Development of novel biosensors to study receptor-mediated activation of the G-protein α subunits Gs and Go1f

Received for publication, June 7, 2017, and in revised form, October 2, 2017 Published, Papers in Press, October 17, 2017, DOI 10.1074/jbc.M117.800698

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Edited by Henrik G. Dohlman

Ga (Gs) and Gaol (Go1f) are highly homologous G-protein α subunits that activate adenylate cyclase, thereby serving as crucial mediators of intracellular signaling. Because of their dramatically different brain expression patterns, we studied similarities and differences between their activation processes with the aim of comparing their receptor coupling mechanisms. We engineered novel luciferase- and Venus-fused Ga constructs that can be used in bioluminescence resonance energy transfer assays. In conjunction with molecular simulations, these novel biosensors were used to determine receptor activation–induced changes in conformation. Relative movements in Ga were consistent with the crystal structure of β2 adrenergic receptor in complex with Gs. Conformational changes in Go1f activation are shown to be similar to those in Ga. Overall the current study reveals general similarities between Ga and Go1f activation at the molecular level and provides a novel set of tools to search for Ga- and Go1f-specific receptor pharmacology. In view of the wide functional and pharmacological roles of Ga- and Go1f-coupled dopamine D1 receptor and adenosine A2A receptor in the brain and other organs, elucidating their differential structural–function relationships with Ga and Go1f might provide new approaches for the treatment of a variety of neuropsychiatric disorders. In particular, these novel biosensors can be used to reveal potentially therapeutic dopamine D1 receptor and adenosine A2A receptor ligands with functionally selective properties between Ga and Go1f signaling.

The Ga family of Ga proteins, comprised of two highly homologous Ga and Go1f subtypes, positively couple to adenylate cyclase (thus, “s” for stimulatory). Upon activation, both Ga and Go1f promote cAMP production and subsequent signaling events such as activation of the PKA cascade. Ga is ubiquitously expressed in most organs, whereas Go1f is mainly restricted to the brain. Moreover, within the brain, Ga and Go1f exhibit distinct expression patterns. Ga is uniformly expressed throughout the brain, except in the striatum where its expression is very low. In contrast, Go1f is highly expressed in the striatum and olfactory tubercle, as well as in the hippocampus and cerebellum to a lesser extent (41). The contrast in tissue expression for Ga and Go1f is quite dramatic and unique among other Ga homologs (e.g. G11 versus Ga, Gq versus G11, and G12 versus G11) (1), making Ga and Go1f fascinating molecular targets with regard to their corresponding functions, particularly in terms of D1 receptor (D1R)-mediated3 and A2A receptor (A2AR)-mediated signaling in the striatum compared with other brain regions.

Conformational changes associated with GPCR activation have been revealed in remarkable detail by the crystal structure of agonist-bound β2 adrenergic receptor (β2AR) in complex with Ga by complementary spectroscopy studies, as well as by related molecular dynamics studies (2–5). However, the extent to which conformational changes in Ga protein are conserved in living cells, as well as across different receptors and different G-protein isoforms, remains unclear. In particular, little is known about the Ga homolog Go1f in terms of its functional similarities and differences. Despite their different expression patterns, the high degree of homology in amino acid sequences (89% identity) has led to the assumption that Ga and Go1f function essentially identically at both the molecular and cellular levels. For this reason, as well as the fact that Go1f expression is typically poor in heterologous cells, Ga functional assays have been used as surrogates for Go1f activation, begging the question of just how similar these processes are. The answer may provide specific ways to target selectively physiological functions mediated by either Ga or Go1f signaling.

In the current study, we first focused on β2AR-Ga activation in intact cells to investigate conformational changes of different domains of Ga. Using bioluminescence resonance energy transfer (BRET)-based assays, we assessed movements both within the Ga protein, as well as between the receptor and Ga subunit.

This work was supported by Intramural funds of the National Institute on Drug Abuse (to S. F.), a fellowship from the Japan Society for the Promotion of Science (to H. Y.), and National Institutes of Health Grants DA022413 and MH54137 (to J. A. J.) and DA026434 (to M. F.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains supplemental Tables S1–S3 and Figs. S1–S7.

