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Direct Coupling of a Seven Transmembrane Span Receptor to a GαiGPR Complex

William G. Robichaux, III, Sukru S. Oner, Stephen M. Lanier and Joe B. Blumer

Department of Cell and Molecular Pharmacology and Experimental Therapeutics (W.G.R., S.S.O., S.M.L., J.B.B.) and Department of Neurosciences (J.B.B.), Medical University of South Carolina, Charleston, SC 29414

Istanbul Medeniyet University, Goztepe Research and Training Hospital, Istanbul, Turkey (S.S.O., present address)

Department of Pharmacology, Wayne State University, 5057 Woodward, Suite 6409, Detroit, MI 48202 (S.M.L. present address)

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Running Title: Direct coupling of a 7TM receptor to GαiGPR

Corresponding Author:

Joe B. Blumer, Ph.D.

Department of Cell and Molecular Pharmacology and Experimental Therapeutics

Medical University of South Carolina

173 Ashley Ave, BSB358, MSC509

Charleston, SC 29425

Tel: (843) 792-3552

Fax: (843) 792-2475

E-mail: blumerjb@musc.edu

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Abbreviations: α_2-AR, α_2 adrenergic receptor; AGS, Activator of G-protein Signaling; AGS3, Activator of G-protein Signaling 3; AGS4, Activator of G-protein Signaling 4; BRET, bioluminescence resonance energy transfer; ERK, extracellular signal-regulated kinase; GFP,

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green fluorescent protein; Gpsm1, G-protein signaling modulator-1; Gpsm3, G-protein signaling modulator 3; GPCR, G-protein coupled receptor; GPR, G-protein regulatory motif; GRK2, G-protein coupled receptor kinase 2; PT, pertussis toxin; RFU, relative fluorescence units; RLU, relative luminescence units; Rluc, *Renilla* luciferase; 7TMR, seven transmembrane span receptor; SDS-PAGE, sodium dodecyl sulfide – polyacrylamide gel electrophoresis; YFP, yellow fluorescent protein

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ABSTRACT

Group II Activators of G-protein Signaling (AGS) proteins contain one or more G-protein regulatory (GPR) motifs, which serve as docking sites for $G\alpha i_{GDP}$ independent of $G\beta\gamma$ and stabilize the GDP-bound conformation of $G\alpha i$ acting as guanine nucleotide dissociation inhibitors. The $G\alpha$GPR interaction is regulated by seven-transmembrane-spanning receptors (7TMR) in the intact cell as determined by bioluminescence resonance energy transfer (BRET). It is hypothesized that a 7TMR directly couples to the $G\alpha$GPR complex in a manner analogous to receptor coupling to $G\alpha\beta\gamma$ heterotrimer. As an initial approach to test this hypothesis we utilized BRET to examine 7TMR-mediated regulation of $G\alpha$GPR in the intact cell when $G\alpha i_2$YFP was tethered to the carboxyl-terminus of the $\alpha_{2A/D}$-adrenergic receptor ($\alpha_{2A/D}$AR-$G\alpha i_2$YFP). AGS3- and AGS4-Rluc exhibited robust BRET with the tethered $G\alpha$iYFP and this interaction was regulated by receptor activation localizing the regulation to the receptor microenvironment. Agonist regulation of the receptor-$G\alpha$i-GPR complex was also confirmed by co-immunoprecipitation and cell fractionation. The tethered $G\alpha i_2\beta\gamma$ was rendered pertussis toxin-insensitive by a C352I mutation and receptor coupling to endogenous $G\alpha i/o\beta\gamma$ was subsequently eliminated by cell treatment with PT. Basal and agonist-induced regulation of $\alpha_{2A/D}$AR-$G\alpha i_2$YFP^{C352I}:AGS3-Rluc and $\alpha_{2A/D}$AR-$G\alpha i_2$YFP^{C352I}:AGS4-Rluc BRET was not altered by PT treatment or $G\beta\gamma$ antagonists. Thus, the localized $G\alpha$GPR interaction appears independent of endogenous $G\alpha i/o\beta\gamma$ suggesting that $G\alpha$iAGS3 and $G\alpha$iAGS4 directly sense agonist-induced conformational changes in the receptor as is the case for 7TMR coupling to $G\alpha\beta\gamma$ heterotrimer. The direct coupling of a receptor to the $G\alpha$iGPR complex provides an unexpected platform for signal propagation with broad implications.

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INTRODUCTION

The discovery of Activators of G-protein Signaling (AGS) proteins, originally identified in a yeast-based functional screen for mammalian cDNAs that activated G-protein signaling in the absence of a receptor, revealed both unexpected regulatory mechanisms for G-protein signaling systems and expanded functional roles for the G-protein subunits (Cao et al., 2004; Cismowski et al., 1999; Sato et al., 2006; Sato et al., 2011b; Takesono et al., 1999). Group I AGS proteins encompass non-receptor guanine nucleotide exchange factors whereas Group II AGS proteins, all of which contain a G-protein regulatory (GPR) motif, engage Gαi/o/t as guanine nucleotide dissociation inhibitors. Group III AGS proteins appear to engage Gβγ, whereas Group IV AGS proteins, which were just recently identified, interact with Gα16 (Sato et al., 2011a).

We recently reported that the Gαi-GPR interaction is regulated by agonist-bound cell surface seven transmembrane span (7TM) receptors in the intact cell as determined by bioluminescence resonance energy transfer (BRET) (Oner et al., 2010a; Oner et al., 2010b). As the GPR motif stabilizes the GDP-bound conformation of Gα free of Gβγ, it was hypothesized that a 7TM receptor may directly couple to the GαGPR complex in a manner that is analogous to direct receptor coupling to the Gαβγ heterotrimer (Figure 1A) (7-9). Indeed, the GαGPR complex appears to be positioned in close proximity to the 7TM receptor and this positioning, which is regulated by agonist, is dependent upon interaction of the GPR protein with Gαi (Oner et al., 2010a; Oner et al., 2010b; Vellano et al., 2011). Alternatively, the regulation of GαGPR observed with receptor activation may be secondary to canonical 7TM receptor coupling to Gαβγ subsequent to G-protein subunit flux within the microenvironment of a signaling complex (Figure 1B). It was also recently postulated that Groups I-III AGS proteins may

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actually represent a signaling triad that parallels that of the well characterized 7TM receptor – Gαβγ – effector system (Blumer and Lanier, 2014).

As part of a broader approach to explore these concepts, we examined the 7TM receptor - mediated regulation of the Gαi-GPR complex when Gαi was actually tethered to the 7TM receptor itself (Figure 1C). Thus the Gαi-GPR interaction would be highly localized and could also be monitored independent of endogenous Gαβγ as the tethered G-protein could be rendered pertussis toxin insensitive by a single point mutation (Figure 1D). The results of these studies suggest direct coupling of a 7TM receptor to the GαGPR complex, which has broad implications for G-protein signal processing.

