Priming GPCR signaling through the synergistic effect of two G proteins

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Although individual G-protein–coupled receptors (GPCRs) are known to activate one or more G proteins, the GPCR–G-protein interaction is viewed as a bimolecular event involving the formation of a ternary ligand–GPCR–G-protein complex. Here, we present evidence that individual GPCR–G-protein interactions can reinforce each other to enhance signaling through canonical downstream second messengers, a phenomenon we term "GPCR priming." Specifically, we find that the presence of noncognate Gq protein enhances cAMP stimulated by two Gs-coupled receptors, β2-adrenergic receptor (β2-AR) and D_1 dopamine receptor (D_1-R). Reciprocally, Gs enhances IP_1 through vasopressin receptor (V_{1A}-R) but not α1 adrenergic receptor (α1-AR), suggesting that GPCR priming is a receptor-specific phenomenon. The C terminus of either the Gαs or Gαq subunit is sufficient to enhance Gα subunit activation and cAMP levels. Interaction of Gαs or Gαq C termini with the GPCR increases signaling potency, suggesting an altered GPCR conformation as the underlying basis for GPCR priming. We propose three parallel mechanisms involving (*i*) sequential G-protein interactions at the cognate site, (*ii*) G-protein interactions at distinct allosteric and cognate sites on the GPCR, and (*iii*) asymmetric GPCR dimers. GPCR priming suggests another layer of regulation in the classic GPCR ternary-complex model, with broad implications for the multiplicity inherent in signaling networks.

GPCR | G protein | cell signaling | ER/K linker | GPCR priming

The G-protein–coupled receptor (GPCR)–G-protein interaction is primarily viewed from the perspective of forming a ternary complex between ligand, GPCR, and cognate G protein (1). Interactions with noncognate G proteins have recently gained significance in the context of functional selectivity, wherein ligands can differentially activate distinct G proteins (2). However, the functional consequences of GPCR–G-protein interactions that do not precipitate G-protein activation remain unappreciated (3). Noncognate interactions, if short-lived, may in fact have no impact on the cognate interaction. Nonetheless, given the emerging conformational heterogeneity of ligand-bound GPCRs (4, 5), noncognate interactions may influence the GPCR conformational landscape with possible consequences for downstream signaling. The cocrystal structure of the GPCR–G-protein interface (6) suggests a 1:1 stoichiometry of this protein interaction. A single, cognate binding site on the GPCR for the G protein implies that long-lived noncognate interactions may competitively suppress canonical signaling. However, a recent study (7) argues for the simultaneous binding of two effectors (G protein and β-arrestin) at distinct sites on the GPCR, leading to a supercomplex that enhances the signaling properties of the GPCR.

The response downstream of a GPCR is strongly dependent on physiological context (8). Expression of receptor isoforms with distinct signaling profiles, relative abundance of GPCRs and G-protein subtypes, and sharing of G-protein pools among receptors are just some of the factors that govern cell type-specific responses (8, 9). The molecular mechanisms underlying cellular GPCR signaling multiplicity remain an outstanding challenge. GPCR–G-protein fusions have been successfully used to compare signaling downstream of distinct GPCR–G-protein interactions. By regulating the stoichiometry of the interaction, these direct GPCR–G-protein fusions have elucidated structural determinants and kinetics of GPCR–G-protein interactions (10). The signaling properties of β2-AR fused to distinct Gα subunits also provided early insights into the multiplicity of GPCR conformations (10). However, in some cases, fusion between GPCR and G proteins show counterintuitive downstream responses. For instance, increased adenylate cyclase activity of a β2-AR–Gαi fusion (11) was interpreted as a consequence of constrained mobility between the receptor and the Gα subunit, impinging on downstream effectors. In this study, we revisit noncognate GPCR–G-protein interactions using a distinct fusion approach. This approach termed systematic protein affinity strength modulation (SPASM) uses an ER/K single α-helical linker to tether the GPCR and the G protein. We have previously reported that tethering with an ER/K linker maintains the effective concentration of the interaction between the proteins at the ends (12). The longer length of the ER/K linker (10–30 nm), compared with direct fusions (<5 nm), is designed to provide 1:1 stoichiometry of the interaction with minimum steric hindrance and serves to modulate the existing bimolecular interactions, rather than enforcing them.

In this study, we use SPASM GPCR–G-protein sensors to understand the interplay between Gs and Gq interactions with signaling downstream of β2 and D_1-R. Given that the influence of noncognate G proteins is likely to be concentration dependent, we used the SPASM system in HEK293 cells to provide equal effective concentrations and to pairwise compare the downstream effects of cognate and noncognate interactions. Surprisingly, Gq enhances Gs activation and cAMP levels in response to agonist stimulation. The C terminus of either Gαq or Gαs is minimally sufficient to augment cAMP levels. We introduce the concept of "GPCR priming" to highlight the ability of noncognate GPCR–G-protein interactions to stimulate canonical signaling. Analysis of concentration–response curves using the operational model of agonism (13) reveals an increase in receptor potency as the underlying basis of GPCR priming.

Significance

In this study, we uncover a G-protein–coupled receptor (GPCR) priming mechanism that results from the synergistic effects of two distinct G proteins. Although recent structural and spectroscopic studies of GPCR structure reveal a broad receptor conformational landscape, G-protein activation and downstream signaling are still viewed through the lens of individual ternary complexes between ligand, receptor, and individual effectors. Instead, our findings suggest positive interference between otherwise-disparate signaling pathways that can impact both the potency of GPCR ligands and their cell type-specific responses.