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3 The abbreviations used are: D1R, dopamine D1 receptor; D2R, dopamine D2 receptor; BRET, bioluminescence resonance energy transfer; A2AR, adenosine A2A receptor; β1AR, β1 adrenergic receptor; β2AR, β2 adrenergic receptor; PDB, Protein Data Bank; M1R, muscarnic M1 receptor; RluC8, Renilla luciferase 8; RET, resonance energy transfer; RMSD, root mean square deviation.
Using a library of novel Ga biosensors with either luciferase or GFP variants inserted at various positions throughout the structure, we studied conformational changes in living cells and compared the results to the crystal structures of the closed and open conformations of Ga (2, 6). We then studied conformational changes in Ga induced by activation of the D1R, A2AR, and β1 adrenergic receptor (β1AR). Next, taking advantage of the significant homology, we created a series of Gol biosensor constructs with insertions at the same nine positions used for Ga. Agonist-induced conformational changes in Golf were compared with those in Ga. Finally, Gol assay optimization was carried out for D1R. Our analysis using these Ga biosensors suggests that conformational changes within the Ga heterotrimer are similar when induced by different Ga-coupled receptors. Comparison between the Ga and Gol biosensor readouts also indicates a very similar regulation of activation by endogenous agonists. Using this set of Ga and Gol biosensors, the efficacy and potency of agonists, as well as the activation preference between Ga and Gol, can be studied in relation to structural changes and subsequent effector activation. Our results with D1R establish that these biosensors represent a novel pharmacological tool to study structure-function relationships comparing Ga and Gol.

**Results**

**Sensor insertion positions to assess the open and closed conformations of Ga**

A dramatic structural change is apparent between the closed (PDB code 1AZT) and open (PDB code 3SN6) states of the Ga heterotrimer, particularly in the α-helical domain (Fig. 1A). To detect such conformational changes upon G-protein activation, biosensors were constructed in which RLuc or mVenus were inserted at nine different insertion positions in the loop motifs of different domains in Ga (Fig. 1, A and B). The insertion positions (i.e. loop regions) were selected to avoid structural perturbations. Position 7 is located between the N terminus and αN. Positions 67 and 71 are situated in the linker-loop motif, which was not resolved in the crystal structures; they are in the hinge domain that connects the Ras-like catalytic domain and α-helical domain. Insertion of GFP at position 71 of Ga has been functionally validated previously (7). Positions 99, 154, and 175 are located in the α-helical domain (position 99, proximal; positions 154 and 175, distal), whereas positions 305, 338, and 349 are located in the Ras-like domain, avoiding the catalytic core. Insertion of mVenus (YFP variant) or RLuc8 at these positions led to similar levels of expression based on levels of fluorescence for the mVenus constructs and luminescence for the RLuc8 constructs (data not shown).

**Ga biosensors detect distinct conformational changes upon activation**

Relative movements between Ga and Gy upon receptor activation were studied, similarly to previous analysis of Ga activation using Ga biosensors with insertions at positions 60, 91, and 122 (aligned with positions 67, 99, and 131 in Ga, respectively) (8, 9). Of the nine insertion constructs we created, when co-expressed with β2AR (Fig. 2A), Ga with insertions at positions 305, 338, and 349 failed to show significant isoproterenol-induced BRET changes, although the fluorescence and luminescence levels were not significantly different from other biosensors. Insertion positions, 67, 71, 99, and 154, on the other hand, produced substantial agonist-induced BRET changes. When co-expressed with β2AR (Fig. 2A), isoproterenol increased BRET between Ga71-RLuc and γ2-GFP10 or Ga67-RLuc and γ2-GFP10, consistent with greater proximity of the sensors in the two subunits. In contrast, Ga99-RLuc and γ2-GFP10 decreased BRET, indicating an increase in distance. When the donor–acceptor pair was reversed, the directions of BRET change in Ga-γ2-Venus-γ2-RLuc remained the same (supplemental Fig. S1). Furthermore, when co-expressed with D1R (Fig. 2B), the directions of change for all the positions were consistent with the β2AR results. Activation of adrenergic β1AR and adenosine A2AR also showed the same directionality as D1R and β2AR (supplemental Fig. S2).