MATERIALS AND METHODS

Materials – Polyethylenimine (PEI) (25 kDa molecular mass, linear form), was obtained from Polysciences, Inc (Warrington, PA). Benzyl-coelenterazine was obtained from NanoLight Technology (Pinetop, AZ). UK14304, pertussis toxin, and β–actin antiserum (A5441) were purchased from Sigma-Aldrich (St. Louis, MO). Gray 96-well Optiplates were obtained from Perkin Elmer (Waltham, MA). GFP antiserum was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). $G\alpha i_{1/2}$ antiserum was kindly provided by Dr. Thomas Gettys (Pennington Biomedical Research Center, Baton Rouge, LA). GRK2 antibody (ab50633) and anti-GFP-Sepharose (ab69314) were obtained from Abcam (Cambridge, MA). n-Dodecyl-β-D-maltoside (DβM) was obtained from Cayman Chemical (Ann Arbor, MI). Protease inhibitor mixture tablets (Complete Mini) were obtained from Roche Applied Science (Indianapolis, IN). AGS3 and AGS4 fused at the carboxyl terminus to *Renilla* luciferase (Rluc) and $\alpha_{2A/D}$AR constructs

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were generated as previously described (Oner et al., 2010a; Oner et al., 2013a; Oner et al., 2010b). $G\alpha i_2$-yellow fluorescent protein ($G\alpha i_2$YFP) was generated by Dr. Scott Gibson (Gibson and Gilman, 2006) and kindly provided by Dr. Nathan Dascal (Tel Aviv University, Tel Aviv, Israel). YFP was inserted within the αB-αC loops in the helical domain of Gαi as described (Gibson and Gilman, 2006; Oner et al., 2010a; Oner et al., 2010b). pcDNA3::GRK2-CT, which encodes amino acids Tyr^{466} – Leu^{689} in the carboxylterminus of GRK2, was kindly provided by Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA). All other reagents and materials were obtained as described elsewhere (Oner et al., 2010a; Oner et al., 2013a; Oner et al., 2010b).

Site-directed Mutagenesis and plasmid construction –The α_{2A}AR – $G\alpha i_2$YFP fusion protein was generated by polymerase chain reaction (PCR) using the rat $\alpha_{2A/D}$AR as template and primer sets containing specific sites for restriction enzyme digest as follows: XhoI, α_{2A}AR forward primer 5'-AAA CTC GAG GCC GCC ACC ATG GGC TCC CTG CAG CCG GAC-3'; EcoRI, α_{2A}AR reverse primer 5'-CAT GAA TTC CTG CAA GCT TCC TCC TCC TCC GGA CAC GAT CCG CTT-3'. The reverse primer also encodes a SGGGS linker between α_{2AD}AR and $G\alpha i_2$YFP. Digestion of pcDNA3::$G\alpha i_2$YFP or pcDNA3::$G\alpha i_2YFP^{C352I}$ constructs at upstream XhoI/EcoRI sites followed by ligation with the digested receptor-linker resulted in in-frame construction of the α_{2A}AR – $G\alpha i_2$YFP fusion proteins. Cysteine 352 (C352) in $G\alpha i_2$, which is the site of ADP-ribosylation by pertussis toxin (PT), was converted to isoleucine to render the protein PT insensitive by site-directed mutagenesis using the pcDNA3::$G\alpha i_2$YFP construct with the following primer set: $G\alpha i_2YFP^{C352I}$ forward primer 5'-AAC AAC CTG AAG GAC ATT

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GGC CTC TTC TGA-3'; $G\alpha i_2YFP^{C352I}$reverse primer 5'-TCA GAA GAG GCC AAT GTC CTT CAG GTT GTT-3'.

Cell Culture, Transfection, Immunoblotting, BRET, Plasmid expression – BRET measurements and immunoblotting were performed as previously described (Oner et al., 2010a; Oner et al., 2013a; Oner et al., 2010b). In experiments measuring BRET between AGS3-Rluc or AGS4-Rluc and $\alpha_{2A/D}AR$-$G\alpha i_2YFP$ or $\alpha_{2A/D}AR$-$G\alpha i_2YFP^{C352I}$, HEK293 cells were transfected with 10 ng $phRLuc_{N3}$::AGS3 or 2 ng $phRLuc_{N3}$::AGS4, respectively and 750 ng pcDNA3::$\alpha_{2A/D}AR$-$G\alpha i_2YFP$ or pcDNA3:: $\alpha_{2A/D}AR$-$G\alpha i_2YFP^{C352I}$. Based upon a series of preliminary experiments we optimized the system to generate levels of $\alpha_{2A/D}AR$-$G\alpha i_2YFP$ and $\alpha_{2A/D}AR$-$G\alpha i_2YFP^{C352I}$ that bracketed the levels of endogenous Gαi2 as determined by immunoblotting. For BRET saturation experiments, AGS3–Rluc and AGS4-Rluc were expressed as above with increasing amounts (0 – 1000 ng) of pcDNA3::$\alpha_{2A/D}AR$-$G\alpha i_2YFP$ or pcDNA3:: $\alpha_{2A/D}AR$-$G\alpha i_2YFP^{C352I}$. Forty-eight hours after cell transfection, cells were dispensed in triplicate at 1×10^5 cells/well in gray 96-well Optiplates (Perkin Elmer (Waltham, MA). Fluorescence and luminescence signals were measured using a TriStar LB 941 plate reader (Berthold Technologies) with MikroWin 2000 software. Cells were incubated with the α_2-AR agonist (UK14304 – 10 μM) or vehicle in Tyrode's solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.37 mM NaH_2PO_4, 24 mM $NaHCO_3$, 10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4 and 0.1% glucose (w/v)) for 5 minutes prior to addition of coelenterazine H. Coelenterazine H (Nanolight Technology, 5 μM final concentration) was added to each well and luminescence measured after two minutes (donor: 480 $\pm$ 20 nm; acceptor: 530 $\pm$ 20 nm) with the TriStar LB 941 plate reader. $G\alpha i_2YFP$ or $\alpha_{2A/D}AR$-$G\alpha i_2YFP$ fusion protein expression was monitored by

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measuring YFP fluorescence (excitation 485 nm, emission 535 nm). AGS3- and AGS4-Rluc expression was monitored by measuring the intensity of the luminescence signal. BRET signals were determined by calculating the ratio of the light intensity emitted by the YFP divided by the light intensity emitted by Rluc. Net BRET values were determined by first calculating the 530 ± 20:480 ± 20 nm ratio and then subtracting the background BRET signal determined from cells transfected with the donor plasmids phRLuc$_{N3}$::AGS3 or phRLuc$_{N3}$::AGS4 alone. Cell lysates and immunoblotting were performed as previously described (Oner et al., 2010a; Oner et al., 2010b). Where indicated, cells were incubated with pertussis toxin (100 ng/ml) for 18 hours prior to BRET measurements. Cellular fractionation of UK14304- or vehicle-treated cells by hypotonic lysis and centrifugation were performed as previously described (Oner et al., 2013b), using HEK293 cells transfected with AGS3 and AGS4 donor plasmids (10 ng and 2 ng, respectively) and $\alpha_{2A/D}$AR-Gαi_2YFP acceptor plasmid (750 ng) as described above.

Immunoprecipitation - HEK293 cells expressing α_{2A}AR-Gαi_2YFP and AGS3Rluc (1.4 μg and 0.1 μg plasmid per well in a 6-well plate, respectively) for 24 h were treated with the α_2AR agonist UK14304 at a final concentration of 10 μM or with vehicle (Tyrode's solution) for 5 min at room temperature and harvested in 4.5 mL Tyrode's solution. Cells were centrifuged at 500 x g for 5 min and resuspended in 0.5 mL immunoprecipitation (IP) buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, protease inhibitor cocktail) and sonicated at 50% amplitude for three intervals at five seconds each. n-Dodecyl-β-d-maltoside (DβM) was added to a final concentration of 2% and membrane proteins extracted by rotating 3 hrs at 4°C followed by centrifugation at 21,000 x g for 30 min at 4°C. The supernatant was collected and an input sample (1/20th vol; 82.5 μg) taken; to the remaining supernatant (1.65 mg) 25 μL 50% anti-GFP-

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Sepharose (Abcam) was added and rotated overnight at 4°C followed by 6 x 500 µL resin washes with IP buffer containing 0.2 % DBM. Twenty-five µL 5X Laemmli sample buffer was added to the washed resin, incubated at room temperature for 5 min and processed for SDS-PAGE (7% polyacrylamide), transferred to PVDF membranes and immunoblotted with AGS3 antisera followed by stripping and re-probing with GFP antisera.

