Agonist-induced formation of unproductive receptor-G_{12} complexes

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G proteins are activated when they associate with G protein-coupled receptors (GPCRs), often in response to agonist-mediated receptor activation. It is generally thought that agonist-induced receptor-G protein association necessarily promotes G protein activation and, conversely, that activated GPCRs do not interact with G proteins that they do not activate. Here we show that GPCRs can form agonist-dependent complexes with G proteins that do not activate. Using cell-based bioluminescence resonance energy transfer (BRET) and luminescence assays we find that vasopressin V_{2} receptors (V_{2}R) associate with both G_{i} and G_{12} heterotrimers when stimulated with the agonist arginine vasopressin (AVP). However, unlike V_{1A}-G_{i} complexes, V_{2}R-G_{12} complexes are not destabilized by guanine nucleotides and do not promote G_{12} activation. Activating V_{2}R does not lead to signaling responses downstream of G_{12} activation, but instead inhibits basal G_{12}-mediated signaling, presumably by sequestering G_{12} heterotrimers. Overexpressing G_{12} inhibits G protein receptor kinase (GRK) and arrestin recruitment to V_{2}R and receptor internalization. Formyl peptide (FPR1 and FPR2) and Smoothened (Smo) receptors also form complexes with G_{12} that are insensitive to nucleotides, suggesting that unproductive GPCR-G_{12} complexes are not unique to V_{2}R. These results indicate that agonist-dependent receptor-G protein association does not always lead to G protein activation and may in fact inhibit G protein activation.

Significance

G protein-coupled receptors (GPCRs) are targeted by a large fraction of approved drugs and regulate many important cellular processes. Association of GPCRs with heterotrimeric G proteins in response to agonist activation is thought to invariably lead to G protein activation. We find instead that G_{12} heterotrimers can associate with agonist-bound receptors in a manner that does not lead to activation. These unproductive agonist–receptor-G_{12} ternary complexes sequester G_{12} heterotrimers and thus inhibit rather than support G_{12} signaling. These findings reveal a mechanism whereby agonist activation of GPCRs can inhibit as well as promote G protein signaling.

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Because receptor-G protein complexes are transient it can be difficult to detect agonist-induced BRET signals between receptors and G proteins when guanine nucleotides are present (6). For example, arginine vasopressin (AVP) did not detectably increase BRET between vasopressin V2 receptors (V2R) and Gq heterotrimers in the presence of GDP (Fig. L4). In contrast, AVP produced large BRET increases in the absence of nucleotides (Fig. L4). Stabilization of agonist–receptor-G protein complexes when Gα subunits are nucleotide-free indicates conventional “productive” allosteric coupling and predicts that agonist-bound receptors will promote GDP release and G protein activation under physiological conditions. Nucleotide-free conditions also enhanced AVP-induced BRET between V2R and G12 or Gq heterotrimers (SI Appendix, Fig. S1). These results are consistent with cognate V2R activation of Gα and Gq heterotrimers (11, 12) and predict some ability to activate G12 heterotrimers. In contrast, we observed surprisingly robust agonist-induced BRET between V2R and G12 heterotrimers in the presence of GDP that was not enhanced by nucleotide depletion (Fig. L4). These results suggest that AVP-bound V2R can form complexes with GDP-bound G12 heterotrimers that do not progress to the nucleotide-free state and therefore are not stabilized when GDP is removed (Fig. L4B). As an index of allosteric coupling we divide the increase in BRET produced by agonist in the presence of GDP (ΔBRET+AVP) by the increase in BRET produced by the combined effect of agonist and nucleotide depletion (ΔBRETαβγapy), and refer to this index as the GDP-resistance ratio, or RGD. RGD values that are less than 1 indicate conventional productive coupling, whereas an RGD value of 1 indicates nucleotide-resistant or unproductive coupling. RGD for V2R and G12 was 0.08 ± 0.12 (mean ± SD; n = 6), whereas RGD for V2R and G12 was 1.01 ± 0.06 (n = 6). In contrast to V2R, we observed more conventional productive coupling of both endothelin A (ETA) and thromboxane A2 (TP) receptors with G12 heterotrimers, with RGD values of 0.66 ± 0.09 (n = 3) and 0.55 ± 0.09 (n = 6), respectively. Both of these receptors also coupled productively with Gq heterotrimers, with RGD values of 0.29 ± 0.08 (n = 3) and 0.11 ± 0.07 (n = 6), respectively (SI Appendix, Fig. S2).

Receptors that couple to one member of a Gs subunit family can usually couple to other members of the same family. Therefore, we examined V2R coupling to G13 heterotrimers, the other member of the G1213 family (13). We found that stimulation with AVP increased BRET between V2R and G13 heterotrimers in the presence of GDP (SI Appendix, Fig. S3). However, we observed that both ETA and Vr receptors were able to associate with G12, the two receptors should compete for a common pool of heterotrimer activation by residual GTP (RGDP = 0.67 ± 0.08; n = 4), indicating productive coupling, consistent with weak V2R-mediated activation of G13 (14). Similar results were obtained with G13 heterotrimers and ETA (RGDP = 0.21 ± 0.03; n = 4) and TP (RGDP = 0.18 ± 0.01; n = 4) receptors (SI Appendix, Fig. S3).