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We propose three parallel "priming" mechanisms based on (*i*) sequential binding of noncognate and cognate G proteins to the GPCR at the cognate site, (*ii*) binding of noncognate and cognate G proteins to two distinct binding sites on the GPCR, and (*iii*) formation of asymmetric dimers between GPCRs bound to cognate and noncognate G protein, respectively.

Results

Noncognate Gαq Binds Weakly to β2-AR Compared with Cognate Gαs. Although noncognate interactions are typically not factored into the ternary-complex model, a systematic measurement of the relative binding affinity of a GPCR for both cognate and noncognate Gα subunits has not been performed. Hence, we used a quantitative coimmunoprecipitation assay to directly compare the relative binding strengths of Gαs and Gαq for β2-AR. The interaction of β2-AR with Gαq was found to be weaker than the interaction with Gαs (Fig. S1 *B–D*). Hence, ER/K-linked sensors (12) were used to fuse receptor and G protein, thereby engineering comparable stoichiometries and effective concentrations in live cells. Under these conditions, we could compare the outcome of the cognate and noncognate interactions with the receptor.

Noncognate G Proteins Augment Canonical Signaling for Select Receptors. To delineate the effects of noncognate interactions on downstream signaling, adrenergic receptor β2-AR and dopamine receptor D_1-R were used. Both receptors are Gs-coupled and stimulate cAMP responses via adenylate cyclase. Sensors were designed to tether either cognate Gαs or noncognate Gαq to chosen GPCRs via an ER/K linker of known length (Fig. 1*A*). The resultant sensors expressed in cells contained the following, from N to C terminus: GPCR, mCitrine, ER/K α-helix, mCerulean, and Gα subunit. Sensors that terminated in a Gly–Ser–Gly × 4 peptide, without the terminal Gα, are indicated by (−) and were used as controls throughout (Fig. 1*A*). Either Gα subunit tethered to the receptors was functional, as observed from increased Gβγ association with membranes from cells expressing the β2-AR–10 nm–Gαs and β2-AR–10 nm–Gαq sensors, compared with controls (Fig. 1*A*). Isoproterenol-stimulated cAMP response was measured in cells expressing the individual sensors and in untransfected cells. The sensors were expressed to equivalent levels as confirmed by mCitrine fluorescence, and comparable cell numbers were used based on absorbance at 600 nm (*Materials and Methods*). Control sensor-expressing cells exhibit a higher cAMP response than untransfected cells (Fig. 1*B*), suggesting that the β2-AR is functional in detecting and relaying isoproterenol stimulation. The β2-AR–10 nm–Gαs sensor exhibits an increase in the cAMP response over the control, suggesting that the tethered Gαs is functional (Fig. 1*B*). Surprisingly, tethering the noncognate Gαq to β2-AR causes a further increase in the cAMP response (Fig. 1*B*). Similarly, D_1-R–10 nm–Gαq sensor shows an increased cAMP response to dopamine stimulation (Fig. 1*C*). The phenomenon of the tethered noncognate G protein augmenting canonical signaling is hereon referred to as GPCR priming. Reciprocally, two Gq-coupled receptors, adrenergic receptor α1-AR and vasopressin receptor V_{1A}-R were used (Fig. S2). Of these receptors V_{1A}-R, but not α1-AR, exhibited an augmented IP_1 response when tethered to the noncognate Gs, compared with cognate Gq (Fig. S2 *B* and *C*). These results suggest that GPCR priming is a receptor-specific phenomenon.

Increasing the ER/K Linker Length Reduces GPCR Priming. To test whether GPCR priming stems from an interaction between GPCR and the tethered Gα protein, the length of the linker connecting β2-AR to the Gα subunit was increased systematically from 10 to 20 and 30 nm (Fig. 1*D*). Isoproterenol-stimulated cAMP response was measured in cells expressing these sensors and compared with the response from control sensors. Equivalent expression and similar cell numbers were confirmed as described earlier. Increasing ER/K linker length systematically decreased cAMP response for β2-AR–Gαs (Fig. 1*E*). This is consistent with a functional interaction between β2-AR and the tethered Gαs subunit. Similarly, increasing ER/K linker length