Data Analysis – Statistical significance for differences involving a single intervention was determined by one-way ANOVA using GraphPad Prism version 4.03 (GraphPad Software, San Diego).

RESULTS AND DISCUSSION

As a first step to address the hypothesis regarding direct receptor coupling to $G\alpha i_2$GPR, we generated a fusion protein in which $G\alpha i_2$YFP was tethered to the carboxyl terminus of the $\alpha_{2A/D}$AR via a flexible glycine linker (Bahia et al., 1998; Bertin et al., 1994; Burt et al., 1998; Seifert et al., 1999; Wise et al., 1997).[1] We also generated a variant of the $\alpha_{2A/D}$AR-$G\alpha i_2$YFP fusion protein that was PT-insensitive ($\alpha_{2A/D}$AR-$G\alpha i_2$YFP^{C352I}). We then examined the ability of GPR proteins to interact with the tethered $G\alpha i_2$. AGS3 and AGS4 were selected as representative members of two distinct subgroups of AGS proteins. AGS3 has four GPR motifs downstream of a series of tetratricopeptide repeat domains (TPR) involved in protein interactions and intramolecular regulatory events, whereas AGS4 is a smaller protein with three full GPR motifs without any clearly defined protein interaction motifs upstream of the GPR motifs.

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Both AGS3 and AGS4 interacted with the tethered WT and PT-insensitive $G\alpha i_2$ as indicated by the robust basal levels of BRET (Figure 2A). Expression and functionality of $\alpha_{2A/D}$AR-$G\alpha i_2$YFP and $\alpha_{2A/D}$AR-$G\alpha i_2$YFP^{C352I} were confirmed by immunoblotting (Figure 2A) and agonist-induced phosphorylation of ERK1/2.[2] $\alpha_{2A/D}$AR-$G\alpha i_2$YFP:AGS3-Rluc BRET and $\alpha_{2A/D}$AR-$G\alpha i_2$YFP:AGS4-Rluc BRET were not observed with the GPR-insensitive $G\alpha i^{N149I}$ mutant or with AGS3 or AGS4 that were rendered incapable of binding $G\alpha i$ by mutation of a conserved glutamate residue in each of the GPR motifs, thus demonstrating the specificity of the interaction (Oner et al., 2010a; Oner et al., 2010b; Peterson et al., 2002; Sato et al., 2004; Willard et al., 2008).[2]

Incubation of cells with the $\alpha_{2A/D}$AR agonist UK14304 reduced the $\alpha_{2A/D}$AR-$G\alpha i_2$YFP:AGS3-Rluc BRET by ~40% (Figure 2B, left panel). Significant agonist-induced reductions in $\alpha_{2A/D}$AR-$G\alpha i_2$YFP:AGS4-Rluc BRET were also observed, although not to the same magnitude as that observed for AGS3-Rluc (Figure 2B, right panel). Both the basal $\alpha_{2A/D}$AR-$G\alpha i_2$YFP:AGS3-Rluc BRET and the magnitude of the agonist-induced decrease in BRET observed for AGS3-Rluc or AGS4-Rluc with tethered $G\alpha i_2$YFP were similar to that observed with untethered $G\alpha i_2$YFP.[1] Thus, these data indicate that a 7TM agonist is regulating a GαGPR complex that is directly anchored to the receptor.

A similar distinction between AGS3 and AGS4 with respect to the magnitude of agonist-induced changes in BRET was also observed with untethered $G\alpha i_1$YFP (Oner et al., 2010a; Oner et al., 2010b). It is not clear if the differences in the magnitude of the agonist-induced changes in GαiYFP:AGS3-Rluc versus GαiYFP:AGS4-Rluc BRET reflect different coupling efficiencies, stoichiometric considerations and/or the relative spatial positioning of the acceptor and donor for

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AGS3 versus AGS4. As an initial approach to address this issue and to verify that the agonist-induced changes in BRET were the result of translocation of GPR proteins away from the receptor-Gαi complex, we monitored the subcellular distribution of AGS3Rluc or AGS4Rluc and α_{2A}AR-Gαi_2YFP by cellular fractionation into crude membranes and cytosol (Figure 3). These data indicate that activation of α_{2A}AR-Gαi_2YFP resulted in translocation of GPR proteins away from the membrane fraction and into the cytosol while α_{2A}AR-Gαi2YFP remained in the membrane fraction, suggesting that the observed agonist-induced changes in BRET result from a physical dissociation of GPR proteins from the receptor-Gα complex. The relative extent of AGS3 and AGS4 translocation was almost directly related to the degree of agonist-induced reductions in BRET between AGS3 or AGS4 and α_{2A}AR-Gαi_2YFP as shown in Figure 2B.

As an additional approach to observe agonist-regulated interaction of GPR proteins with Gαi-coupled 7TMRs, we asked if AGS3 co-immunoprecipitates with α_{2A}AR-Gαi2YFP and if this complex was also regulated by agonist. Indeed, AGS3Rluc co-immunoprecipitated with α_{2A}AR-Gαi_2YFP (Figure 4). AGS3-Q/A-Rluc, which cannot bind Gαi (Oner et al., 2010a), did not co-immunoprecipitate with α_{2A}AR-Gαi_2YFP, thus serving as an important internal negative control. Treatment with the α_{2A}AR agonist UK14304 resulted in a ~30% decrease in co-immunoprecipitation of AGS3-Rluc with α_{2A}AR-Gαi_2YFP compared to vehicle treatment. These data further support our hypothesis of an agonist-sensitive 7TMR-Gαi-GPR complex and are consistent with the degree of agonist effect in our BRET system (Figure 2B).

Regulation of the $\alpha_{2A/D}$AR-Gαi_2YFP:GPR-Rluc complex by agonist may reflect the ability of the Gαi_2GPR cassette to directly sense agonist-induced conformational changes in the receptor (Figure 1C) as is the case for 7TM receptor coupling to Gαβγ heterotrimer. Alternatively, the

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agonist-induced reduction of $\alpha_{2A/D}$AR-Gαi_2YFP:GPR-Rluc BRET may reflect displacement of AGS3- or AGS4-Rluc from the 7TM receptor-Gαi_2YFP fusion protein by G$\beta\gamma$ or Gα subsequent to receptor coupling to either endogenous G$\alpha\beta\gamma$ heterotrimer (Burt et al., 1998) or the $\alpha_{2A/D}$AR-Gαi_2YFP fusion protein where endogenous G$\beta\gamma$ is bound to the tethered Gαi_2YFP (Figure 1E).