Because nucleotide-resistant V2R-G12 association was unexpected we performed additional experiments to rule out the possibility that our standard BRET assay was simply detecting an agonist-induced change in V2R-RLuc8 conformation. We reasoned that if both ETA and Vr receptors were able to associate with G12, the two receptors should compete for a common pool of heterotrimers. Indeed, we found that stimulation of unlabeled ETA receptors inhibited AVP-induced BRET between V2R-RLuc8 and G12 heterotrimers in intact cells (SI Appendix, Fig. S4A). Conversely, stimulation of unlabeled Vr receptors inhibited endothelin-1–induced BRET between ETA-RLuc8 and G12 heterotrimers (SI Appendix, Fig. S4B). Second, we found that stimulation of unlabeled Vr receptors increased BRET between G13-RLuc8 and GpV-Venus in intact cells (SI Appendix, Fig. S5 A and B). This increase persisted in permeabilized cells that were treated with apyrase and supplemented with GDP±PS to prevent the possibility of heterotrimer activation by residual GTP (SI Appendix, Fig. S5B). This suggests that active Vr receptors may impose a conformational change in G13 heterotrimers that does not require GTP binding or G13 activation. In contrast, stimulation of unlabeled ETA and TP receptors decreased BRET between G13-RLuc8 and GpV-Venus, and these decreases were largely blocked in permeabilized cells when only GDP±PS was present (SI Appendix, Fig. S5C). Finally, we found that AVP increased luciferase complementation when a small fragment (SmBit) of Nanoluc was fused to Vr, and a large fragment of Nanoluc was fused to G12, and these proteins were coexpressed with unlabeled G13 and GpV (SI Appendix, Fig. S6). These results are consistent with AVP-induced association of Vr receptors and G12 heterotrimers.

Additional experiments revealed that V2R recruited Gs and G12 heterotrimers at similar rates (Fig. 1C) and that agonist-induced V2R-G12 complexes were equally stable in the presence of GDP or GTP (Fig. 1D). Stimulation of V2R with the agonist oxytocin produced similar responses to AVP, indicating...
that nucleotide-insensitive V2R-G12 interactions are not restricted to AVP (SI Appendix, Fig. S7A), and AVP-induced responses were inhibited by the antagonist mozapatan (SI Appendix, Fig. S7B).

**V2R Does Not Activate G12 Heterotrimers.** The above results suggested that AVP-stimulated V2R should not activate G12 heterotrimers. To test this prediction we turned to sensitive assays that monitor signaling downstream of G12 activation. We first examined translocation of full-length p115-RhoGEF and a fragment (amino acids 281–483) of PDZ-RhoGEF from the cytosol to the plasma membrane using bystander BRET assays (15, 16). These proteins bind to activated G12 subunits at the plasma membrane to regulate Rho GTPase activity and actin fiber formation (17, 18). ETA and TP receptors robustly recruited p115-RhoGEF to the plasma membrane in a G12-dependent manner (Fig. 2A). In contrast, stimulation of V2R receptors failed to recruit p115-RhoGEF and instead decreased the baseline abundance of this reporter at the plasma membrane (Fig. 2A). Similar results were obtained with V2R and TP receptors and PDZ-RhoGEF recruitment to the plasma membrane (Fig. 2B) and direct recruitment of the RGS homology domain (amino acids 1–246) of p115-RhoGEF, p115-RGS-GFP, to Gq12-RlucII (Fig. 2C). The AVP-induced decrease in p115-and PDZ-RhoGEF at the plasma membrane suggests that active V2R may sequester G12 heterotrimers, preventing activation by endogenous receptors. Consistent with this suggestion, we found that activation of V2R could significantly reduce p115-RhoGEF recruitment mediated by activation of ETA receptors (Fig. 2D). A second sensitive assay of G12 activity is gene transcription driven by activation of the serum response element (SRE) (19). Stimulation of V2R receptors failed to activate SRE-dependent gene transcription, whereas stimulation of both ETA and TP receptors could activate SRE in a G12-dependent manner (Fig. 2E). A similar trend was observed with transcription driven by nuclear factor kappa-light-chain enhancer of activated B cells (NFκB; Fig. 2F). These results demonstrate that active V2R receptors do not detectably activate G12 heterotrimers, even though the two proteins interact in an agonist-dependent manner.

