Correction

PHARMACOLOGY


The authors note that Fig. 1 appeared incorrectly. They also wish to note the following: “Upon further evaluation of the unbiased electron density maps for the structural models of Gαi1(G202A)-GDP (PDB id 2PZ2) and Gαi1(G202A)-GDP-AIF₄⁻ (PDB id 2PZ3), there is a lack of clear and continuous density to support an entirely ordered switch II region or to support the presence of aluminum tetrafluoride in the latter structure. We have therefore obsoleted the x-ray structure model PDB 2PZ3 from the Protein Data Bank. We have replaced PDB 2PZ2 with PDB 3UMS in the Protein Data Bank to reflect the more accurate refinement of the Gαi1(G202A)-GDP structural model.”

The corrected figure and its corresponding legend appear below. This error does not affect the conclusions of the article.

![Corrected Figure](image-url)

Fig. 1. G202A substitution in Gαi1 switch II leads to a pretransition state that accelerates intrinsic GTPase activity. (A) Single-turnover GTP hydrolysis assays were performed on ice using indicated recombinant wildtype and mutant Gα proteins, demonstrating the enhanced intrinsic GTPase rate of the Gαi1(G202A) mutant. The nearly order of magnitude GTPase rate enhancement observed with the G202A mutation is consistent with that reported by Thomas et al. (22); nevertheless, it must be acknowledged that some RGS proteins have been observed to accelerate Gα-mediated GTP hydrolysis by orders of magnitude under optimal conditions (24, 45). (B) Ribbon Cα tracing of the proposed transition state for GTP hydrolysis from the published structural model of wild-type Gαi1·GDP·AIF₄⁻ [PDB id 1GFI; (23)], highlighting the disposition of the three residues involved in GTP hydrolysis (Arg-178, Thr-181, Gin-204; yellow sticks), as well as the position of glycine-202 (red). GDP is colored magenta with the AIF₄⁻ and magnesium ions colored teal and green, respectively. (C) Ribbon Cα tracing of our 2.34 Å structural model of GDP-bound Gαi1(G202A) derived from x-ray crystallography (PDB id 3UMS). Switch regions (SI–SIII) are colored red, with the catalytic residues Arg-178 and Thr-181 depicted in red sticks and the mutant alanine-202 residue in black sticks.

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Regulators of G-protein Signaling accelerate GPCR signaling kinetics and govern sensitivity solely by accelerating GTPase activity

Nevin A. Lambertb,1, Christopher A. Johnstonb,2, Steven D. Cappellb, Sudhakiranmayi Kuravi, Adam J. Kimpleb, Francis S. Willardb,1,3, and David P. Siderovskiab,1

*bDepartment of Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA 30912; and 3Department of Pharmacology, Lineberger Comprehensive Cancer Center, and UNC Neuroscience Center, University of North Carolina, Chapel Hill, NC 27599

Edited by Lutz Birnbaumer, National Institute of Environmental Health Sciences, Research Triangle Park, NC, and approved March 5, 2010 (received for review November 9, 2009)

G-protein heterotrimers, composed of a guanine nucleotide-binding Gα subunit and an obligate Gβγ dimer, regulate signal transduction pathways by cycling between GDP- and GTP-bound states. Signal deactivation is achieved by Gα-mediated GTP hydrolysis (GTPase activity) which is enhanced by the GTPase-accelerating protein (GAP) activity of “regulator of G-protein signaling” (RGS) proteins. In a cellular context, RGS proteins have also been shown to speed up the onset of signaling, and to accelerate deactivation without changing amplitude or sensitivity of the signal. This latter paradoxical activity has been variably attributed to GAP/enzymatic or non-GAP/scaffolding functions of these proteins. Here, we validated and exploited a Gα switch-region point mutation, known to engender increased GTPase activity, to mimic in cis the GAP function of RGS proteins. While the transition-state, GDP-AlF4−-bound conformation of the G202A mutant was found to be nearly identical to wild-type, Gαi1(G202A)-GDP assumed a divergent conformation more closely resembling the GDP-AlF4−-bound state. When placed within Saccharomyces cerevisiae Gα subunit Gpa1, the fast-hydrolysis mutation restored appropriate dose–response behaviors to pheromone signaling in the absence of RGS-mediated GAP activity. A bioluminescence resonance energy transfer (BRET) readout of heterotrimer activation with high temporal resolution revealed that fast intrinsic GTPase activity could recapitulate in cis the kinetic sharpening (increased onset and deactivation rates) and blunting of sensitivity also engendered by RGS protein action in trans. Thus, Gα-directed GAP activity, the first biochemical function ascribed to RGS proteins, is sufficient to explain the activation kinetics and agonist sensitivity observed from G-protein–coupled receptor (GPCR) signaling in a cellular context.