To address these questions, we conducted two sets of experiments. First, we studied the effect of agonist on $\alpha_{2A/D}$AR-Gαi_2YFP:GPR-Rluc BRET after rendering the tethered Gα subunit PT insensitive by mutation of the cysteine that is actually ADP ribosylated by pertussis toxin (Figure 1D). Such an approach would allow us to eliminate receptor coupling to endogenous G$\alpha\beta\gamma$, but retain the coupling integrity of the $\alpha_{2A/D}$AR-Gαi_2YFP^{C352I} fusion protein (Bahia et al., 1998). Thus, we have an experimental platform that provides a highly localized readout of receptor-mediated regulation of Gαi_2GPR.

The agonist-induced regulation of $\alpha_{2A/D}$AR-Gαi_2YFP:AGS3-Rluc or $\alpha_{2A/D}$AR-Gαi_2YFP:AGS4-Rluc BRET observed with untethered[2] or tethered Gα was completely blocked by incubation of cells with PT (Figure 2B). However, the agonist-induced regulation of untethered[2] or tethered Gαi^{C352I} was not altered by PT pretreatment, which blocked receptor coupling to endogenous Gαi/o$\beta\gamma$ (Figure 2B). These data indicate that the agonist-induced regulation of $\alpha_{2A/D}$AR-GαiYFP:AGS3-Rluc or $\alpha_{2A/D}$AR-GαiYFP:AGS4-Rluc BRET is spatially localized and not likely due to exchange of endogenous Gαi/o for GαYFP bound to the GPR protein or to the displacement of GαYFP bound to the GPR protein by G$\beta\gamma$ subsequent to receptor-mediated coupling to G$\alpha\beta\gamma$ heterotrimer.

In addition to interacting with the GPR proteins AGS3 and AGS4, the $\alpha_{2A/D}$AR-Gαi_2YFP fusion protein may also interact with endogenous G$\beta\gamma$. Agonist induced activation of the

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$\alpha_{2A/D}$AR-Gαi_2YFP:G$\beta\gamma$ complex may "release" G$\beta\gamma$, which could potentially displace AGS3 or AGS4 from the $\alpha_{2A/D}$AR-Gαi_2YFP fusion protein reducing $\alpha_{2A/D}$AR-Gαi_2YFP:GPR-Rluc BRET (Figure 1E). To address this issue, we used the carboxyl terminus of G-protein coupled receptor kinase 2 (GRK2-CT) to scavenge any G$\beta\gamma$ that may be "released" by agonist-induced activation of $\alpha_{2A/D}$AR-Gαi_2YFP:G$\beta\gamma$ (Figure 5). GRK-CT expression was confirmed by immunoblotting (Figure 5B). Expression of GRK2-CT did not alter the agonist induced regulation of the BRET observed with AGS3-Rluc or AGS4-Rluc and the untethered[2] or tethered Gαi_2YFP (Figure 5A). Under similar experimental conditions with untethered Gαi_2YFP, expression of G$\beta\gamma$ reduces basal Gαi_2YFP:GPR-Rluc BRET (Oner et al., 2010a; Oner et al., 2010b) and this effect of G$\beta\gamma$ was reversed by GRK2-CT providing an internal control that indicates effective G$\beta\gamma$ scavenging (Figure 5B). The lack of effect of GRK2-CT on agonist-induced regulation of the interaction of GPR proteins with the tethered GαiYFP is consistent with previous observations using untethered GαiYFP (Oner et al., 2010a). Furthermore, the G$\beta\gamma$ inhibitor gallein also did not alter the basal or agonist-regulated BRET between AGS3-Rluc or AGS4-Rluc and either Gαi_2YFP or the $\alpha_{2A/D}$AR–Gαi_2YFP fusion protein.[2] These data suggest that the agonist induced regulation of the interaction of Gαi with GPR proteins does not involve subunit flux subsequent to receptor coupling to G$\alpha\beta\gamma$.

Our data suggest that a 7TM receptor couples directly to a GαiGPR complex, ostensibly promoting exchange of GDP for GTP in a manner that may be similar to 7TM receptor engagement of G$\alpha\beta\gamma$ heterotrimer. Agonist-mediated activation of a 7TM receptor coupled to GαiGPR apparently results in reversible dissociation of the GPR protein from Gαi (Oner et al., 2010a; Oner et al., 2010b). Upon termination of agonist-induced activation, the GPR protein

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then re-associates with $G\alpha i_{GDP}$, representing a cycle that is conceptually analogous to the $G\alpha\beta\gamma$ activation – deactivation cycle (Oner et al., 2010a; Oner et al., 2013a; Oner et al., 2010b; Oner et al., 2013c). There are several interesting conceptual thoughts that emanate from this work. As the regulation of both the GαiGPR complex and the $G\alpha i\beta\gamma$ heterotrimer is PT sensitive (Figures 2, 3) (Oner et al., 2010a; Oner et al., 2010b), this raises the intriguing possibility that functional effects associated with PT may be mediated in part by 7TM regulation of Gαi-GPR complexes. Secondly, as Group II AGS proteins may complex with multiple Gα subunits simultaneously (Adhikari and Sprang, 2003; Bernard et al., 2001; Jia et al., 2012; Kimple et al., 2004), AGS3 and AGS4 may scaffold receptors and Gα subunits within a larger signaling complex (Blumer and Lanier, 2014; Jahangeer and Rodbell, 1993). It is interesting to speculate on the relative ratio of receptors coupling to $G\alpha\beta\gamma$ versus GαGPR. Regulation of GPR protein expression levels may play a role in determining this stoichiometry, as AGS3 and AGS4 levels are responsive to changes in environmental and pathophysiological conditions, including withdrawal from drugs of abuse, ischemia/reperfusion injury, and leukocyte activation (Bowers et al., 2008; Bowers et al., 2004; Branham-O'Connor et al., 2014; Giguere et al., 2013; Kwon et al., 2012; Nadella et al., 2010; Regner et al., 2011; Yao et al., 2005).[2] Additional signals regulating the Gα-GPR interaction and subcellular distribution of GPR proteins may also be involved and may provide more rapid and dynamic control of cellular responses (An et al., 2008; Blumer et al., 2003; Giguere et al., 2012; Nadella et al., 2010; Oner et al., 2010a; Oner et al., 2010b; Oner et al., 2013c; Vural et al., 2010). Finally, of particular interest, the coupling of a receptor to the GαGPR complex or the $G\alpha\beta\gamma$ heterotrimer may be differentially regulated by hormones, neurotransmitters and small molecules.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Robichaux, Oner, Lanier, and Blumer.

Conducted experiments: Robichaux, Oner, and Blumer.

Contributed new reagents or analytic tools: Robichaux, Oner, Lanier, and Blumer.

Performed data analysis: Robichaux, Oner, Lanier, and Blumer.

Wrote or contributed to the writing of the manuscript: Robichaux, Oner, Lanier, and Blumer.

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FOOTNOTES

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Send reprint requests to:

Joe B. Blumer, Ph.D.

Department of Cell and Molecular Pharmacology and Experimental Therapeutics

Medical University of South Carolina

173 Ashley Ave, BSB358, MSC509

Charleston, SC 29425

Tel: (843) 792-3552

Fax: (843) 792-2475

E-mail: blumerjb@musc.edu

[1] Robichaux, III., W.G. and Blumer, J.B. Similar results were obtained with the $\alpha_{2A/D}$-AR – $G\alpha i_1$-YFP fusion protein.

[2]Robichaux, III., W.G. and Blumer, J.B., unpublished observations.