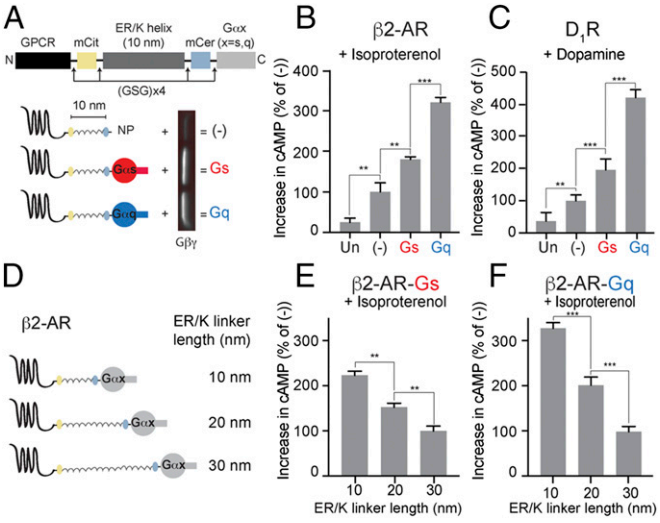

Fig. 1. Effect of tethered Gα subunits on signaling via Gs-coupled receptors. (*A*) Schematics of GPCR G-protein sensors used here. The GPCR (β2-AR or D_1-R), mCitrine, 10-nm ER/K linker, mCerulean, and Gα subunit (Gαs, red, or Gαq, blue) are expressed as a single polypeptide, separated from each other by Gly–Ser–Gly (GSG) × 4 linkers. Sensors that terminated at a Gly–Ser–Gly × 4 peptide without Gα (NP, no peptide at the end) are indicated as (–) and were used as controls. Western blot of membranes purified from sensor-expressing cells, probed with Gβ antibody, reveal interaction of tethered Gα subunits with endogenous Gβγ. (*B* and *C*) Increase in cAMP levels between buffer-treated and agonist-treated (*B*, 10 μM isoproterenol; *C*, 10 μM dopamine) HEK293T cells expressing equivalent amount of GPCR G-protein sensors. Gαq tethered to the receptors via 10-nm ER/K linker exhibits the greatest increase in cAMP, a phenomenon we term GPCR priming. (*D*) Schematics of β2-AR sensors tethered to Gα subunits through ER/K linker length varied sequentially from 10 to 30 nm. (*E* and *F*) Effect of linker length used for tethering Gα subunit to β2-AR on cAMP levels between isoproterenol (10 μM) and buffer-treated HEK293T cells expressing equivalent levels of sensors. (*B*, *C*, *E*, and *F*) Values are mean ± SEM from $n \geq 10$ observations over at least three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.005$ by unpaired *t* test.

systematically decreased cAMP response for β2-AR–Gαq (Fig. 1*F*). This indicates that GPCR priming arises due to an interaction between β2-AR and the tethered Gαq subunit.

Canonical Pathways Downstream of the Noncognate G Protein Are Not Measurably Activated During Priming. There is a possibility that effectors downstream of tethered Gαq could influence adenylate cyclase activity, leading to observed effects on cAMP (14). To investigate this possibility, canonical signaling via the Gαq–PLC pathway was monitored by measuring IP_1 levels. IP_1 responses following phenylephrine stimulation of α1-AR sensors (Fig. S2*A*) were used as references (Fig. S2*B*). Cells expressing α1-AR control sensor exhibit an increase in IP_1 response compared with untransfected cells, indicating functionality of α1-AR sensors. α1-AR–10 nm–Gαq-expressing cells exhibit a further increase in IP_1, indicating that the tethered Gq is a signaling-competent entity. However, cells expressing β2-AR–10 nm–Gαq exhibit no measurable increase in IP_1 levels following isoproterenol stimulation (Fig. S2*B*). Because there is no measurable activation of Gq following isoproterenol stimulation of the β2-AR–Gαq sensor, effectors downstream of Gαq are unlikely to contribute to β2-AR priming. This further supports a role for interaction of the tethered Gαq with the β2-AR in priming.

GPCR Priming Is Not Affected by the Cytoplasmic Tail of the Receptor or Membrane Microdomain Organization. Different ligands are known to trigger distinct signaling outcomes via the same GPCR. This functional selectivity has been partially attributed to the ability of the C-terminal cytoplasmic tail region of GPCRs to function as a scaffold for effectors like PKA (15) and β-arrestin

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(16). To test whether the tethered Gαq subunit exerted its effect on GPCR priming via scaffolded effectors, β2-AR was truncated at position 350 to remove the tail domain that causes scaffolding. Sensors in which truncated β2-AR was tethered to Gαq continued to display GPCR priming following isoproterenol stimulation (Fig. S3*A*). This suggests that scaffolding activity of β2-AR, as well as interactions with scaffolded effectors, are dispensable for GPCR priming. There is evidence that β2-AR, Gs, and adenylate cyclase colocalize with caveolae in the plasma membrane (17, 18). This colocalization is proposed to assist signal transduction. To test whether the caveolar organization is important for GPCR priming, caveolae were disrupted by filipin treatment (17). Cells expressing the β2-AR–10 nm–Gαq sensor displayed a similar extent of GPCR priming even on treatment with filipin. (Fig. S3*B*). Hence, GPCR priming does not appear to originate from the induced proximity of the tethered Gαq to downstream signaling components in caveolae, further strengthening a direct role for GPCR–Gα-protein interaction in GPCR priming.

The C-terminal α5 Helix of the Gα Protein Is Minimally Sufficient to Cause Priming. The data imply that direct interaction between tethered Gαq and β2-AR/D_1-R causes GPCR priming. It is known that the α5 helix from Gα C terminus interacts with β2-AR (19). To test whether the same α5 peptide plays a role in GPCR priming, β2-AR or D_1-R were tethered via 10-nm ER/K linker to the α5 peptide derived either from Gαs (s-pep) or from Gαq (q-pep) (Fig. 2*A*). Isoproterenol-stimulated cAMP response was measured in cells expressing these peptide sensors to equivalent levels. Control sensor-expressing cells are found to exhibit a higher cAMP response than untransfected cells (Fig. 1*B*). The β2-AR–s-pep sensor exhibited an increase in the cAMP response over the control (Fig. 2*B*). β2-AR–q-pep sensor caused a further increase in cAMP (Fig. 2*B*). Thus, specific interaction with the tethered α5 peptide is sufficient for GPCR priming. GPCR priming is also observed upon dopamine stimulation of D_1-R–s-pep and D_1-R–q-pep sensors (Fig. 2*C*).The difference in magnitude of GPCR priming between s-pep and q-pep sensors for the same GPCR suggested a role for the sequence of the tethered C terminus peptide in this phenomenon. To test the sequence dependence, a scrambled sequence of the s-pep was tethered to β2-AR. Isoproterenol stimulation of the resultant β2-AR–scram sensor (Fig. 2*A*) led to an increase in cAMP response compared with controls. However, the magnitude of the increase was less than that caused by the β2-AR–s-pep sensor (Fig. 2*B*). Thus, sequence-specific interactions between the tethered α5 peptide and β2-AR mediate GPCR priming.

