, how this interaction is regulated, or where the interaction takes place. As an initial approach to these questions, we evaluated the interaction of AGS4 with Goαi, in living cells using bioluminescence resonance energy transfer (BRET). AGS4 and Goαi, reciprocally tagged with either Renilla luciferase (RLuc) or yellow fluorescent protein (YFP) demonstrated saturable, specific BRET signals. BRET signals observed between AGS4-RLuc and Goαi-YFP were reduced by G-protein-coupled receptor activation, and this agonist-induced reduction in BRET was blocked by pertussis toxin. In addition, specific BRET signals were observed for AGS4-RLuc and α2-adrenergic receptor-Venus, which were Goαi-dependent and reduced by agonist, indicating that AGS4-Goαi complexes are receptor-proximal. These data suggest that AGS4-Goαi complexes directly couple to a G-protein-coupled receptor and may serve as substrates for agonist-induced G-protein activation.

Activators of G-protein signaling (AGS)3 proteins were identified using a yeast-based functional screen of mammalian cDNA libraries for cDNAs that activated G-protein signaling in the absence of a GPCR (1–4). Group II AGS proteins all contain at least one G-protein regulatory (GPR) motif (3, 5) (also termed the GoLoco motif (6)), a 20–25-amino acid motif that contains and competes with Gβγ for Goαi binding (reviewed in Ref. 5). Proteins with multiple GPR motifs can bind to multiple Goαi subunits simultaneously, which presents a unique opportunity to act as a scaffold to organize a signaling complex (7, 8).

Functional studies indicate crucial roles for GPR proteins beginning with the original observations in model organisms describing a role for GPR proteins and their interaction with G-proteins in an asymmetric cell division (5, 9). Additional functional studies with GPR proteins indicate further functional diversity with roles observed in blood pressure control, fat deposition and energy expenditure, neuronal outgrowth, drug addiction and relapse behavior, autophagy, G-protein-coupled inwardly rectifying potassium channel regulation, and transport of membrane proteins to the cell surface (10–18). These observations indicate crucial functionality of the GPR motif in biological systems and implicate Goαi-GPR complexes in the regulation of G-protein signaling in unexpected, albeit poorly understood ways. In the context of the group II AGS proteins, which contain multiple GPR motifs, many outstanding questions remain to be addressed. Chief among them is what regulates the formation and disassembly of GPR-Goαi complexes? Is their interaction with G-protein influenced by GPCR activation or other signals?

AGS4 was identified in the yeast based functional screen for receptor-independent G-protein activators from a human prostate leiomyosarcoma cDNA library (1), and apart from initial reports describing its interaction with Goαi subunits from cell lysates and purified proteins in vitro, little is known regarding its interaction with Goαi in the intact cell or how this interaction is regulated. Although AGS3 and AGS5/Leu-Gly-Asn repeat-enriched protein (LGN) both contain seven tetra-tripeptide repeats upstream of their four GPR motifs and are broadly expressed in many tissues, AGS4 has a much different domain organization with a proline-rich N terminus followed by a proline repeat-enriched protein (LGN) both contain seven tetra-tripeptide repeats upstream of their four GPR motifs and are broadly expressed in many tissues, AGS4 has a much different domain organization with a proline-rich N terminus followed by a proline repeat-enriched protein.
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EXPERIMENTAL PROCEDURES

Materials—Polyethyleneimine (PEI) (25 kDa molecular mass, linear form), was obtained from Polysciences, Inc. Benzoyl-co-elongerzine was obtained from NanoLight Technology (Pinetop, AZ). UK14304, rauwolscine HCl, and pertussis toxin were purchased from Sigma. Materials for cell culture were purchased from Invitrogen. Gray 96-well Optitlases were obtained from PerkinElmer Life Sciences. Anti-Gαi1 and anti-

Renilla luciferase were purchased from Millipore (Billerica, MA). Anti-Gαi3 and Gαi5 were kindly provided by Dr. Thomas Gettys (Pennington Biomedical Research Center, Baton Rouge, LA). AGS4 GPR mutants, Gαi1-YFP-G202T, Gαi1-YFP-Q204L, and Gαi5-YFP-N149I were generated by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA). Gαi3, Gαi3-G202T, and Gαi3-Q204L CDNAs were obtained from the cDNA Resource Center (University of Missouri, Columbia, MO). pcDNA3.1-Gαi3-YFP was generated by Dr. Gibson (20) and provided by Gregory G. Tall (University of Rochester School of Medicine and Dentistry). Construction of expression vectors for α2a-AR, β2-AR, and μ-opioid receptor (MOR) were previously described (21–23). MOR-YFP plasmid was kindly provided by Dr. Lakshmi A. Devi (Mount Sinai Medical Center, New York, NY). All other reagents and materials were obtained as described elsewhere (22, 24).