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FIGURE LEGENDS

Figure 1. Predicted effects of receptor activation on GPR-Rluc – GαiYFP BRET. Agonist-induced reductions in GαiYFP – GPR-Rluc BRET may reflect the following: *A*, Direct coupling of the receptor to the GαiGPR module, which is regulated by agonist-induced nucleotide exchange on GαiYFP, or *B*, competitive inhibition of Gαi binding to GPR proteins by endogenous Gα or Gβγ liberated subsequent to receptor coupling to endogenous Gαβγ. *C*, Agonist-induced regulation of an $\alpha_{2A/D}$AR-Gαi_2YFP fusion protein complexed with the GPR proteins AGS3 or AGS4. *D*, To determine the influence of endogenous, Gαβγ on basal and agonist-induced regulation of $\alpha_{2A/D}$AR-Gαi_2YFP:GPR-Rluc BRET, Cys352 in Gαi_2 was mutated to Ile (C352I) rendering the tethered Gαi_2YFP insensitive to pertussis toxin. Receptor coupling to endogenous Gαβγ could then be blocked by cell treatment with PT (Burt et al., 1998). *E*, Endogenous Gβγ subunits may also engage the $\alpha_{2A/D}$AR-Gαi_2YFP fusion protein (Burt et al., 1998). GRK2-CT was expressed as a scavenger for free Gβγ released subsequent to receptor activation of an $\alpha_{2A/D}$AR-Gαi_2YFP fusion protein complexed with endogenous Gβγ. Agonist bound to the receptor is denoted by an asterisk (*).

Figure 2. Agonist-induced regulation of an $\alpha_{2A/D}$AR-Gαi_2 fusion protein complexed with the GPR proteins AGS3 and AGS4. *A*, Left panel – HEK293 cells expressing a fixed amount of AGS3-Rluc (left) or AGS4-Rluc (right) and increasing amounts of $\alpha_{2A/D}$AR-Gαi_2YFP (squares) or $\alpha_{2A/D}$AR-Gαi_2YFP^{C352I} (triangles) were processed for BRET measurements as described in "Materials and Methods." Right panel - Lysates (50 μg) from control HEK293

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cells or HEK293 cells expressing $G\alpha i_2$, $G\alpha i_2$YFP, $\alpha_{2A/D}$AR-$G\alpha i_2$YFP or $\alpha_{2A/D}$AR-$G\alpha i_2$YFP^{C352I} (750 ng each plasmid) were subjected to sodium dodecyl sulfide – polyacrylamide gel electrophoresis (SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane and immunoblotted with GFP antiserum. *B*, HEK293 cells expressing AGS3-Rluc (left panel) or AGS4-Rluc (right panel) and $\alpha_{2A/D}$AR-$G\alpha i_2$YFP or $\alpha_{2A/D}$AR-$G\alpha i_2$YFP^{C352I} were incubated in the absence or presence of pertussis toxin (PT,100 ng/mL) for 18 hours as described in "Materials and Methods." Cells were then washed and incubated with vehicle (Tyrode's solution) or $\alpha_{2A/D}$AR agonist UK14304 (10 μM) for five minutes followed by fluorescence and luminescence readings to obtain net BRET signals as described in "Materials and Methods." *B* (Left panel) AGS3-Rluc relative luminescence units (RLU): AGS3-Rluc + $\alpha_{2A/D}$AR-$G\alpha i_2$YFP – 335,234 ± 9,929; AGS3-Rluc + $\alpha_{2A/D}$AR-$G\alpha i_2$YFP + PT – 327,626 ± 15,110; AGS3-Rluc + $\alpha_{2A/D}$AR-$G\alpha i_2$YFP^{C352I} – 385,996 ± 22,073; AGS3-Rluc + $\alpha_{2A/D}$AR-$G\alpha i_2$YFP^{C352I} + PT – 373,388 ± 17,790. Relative fluorescence units (RFU): $\alpha_{2A/D}$AR-$G\alpha i_2$YFP – 111,523 ± 3,246; $\alpha_{2A/D}$AR-$G\alpha i_2$YFP + PT – 112,991 ± 2,545; $\alpha_{2A/D}$AR-$G\alpha i_2$YFP^{C352I} – 110,420 ± 2,416; $\alpha_{2A/D}$AR-$G\alpha i_2$YFP^{C352I} + PT – 112,565 ± 3,072. *B (*right panel) AGS4-Rluc RLU: AGS4-Rluc + $\alpha_{2A/D}$AR-$G\alpha i_2$YFP – 87,143 ± 6,516; AGS4-Rluc + $\alpha_{2A/D}$AR-$G\alpha i_2$YFP + PT – 71,193 ± 5,723; AGS4-Rluc + $\alpha_{2A/D}$AR-$G\alpha i_2$YFP^{C352I} – 148,939 ± 7,362; AGS4-Rluc + $\alpha_{2A/D}$AR-$G\alpha i_2$YFP^{C352I} + PT – 133,482 ± 11,038. RFU: $\alpha_{2A/D}$AR-$G\alpha i_2$YFP – 106,882 ± 5,325; $\alpha_{2A/D}$AR-$G\alpha i_2$YFP + PT – 109,976 ± 5,497; $\alpha_{2A/D}$AR-$G\alpha i_2$YFP^{C352I} – 142,380 ± 2,980; $\alpha_{2A/D}$AR-$G\alpha i_2$YFP^{C352I} + PT – 166,057 ± 8,005. All BRET data are expressed as means ± S.E. from at least 3 independent experiments with triplicate determinations and immunoblots are representative of three independent experiments. *, $p < 0.05$ compared with vehicle treated control group.

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Figure 3. Agonist-induced changes in GPR protein distribution. AGS3Rluc (A, B) or AGS4Rluc (C, D) and $\alpha_{2A/D}$AR-Gαi_2YFP were expressed in HEK293 cells as described in "Materials and Methods." Cells were incubated with vehicle (Tyrode's solution) or UK14304 (10 μM final concentration) for 5 minutes followed by hypotonic lysis and AGS3Rluc (A) or AGS4Rluc (C) relative luminescence units (RLU) and $\alpha_{2A/D}$AR-Gαi_2YFP relative fluorescence units (RFU, B, D) were measured in supernatant (S) and pellet (P) fractions representing crude cytosol and membrane fractions, respectively. *, $p < 0.05$ for UK14304-treated samples compared to vehicle.

Figure 4. Co-immunoprecipitation of the 7TMR – Gαi – GPR complex is regulated by agonist. Left panel – HEK293 cells expressing α_{2A}AR-Gαi_2YFP and AGS3Rluc for 24 h were treated with α_2AR agonist UK14304 at a final concentration of 10 μM or with vehicle (Tyrode's solution) for 5 min at room temperature as described in "Materials and Methods." Cell pellets were sonicated in IP buffer and cell membranes extracted with 2% DβM followed by immunoprecipitation with anti-GFP-Sepharose overnight at 4°C. Immunoprecipitates were washed and resolved by SDS-PAGE and immunoblotted with AGS3 antisera (upper panel) followed by stripping and re-probing with GFP antisera (lower panel) as described in "Materials and Methods." "Input" represents $1/20^{th}$ of the total volume of cellular lysate taken prior to immunoprecipitation. Right panel – densitometric analysis from the means of two independent immunoprecipitation experiments as shown in the left panel with pixel density set relative to the AGS3-WT vehicle-treated input.