Dependence of GPCR priming on the sequence of the tethered C-terminal peptide was additionally tested using a chimera. The C terminus α5 peptide of a Gαs subunit was substituted by the corresponding peptide from Gαq, resulting in a chimeric protein designated Gαs/q. Chimeric Gαs/q bound to BODIPY-FL–GTPγS with similar efficiency as Gαs, indicating that the chimeric Gαs/q was functional (Fig. 2*E*). The chimeric Gαs/q was tethered to β2-AR generating a β2-AR–Gαs/q sensor (Fig. 2*D*), which exhibited GPCR priming compared with β2-AR–Gαs (Fig. 2*F*). This strongly supports the interpretation that sequence of the tethered α5 peptide determines the magnitude of GPCR priming. Simultaneously, the signaling profile of events downstream from the receptor does not change due to priming.

GPCR Priming Can Be Reconstituted in Vitro. To address the possibility that GPCR priming is an artifact of the tethered nature of sensors, a reconstitution approach was used (Fig. 3*A*). Concomitantly, the influence of the ER/K linker on GPCR priming was also tested (Fig. S4*A*). To this end, the ligand-dependent increase in the fluorescence of BODIPY-FL–GTPγS was monitored in a reaction containing GPCRs in a urea-treated membrane and exogenously added G proteins (*Materials and Methods*). Upon fenoterol stimulation of β2-AR control sensor-containing membranes (Fig. 3*B*), the reaction containing Gαq did not show an increase in fluorescence, as is expected for the Gs-coupled β2-AR. Simultaneously, Gαs showed an increase in fluorescence, indicating that the

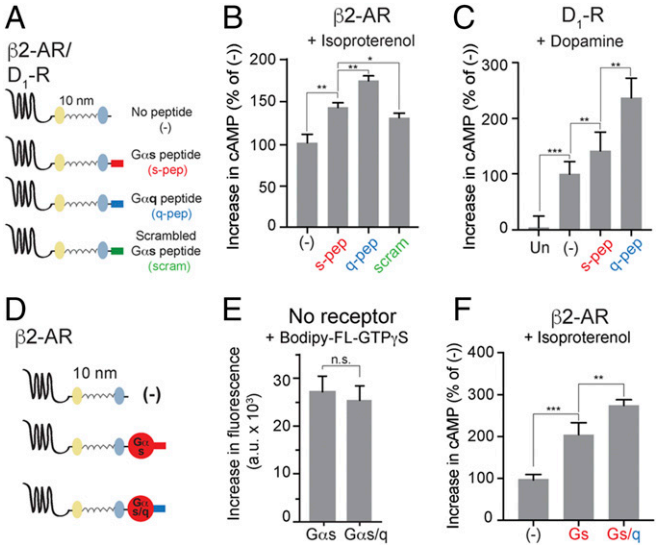


Fig. 2. Tethered Gα C terminus peptide is sufficient for GPCR priming. (*A*) Schematics of the peptide sensors used. The GPCR (β2-AR or D1-R), mCitrine, 10-nm ER/K linker, mCerulean, and α5 peptide from the Gα C terminus of either Gαs (s-pep, red) or Gαq (q-pep, blue) or scrambled sequence from Gαs peptide (scrambled, scram, green) are expressed as a single polypeptide, separated from each other by Gly–Ser–Gly (GSG) × 4 linkers. (*B* and *C*) Increase in cAMP levels between buffer-treated and agonist-treated (*B*, 10 μM isoproterenol; *C*, 10 μM dopamine) HEK293T cells expressing equivalent amount of peptide sensors. Tethered q-pep exhibits the greatest increase in cAMP. (*D*) Chimeric Gαs/q constructed by swapping the C terminus α5 peptide of Gαs with the corresponding peptide from Gαq. Comparison of sensors tethering β2-AR to Gαs and chimeric Gαs/q. (*E*) GTP-binding ability of Gαs and Gαs/q. Incorporation of BODIPY-FL–GTPγS into Gα subunits measured as an increase in fluorescence between BODIPY-FL–GTPγS alone, and BODIPY-FL–GTPγS with indicated Gα subunit. (*F*) Increase in cAMP levels between buffer-treated and isoproterenol-treated (10 μM) HEK293T cells expressing equivalent amount of indicated sensors. Tethered chimeric Gαs/q exhibits the greatest increase in cAMP. (*B*, *C*, and *F*) Values are mean ± SEM from $n \geq 10$ observations over at least three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ by unpaired *t* test.