Cell Culture and Transfection—HEK-293 cells were maintained in Dulbecco’s minimal essential medium (high glucose, without phenol red) containing 5% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were grown in the presence of 5% CO2 at 37 °C in a humidified incubator. For transfection, 8 x 105 cells/well were seeded on 6-well plates and cultured overnight at 37 °C. BRET donor and acceptor plasmids were diluted for transfection with PEI (1 mg/ml in distilled H2O) at a DNA:PEI ratio of 1:4. PEI and plasmid DNA were diluted in separate tubes with 100 μl of serum-free medium. DNA and PEI solutions were vortexed at maximum speed for 3–5 s and incubated for 15 min at room temperature prior to addition to the cells. Cells were incubated for 48 h prior to collection for experiments. Cell lysates and immunoblotting were performed as described previously (24).

BRET—Initial experiments were performed to optimize the BRET system for AGS4-Gαi3 interactions and to ensure the specificity of observed signals. All studies involved saturation BRET analysis, altering donor/acceptor ratios and/or time course analysis. Forty-eight hours after transfection, the cells were washed once with phosphate-buffered saline and harvested with Tyrod’s solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 0.37 mM NaH2PO4, 24 mM NaHCO3, 10 mM HEPES, pH 7.4, and 0.1% glucose (w/v)). Cells were distributed in triplicate at 1 x 105 cells/well into gray 96-well plates. Fluorescence and luminescence signals were measured with a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). BRET saturation curves and statistical analyses were measured using GraphPad Prism (GraphPad Software, San Diego, CA). Data were analyzed by analysis of variance with significant differences between groups determined by Tukey’s post-hoc test.

RESULTS AND DISCUSSION

Key questions in the field and for AGS4 in particular are what regulates the formation and disassembly of AGS4-Gαi complexes and is the AGS4-G-protein interaction influenced by GPCR activation or other signals? As an initial approach to address these questions, we used BRET with contingent binding proteins tagged with RLuc or yellow fluorescent protein (YFP). The enzymatic oxidation of luciferase substrates such as coelenterazine and subsequent non-radiative emission can excite YFP if the two proteins are in close proximity (< 100 Å). The strength of the BRET signal for two proteins fused with RLuc and YFP, respectively, depends upon distance between the fluorophores, their relative orientations, and the relative expression levels of donor (RLuc) and acceptor (YFP). Specific interactions exhibit saturation of BRET signals using a constant amount of the luciferase donor and increasing amounts of the YFP acceptor (25). AGS4 was tagged with either YFP or RLuc, and YFP or RLuc was inserted into the loop connecting helices αB and αC of the helical domain in Gαi1, which confers nucleotide binding and hydrolysis properties similar to the untagged protein (20, 22).