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Figure 5. Influence of a Gβγ scavenger on the agonist-induced regulation of GαiGPR where Gαi is tethered to the receptor. *A*, Net BRET values obtained from HEK293 cells expressing AGS3-Rluc (left panel) or AGS4-Rluc (right panel) and $\alpha_{2A/D}$AR-Gαi_2YFP as described in Figure 2 and "Materials and Methods." Where indicated, cells also expressed GRK2-CT. Cells were incubated with vehicle (Tyrode's solution) or UK14304 (10 μM) for 5 minutes. For experiments involving PT, cells were incubated with PT (100 ng/mL) for 18 hours prior to agonist exposure. *A* (left panel): RLU: AGS3-Rluc – 195,791 ± 15,175; AGS3-Rluc + PT – 178,887 ± 24,596; AGS3-Rluc + GRK2-CT – 218,392 ± 12,663; AGS3-Rluc + GRK2-CT + PT – 220,238 ± 19,824. RFU: $\alpha_{2A/D}$AR-Gαi_2YFP – 110,414 ± 2,294; $\alpha_{2A/D}$AR-Gαi_2YFP + PT – 104,532 ± 2,263; $\alpha_{2A/D}$AR-Gαi_2YFP + GRK2-CT -106,967 ± 2,562; $\alpha_{2A/D}$AR-Gαi_2YFP + GRK2-CT + PT – 116,045 ± 3,266. *A* (right panel) RLU: AGS4-Rluc – 147,140 ± 7,740; AGS4-Rluc + PT – 150,290 ± 8,165; AGS4-Rluc + GRK2-CT – 155,576 ± 8,972; AGS4-Rluc + GRK2-CT + PT – 147,944 ± 10,565. RFU: $\alpha_{2A/D}$AR-Gαi_2YFP – 109,090 ± 2,942; $\alpha_{2A/D}$AR-Gαi_2YFP + PT – 112,983 ± 3,019; $\alpha_{2A/D}$AR-Gαi_2YFP + GRK2-CT – 124,288 ± 2,273; $\alpha_{2A/D}$AR-Gαi_2YFP + GRK2-CT + PT – 112,371 ± 2,189. *, $p < 0.05$ compared with vehicle treated control group. *B*, far left panel – Lysates (50 μg) from a representative experiment as described in *A* were subjected to SDS-PAGE and immunoblotting with GRK2 and β-actin antisera as