β2-AR in urea-treated membranes was functionally active. Increasing the concentration of Gαs caused a further increase in fluorescence, consistent with canonical signaling downstream of β2-AR proceeding via Gαs. Stimulation of a mixture containing both Gαs and Gαq led to a synergistic increase in fluorescence, mimicking GPCR priming. Because Gαq showed minimal activation downstream of β2-AR, the synergism indicated that presence of Gαq greatly increased the activation of Gαs. Thus, GPCR priming can be reconstituted in vitro without tethering the Gα subunit to β2-AR. In agreement with the observations in live cells (Fig. 2*B*), exogenously added s-pep, in combination with Gαs, augmented the fluorescence increase over that observed with Gαs alone (Fig. 3*B*, dark bars). q-pep addition caused a further increase in fluorescence. Thus, the pattern of q-pep exhibiting a greater magnitude of GPCR priming than s-pep was also recapitulated in vitro. However, Gαq did not show an increase in fluorescence (Fig. 3*B*), even in presence of q-pep (data not shown, for clarity). A similar pattern of Gαs activation was observed when β2-AR–mCer-containing membranes were used (Fig. S4*B*). Results from the β2-AR–mCer fusion indicate that the ER/K linker does not influence GPCR priming. Together, our results suggest that interaction of the α5 peptide with β2-AR increases activation of cognate Gαs, contributing to GPCR priming.

Endogenous Gαs Is Required for GPCR Priming. Extrapolating the in vitro data (Fig. 3*B*) to previous results (Fig. 2*B*) suggested that GPCR priming would depend on endogenous Gαs. shRNA directed to Gαs caused a 53% reduction in endogenous Gαs protein, compared with cells with an empty vector (vector, Fig. 3*C*).

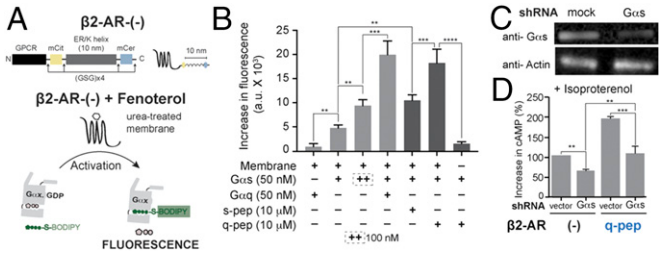


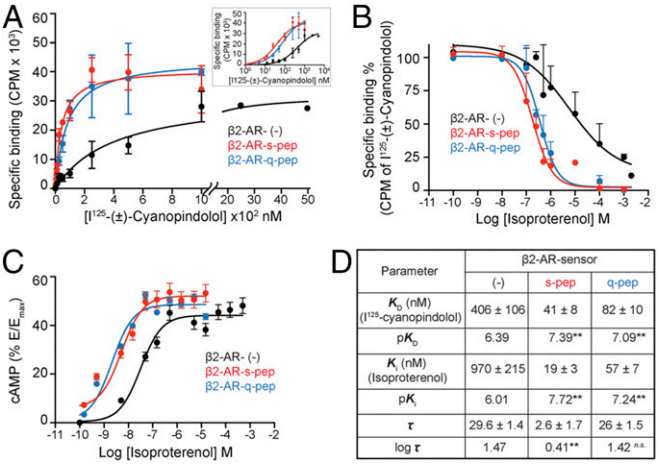
Fig. 3. Synergism between Gαs and a C terminus peptide causes GPCR priming. (*A*) Schematic representation of Gα subunit activation measured in vitro by increase in fluorescence of BODIPY-FL–GTPγS. Activation is triggered by addition of fenoterol to urea-treated membranes containing the β2-AR control sensor (–), harboring an ER/K linker. (*B*) Effect of Gα proteins (+, 50 nM; ++, 100 nM) and soluble Gα C terminus peptides (10 μM) on the in vitro activation of Gαs by fenoterol treatment of β2-AR. Gαq causes synergistic activation of Gαs. s-pep and q-pep increase the activation of Gαs, with q-pep showing an augmented increase. (*C*) Western blot of lysates from HEK293T cells expressing Gαs shRNA compared with vector-transfected cells (mock). At equivalent loading (anti-Actin), shRNA-expressing cells are depleted of Gαs protein (anti-Gαs). (*D*) Change in cAMP levels due to Gαs depletion in cells expressing either β2-AR control (–) or β2-AR-q-pep sensors. In both sensors, depletion of Gαs reduces cAMP levels. Values are mean ± SEM from $n \geq 5$ observations from three independent experiments (*B*) and $n \geq 10$ observations from three independent experiments (*D*). $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.001$ by unpaired *t* test.

In cells expressing the control sensor, depletion of endogenous Gαs led to a reduction in the cAMP response relative to vector-treated cells (Fig. 3*D*). Reduction in the cAMP response and decline in Gαs protein had similar magnitude (~50%). Among vector-treated cells, β2-AR–q-pep sensor exhibited GPCR priming compared with control sensor expression. Simultaneous depletion of endogenous Gαs and expression of β2-AR–q-pep sensor led to a reduction in GPCR priming. The reduction in cAMP in cells depleted of Gαs, even when expressing β2-AR–q-pep sensor, strengthens the idea that GPCR priming is manifested via endogenous Gαs.