**V2R Activates G12 Chimeras and Mutants.** Canonical GPCR-mediated activation of G proteins involves extension of the Ga subunit C terminus (helix 5; H5) into the active receptor core (20, 21). This region of Ga is necessary for productive coupling and is also a key determinant of receptor-G protein selectivity. Therefore, we hypothesized that changing the Gα12 C terminus with C-terminal peptides from other Ga subunits might allow productive coupling with V2R. Indeed, we found that G12 mutants bearing the last 10 amino acids of either Ga12 or Gaq (Fig. 3A) interacted with AVP-activated V2R in a GDP-sensitive manner; RGGDP values were significantly less than 1 for Gα12 and Gαq heterotrimers and were similar to RGGDP for Gα and Gq heterotrimers (Fig. 3B). Gα12 and Gαq12 chimeras also supported V2R-mediated translocation of p115-RhoGEF to the plasma membrane, consistent with productive coupling to these heterotrimers and activation of G12 signaling pathways (Fig. 3C). Conversely, we found that replacing the C-terminal peptides of either Gaq or Gaqα with that of Gα12 (Fig. 3D) dramatically increased RGGDP, indicating much less productive coupling to Gα12 and unproductive coupling to Gαq12 (Fig. 3B). We next made point mutations in the Gα12 C-terminus to introduce residues with properties shared by the corresponding residues in Gαq and Gα12 (Fig. 3A). We found that Gα12 mutants with a hydrophobic residue in the −1 position (Q381L and Q381V) still coupled unproductively with V2R (Fig. 3 D and E). In contrast, Gαq12 mutants with a tyrosine in the −4 position (I378Y) coupled productively with V2R; RGGDP was less than 1 (Fig. 3D), and I378Y supported V2R-mediated translocation of p115-RhoGEF (Fig. 3E). Similar weak but productive coupling to V2R was observed when Gα12 was simply truncated by a single amino acid (A1; Fig. 3 D and E). These results indicate that the Gα12 C terminus is required for unproductive coupling to active V2R. Together with the observation that subtle modifications of the Gα12 C terminus overcome the barrier to productive coupling, this result suggests that G12 heterotrimers are likely to interact with active V2R in a manner that is structurally similar to canonical GPCR-G protein complexes.

**The V2R-Gq11 Interaction Interferes with Other Transducers.** The robust agonist-induced BRET signal between V2R receptors and G12 heterotrimers in the presence of nucleotides suggested that this interaction might be stable enough to interfere with recruitment of other intracellular transducer molecules to V2R. As V2R receptors cannot activate Gα12 heterotrimers (11), we first asked how overexpressing G12 would influence activation of adenyl cyclase and cAMP accumulation. We found that overexpressing G12 resulted in modest inhibition of Gα12 activation, as
Fig. 3. Role of the G\textsubscript{12} C terminus in unproductive coupling with V\textsubscript{2}R. (A) Amino acid sequences of the distal C terminus of G\textsubscript{12} (green), G\textsubscript{i6} (blue), G\textsubscript{i4} (black), and the chimeras and mutants used in 
B–E. Boxes indicate residues whose properties are shared between G\textsubscript{12} and G\textsubscript{i6}, but not G\textsubscript{i4}. (B) GDP resistance \((R_{GDP})\) of interactions between V\textsubscript{2}R-Rluc8 and heterotrimers incorporating the indicated G\textsubscript{12} subunits; n.s., not significant; all other groups significantly different from one; \(P < 0.05\); one-sample \(t\) test; \(n = 4–10\). (C) Activation of V\textsubscript{2}R decreases bystander BRET between p115RhoGEF-Rluc8 and Venus-Kras when G\textsubscript{12} is expressed, but increases bystander BRET when G\textsubscript{i4} or G\textsubscript{i6} are expressed, indicating receptor-mediated activation of these chimeras; all groups were significantly different from mock-transfected controls (\(\cdot\)). \(P < 0.05\); one-way ANOVA (Dunnett’s test); \(n = 7\). (D) GDP resistance of interactions between V\textsubscript{2}R-Rluc8 and G\textsubscript{12} heterotrimers bearing the indicated point mutations; n.s., not significant; all other groups significantly different from one; \(P < 0.05\); one-sample \(t\) test; \(n = 3–14\). (E) Activation of V\textsubscript{2}R decreases p115RhoGEF-Rluc8 translocation when G\textsubscript{12} wt, Q381L, or Q381V are expressed, but increases translocation when G\textsubscript{i4} I378Y or \(\Delta 1\) are expressed; all groups were significantly different from mock-transfected controls (\(\cdot\)); \(P < 0.05\); one-way ANOVA (Dunnett’s test); \(n = 3–13\).