Although the β1 and γ2 subunits are widely expressed in the brain, β2 and γ7 subunits have been reported to be enriched in the striatum where they play an important role in D1R- and A2AR-mediated signaling (10–13). Therefore, β1/γ2-Venus or β1/γ2-GFP10 was replaced with β2/γ7-Venus or β2/γ7-GFP10 to study D1R activation. The directionalities of BRET change were the same for the β1/γ2 and β2/γ7 pairs (supplemental Fig. S1). When tested for β2AR with β1/γ2 or β2/γ7, the same directionalities were also observed (supplemental Fig. S1).

**Simulated conformational trajectories reveal a movement in the hinge loop**

To provide a structural context to our BRET results, we used the closed crystal structure of Ga (6) and the open conformation of the Ga crystal structure in complex with β2AR (2) as beginning and ending structures, respectively, to simulate domain movement between the closed and open crystal structures of Ga (Fig. 2, C and D). A missing loop (residues 66–72) of the closed Ga crystal structure was built using the Rosetta loop prediction algorithm. The best scoring conformation was extracted and equilibrated in the context of the protein by a 20-ns all-atom MD simulation. Adiabatic biased MD was then employed to generate a continuous, low-energy transition path starting from the closed Ga crystal structure and reducing the root mean square deviation from the open Ga crystallographic conformation. Distances between the Cα atoms of experimental insertion points for the different configurations are reported in supplemental Table S1, showing the general agreement with changes in BRET values. The hinge loop (positions 67 and 71), buried partially between Ras-like and α-helical domains in closed state, opens and moves closer to the γ subunit in the open state. In contrast, the α-helical domain (positions 99 and 154) moves away from the γ subunit.
As mentioned above, the G\(_{\text{olf}}\) subunit is widely expressed in the striatum, where it is critical to the function of D1R. Taking advantage of its 89% sequence identity to G\(_{\text{s}}\), luciferase or mVenus was inserted at the same nine positions explored above (Fig. 1B). Given their enriched expression in striatum (12, 14–17), identical and homologous residues are highlighted in yellow and green, respectively. Insertion positions for G\(_{\text{s}}\) as well as G\(_{\text{olf}}\) are enclosed by rectangles, and the residue numbers for G\(_{\text{s}}\) are shown above.

**G\(_{\text{olf}}\) movement extrapolated from novel G\(_{\text{olf}}\) biosensors corresponds to that of G\(_{\text{s}}\)**

As mentioned above, the G\(_{\text{olf}}\) subunit is widely expressed in the striatum, where it is critical to the function of D1R. Taking advantage of its 89% sequence identity to G\(_{\text{s}}\), luciferase or mVenus was inserted at the same nine positions explored above (Fig. 1B). Given their enriched expression in striatum (12, 14–17), \(\beta_2\) and \(\gamma_7\) constructs were used to study G\(_{\text{olf}}\) activation. Similar to the G\(_{\text{s}}\) results, both G\(_{\text{olf}}\)-Rluc-\(\gamma_7\)-GFP10 and G\(_{\text{olf}}\)-Venus-\(\gamma_7\)-Rluc configurations revealed an increase in BRET values at the hinge region (position 69) and a decrease in BRET values or a lack of response in the \(\alpha\)-helical domain (positions 100 and 155) for \(\beta_2\)AR receptor activation (Fig. 3A and supplemental Table S2). The same directionalities were observed for D1R, further supporting the conservation of domain movements of these homologous G proteins when activated by different receptors (Fig. 3A and supplemental Table S2). The results are also consistent with a large displacement of the \(\alpha\)-helical domain in G\(_{\text{olf}}\), similar to that observed in G\(_{\text{s}}\) (Fig. 2, C and D) and to the crystal structure of the active complex (2).