Analysis of different AGS4 and Gαi BRET donor/acceptor pair combinations revealed that the AGS4-RLuc-Gαi1-YFP (YFP at position 122) BRET pair yielded the strongest BRET signal and the highest signal-to-noise ratio (data not shown). The higher BRET signals detected by C-terminal tagged AGS4-RLuc and Gαi1-YFP likely reflect the ability of the donor AGS4-RLuc to simultaneously bind more than one acceptor Gαi1-YFP due to the presence of three GPR motifs in AGS4. Robust, specific, and saturable BRET signals were observed between AGS4-RLuc and Gαi1-YFP (Fig. 1, A and B). BRET signals were not observed in cells expressing AGS4-Q/A, which contains Gln-Ala mutations in each of the three GPR motifs in AGS4, rendering it unable to bind Gαi (26) (Fig. 1, A and B), thus confirming the specificity of the BRET signal. AGS4-RLuc-Gαi1-YFP BRET signals were also decreased following co-expression of Gβγ subunits, consistent with the ability of Gβγ subunits to compete with GPR motifs for Gαi binding (8, 27) (Fig. 1B). AGS4-RLuc-Gαi1-YFP BRET signals were markedly reduced upon introduction of the Q204L mutation in Gαi1-YFP, which alters GTP hydrolysis consistent with the known preference of GPR motifs for GDP-bound Gαi subunits. The N149I mutation, which renders Gαi incapable of binding GPR motifs (28), also predictably reduced the AGS4-Gαi BRET signal (Fig. 1C). Interestingly, treatment of cells with pertussis toxin, which ADP-ribosylates a cysteine residue in Gαi1 subunits four amino acids from the C terminus and renders Gαi incapable of
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FIGURE 1. Interaction of AGS4 and Gαi in living cells using BRET. A, emission spectra for luminescence in HEK cells transfected with 10 ng of phRLuc-AGS4 or phRLuc-AGS4-Q/A and 750 ng of pcDNA3-Gαi-YFP. RLU, relative luminescence units. B, HEK cells were transfected with a fixed amount (2 ng) of phRLuc-AGS4 (open squares) or phRLuc-AGS4-Q/A (open triangles) and increasing amounts of pcDNA3-Gαi-YFP (0–750 ng) without or with 500 ng of pcDNA3-Gβi, and 500 ng of pcDNA3-Gγi (Gβγ, open circles), and BRET signals were measured as described under "Experimental Procedures." C, net BRET signals generated from HEK cells transfected with 2 ng of phRLuc-AGS4 and 750 ng of pcDNA3-Gαi-YFP in the presence or absence of 100 ng/ml pertussis toxin (PTX) pretreatment for 18 h or of Gαi1-N149I-YFP or Gαi1-Q204L-YFP. Mean acceptor/donor ratios are: Gαi1, 2.2; Gαi1 + pertussis toxin, 1.6; Gαi1-N149I, 1.6; Gαi1-Q204L, 2.3. *, p < 0.05 as compared with Gαi1. D, net BRET signals generated from HEK cells transfected with 2 ng of phRLuc-AGS4, AGS4-NT (Met1–Ser56), AGS4-CT (Leu57–Cys160), and AGS4-Q/A, 1.6. *, p < 0.05 as compared with AGS4-RLuc WT, wild type. E, net BRET signals generated from HEK cells transfected with 750 ng of pcDNA3-Gαi-YFP and 2 ng of phRLuc3, containing AGS4 or AGS4 variants with single residue mutations in each of the three AGS4-GPR motifs (GPRI-Q80A; GPRII-Q122A; GPRIII-Q151A) that inhibit GPR motif-Gαi binding. Mean acceptor/donor ratios are: wild type, 1.6; GPRI–III, 1.3; GPRI, 1.6; GPRII, 1.3; GPRII–III, 1.2; GPRI–II, 1.0. *, p < 0.05 as compared with AGS4-WT. **, p < 0.05 as compared with GPRI. ***, p < 0.05 as compared with GPRI–III. #, p < 0.05 as compared with GPRI–II. All data presented are representative of 3–9 experiments, and individual values were the average of triplicate determinations. BRET saturation curves were fitted using a non-linear regression equation assuming one-site binding. Error bars in B–E indicate S.E.

being activated by GPCRs, had no effect AGS4-RLuc-Gαi1-YFP BRET signals (Fig. 1C).

Truncations of AGS4 revealed that the AGS4-RLuc-Gαi1-YFP BRET signals require the AGS4 C-terminal GPR domain (AGS4-CT-Leu122-Cys160) (Fig. 1D). However, although no BRET signals were observed between the AGS4 N terminus (AGS4-NT-Met1–Ser56) and Gαi1-YFP, the reduction in the overall magnitude of the BRET signals of AGS4-CT-RLuc as compared with full-length AGS4-RLuc suggests that the N terminus of AGS4 may influence the ability of AGS4 to interact with Gαi1, consistent with the idea that residues outside of the core GPR motif may influence the GPR-Gαi interaction (1, 19, 26, 29).