indicated. *B*, left panel – HEK293 cells expressing AGS3-Rluc (10 ng plasmid) and $\alpha_{2A/D}$AR-Gαi_2YFP (250 ng plasmid) in the absence and presence of Gβ_1, Gγ_2 and/or GRK2-CT (500 ng each plasmid) as indicated were subjected to BRET measurements as described in "Experimental Procedures." *B*, right panel – Lysates (50 μg) from a representative experiment as described in the upper panel of *B* were subjected to SDS-PAGE and immunoblotting with GRK2 and β-actin antisera as indicated. *B* far right panel – HEK293 cells expressing AGS4-

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Rluc (2 ng plasmid) and $\alpha_{2A/D}$AR-Gαi_2YFP (250 ng plasmid) in the absence and presence of Gβ_1, Gγ_2 and/or GRK2-CT (500 ng each plasmid) as indicated for 48h were subjected to BRET measurements as described in "Materials and Methods." All BRET data are expressed as means ± S.E. from at least 3 independent experiments with triplicate determinations and immunoblots are a representative image of three independent experiments. *, $p < 0.001$ compared with control group. †, $p < 0.001$ compared with G$\beta_1\gamma_2$-expressing group.

Figure 1

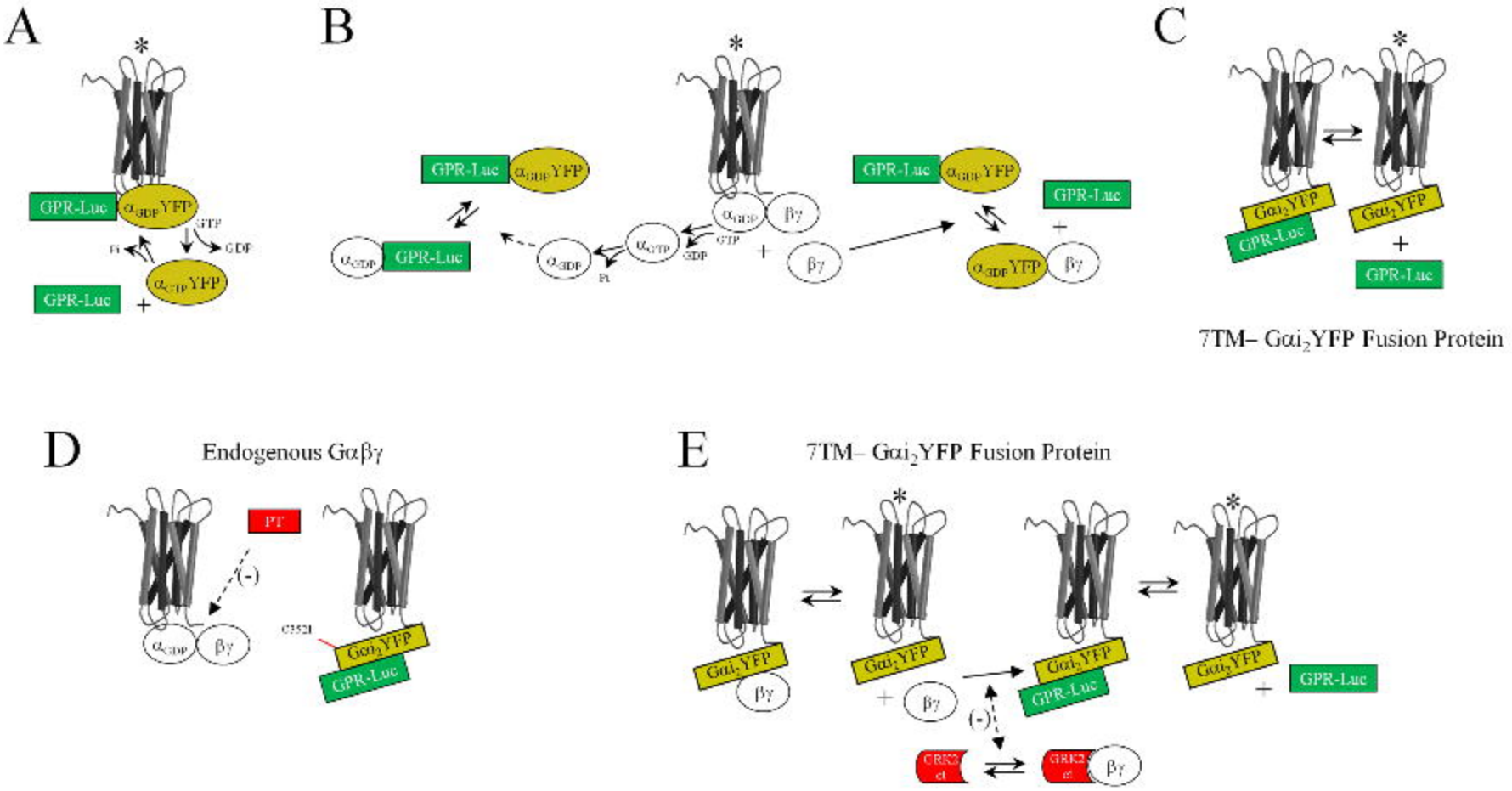
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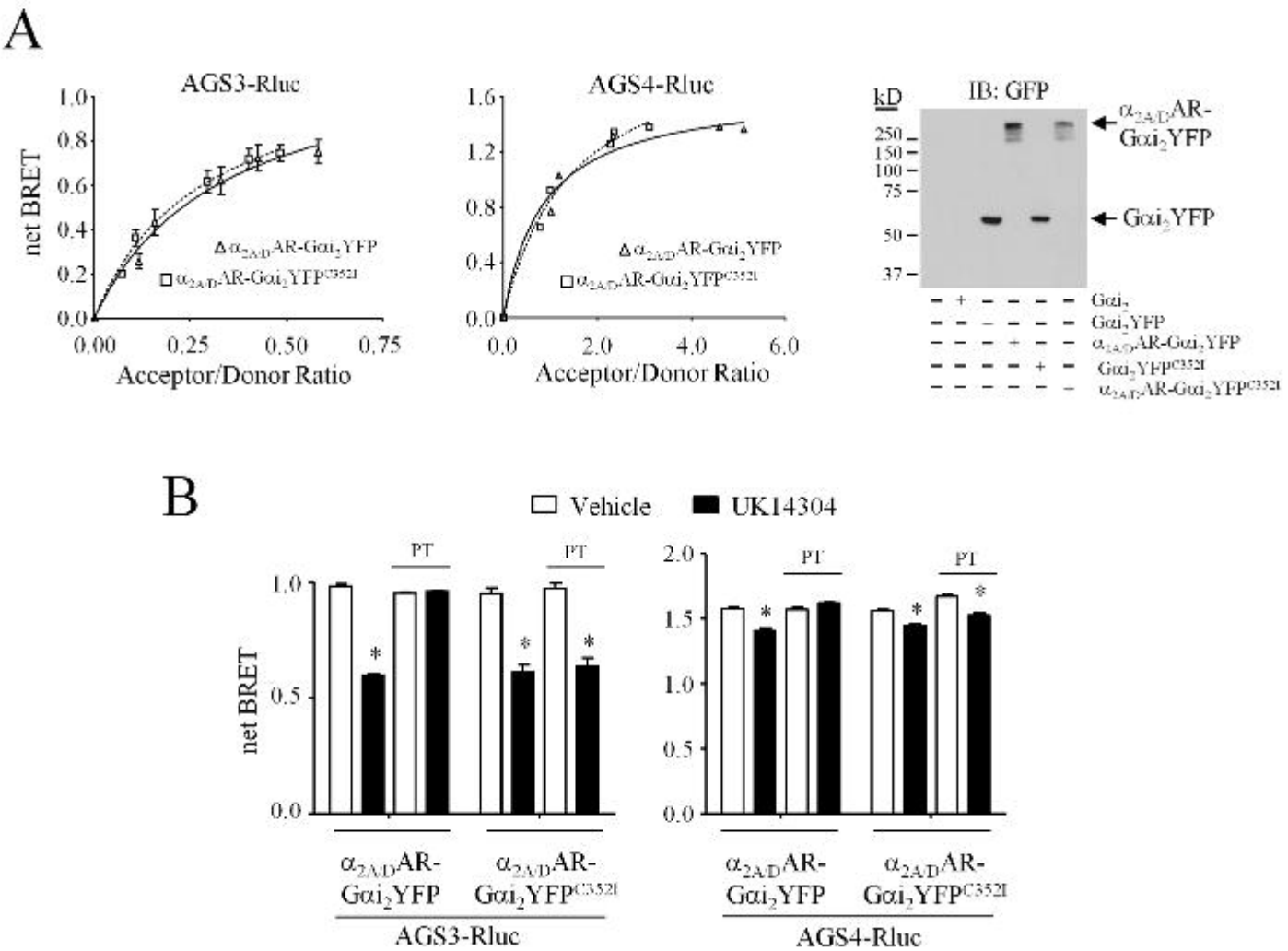
Figure 2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AGS4-Rluc
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IB: GFP
250
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100
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$\alpha_{2A/D}$AR-Gαi$_2$YFP
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Figure 3