indicated by a G\textsubscript{s} biosensor (SI Appendix, Fig. S8A). Surprisingly, this did not lead to detectable inhibition of V\textsubscript{2}R-mediated cAMP accumulation, as indicated by two different cAMP sensors (SI Appendix, Fig. S8 B and C). Active V\textsubscript{2}R receptors are phosphorylated by G protein receptor kinases (GRKs), and phosphorylated V\textsubscript{2}R bind tightly to \(\beta\)-arrestins (22). Remarkably, we found that overexpressing G\textsubscript{i4} significantly reduced AVP-induced BRET between V\textsubscript{2}R-Rluc8 and \(\beta\)-arrestin2–Venus (Fig. 4 A and B). A much smaller but still significant reduction was observed after overexpressing G\textsubscript{i6} heterotrimers (Fig. 4 A and B). In contrast, overexpressing G\textsubscript{i12} did not significantly reduce \(\beta\)-arrestin2 recruitment to ET\(_{\text{A}}\), \(\beta\)-adrenergic, or angiotensin AT\(_{1}\) receptors (SI Appendix, Fig. S9). Because V\textsubscript{2}R–\(\beta\)-arrestin interactions are very stable and because phosphorylated V\textsubscript{2}R can accommodate G protein and arrestin binding simultaneously (23), we suspected that G\textsubscript{i12} overexpression was acting upstream of arrestin binding to inhibit V\textsubscript{2}R interactions with GRKs. Consistent with this hypothesis, we found that G\textsubscript{i12} overexpression greatly reduced the AVP-induced interaction of V\textsubscript{2}R and GRK2 (SI Appendix, Fig. S10). Because arrestin binding is critical for agonist-dependent V\textsubscript{2}R internalization (22) we then asked if G\textsubscript{i12} overexpression would inhibit receptor endocytosis. Indeed, overexpression of G\textsubscript{i12} but not G\textsubscript{i4} heterotrimers inhibited V\textsubscript{2}R trafficking from the plasma membrane to the endosomal compartment as assessed by enhanced bystander BRET (eBRET; Fig. 4 C and D). Conversely, there was a small but significant enhancement of V\textsubscript{2}R internalization in cells lacking G\textsubscript{i12} and G\textsubscript{i13} subunits (SI Appendix, Fig. S11).

Other Receptors Also Form Unproductive Complexes with G\textsubscript{12}. In the course of experiments examining coupling of multiple different GPCRs to G proteins we encountered three additional examples of receptors that interact with G\textsubscript{12} heterotrimers in a nucleotide-resistant, unproductive manner. Smoothened (Smo) displays constitutive activity when the sterol transporter Patched is inhibited by Hedgehog or is not present, as is the case in HEK 293 cells. Smo is known to couple to and activate G\textsubscript{12} heterotrimers (24). We found that unliganded Smo-Rluc8 did indeed interact with G\textsubscript{12} heterotrimers in BRET assays, and this interaction was inhibited by either the inverse agonist cyclopamine or GDP, indicative of productive coupling \((R_{GDP} = 0.19 \pm 0.10\); \(n = 3\)). In contrast, BRET between Smo-Rluc8 and G\textsubscript{i12} heterotrimers was inhibited by cyclopamine but not GDP, indicative of unproductive coupling \((R_{GDP} = 1.01 \pm 0.22\); \(n = 3\); Fig. 5A). Similarly, activation of formyl peptide 2 receptors (FPR2) with the agonist peptide WKYMVm (WKY) promoted productive coupling with G\textsubscript{i12} heterotrimers \((R_{GDP} = 0.30 \pm 0.07\); \(n = 4\)), but unproductive coupling with G\textsubscript{i12} heterotrimers \((R_{GDP} = 0.92 \pm 0.04\); \(n = 4\); Fig. 5B). Although neither of these two receptors is known to activate G\textsubscript{i12} we directly assessed activation of downstream G\textsubscript{12} signaling pathways by FPR2. As was the case with V\textsubscript{2}R, activation of FPR2 failed to recruit p115-RhoGEF to the plasma membrane and failed to activate SRE-dependent gene transcription (SI Appendix, Fig. S12). Formyl peptide 1 receptors (FPR1) are highly homologous with FPR2 (68% identical), and we found that FPR1 also coupled productively with G\textsubscript{i12} heterotrimers \((R_{GDP} = 0.26 \pm 0.01\); \(n = 3\)), but unproductively with G\textsubscript{i12} heterotrimers \((R_{GDP} = 0.96 \pm 0.02\); \(n = 3\)).

When we examined association of GPCRs with G proteins from all four G\textsubscript{a} subtype families, we found that highly GDP-resistant interactions \((R_{GDP} > 0.5)\) were restricted to G\textsubscript{i12} heterotrimers (Fig. 6). For 19 of the 20 interactions that were studied with G\textsubscript{a6}, G\textsubscript{i4}, and G\textsubscript{i3} heterotrimers, R\textsubscript{GDP} was <0.3, whereas this was the case for only two of the nine interactions we studied with G\textsubscript{i12} heterotrimers. These results suggest that receptor-G\textsubscript{i} complex may generally be more stable than other receptor-G protein complexes when G proteins are bound to GDP.
Fig. 4. Overexpression of G12 inhibits arrestin recruitment to V2R and receptor internalization. (A and B) Time course and concentration-dependence of BRET between V2R-Rluc8 and β-arrestin2–Venus in response to AVP (1 μM in A; mean ± SEM; n = 3). Overexpression of G12 but not Gs heterotrimers inhibits arrestin recruitment. (C and D) AVP-induced changes in BRET between V2R-RlucII and the plasma membrane marker rGFP-CAAX (C) and the endosome marker rGFP-FYVE (D), indicating trafficking of V2R-RlucII from the plasma membrane to endosomes (mean ± SEM; n = 6–8). Overexpression of G12, but not Gs heterotrimers inhibits AVP-induced internalization of V2R-RlucII.