bioluminescence resonance energy transfer | GTPase-accelerating protein activity | Regulator of G-protein Signaling proteins | signal onset and recovery | signal sensitivity

Heterotrimetric G proteins regulate numerous signaling pathways that elicit critical physiological responses in many organisms from Dictyostelium and fungi to plants and metazoans (1–3). The G-protein heterotrimer consists of a guanine nucleotide-binding Gα subunit that, in its GDP-bound state, is tightly associated with an obligate Gβγ dimer (4, 5). Agonist binding to seven-transmembrane, G-protein–coupled receptors (GPCRs) activates this complex by catalyzing exchange of GDP for GTP in Gα that leads to a conformation change and Gβγ dissociation (6–8). Proper responses rely on timely deactivation of heterotrimer signaling, achieved by intrinsic Gα-mediated hydrolysis of GTP to GDP (GTPase activity). This hydrolysis is enhanced by “regulator of G-protein signaling” (RGS) proteins that serve as GTPase-accelerating proteins (GAPs) (9, 10). The RGS domain common to these proteins elicits GAP activity by stabilizing Gα in its transition-state intermediate form, thus lowering the required reaction free energy for GTP hydrolysis and subsequent return to the Gα-GDP state (11, 12).

Early studies of the cellular effects of RGS proteins on GPCR signaling described accelerated signal onset as well as accelerated decay, particularly for Gβγ-gated ion channel responses measured by whole-cell electrophysiological recordings, as reviewed elsewhere (13). For example, the slow, non-physiological rate of K+ current onset, normally seen with inward-rectifying potassium (GIRK/Kir3) channels expressed in heterologous systems (CHO-K1 cells, Xenopus oocytes), was dramatically sharpened upon coexpression of RGS proteins such as RGS4 and RGS8. Surprisingly, no demonstrable changes in signal amplitude nor agonist sensitivity were apparent in these early reports (14, 15). Both groups labeled these findings paradoxical (14, 15) in light of the negative regulatory role first ascribed to RGS proteins (i.e., Gα-directed GAP activity).

Models have been put forth to account for this “paradoxical” regulatory role of RGS proteins in GPCR signaling. Many RGS proteins have multiple, modular protein domains in addition to the signature RGS domain, as previously reviewed (9, 16). Thus, a model of “physical scaffolding” (17, 18) suggests that RGS proteins use these accessory protein domains (and/or non-GAP motifs within the RGS domain) to mediate interactions that modulate receptor/heterotrimer coupling or other aspects of receptor/G-protein effector signaling output (19). Alternatively, the “kinetic scaffolding” or “spatial focusing” (20) model eschews evoking additional functions for RGS proteins and relies on their GAP activity alone. This model suggests that G-protein activation becomes saturated near spatially constrained “clusters” of agonist-activated GPCRs, causing GTP hydrolysis, rather than GDP release, to become rate limiting in the nucleotide cycle. RGS domain GAP activity is proposed to enhance steady-state pathway activation by preventing local depletion of Gα-GDP, thus providing receptors with additional Gβγ heterotrimers to activate. Therefore, by promoting continuous cycling of G-protein activity, as opposed to prolonging the Gα-GTP state, rapid GTP hydrolysis alone may provide a mechanism of...
accelerating both signal onset and decay and shaping signal sensitivity (19–21).