Gα C Terminus Peptide Interaction with GPCR Increases Receptor Potency. To gain insights into the mechanism of priming, a combination of radioligand binding and concentration–response analyses were performed. Equilibrium dissociation constants for the orthosteric antagonist [^{125}I]cyanopindolol and the orthosteric agonist isoproterenol were determined by saturation binding (Fig. 4*A*) and competition (Fig. 4*B*) assays, respectively. Compared with control sensor, the peptide sensors had greatly increased affinities for [^{125}I]cyanopindolol (pK_D) (Fig. 4 *A* and *D*) as well as for isoproterenol (pK_i) (Fig. 4 *B* and *D*). However, β2-AR–s-pep and β2-AR–q-pep sensor had similar affinity for [^{125}I]cyanopindolol as well as isoproterenol (Fig. 4*D*). To understand the influence of the increased receptor–ligand affinity on cellular response, cells expressing equivalent levels of β2-AR peptide sensors were exposed to increasing isoproterenol concentration (Fig. 4*C*). The resulting increase in cAMP was expressed as a percentage of the maximum cAMP (E_{max}) that could be generated by each sensor upon forskolin stimulation. Comparison of the concentration–response curves indicated that the β2-AR peptide sensors had greater potency than the control sensor. Further analysis of these concentration–response curves was performed in the framework of the operational model of agonism (13). An operational measure of receptor efficacy (log τ, Fig. 4*D*) was obtained for each sensor by constraining the equilibrium dissociation constant for the interaction between each β2-AR sensor and isoproterenol (K_i) (Eqs. **S1**–**S4**, *SI Materials and Methods*). The Gαs peptide, but not the Gαq peptide, substantially decreases the efficacy of signal transduction. Hence, the combination of enhanced receptor–ligand binding affinity without a decrease in receptor efficacy presents a potential mechanism for GPCR priming.

Discussion

The influence of one G-protein subtype upon signaling through another G-protein pathway, has remained unappreciated, despite the presence of multiple G-protein subtypes that can interact with the same GPCR (2). The only published GPCR–G-protein structure suggests a steric 1:1 stoichiometry in the GPCR–G-protein interaction (6). Hence, the binding of one G protein can be expected to competitively inhibit a simultaneous interaction with another G protein of the same or different subtype. Here, we find instead that GPCR interactions with one G-protein subtype can stimulate signaling through a distinct G-protein subtype, a phenomenon we term GPCR priming. Specifically, we report that interactions between either β2-AR or D_1-R and Gαq enhance cAMP signaling through Gαs (Fig. 1 *B* and *C*). GPCR priming is not limited to Gs-coupled receptors, because the Gq-coupled V_{1A}-R exhibits enhanced IP_1 signal upon interaction with Gαs (Fig. S2*C*). Minimally, interactions between β2-AR or D_1-R and a peptide derived from the C terminus of the Gα subunit of either Gs or Gq are sufficient to observe this enhanced signaling (Fig. 2 *B* and *C*). The increased signaling is specific to the sequence of the peptide, as a scrambled peptide was less efficient (Fig. 2*B*). Synergistic G-protein activation in vitro by agonist-stimulated receptor, in the presence of both Gαs and Gαq, argues that GPCR priming is a characteristic of interaction multiplicity and not simply a tethering artifact (Fig. 3*B* and Fig. S4*B*). Last, radioligand binding assays, concentration–response studies, and analysis in



Parameter	β2-AR-sensor		
	(-)	s-pep	q-pep
K_D (nM) (I^{125}-cyanopindolol)	406 ± 106	41 ± 8	82 ± 10
pK_D	6.39	7.39**	7.09**
K_i (nM) (Isoproterenol)	970 ± 215	19 ± 3	57 ± 7
pK_i	6.01	7.72**	7.24**
τ	29.6 ± 1.4	2.6 ± 1.7	26 ± 1.5
log τ	1.47	0.41**	1.42 n.s.

Fig. 4. Gα C terminus peptides increase the potency of cAMP response. (*A*) Radioligand binding to purified membranes. Saturation binding of (±)-[^{125}I]iodocyanopindolol to 10 μg of purified membranes from cells expressing β2-AR sensors was measured from bound radioactivity as a function of increasing radioligand concentration and used to calculate the equilibrium dissociation constant (K_D). Membranes containing β2-AR control sensors require greater radioligand to display saturation (displayed with broken axis). (*Inset*) Radioligand binding data represented on a logarithmic *x* axis. (*B*) Competition of radioligand binding to β2-AR sensors by isoproterenol. Purified membranes containing 20 fmol of indicated β2-AR sensors were incubated with increasing isoproterenol in the presence of excess (±)-[^{125}I]iodocyanopindolol (peptide sensors, 500 nM; control sensor, 2 μM). Bound radioactivity was measured as a function of increasing isoproterenol concentration to estimate equilibrium dissociation constant (K_i). (*C*) cAMP accumulation in HEK293T cells expressing equivalent amounts of β2-AR sensors. Cells expressing control or peptide sensors were stimulated with varying concentration of isoproterenol (0.1 nM to 1 mM) and the cAMP response measured. Increase in cAMP was expressed as a percentage of the cAMP response from forskolin stimulation (E_{max}), for each sensor. Resulting concentration–response curves were fitted to the operational model to estimate receptor efficacy (log τ), with K_i (from *B*) as a constraining parameter. (*D*) Values are mean ± SEM obtained by analysis of the three independent experiments. $^{**}P < 0.01$, by unpaired *t* test, comparing values for each peptide to the control sensor.

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the framework of the operational model reveal that a peptide from the C terminus of either Gα subunit causes an increase in ligand binding affinity of the receptor (Fig. 4). We propose that this increase in ligand binding affinity, without compromising receptor efficacy, leads to effective GPCR priming.