### Discussion

Taken together, our results suggest that several GPCRs bind to G12 heterotrimers in an activation-dependent manner, but the resulting GPCR-G12 complexes are insensitive to guanine nucleotides. These interactions do not activate G12 signaling, but may instead have a negative effect on RhoGEF recruitment and signaling by sequestering G12, thus preventing activation by other receptors. These interactions may also interfere with recruitment of other intracellular transducers and thus change signaling or trafficking of receptors that recruit but fail to activate G12 heterotrimers. Whether or not these inhibitory effects occur under physiological conditions will depend on several factors, most notably the local abundance of G12 heterotrimers and the stoichiometry of receptors and intracellular transducers. The normal physiological role of the V2R is to enhance water reabsorption in the kidney by stimulating Gs, which ultimately leads to incorporation of aquaporin-2 water channels to the luminal surface of collecting duct cells (25). An inhibitory effect of V2R activation on G12 signaling could conceivably contribute to the physiological activity of this receptor, as Rho activity has been reported to act as an inhibitor of aquaporin transport (26). An inhibitory effect of the V2R-G12 interaction on arrestin recruitment could also play a regulatory role to limit receptor internalization. Although we found that G12 overexpression weakly inhibited V2R-mediated Gs activation when assessed using a direct Gs activation assay, we were surprised to find that this did not lead to a detectable decrease in cAMP accumulation. It is possible that GRK and arrestin recruitment are more sensitive to competition with G12 than cAMP accumulation because cAMP signals are amplified downstream of Gs. Similar observations have been made after expression of some intrabodies that recognize the active state of β2-adrenergic receptors (27). Further studies with native systems will be required to determine if unproductive GPCR-G12 association has physiological significance.

At present, our findings significantly change the current model of GPCR coupling by demonstrating robust agonist-induced receptor-G protein interactions that do not lead to nucleotide exchange and G protein activation. GPCRs are thought to have access to all G protein subtypes expressed in a given cell, but possible interactions with noncognate heterotrimers (defined as G proteins that cannot be activated by a given GPCR) have, with a few exceptions (28), been overlooked. It is commonly assumed that stable agonist-induced GPCR-G protein interactions are restricted to cognate G proteins and are associated with G protein activation. One implication of this idea is that the conventional selection process whereby receptors reject noncognate G proteins occurs at an early stage of receptor-G protein association, such that complexes with noncognate G proteins do not progress past weak and transient encounter complexes. This seems to be true in the majority of cases, as several previous studies using sensitive methods have shown that interactions between GPCRs and noncognate G proteins are usually undetectable (7, 29). In contrast, our results suggest that some receptors functionally reject G12 heterotrimers despite forming relatively stable GPCR-G12 complexes. It is thought that GPCR-G protein complexes evolve through multiple intermediate conformations prior to receptor-stimulated nucleotide release (30–34). It is possible that receptors such as V2R and FPR2 form similar intermediate complexes with G12 heterotrimers that are unusually stable (Fig. 1B) and are unable to promote the changes in G12 that lead to GDP release. Spontaneous GDP release from

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is necessary for unproductive complexes with V2R, implying that interaction wherein agonist-activated receptors bind to G12 heterotrimers are individually identified. Additional receptors are genes and ligands is provided in Gurevich (Vanderbilt University, Nashville, TN). V2R-SmBit and ETAR-SmBit and GRK2-Venus-Kras R587Q were generated by appending Venus fused to the C terminus of each receptor behind a GGRGGGGSG linker. Plasmids encoding hGRK2 from the former and GFP10 from the latter. hGRK2 was subsequently ligated in frame with pcDNA3.1/Hygro(+) RlucII db v.2 to produce a C-terminal RlucII construct. All plasmid constructs were verified by Sanger sequencing.

Cell Culture and Transfection. HEK 293 cells (ATCC) were propagated in plastic flasks and on 6-well plates according to the supplier’s protocol. HEK 293 cells with targeted deletion of GNAS and GNA1 (Gs, knockouts; GSKO), targeted deletion of GNAS, GNA1, GNAQ, GNA11, and GNA12 (G protein three family knockouts; 3GKO), and HEK 293 cells with additional targeted deletions to the GNA1, GNA2, GNA11, GNA12, GNA13, GNA15, and GNAO1 (G protein four family knockouts; 4GKO) were derived, authenticated, and propagated as previously described (9, 10). HEK 293 cells with additional targeted deletion of ARRB1 and ARRB2 (beta-arrestin knockouts; ARBBKO) were derived, authenticated, and propagated as previously described (37, 38). Cells were transfected in growth medium using linear PEI MAX (MW 40,000) at a nitrogen/phosphate ratio of 20 and were used for experiments 12–48 h later. Up to 3.0 μg of plasmid DNA was transfected in each well of a 6-well plate. For ebBRET experiments, up to 1.0 μg of plasmid DNA was transfected in suspension to a cell density of 350,000 cells/mL in white 96-well plates.