As an initial approach to define the relative influence of each of the GPR motifs in AGS4 on Gαi binding, we tested AGS4-RLuc constructs with the Q/A substitution in each of the AGS4-GPR motifs (Q80A, Q122A, and Q151A) in AGS4-RLuc-Gαi1-YFP BRET experiments (Fig. 1E). These data are interesting in a number of aspects. First, individually mutated AGS4-GPR motifs show a progressive decrease in Gαi1-YFP BRET signals with GPRIII-Q/A having the most significant effect. In addition, AGS4-RLuc constructs with mutations in GPRIII in combination with either GPRI (GPRIII-Q/A) or GPRII (GPRII–III-Q/A) have significantly lower BRET signals with Gαi1-YFP than AGS4-Q/A mutations in the first two GPR motifs (GPRII–II–Q/A). Taken together these data suggest that GPR-III is important for Gαi binding in cells. Alternatively, the decreased BRET signals generated in GPR-III Q/A mutants may reflect the proximity of the C-terminal RLuc tag to GPR-III and increased resonance energy transfer to Gαi1-YFP. Secondly, Q/A mutations in both GPR-I and GPR-III of AGS4 (GPRIII) still yield significant Gαi1-YFP BRET signals with GPR-II as the sole GPR motif, suggesting that in the intact cell, GPR-II does indeed contribute to Gαi binding consistent with earlier observations (1). This is in contrast to a previous report in which a glutathione S-transferase fusion protein containing AGS4-GPRII was essentially inactive as a guanine nucleotide dissociation inhibitor for Gαi1 unless Ala421 was changed to Asp (19).
We then sought to determine the influence of GPCR activation on the AGS4-Gαi interaction. Co-expression of the α2A-adrenergic receptor had no effect on AGS4-RLuc-Gαi-YFP BRET signal generation (Fig. 2A), and BRET signals were detected at levels of Gαi1-YFP that were similar to the endogenous level of Gαi1 detected by immunoblotting (Fig. 2B, lower panel). However, when the α2-AR agonist UK14304 was added, an ~30% reduction in AGS4-RLuc-Gαi-YFP BRET signal was observed (Fig. 2A, B). This agonist-mediated effect was dose-dependent (Fig. 2C), occurred within 2 min of agonist treatment, and persisted for up to 30 min (Fig. 2D). The UK14304-mediated reduction in AGS4-RLuc-Gαi1-YFP BRET was blocked by the α2-AR antagonist rauwolscine (Fig. 2E). Similar reductions in AGS4-RLuc-Gαi1-YFP BRET were observed upon co-expression of CXCR4 and the mu-opioid receptor (MOR) after treatment with CXCL12 and [d-Ala2, N-MePhe4, Gly-ol]-enkephalin, respectively (data not shown). Similar reductions in BRET signals were observed between the GPR protein AGS3 fused to Renilla luciferase and Gαi1-YFP in cells expressing Gαi-coupled GPCRs upon treatment with agonist.

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The effect of receptor activation on the AGS4-Gαi interaction led us to ask whether AGS4-Gαi complexes were actually substrates for receptor-stimulated activation of Gαi at the cell surface. We first examined the subcellular distribution of AGS4-YFP and Gαi to determine whether their localization might overlap with that of a receptor present at the cell surface.
Receptor Regulation of AGS4-\(\alpha_i\) Complexes

![Graphs and images related to the study of AGS4-\(\alpha_i\) complex regulation](image)