We propose three parallel mechanisms that lead to GPCR priming. First, G-protein binding to the cognate site triggers a conformational change in the GPCR that persists following initial G-protein dissociation. Because this "primed" conformation has greater ligand binding affinity, it exhibits a higher potency for subsequent activation of cognate G proteins. GPCR priming through cognate site interactions (Fig. S5, Cognate site interaction) requires temporal persistence of an altered GPCR conformation as has been very recently reported (20). Second, GPCR priming occurs through the interaction of G proteins with distinct sites on the GPCR, one at the cognate site (6) and the second at a distal allosteric site. The interaction of a G protein with the allosteric site could influence GPCR conformation to increase the ligand-binding affinity, leading to enhanced activation at the cognate site. Considering the large surface area of the G protein relative to the interface provided by the GPCR, as observed in the recent GPCR–G-protein crystal structure (6), it is unclear how a cognate and a separate allosteric site would be accommodated. Nonetheless, a recent report provides evidence for the simultaneous binding of G protein and β-arrestin to the activated GPCR (7). A similar supercomplex with two G proteins, one at the cognate site and another at the distal allosteric site could contribute to GPCR priming (Fig. S5, Allosteric site interaction). Third, cognate G-protein activation is influenced by the formation of an asymmetric oligomer. This oligomer comprises a cognate receptor G-protein pair transiently interacting with a noncognate pair (Fig. S5, Asymmetric oligomers). Asymmetric dimers of laterally associated GPCRs, where the monomers exist in distinct conformational states, enhance the activation of a single G protein (21). It may be hypothesized that the ability of a noncognate pair to induce an active conformation in the cognate pair by lateral allosterism may lead to an increase in G-protein activation. Because these possibilities are not mutually exclusive, further investigations are necessary to define the molecular basis of GPCR priming.

GPCR priming arises as a consequence of interactions between the receptor and Gα subunits, involving the C terminus peptide of the Gα subunit. We find that the noncognate peptide primes better than the cognate peptide. Although both peptides independently increase the ligand binding affinity, they have differing effects on receptor efficacy (Fig. 4*D*). We have recently reported that the cognate Gα C terminus peptide binds to the receptor with high affinity and stabilizes receptor conformation (22), which is consistent with an early study suggesting that a similar peptide–receptor interaction increases the "high-affinity agonist binding" form of the receptor (23). This stable complex, composed of agonist, receptor, and Gαs C terminus peptide, could limit subsequent G-protein activation, contributing to observed changes in efficacy (Fig. 4*D*). In contrast, the noncognate G protein can interact only weakly with the receptor (Fig. S1), consistent with a lower binding energy (22). Thus, the weaker and hence more transient noncognate GPCR–G-protein interaction increases ligand binding affinity, while maintaining receptor efficacy, leading to effective GPCR priming. We propose that the difference in the magnitude of GPCR priming by cognate and noncognate G proteins can be explained by the stability of their interaction with the GPCR.

The positive interference of multiple G-protein interactions with a GPCR reported here, represents a fundamental shift in our view of GPCR signaling. The ternary-complex model is a mathematical description of the interactions between ligand, receptor, and a single, cognate G protein that precipitates G-protein activation and consequent physiological responses. The ternary-complex model posits that ligand-bound receptor has increased coupling with a G protein. Conversely, G-protein–bound receptor has increased affinity for agonists (24). Our data suggest another layer of regulation, wherein noncognate G proteins interact with the receptor allosterically (25, 26) or using different binding modes at the cognate site (22) to modulate the ligand-binding affinity without compromising receptor efficacy. Such interactions between receptors and noncognate effectors present proximal factors that can drive cell type-specific responses.

Materials and Methods

Reagents. GPCR ligands were purchased from Sigma-Aldrich or Tocris (*SI Materials and Methods*). (±)-[^{125}I]Iodocyanopindolol was purchased from PerkinElmer and used under appropriate containment. BODIPY-FL–GTPγS was from Thermo Fisher/Life Technologies. *n*-Dodecyl-β-D-maltopyranoside, anagrade (DDM), was bought from Anatrace. DNA sources were described previously (22, 27). Purified Gαq (*Mus musculus*) and Gαs long (*Rattus norvegicus*) were obtained from Kerafast. Primary antibodies were from Santa Cruz Biotechnology and secondary from Jackson ImmunoResearch Laboratories (*SI Materials and Methods*).

Constructs. GPCRs (β2-AR, D_1-R, α1-AR, or V_{1A}-R) were linked to Gα or α5 peptide from Gα C terminus in pcDNA5/FRT via a module containing mCitrine, ER/K α-helix, and mCerulean (*SI Materials and Methods*). To generate truncated β2-AR sensors, the full-length β2-AR sequence was replaced with β2-AR residues 1–350. All constructs were confirmed by sequencing. Control sensors terminated in repeating $(Gly\text{-}Ser\text{-}Gly)_4$ residues after mCerulean. 6×His-β2-AR sensor without Gα subunit, Flag-tagged-Gαs, and Flag-tagged-Gαs/q chimera were cloned into pBiex-1 (*SI Materials and Methods*).

Synthetic Peptides. Peptides corresponding to s-pep, DTENIRRVFNDCRDIIQRMHLRQYELL, and q-pep, DTENIRFVFAAVKDTILQLNLKEYNLV, were custom-synthesized by GenScript. Concentration was determined by UV absorbance at 280 nm of aqueous solutions.

Cells, Cell Culture, and Transfection. HEK293T-Flp-In (hereafter HEK293T; Thermo Fisher/Life Technologies) cells were cultured, transfected using XtremeGENE HP (Roche), and evaluated as described previously (22, 27). Fluorescence and absorbance were monitored for the cells following resuspension in PBS plus 0.02% glucose plus 800 μM ascorbic acid. Each experiment was performed at equivalent sensor expression and cell density. Sf9 cells (Thermo Fisher/Life Technologies) were cultured in suspension in Sf900-II media (Thermo Fisher/Life Technologies) and transiently transfected using Escort IV transfection reagent (Sigma-Aldrich) as per the manufacturer's instructions. Seventy-two hours posttransfection, cultures were pelleted and used for protein purification (*SI Materials and Methods*).