BRET and Luminescence Assays. Measurement of coupling between receptor and G protein in nucleotide-depleted cells. Cells were transfected with a GPCR-Rluc8 and Gα subunit pair, Venus-1–155-Gt27, Venus-155–239-Gp12, and pcDNA3.1(+ or PTX-S1 in a (1:3:1:1:1) ratio. Experiments with Gαi were conducted in 4GKO cells for Gαi cognate receptors and in 3GKO cells for all other receptors. Experiments with Gαi were conducted without PTX-S1; all other Gα subunits were cotransfected with PTX-S1. After a 48-h incubation, cells were washed twice with permeabilization buffer (KPS) containing 140 mM KCl, 10 mM NaCl, 1 mM MgCl2, 20 mM HEPES (pH 7.2); harvested by trituration; permeabilized in KPS buffer containing 10 μg mL−1 high-purity digitonin; and transferred to opaque black 96-well plate. Measurements were made from permeabilized cells supplemented either with 100 μM GDP or 2U mL−1 apyrase, in both cases with or without agonist (SI Appendix, Table S1). Luciferase complementation. Cells were transfected with a GPCR-SmBit, Gai, Gai, and pcDNA3.1(+) or PTX-S1 in a (1:5:4:1:2:3:3) ratio. After a 48-h incubation, cells were washed twice with permeabilization buffer (KPS) containing 140 mM KCl, 10 mM NaCl, 1 mM MgCl2, 20 mM HEPES (pH 7.2); harvested by trituration; permeabilized in KPS buffer containing 10 μg mL−1 high-purity digitonin; and transferred to opaque black 96-well plate. Measurements were made from permeabilized cells supplemented either with 100 μM GDP or 2U mL−1 apyrase.

G protein BRET conformational biosensor. HEK 293 cells were transfected with an untagged GPCR or pcDNA3.1(+) GPCR-Rluc8, Gαi1, and Venus-1–155-Gt27, Venus-155–239-Gp12, and PTX-S1 in a (10:1:2:2:4) ratio. After a 48-h incubation, cells were washed twice with DPBS, harvested by trituration, and transferred to opaque black 96-well plates.

Materials and Methods. Materials. Trypsin, DPBS, PBS, HBSS, FBS, MEM, DMEM, penicillin/streptomycin, and l-glutamine were from Gibco (ThermoFisher Scientific). Polyclonal antibodies were purchased from Polysciences, Inc. Some receptor ligands, luciferin-D, and forskolin were purchased from Cayman Chemical. The remaining receptor ligands, digitonin, apyrase, GDP, GTP, GDP, and GTPS were purchased from MilliporeSigma. Coelenterazine h and coelenterazine 400a were purchased from Nanolight Technologies. NanoGlo luciferase substrate was purchased from Promega.