**Development of a D1R-G\(_{\text{olf}}\) assay**

To establish a reliable assay for drug screening at the D1R with regard to G\(_{\text{olf}}\) coupling, different configurations of BRET...
were tested (supplemental Table S2 and Fig. 4). Fig. 4 shows direct comparisons between G_{s} and G_{olf} biosensors in the activation and engagement modes. For G_{s}-\gamma activation assays, the relative potency and efficacy differences between dopamine, a full agonist, and norepinephrine, a less potent agonist, were tested. The potency differences between the two agonists were similar for activation of G_{s} and G_{olf} (Fig. 4, A and B, and supplemental Table S3). The engagement assays also demon-

Figure 2. A and B, dose-response curves of G_{s} protein activation BRET for \( \beta 2\)AR with isoproterenol (A) and for D1R with dopamine (B). Different colors represent insertion positions for Rluc: black, 7–8 amino acids; orange, 67–68 amino acids; blue, 71–72 amino acids; red, 99–100 amino acids; green, 154–155 amino acids; yellow, 175–176 amino acids. The dose-response curves represent the means ± S.E. of more than five experiments performed in triplicate. C, composite pictures (five frames) of the simulated transition from an inactive/closed (modeled by the 1AZT crystal structure) to an active/open (modeled by the 3SN6 crystal structure) state of the G_{s} subunit. Computer simulations predict transitional movement (frames 2–4) between the \( \alpha \)-helical domain containing 99 and 154 amino acids and hinge domain (with 67 and 71 amino acids) linking the \( \alpha \)-helical and Ras-like domains. The \( \alpha \)-helical and Ras-like domains of G_{s} are in blue and red, respectively, whereas G_{olf} and G_{olf} are in light gray and dark gray. D, superposition of the closed and open crystal structures of the G_{s} protein, highlighting the movement of the G_{s} \( \alpha \)-helical domain (light and dark blue, respectively, in the inactive and active conformations), with respect to the Ras-like domain (in light and dark red, respectively, in the two conformations), hinged on the region of probes 67 and 71. The black sphere indicates where the acceptor GFP10 is fused at the N terminus of G-\gamma subunit. The location of four selected probes introduced at residues 67 (orange), 71 (blue), 99 (red), and 154 (green) are indicated with spheres.

Figure 3. A and B, dose-response curves of G_{olf} protein activation BRET for \( \beta 2\)AR with isoproterenol (A) and for D1R with dopamine (B). Different colors represent insertion positions for Rluc: orange, 69–70 amino acids; blue, 72–73 amino acids; red, 100–101 amino acids; green, 155–156 amino acids. The dose-response curves represent the means ± S.E. of more than five experiments performed in triplicate. EC_{50} values between \( \beta 2\)AR and D1R for each of 69, 72, and 155 positions of G_{olf} sensors were compared using one-way analysis of variance with post hoc Tukey test analysis and did not reach statistical significance.
strated a tight agreement of relative potency and efficacy between dopamine and norepinephrine in Gs and Golf (Fig. 4, C and D, dose-response curves of dopamine (black curve) or norepinephrine (blue curve) induced BRET between D1R-Rluc and Gs-Venus (C) or Golf-Venus (D). Corresponding schemes are illustrated in insets. The dose-response curves represent the means ± S.E. of more than five experiments performed in triplicate. DA, dopamine; NE, norepinephrine.

The engagement (i.e. D1R-Rluc-Golf-Venus) BRET configuration was pursued for optimization because of its larger dynamic window. Different β-γ subunit combinations were tested (supplemental Fig. S5). Although the β1-γ7 and β2-γ7 combinations showed a similar dynamic range, β2-γ7 was chosen for the rest of the studies because of the established expression overlap in striatum (10–13). One of the crucial factors for successful Gαolf BRET assay regardless of configuration was co-expression of the G-protein chaperone Ric8 (18–20), which robustly enhanced the dynamic range of agonist-induced BRET (supplemental Fig. S4). Because luciferase expression, detected by luminescence, does not differ significantly with and without Ric8 co-expression, the dramatic change in dynamic range of BRET may have to do with chaperone activities of Ric8, possibly rescuing misfolding or aiding proper localization of the Gαolf biosensor to the receptor complex rather than simply enhancing its expression (supplemental Fig. S4). Overall, cross-comparison of the D1R-Ga engagement and Gα-γ activation assays validates the potency and efficacy range of the four assays tested and thus their utility in pharmacological characterization of D1R activation.