**FIGURE 3. AGS4 forms a \(\alpha_i\)-dependent complex with GPCRs that is regulated by agonist.** A, net BRET signals generated from HEK cells transfected with 2 ng of phRLuc\(_{\alpha_i}\)-AGS4 and 500 ng of pcDNA3-\(\alpha_{2A}\)-AR-Venus in the presence or absence of 750 ng of pcDNA3-\(\alpha_i\)-G\(_i\). Cells were treated with vehicle (−), the \(\alpha_{2A}\)-AR agonist UK14304 (1 \(\mu\)M), and/or the \(\alpha_{2A}\)-AR antagonist rauwolscine (10 \(\mu\)M) for 15 min and processed for BRET as described under "Experimental Procedures." *, \(p < 0.05\) as compared with vehicle treatment. B, BRET saturation signals from HEK cells transfected with a fixed amount (2 ng) of phRLuc\(_{\alpha_i}\)-AGS4 and increasing amounts of pcDNA3-\(\alpha_{2A}\)-AR-Venus (0–1 \(\mu\)g) in the presence or absence of 750 ng of pcDNA3-\(\alpha_i\)-G\(_i\). Cells were treated with vehicle or 10 \(\mu\)M UK14304 as indicated in the figure for 15 min and processed for BRET measurements. **, \(p < 0.05\) as compared with vehicle treatment. Bottom panel, representative immunoblot of HEK cells (50 \(\mu\)g protein/lane) untransfected (−) or transfected (+) with 250 ng of pcDNA3-\(\alpha_{2A}\)-AR-Venus (8B-Venus), 500 ng of pcDNA3-\(\alpha_i\)-G\(_i\), and 2 ng of phRLuc\(_{\alpha_i}\)-AGS4. C, net BRET signals generated from HEK cells transfected with 2 ng of phRLuc\(_{\alpha_i}\)-AGS4, 500 ng of pcDNA3-\(\alpha_{2A}\)-AR-Venus, and 750 ng of pcDNA3-\(\alpha_i\)-G\(_i\), \(\alpha_{2A}\)-AR-Venus, and 750 ng of pcDNA3-\(\alpha_{2A}\)-AR-Venus, and 750 ng of pcDNA3-\(\alpha_i\)-G\(_i\), G\(_i\)-Q204L, G\(_i\)-G202T, or G\(_i\). Cells were treated with vehicle or 10 \(\mu\)M UK14304 as indicated in the figure for 15 min and processed for BRET measurements. *, \(p < 0.05\) as compared with vehicle treatment. Bottom panel, \(\alpha_i\)- and \(\alpha_{2A}\)-immunoblot (75 \(\mu\)g of protein lysate per lane). D and E, HEK cells were transfected with 2 ng of phRLuc\(_{\alpha_{2A}}\)-AGS4, 500 ng of pcDNA3-\(\alpha_{2A}\)-AR-Venus, and 750 ng of pcDNA3-\(\alpha_i\)-G\(_i\). Cells were incubated with increasing amounts of UK14304 (10 \(-\) M) for 15 min (D) or treated with 10 \(\mu\)M UK14304 for 0–30 min (E) and processed for BRET measurements. *, \(p < 0.05\) as compared with vehicle treatment (D) or 0 time point (E). F, HEK cells were transfected with 2 ng of phRLuc\(_{\alpha_{2A}}\)-AGS4, 500 ng of pcDNA3-\(\alpha_{2A}\)-AR-Venus, and increasing amounts of pcDNA3-\(\alpha_i\)-G\(_i\), (0–750 ng). Cells were incubated in the presence or absence of 100 ng/ml pertussis toxin (PTX) for 18 h and then treated with vehicle or 10 \(\mu\)M UK14304 for 15 min. *, \(p < 0.05\) as compared with control in each group. **, \(p < 0.05\) as compared with UK14304 treatment in each group. G, HEK cells were transfected with 2 ng of phRLuc\(_{\alpha_{2A}}\)-AGS4 and 500 ng of pcDNA3-\(\alpha_{2A}\)-AR-Venus, MOR-YFP, or \(\beta_2\)-AR-Venus in the absence or presence of 750 ng of pcDNA3-\(\alpha_i\)-G\(_i\) as indicated in the figure. Cells were then treated with 10 \(\mu\)M UK14304, 10 \(\mu\)M DAMGO, or 10 \(\mu\)M isoproterenol for 15 min as indicated in the figure. *, \(p < 0.05\) as compared with vehicle treatment for each group. All data presented are representative of 4–10 experiments, and individual values were the average of triplicate determinations. Error bars in A–G indicate S.E.

cell surface. Although AGS4 is primarily localized to the cytosol (supplemental Fig. S1) (1), co-expression with \(\alpha_i\) resulted in a dramatic recruitment of AGS4-GFP to the cell cortex (supplemental Fig. S1), suggesting that AGS4-\(\alpha_i\) complexes are at least within the same subcellular compartment as GPCRs, i.e., at the cell surface. \(\alpha_i\)-mediated recruitment of AGS4-GFP to the cell cortex was not observed for AGS4-Q/A-GFP, nor in the context of the GTP hydrolysis-deficient \(\alpha_{13}\)-Q204L variant (supplemental Fig. S1). As AGS4-RLuc-G\(_i\)-YFP BRET signals were dramatically reduced or absent in the context of the \(\alpha_{2A}\)-Q204L and AGS4-Q/A variants (Fig. 1), the co-localization of wild-type AGS4 and \(\alpha_i\) suggests that their co-localization at the cell surface likely results from their interaction. We then measured agonist-induced changes in AGS4-RLuc and G\(_i\)-YFP distribution in crude membrane and cytosol fractions (supplemental Fig. S2). Receptor activation decreased the amount of AGS4-RLuc in the membrane fraction with a concomitant increase in the cytosol fraction as measured by both luminescence and immunoblotting (supplemental Fig. S2A), whereas G\(_i\)-YFP distribution was unaltered (supplemental Fig. S2B). These data suggest that AGS4 and \(\alpha_i\) physi-
cally dissociate after receptor activation, with AGS4 moving into the cytosol and Go1 remaining at the plasma membrane. We then sought to determine whether AGS4 and Go1 were actually forming a complex with receptors. Under basal conditions, specific BRET signals were not observed between AGS4-RLuc and α2AR-AR-Venus (Fig. 3, A and B). However, when co-expressed with Go1, dramatic and specific BRET signals were observed (Fig. 3, A and B). We did not observe significant alterations in either the basal or the agonist-induced changes in BRET observed between AGS4-RLuc and α2AR-AR-Venus when either Go1 or Goαi was used (data not shown). BRET signals were not observed between AGS4-RLuc and α2AR-AR-Venus in the context of the Goαi-Q204L or Goαi-G202T mutations or Goαi (Fig. 3C), nor were they observed with the AGS4-Q/A-RLuc variant, which cannot bind Go1 (data not shown), indicating that the AGS4-Goαi interaction is required for the BRET signals observed between AGS4-RLuc and α2AR-AR-Venus. In addition, the Goαi-dependent AGS4-RLuc-α2AR-Venus BRET signals were reduced by ~40–50% upon treatment with the α2-AR agonist UK14304 (Fig. 3, A and B). As was observed for the agonist-regulated BRET signals between AGS4-RLuc and Goαi2AR-YFP (Fig. 2), the reductions in Go1-dependent BRET signals between AGS4-RLuc and α2AR-Venus exhibited dose-dependent and occurred on a similar timescale (Fig. 3, D and E). The agonist-mediated reduction in AGS4-RLuc-α2AR-YFP BRET signals was blocked by the antagonist rauwolscine (Fig. 3A) and by pertussis toxin pretreatment (Fig. 3F). Similar Goαi-dependent and agonist-induced reductions in BRET signals were also observed between AGS4-RLuc and MOR-YFP but were not observed with AGS4-RLuc and β2-AR-Venus, which is primarily a Go1-coupled receptor (Fig. 3G).