Plasmid DNA Constructs. GPCRs plasmids were purchased from cdna.org (Bloomouth University) or were provided by Bryan Roth (PRESTO-Tango Kit #1000000068, Addgene). The V1R-Rluc8 plasmid was received as a gift from Kevin Pfleger (Harry Perkins Institute of Medical Research, Niedlands, Western Australia). A plasmid encoding β-arrestin2–Venus was a gift from Vsevolod Gurevich (Vanderbilt University, Nashville, TN). V1R-SmBit and ETAR-SmBit were generated by replacing the GPCR coding sequence in βAR-SmBit digested with EcoRI and NotI, which appended the SmBit peptide to the C terminus of each receptor behind a GGRGGGGSG linker. Plasmids encoding Gα1 subunits, Gβ12, and Gγ22 were purchased from cdna.org. Gαi1-Rluc8 was generated by inserting Rluc8 (flanked by GSGG linkers) between a residues N136 and K137 of Gαi1 using Quikchange mutagenesis. GRK2-Venus-Kras and GRK2-Venus-Kras R87Q were generated by appending Venus fusion to the last 25 amino acids of Kras to the C terminus of bovine GRK2 or GRK2 R87Q using Quikchange mutagenesis. Plasmids encoding the β1 subunit of pertussis toxin (PTX-S1) and LbG β12-Gy27 were kindly provided by Stephen R. Ikeda (NIAAA, Rockville, MD), and the Nlu-EPAC-VV plasmid was provided by Kirill Martere (JLU, FL). The Gloserson-22F cAMP plasmid (E2301) was obtained from Promega. Plasmids encoding Gα11, Venus-Kras, Venus-1–155-Gt27, and Venus-155–239-Gp12, GPCR-luciferase constructs, and p115RhoG-GEF-RLuc8 have been described previously (6, 15, 36). Plasmids encoding rGFP-CAAX, rGFP-FYVE, and Vγ-RlucII have been described previously (16). PDZ-RhoG-GEF-RlucII was generated by amplifying the cytosolic G12R13 interacting domain of PDZ-RhoG (aa 281–483) with linkerD (GIRLREALKLPAT) on its C terminus which was then cloned onto the N terminus of RlucII in pcDNA3.1/geo(+) by Gibson assembly. GRK2-RlucII D110A was generated by digesting hGRK2-FP10-D110A and pcDNA3.1/Hygro(+) FP10-RlucII db v.2 with NheI and HindIII to excise hGRK2 from the former and FP10 from the latter. hGRK2 was subsequently ligated in frame with pcDNA3.1/Hygro(+) RlucII db v.2 to produce a C-terminal RlucII construct. All plasmid constructs were verified by Sanger sequencing.
incubation for 1 d, transfected cells were harvested with 0.5 mM EDTA-containing DPBS, centrifuged and suspended in 4 mL of HBSS containing 0.01% BSA (fatty acid-free grade; SERVA) and 5 mM Hepes (pH 7.4) (assay buffer). The cell suspension was dispensed in a white 96-well plate at a volume of 80 μL per well and loaded with 20 μL of 50 μM coelenterazine (Carbosynth) diluted in the assay buffer. After 2 h incubation at room temperature, the plate was measured for baseline luminescence (Spectramax L, Molecular Devices), and 20 μL of titrated ligand (AVP) were manually added. The plate was immediately read at room temperature for the following 10 min at a measurement interval of 20 s with an accumulation time of 0.17 s per read. The luminescence counts over 5–10 min after ligand addition were averaged and normalized to the initial count. The fold changes were further normalized to that of vehicle-treated samples.

**Translocation of p115RhoGEF.** Cells were transfected with an untagged GPCR, Gs, Gβγ2, Gβγ1, p115RhoGEF-Rluc8, Venus-Kras, and PTX-S1 in a (2:1:2:4:1:6:2) ratio. After a 48-h incubation, cells were washed twice with DPBS, harvested by trituration, and transferred to opaque black 96-well plates.

**Translocation of PDZ-RhoGEF.** ΔβARR1/2 HEK 293 cells were transfected with either FLAG–VγR or HA–TrpX, Gβγ2-Rluc8, PDZ-RhoGEF-Rluc8 and gRFP-CAAX in a (8:4:1:2) ratio. After a 48-h incubation, cells were washed once with Tyrode’s buffer (140 mM NaCl, 2.7 mM KCl, 1 mM CaCl2, 12 mM NaHCO3, 5.6 mM o-glucose, 0.5 mM MgCl2, 0.37 mM NaH2PO4, 25 mM Hepes [pH 7.4]) and maintained in the same buffer. Cells were stimulated with 5 μM agonist for 3 min. A BioLuminescence reader was used to perform the experiment.

**p115-RGS-GFP biosensor to monitor Gαi/i activity.** A BRET-based biosensor composed of RGS homology (RH) domain (amino acids 1–246) of p115RhoGEF fused to GFP10 was used to measure Gαi/i activity. HEK 293 cells were transfected with 40 ng of Gα12-15-RlucII, 500 ng of p115-RGS-GFP, and 300 ng of gRFP receptor of per row of a 96-well plate. BRET was monitored 2 min from agonist addition.

**SRE transcriptional reporter assay.** Cells were transfected with a GPCR, Gαs subunit, SRE3, and PTX-S1 in a (10:1:100:25) ratio. Medium was exchanged to serum-free 2 h after transfection. After a 24-h incubation, cells were treated with or without agonist for 5 h. Cells were washed twice with DPBS, harvested by trituration, centrifuged at 500 × g for 3 min, and resuspended in equilibration buffer (1x HBSS, 20 mM NaHEPES, pH 7.5) supplemented with 10% BSA by volume. Cells were pelleted by centrifugation. Cells were equilibrated in this solution at room temperature for 30 min and transferred to opaque white 96-well plates.

**NFR8 transcriptional reporter assays.** Cells were transfected with a GPCR, Gs subunit, NFR8-Luc, and empty vector in a (300:1:300:199) ratio. After a 24-h incubation, cells were treated with or without agonist for 5 h. Cells were washed twice with DPBS, harvested by trituration, centrifuged at 500 × g for 3 min, and resuspended in equilibration buffer (1x HBSS, 20 mM NaHEPES, 0.1% wt/vol BSA, pH 7.5) and transferred into 96-well black/white isoplates (Perkin-Elmer). Cells were incubated with 2 mM D-luciferin for 30 min before reading luminescence emission at 525 nm after 30 min of incubation using a PHARASTAR FS (BMG LABTECH).