**Creation of novel homology-based Gi1 and Gq biosensors**

Because the Ras-like domain, hinge region, and α-helical domain are well-conserved in other classes of Gα subunits (21), the relative movements upon activation were compared in other Gα subunits. The same three sensor insertions (i.e. Gs equivalent of positions 67, 99, and 154) were made in Gi1 and Gq at the aligned amino acid residues (Fig. 5A). Upon transfection with dopamine D2 receptor (D2R) and using dopamine as ligand, for Gi1, the hinge region (position 60) moves closer to the γ subunit, whereas the α-helical domain (positions 91 and 145) moves away from the γ subunit (Fig. 5B), similar to our results in Gs (Fig. 2). The conformational changes in the hinge and α-helical domains of Gi1 are consistent with previous

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**Figure 4.** A and B, dose-response curves of Gs (A) or Gαolf (B) protein activation BRET for D1R with dopamine (black curve) or norepinephrine (blue curve). C and D, dose-response curves of dopamine (black curve) or norepinephrine (blue curve) induced BRET between D1R-Rluc and Gs-Venus (C) or Golf-Venus (D). Corresponding schemes are illustrated in insets. The dose-response curves represent the means ± S.E. of more than five experiments performed in triplicate. DA, dopamine; NE, norepinephrine.
reports with insertions at positions 60 and 91 (8, 9). Upon transfection with muscarinic M1 receptor (M1R) and using carbachol as ligand, Gq sensors also revealed the same directionalities of BRET change for the H9251-helical domain (positions 97 and 150) (Fig. 5C) but with a very robust dynamic range for drug response with the position 97 sensor. However, the insertion at position 66 (equivalent to 67 in Gs) yielded very little agonist-induced BRET (Fig. 5C, orange curve), although the luminescence was similar (data not shown), suggesting a possible structural difference in the hinge loop of Gq.

Finally, using the Venus-fused Gi1 or Gq sensors, receptor–Gα engagement BRET was assessed for D2R or M1R (supplemental Fig. S6). Consistent with the Gs results, insertion at position 150 of Gq (aligned to 154 in Gs) gave the largest efficacy window, as well as higher potency when compared with the previously characterized position 97 (22) (supplemental Fig. S6B). This trend was not maintained with G14, where insertion at position 91 (aligned with position 99 in Gs) produced the most robust sensor (supplemental Fig. S6A). Taken together, these results with G-protein activation BRET have established generally conserved movements of the α-helical domain in three different classes of Gα subunits, albeit with subtle differences.

Discussion

The seminal work leading to the crystal structure of the active β2AR-Gi heterotrimer complex has enabled comparison between open and closed structures of the G protein, as well as interactions of the G protein with the receptor, providing molecular details of key conformational changes associated

Figure 5. A, amino acid sequence alignment among Gs, Gi2, Gi3, GoA, Gq short, and Gq. Identical, highly homologous, and homologous residues are highlighted in yellow, blue, and green, respectively. Insertion positions equivalent to Gs short positions 67, 99, and 154 are indicated by arrows. Novel constructs made for this study are indicated by check marks. B and C, dose-response curves of Gi1 protein activation BRET for D2R with dopamine (B) and Gq protein activation for muscarinic M1R with carbachol (C). Different colors represent insertion positions for Rluc: orange, 60–61 [Gi1] or 66–67 [Gq] amino acids; red, 91–92 [Gi1] or 97–98 [Gq] amino acids; green, 145–146 [Gi1] or 150–151 [Gq] amino acids. The dose-response curves represent the means ± S.E. of more than five experiments performed in triplicate.
with the activation process (2, 6, 23). A series of relevant structure–function studies have pointed to the large displacement of the α-helical domain as a central mechanism, albeit not sufficient, for the promotion of GDP–GTP exchange (3–5). Although the α-helical domain may undergo spontaneous fluctuation between the open and closed states, insertion of the α5 helix of Gq into the intracellular vestibule of the β2AR promotes opening of the α-helical domain. The pronounced decrease in BRET values in living cells indicates a distancing event between the α-helical domain and the γ subunit, consistent with an opening movement from three different amino acid positions of the α-helical domain (positions 99, 154, and 175). The movement in the loop structures, which serve as a hinge between the α-helical and Ras-like domains, is therefore an important feature that links the displacement of the α-helical domain with Gₐ activation. Presumably because of the highly flexible nature of the linker loop, this region was not resolved in the crystal structure (2, 6). Based on our MD simulations, we hypothesize that the transition between the closed and open states of the G-protein subunit may involve an outward protruding movement of the linker loop (positions 67, 71), along with the overall structural changes that enable α-helical domain opening. The negative BRET change with the myristoylated αN loop (position 7) and γ subunit is also consistent with the displacement of αN between the opened and closed Gₐ crystal structures.