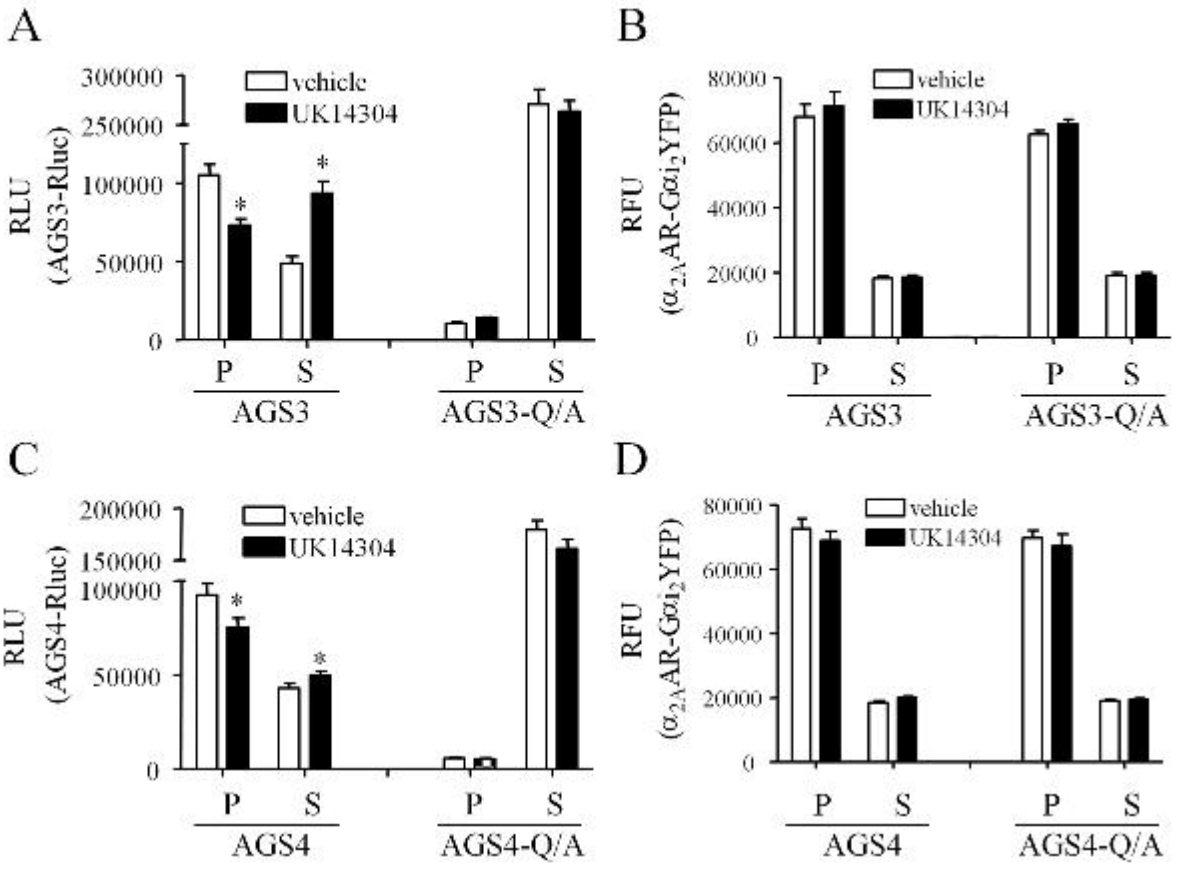
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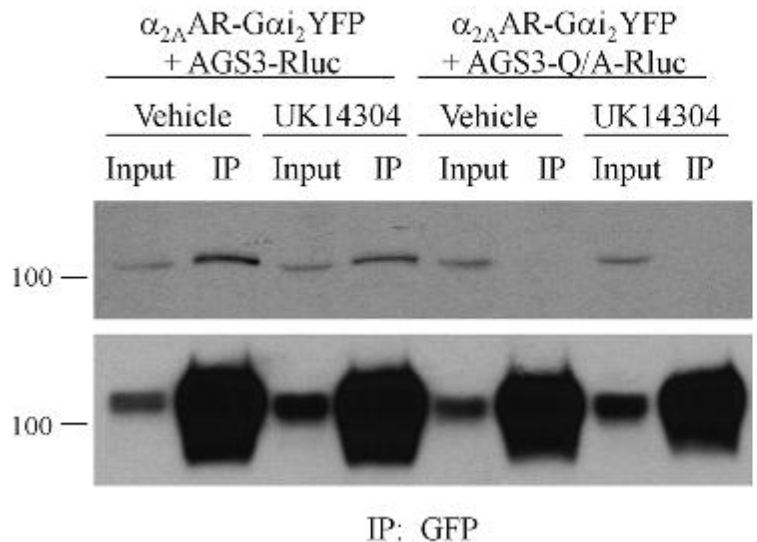
Figure 4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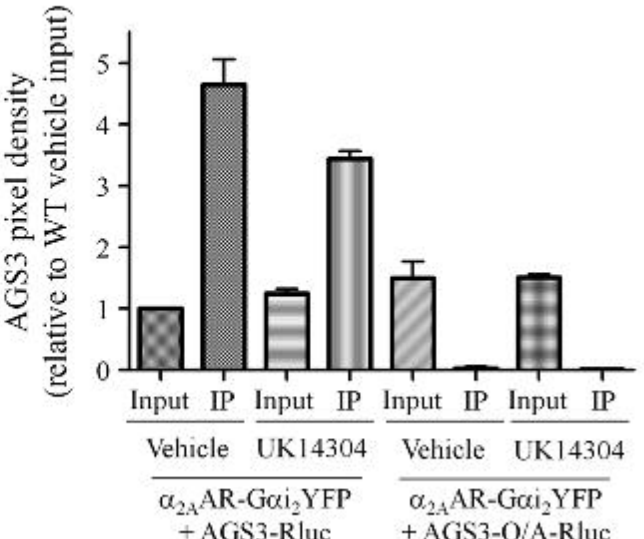
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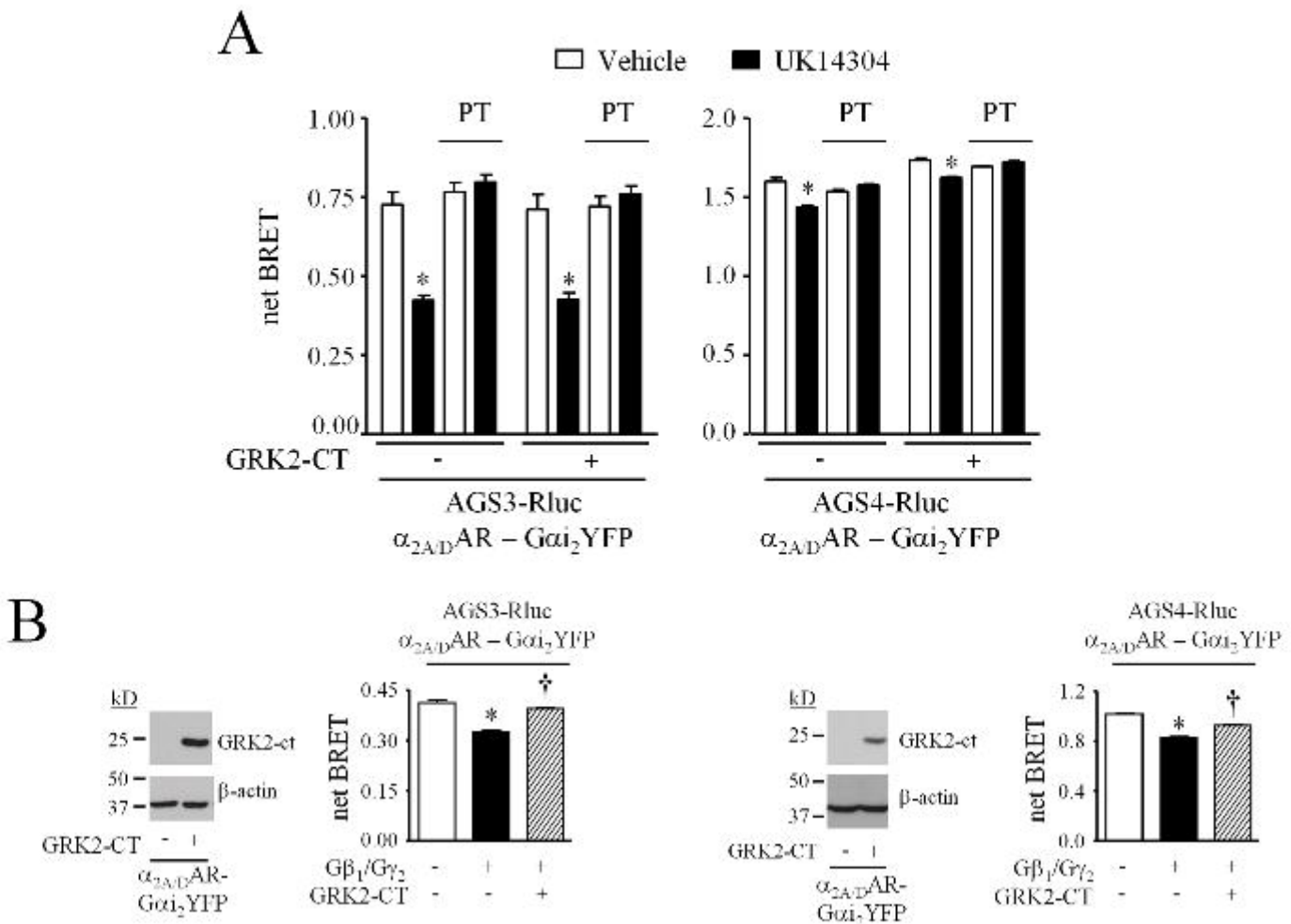
UK14304
α2AAR-Gαi2YFP
+ AGS3-Rluc
α2AAR-Gαi2YFP
+ AGS3-Q/A-Rluc

Figure 5


A
Vehicle
UK14304
PT
PT
PT
PT
net BRET
1.00
0.75
0.50
0.25
0.00
2.0
1.5
1.0
0.5
0.0
GRK2-CT
-
+
-
+
AGS3-Rluc
$\alpha_{2A/D}$AR – Gαi_2YFP
AGS4-Rluc
$\alpha_{2A/D}$AR – Gαi_2YFP
B
AGS3-Rluc
$\alpha_{2A/D}$AR – Gαi_2YFP
kD
25
50
37
GRK2-ct
β-actin
GRK2-CT
$\alpha_{2A/D}$AR-
Gαi_2YFP
net BRET
0.45
0.30
0.15
0.00
Gβ_1/Gγ_2
GRK2-CT
AGS4-Rluc
$\alpha_{2A/D}$AR – Gαi_2YFP
1.2
0.8
0.4
0.0