**Niuc-EPAC-VC AMP assay.** Cells were transfected with a pcDNA3.1(+), a GPCR, Gαs subunit or pcDNA3.1(+), Gβγ2, Gβγ1, and Niuc-EPAC-VC in a (39:15.5:15:10:10:1) ratio. After a 24-h incubation, cells were washed twice with DPBS, harvested by trituration, and transferred to opaque black 96-well plates.

**Glosensor CAMP assay.** GSKO cells were transfected with a GPCR, Gαs subunit, Gβγ2, Gβγ1, Glosensor 22F, and either pcDNA3.1(+) or PTX-S1 in a (1:1:1:1:4:1) ratio. After a 24-h incubation, cells were washed twice with DPBS and treated with trypsin-EDTA (0.05%). Detached cells were harvested and centrifuged at 250 × g for 5 min, and the cell pellet was resuspended in equilibration buffer supplemented with 10% FBS by volume and 2 mM o-luciferin. Cells were incubated at room temperature for 1 h and then distributed to opaque white 96-well plates. Luminescence measurements were made from cells treated with vehicle, agonist, or 100 μM forskolin.

**Arrestin recruitment.** HEK 293 cells were transfected with a GPCR-Rluc8, Gs, Gβγ2, Gβγ1, and j-aretin2-2-Venus in a (1:2:1.1:1) ratio. After a 24-h incubation, cells were washed twice with DPBS, harvested by trituration, and transferred to opaque black 96-well plates.

**Bystander BRET VγR trafficking.** HEK 293 cells were transfected with VγR-Rluc8, Gs, and either gRFP-CAAX or gRFP-FYVE in a (1:2:40) ratio. After a 48-h incubation, cells were washed once with Tyrode’s buffer and maintained in the same buffer. Cells were stimulated for 30 min with agonist before BRET measurements.

**HiBiT-based VγR internalization.** Parental HEK 293 cells, G12[G]i2,-deficient HEK 293 cells (39), or β-aretin2-12-deficient HEK 293 cells (37) in growth phase were seeded in a 6-well culture plate at a concentration of 2 × 104 cells mL−1. Cells were transfected with 100 ng of HiBiT-VγR, which contained an Interleukin 6-derived signal sequence followed by a HiBiT sequence and a linker at the N terminus (MSNSFTSFAPGVPASGLLLVLPAPAIVPGWRLFKKSGSSGGSG; green-synthesized with codon optimization) and an unintended SmBiT tag at the C terminus. After 1 d, cells were harvested, suspended in 1 mL of assay buffer, dispensed in a white 96-well half-area plate at a volume of 25 μL per well, and mixed with 25 μL of 2x substrate buffer consisting of 1:200 of a LgBiT stock solution (Promega) and 20 μM furimazine in the assay buffer. After 40 min at room temperature, the plate was measured for baseline luminescence, and a titrated ligand (10 μL) diluted in the 1x substrate buffer was manually added. The plate was immediately read at room temperature for the following 30 min at a measurement interval of 30 s with an accumulation time of 0.4 s per read. The luminescence counts over 27–30 min after ligand addition were averaged and normalized to the initial count.

**GREK2 recruitment.** For the experiments shown in **SI Appendix, Fig. S10A, ΔβARR1/2 HEK 293 cells were transfected with FLAG–VγR, GREK2-12-D110A, Gβγ2, and gRFP-CAAX in a (1:2:1:2:6) ratio in suspension and distributed into white 96-well plates. After a 48-h incubation, cells were washed once with Tyrode’s buffer and maintained in the same buffer. Cells were stimulated with agonist immediately after addition of coelenterazine 400a. For the experiments shown in **SI Appendix, Fig. S10B, ARRKO cells were transfected with a GPCR-Rluc8, Gs subunit, Gβγ2, Gβγ1, and GSK2-Venus in a (1:1:1:1:3) ratio. After a 48-h incubation, cells were washed twice with DPBS, harvested by trituration, centrifuged at 250 × g for 5 min, and resuspended in equilibration buffer (1x HBSS, 20 mM NaHEPES, pH 7.5) supplemented with 10% BSA by volume and 2 mM o-luciferin. Cells were pelleted by centrifugation. Cells were equilibrated in this solution at room temperature for 30 min and transferred to opaque 96-well plates.

**BRET, luminescence measurements.** Steady-state BRET and luminescence measurements were made using a Mithras LB940 photon-counting plate reader (Berthold Technologies GmbH). Kinetic BRET and luminescence time course measurements were made using a Polarstar Optima plate reader (BMG Labtech). Coelenterazine h (5 μM; Nanolight) or furimazine (Nanoligo; 1:100, Promega) were added to all wells immediately prior to making measurements with Rluc8 and Niuc, respectively. Raw BRET signals were calculated as the raw BRET signal minus the raw BRET signal measured from cells expressing only the Rluc8 donor.

**Data Availability.** All study data are included in the article and **SI Appendix**.

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