Membrane Preparations. For Western blotting and radioligand assays, membranes were prepared as described in detail previously (27) with modifications (*SI Materials and Methods*). For the in vitro reconstitution assay, membranes were prepared and treated with urea, and subsequently stored at −80 °C following a protocol by Lim and Neubig (28) (*SI Materials and Methods*).

cAMP Measurements. cAMP levels were measured in transfected HEK293T cells using the cAMP Glo luminescence-based assay (Promega) as described previously (27) (*SI Materials and Methods*). For dose–response curves, cells were exposed to varying concentrations (0.3 nM to 10 mM) of isoproterenol (3 min, 23 °C). Cholesterol sequestration and membrane disruption were achieved by preincubation of cells with 2 μg/mL filipin for 30 min at 37 °C, before isoproterenol addition.

IP_1 Assay. Twenty to 28 h posttransfection, HEK293T cells expressing the indicated sensor were harvested to assess IP_1 levels using the IP-One HTRF assay kit (Cisbio) as per the manufacturer's protocol (*SI Materials and Methods*).

In Vitro Reconstitution of Gα Activation. Urea-treated membranes (28) were prepared from β2-AR control sensor or β2-AR–mCer fusion expressing HEK293T cells (*SI Materials and Methods*). Reconstitution reactions were assembled on ice with 10 μg of membrane in 194 μL of 20 mM Hepes, 100 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 100 μM GDP, 0.3 mg/mL BSA, and 1 mM DTT. Indicated Gα subunit and/or soluble α5 peptides were added to the concentration indicated. BODIPY-FL–GTPγS (final, 100 nM) and fenoterol (final, 10 μM) were added sequentially. Spectra were acquired before and after fenoterol stimulation, using 470-nm excitation and 511-nm emission.

Radioligand Binding and Competition Assays. Purified membranes containing 10 μg of protein, from cells expressing β2-AR sensors were incubated with an increasing concentration of (±)-[^{125}I]iodocyanopindolol (peptide sensors, 0–500 nM; control sensor, 0–5 μM) in a buffer containing 50 mM Hepes, pH 7.4, 12.5 mM $MgCl_2$, 2 mM EDTA, 100 mM NaCl, 0.05% BSA, and 1 mM ascorbic acid for 90 min at ambient temperature. Nonspecific binding was

defined in the presence of excess alprenolol and found to be less than 1% (*SI Materials and Methods*). Competition assays were performed under the same conditions using 500 nM (±)-[^{125}I]iodocyanopindolol and increasing concentrations of isoproterenol (0–2 mM). Assays were terminated by rapid filtration through GF/C filters, followed by washing with ice-cold Tris-buffered saline (50 mM Tris, pH 7.4, 150 mM NaCl). Filters were allowed to dry, and the bound radioactivity was measured using a Wizard^2 automatic gamma counter (PerkinElmer).

Protein Purification from Sf9 Cells. Purification of N-terminal His-tagged β2-AR (–) control sensor from Sf9 membranes followed previously published protocol (29) (*SI Materials and Methods*). Purification of Flag-tagged Gαs and Gαs/q chimera was performed following Ritt and Sivaramakrishnan (30) (*SI Materials and Methods*).

In Vitro Pull Down Assay. Equivalent amounts of His–β2-AR control sensor was bound to Ni^{2+}–NTA resin and incubated with increasing concentration of either Gαs or Gαq, purified protein (Kerafast) in a buffer containing 50 mM NaCl, 5 mM $MgCl_2$, 20 mM imidazole, 0.1 mM ascorbic acid, 100 μM isoproterenol, 100 μM GDP, 0.1% BSA, 0.1% DDM, protease inhibitors, and 20 mM Hepes, pH 7.45. Unbound Gα proteins were washed away in BSA-free buffer. His–β2-AR and the bound Gα fraction was coeluted with 200 mM imidazole and subjected to fluorescent imaging to detect His–β2-AR (Typhoon gel imager; GE Healthcare) and Western blotting with anti-Gαs or anti-Gαq for quantification (*SI Materials and Methods*).

Western Blotting and Quantitative Analysis. Samples (membrane, cell lysate or eluted protein, and Gα standards) were separated by 10% (wt/vol) SDS/PAGE; transferred to PVDF membranes for 3 h at 300 mA. Membranes were sequentially blocked, washed, and probed with primary antibodies to Gα or Gβ subunits (*SI Materials and Methods*). Blots were washed, probed with secondary antibody, washed again, and developed with Immobilon Western Chemiluminescent HRP substrate (Millipore). Blots were documented using an Odyssey system (Li-Cor Biosciences). For the coimmunoprecipitation assay, gel analysis and measure tools in ImageJ (NIH) were used to calculate mean intensity values for purified Gαx standards. These standards were used to determine the amounts in eluted samples by linear regression.

Analysis of Concentration–Response Curves and Radioligand Assays. Data analysis was performed using Prism (GraphPad Software) following the method of Nguyen et al. (31). Equilibrium dissociation constant of (±)-[^{125}I]iodocyanopindolol (K_D) was determined from saturation binding, and the equilibrium dissociation constant of isoproterenol (K_i) was calculated from competition binding (*SI Materials and Methods*). Receptor efficacy (τ) was estimated by fitting isoproterenol concentration–cAMP response curves to the operational model of agonism (13), using the K_i values as a constraining parameter (*SI Materials and Methods*).

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