Similarly, α-helical domain displacement has been proposed for the Gₛ and Gₐₛ proteins as an activation mechanism (21). In addition to previously studied positions (Gₛₐ₃, Gₐₙ₀, Gₛₙ₇, and Gₐₙ₇) (7–9, 22, 24), we have created novel fusion constructs at Gₛ positions 67, 99, and 154 and equivalent positions in Gₐ and Gₐₛ. Because of the conserved structural domains (i.e., α-helical, linker loop, Ras-like domains), not surprisingly, our results mostly coincide with previous studies. In the activation configuration, only Gₐₙ₇ failed to display positive BRET changes compared with Gₛₐₙ₇ or Gₐₙ₇, possibly because of a difference in the linker-loop structure that does not generate a protruding movement in Gₛ. Overall, the Gₛ-αγ BRET assay demonstrates the conserved nature of α-helical domain movement across three different Gₛ protein subtypes and strengthens the cases for these assays as robust sensors of agonist-induced activation in living cells.

In line with its specific brain distribution, Gₒᵤᵤ is involved in olfaction and basal ganglia function (12, 25, 41). Mutations in the GNAL gene encoding Golf have been implicated in movement disorders in humans (26–29). Because of their high homology, Golf is generally considered to function similarly to Gₛ in terms of its ability to stimulate adenylate cyclase. Although some kinetic difference in GTP hydrolysis has been suggested between Gₛ and Gₒᵤᵤ in β2AR (30), to our knowledge, there has not been a thorough molecular study of its activation. The current study is the first to focus on direct comparison of D1R-Gₛ and D1R-Gₒᵤᵤ coupling and activation. Our new findings indicate that: 1) conformational changes upon activation are similar for Gₛ and Gₒᵤᵤ; 2) Ric8B is required for heterologous expression of Gₒᵤᵤ biosensors, as reported previously (18–20); and 3) the β2–γ7 pair confers the largest dynamic range for Gₒᵤᵤ engagement BRET in agreement with previous studies showing a dependence on co-expression of Gₒᵤᵤ β2 and γ7 subunits for striatal D1R and A2AR signaling (10, 13). The directionality of the Gₒᵤᵤ activation BRET at different insertion positions are for the most part consistent with the Gₛ results, but overall the dynamic range of agonist response is not as robust as for Gₛ. It is worth considering that there may be subtle differences between β2AR and D1R in Gₒᵤᵤ activation because their EC₅₀ values for the 69, 72, and 155 position sensors show a trend of difference, although these did not reach statistical significance.

Notably, although the Rluc or Venus expression level (measured by luminescence or fluorescence) is similar between the Gₛ and Gₒᵤᵤ biosensors, the efficiency of folding or localization of the Gₒᵤᵤ sensors may be impaired because the basal BRET is lower for D1R-Rluc-Gₒᵤᵤ-Venus than for D1R-Rluc-Gₛ-Venus. This may explain the lack of agonist response for the position 100 insertion in Golf-Rluc. The expression of Gₒᵤᵤ and Gₒᵤᵤ fusion constructs is likely challenging because the accessory molecules that are present in neurons may be missing in heterologous cells. Studies have indicated that co-expression of Ric8B and HSP70, both chaperone proteins, enhance the expression of both olfactory receptor and its Golf signaling (31). In our hands, Ric8B increased the BRET dynamic range of BRET of Gₒᵤᵤ constructs, although HSP70 did not. Perhaps expression of other accessory proteins might help to increase further the dynamic range of the Gₒᵤᵤ assays.