In the case of AGS4-RLuc-Goαi2AR-YFP BRET signals, receptor activation resulted in a reduction in BRET that could result either due to dissociation of AGS4-RLuc-Go1-YFP complexes or from a conformational rearrangement of the complex. The observation that receptor activation leads to a redistribution of AGS4 from the plasma membrane to the cytosol supports the first possibility. The reduction in agonist-modulated AGS4-RLuc-Goαi2AR-YFP BRET signal suggests that the agonist-regulated effect either occurs in a spatially restricted manner (e.g., at the plasma membrane) or is the result of second messengers. It is also possible that the agonist-induced reductions in BRET may arise from subunit exchange between the Goα1-YFP complexed with AGS4-RLuc and wild-type, endogenous (i.e., untagged) Go1 from Goβγ heterotrimers or that "free" Goβγ released from activated Goβγ heterotrimers competes with AGS4-RLuc for Goα1-YFP binding. Regarding the latter scenario, although data indicate that increased expression of Goβγ subunits does indeed reduce AGS4-RLuc-Goα1-YFP BRET (Fig. 1B), this is likely due to an overall reduction in the amount of AGS4-RLuc-Goα1-YFP complexes being formed in the presence of excess Goβγ rather than the release of free Goβγ subunits occurring via receptor activation.

The data therefore suggest that AGS4-Goαi complexes are directly coupled to and are regulated by a GPCR. The agonist-induced reduction in Goαi-dependent BRET signals between AGS4-RLuc and α2AR-AR-Venus (Fig. 3) and the agonist-induced reductions in AGS4-RLuc-Goαi2AR-YFP (Fig. 2) are consistent with nucleotide exchange occurring on Go1 while complexed with AGS4-RLuc and suggest that once bound to GTP, the Go1 subunit dissociates from AGS4-RLuc, resulting in the decrease in BRET signals in both cases. The pertussis toxin blockade of these agonist-regulated BRET signals suggests that the mechanism of activation of an AGS4-Goαi complex is similar to that of receptor-mediated activation of Goαiβγ heterotrimers and that GPCRs provide one mode of regulation of AGS4-Goαi complexes. As AGS4 and other proteins containing more than one GPR motif can bind multiple Goαi subunits simultaneously (7, 8, 19), these data have far reaching implications for G-protein signal processing and may provide cells with additional flexibility to modulate signal efficiency, specificity, duration, and location in ways previously unappreciated.

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7 Attempts to isolate an α2AR-AR-Venus-Goαi-AGS4-RLuc complex by co-immunoprecipitation revealed α2AR-AR-Venus-Goαi complexes (data not shown); however, the presence of AGS4-RLuc within this larger complex was inconsistent, suggesting either that the interaction of AGS4-Goαi complexes with α2AR is too transient to be consistently observed by co-immunoprecipitation or that the complex is sensitive to the conditions required to solubilize the receptor from the membrane.
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