In summary, our novel Gₒᵤᵤ assay represents a useful screening method for Golf signaling in heterologous cells. The Gₛ and Gₒᵤᵤ assays presented herein can be used in parallel for pharmacological investigation of receptors relevant in neuropsychiatric disorders, including both D1R and A2AR.

Experimental procedures
DNA constructs and transfection

For all the receptor constructs, a signal peptide followed by a FLAG epitope tag was fused to the N terminus for enhanced cell surface expression (32) and detection (33). The human receptor constructs used were A2AR, D1R, β2AR, β1AR, dopamine D2 short receptor, and M1R (34). For the D1R, D2R, and M1R fusion constructs, the cDNA encoding full-length Rluc8 (provided by Dr. S. Gambhir, Stanford University, Stanford, CA) was fused in-frame to the C terminus of the receptors as reported (35). The following human G-protein constructs were used: Gₛ-mVenus, Golf-mVenus, Gₛ-Rluc, Golf-Rluc, Gₓ₉₄-Rluc, and Gₛₐₙₖ-Rluc whose various insertion positions were specified below. For Gₓ₉₄-Rluc, Rluc was inserted at position 60, 91, or 145. For Gₛₐₙₖ-Rluc, Rluc was inserted at position 66, 97, or 150. For Gₛₐₙₖ-mVenus, Golfₙ₉₄-mVenus, Golfₙ₉₄-Rluc, Golfₙ₉₄-Rluc, and Gₛₐₙₖ-Rluc constructs, mVenus or Rluc was inserted at positions 7 (8), 67 (69), 71 (72), 99 (100), 131 (132), 154 (155), 175 (176), 305 (306), 338 (339), 349 (350) (Gₒᵤᵤ numbering in parentheses). For Gᵧ₂ and Gᵧ₇ fusion constructs, full-length mVenus, GFP10, or Rluc was fused at its N terminus. Untagged βγ subunits Gβ1, Gβ2, Gβ3, Gβ4, Gγ₂, and Gγ₇ were also used for co-transfection. The G-protein chaperone Ric8B was co-transfected with Gₛ and Golf constructs. Ric8A was co-transfected with Gₛₐₙₖ constructs. Ric8 plasmids were kind gifts from Dr. Gregory Tall (20, 36). All the constructs were confirmed by sequence analysis. A constant amount of total
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plasmid cDNA (15 μg) was transfected into human embryonic kidney cells 293T (HEK-293T) using polyethylenimine (Sigma–Aldrich) in a 1:2 ratio in 10-cm plates. The cells were maintained in culture with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and kept in an incubator at 37 °C and 5% CO2. The transfected amount and ratio among the receptor and heterotrimeric G proteins were tested for optimized dynamic range in agonist-induced BRET. For instance, in activation and engagement BRET described below, the ratios of 3:3:4:5:2.5 (receptor:Gα–Rluc:Gβ–GFP10: Ric8B) and 0.25:5:4:2.25 (receptor–Rluc:Gα–Venus:Gβ–Gγ: Ric8B) were used respectively. Experiments were performed ~48 h post-transfection.

BRET assay

Three modes of BRET assays were performed to detect receptor ligand-induced events for 1) Gα–γ protein activation, 2) Gγ–α protein activation, and 3) receptor–Gα engagement. 1) The Gα–γ protein activation assay uses a RLuc-fused Gα-protein subunit and GFP10-fused Gγ protein for a resonance energy transfer (RET) pair. FLAG-tagged receptor and untagged Gβ constructs were co-transfected. 2) Similarly the Gγ–α protein activation assay uses a RLuc-fused Gγ protein subunit and GFP10-fused Gα protein for a RET pair. FLAG-tagged receptor and untagged Gβ constructs were co-transfected. 3) The receptor–Gα engagement assay uses RLuc-fused receptor and mVenus-fused Gα protein for the RET pair. Untagged Gβ and Gγ constructs were co-transfected. As reported previously (35), cells were harvested, washed, and resuspended in PBS. Approximately 200,000 cells/well were distributed in 96-well plates, and 5 μM coelenterazine H (substrate for BRET1) or 5 μM coelenterazine 400a (substrate for BRET2) was added to each well. Three minutes after addition of coelenterazine, ligands (dopamine (Sigma), 1-(-)-norepinephrine (Sigma), 5’-N-ethylcarboxamidoadenosine (Tocris), isoproterenol (Tocris), or carbachol (Tocris)) were added to each well. The fluorescence of the acceptor was quantified for Venus excitation at 500 nm and emission at 530 nm for 1-s recording or for GFP10 excitation at 405 nm and emission at 515 nm for 1-s recording for GFP10) in a Mithras LB940 (Berthold Technologies, Bad Wildbad, Germany) to confirm constant expression levels across experiments. In parallel, luminescence and BRET1 signal from the same batch of cells was determined as the ratio of the light emitted by Venus (530 nm) over that emitted by coelenterazine H (485 nm) or luminescence and BRET2 signal from the same batch of cells was determined as the ratio of the light emitted by GFP10 (515 nm) over that emitted by coelenterazine 400a (400 nm). The results are calculated for the BRET change (BRET ratio for the corresponding ligand minus BRET ratio in the absence of the ligand). Fmax values are expressed as the basal subtracted BRET change in the dose-response graphs. The fluorescence and luminescence counts (arbitrary units) were similar in different experiments using the same construct. The data and statistical analyses were performed with Prism 5 (GraphPad software).

Sequence homology alignment

Amino acid sequence homology analysis was performed using Vector NTI Advance (Invitrogen). Identical residues are highlighted yellow, and homologous residues are highlighted green.

Molecular modeling and computer simulations

The closed (PDB code 1AZT), and open (PDB code 3SN6, chains A, B, and G) crystal structures of Gα were used for MD simulations. Missing loop residues (at positions 66–72) of the closed Gα crystal structure were built using the Rosetta loop prediction algorithm (37). The best scoring conformation was extracted and equilibrated in TIP3P waters by a 20-ns MD simulation using all-atom description and the Charmm27 force field (38). The open and closed structures were used as templates to model Gαolf by homology (39). To investigate the changes in conformation between the inactive and active conformations, an adiabatic biased MD simulation (40) was performed starting from the protein inactive state, using the RMSD from the active state model as a collective variable. Briefly, a steep repulsive bias was applied when the RMSD from the target state increased above the minimum value reached during the simulation. Specifically, the applied potential was

\[ V(R(t)) = \frac{K}{2} (CV(R(t)) - CV_{\text{min}}(R(t)))^2 \]  

(Eq. 1)

where the collective variable is the RMSD to the active state, and

\[ CV_{\text{min}}(R(t)) = \min_{0 < s < t} CV(R(s)) + \eta(t) \]  

(Eq. 2)

Similar to a ratchet and pawl system, propelled by thermal motion, the biasing potential does not exert work on the system and ensures that the obtained trajectory is a low-free energy path connecting the initial and final states. The simulation was stopped after 20 ns. Simulations were performed with Gromacs 4.6 with Plumed 2.0. The simulation was carried out in the NPT ensemble, using v-rescale thermostat and Parrinello–Rahman barostat to maintain temperature and pressure constant. Electrostatics was calculated with the particle-mesh Ewald algorithm, and non-bonded interactions were cut-off at 1.2 nm. A time step of 2 fs was used. Distances between the C atoms of insertion points of the experimental probes were monitored during simulation.

Author contributions—H. Y. designed, conducted, and analyzed the molecular biology and BRET experiments. D. P. and M. F. conducted and analyzed the molecular dynamics simulation. N. S. C. performed the molecular biology work. H. Y. and J. A. J. wrote the manuscript. All authors contributed to reviewing the results and writing the manuscript.

Acknowledgments—We thank Gregory Tall for the Ric8 constructs; Nevin Lambert for the Gαs_91_mVenus and Gαolf_97_mVenus constructs and helpful comments on cloning and Céline Gales for Gαs_60_RLuc8, Gαs_91_RLuc8, and Gαolf_97_RLuc8 constructs. Computer simulations were run on resources available through the Scientific Computing Facility at Mount Sinai and the Extreme Science and Engineering Discovery Environment under Program MCB080077, which is supported by National Science Foundation Grant ACI-1053575.
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