We sought evidence from whole-cell GPCR signaling that could distinguish the contribution(s) of GAP and non-GAP functions of RGS proteins to the observations of accelerated kinetics, yet paradoxical lack of amplitude or sensitivity changes, attributed to RGS proteins. Our strategy was to impose the structural and functional changes in Gαi brought about by RGS protein binding that enhance GTP hydrolysis, while avoiding other possible consequences of RGS protein binding (e.g., physical scaffolding). We used a Gαi switch II point mutation (G202A in human Gα4i, G203A in human Gα4o, G321A in Saccharomyces cerevisiae Gpa1) previously found to increase greatly the intrinsic rate of GTP hydrolysis (22). Our structural models obtained by x-ray crystallography show that the glycine-to-alanine substitution promotes a pretransition state mimicked by the Gα-GDP-AlF4− complex (23), i.e., the same state most favored for RGS protein binding (11, 24). Placing this fast-GTPase mutation into the Gα integral to yeast pheromone sensing was sufficient to restore wild-type dose-responsive behavior in the absence of RGS domain GAP activity. Reconstituting neurotransmitter receptor signaling with fast-GTPase Gα accelerated both signal onset and decay and blunted sensitivity. This study thus provides experimental evidence that faster-than-wild-type GTP hydrolysis by Gα, whether accomplished via RGS protein GAP activity in trans or via accelerated intrinsic GTPase activity in cis, is sufficient to support rapid signal onset and altered sensitivity of agonist activation of GPCRs.

Results

Structures of Gα4i(G202A) Mutant Reveal a Pretransition State for GTP Hydrolysis. A glycine-to-alanine mutation at position 202 of the Gα4i switch II region was originally identified as increasing the intrinsic GTPase activity at least 10-fold in vitro (22). We confirmed this enhanced GTPase activity in single-turnover [γ−32P]GTP hydrolysis assays (Fig. L4). RGS domains not only accelerate wild-type Gαi GTPase activity, but also preferentially bind their transition-state mimetic form (Gαi-GDP-AlF4−) over the ground state (Gαi-GDP) (24). Therefore, evidence of negligible RGS4-mediated enhancement of GTPase rate as well as increased RGS4 affinity for Gα4i(G202A)-GDP (Kd 18 μM) vs. wild-type Gα4i-GDP (Kd 430 μM) led Thomas et al. (22) to hypothesize that the G202A mutant, like RGS domain-bound Gαi, is stabilized in a pretransition state for GTP hydrolysis, explaining its enhanced intrinsic GTPase activity. Here, to ascertain the structural determinants of enhanced GTP hydrolysis, we established structural models derived from x-ray crystallography for the GDP- and GDP-AlF4−-bound forms of Gα4i(G202A) to 2.6 Å and 2.42 Å resolution, respectively; data collection and refinement statistics are listed in Table S1, and illustrations of model fit to experimental electron density are presented in Fig. S1.

Previous Gα structures, reviewed elsewhere (5, 7, 25), have detailed the conformational changes that three switch regions undergo during transition between GTP- and GDP-bound states. Key catalytic residues have also been identified, including a phosphate-neutralizing arginine and magnesium-coordinating threonine in switch-I (R178 and T181 in Gα4i), and a water-coordinating glutamine in switch-II (Q204 in Gα4i). The side chains of these three residues are observed in their hydrolysis-coordinating roles within the structure of Gα4i bound to GDP and to the planar ion AlF4−, which induces a stable, transition-state mimetic form (PDB id 1GFI, ref (23); Fig. 1B). The Gα4i-AlF4−-bound conformation of the G202A mutant is nearly identical to wild-type Gα4i-GDP-AlF4− (overall RMSD 0.93 Å; Fig. S2), with only slight alterations in the three switch regions.

Minor displacements in both the f2/a2 loop and switch I likely result from the G202A mutation itself to compensate for the
adopts an orientation similar to dimer (αG302S/G321A) activation, we used a and resulted in no change in www.pnas.org/cgi/doi/10.1073/pnas.0912934107

G321A luciferase-labeled, membrane-associated C-(i.e., an orientation fully competent = 13) performed in triplicate. Error bars, trans to subunit, Gpa1, is coupled to fi

Fig. S5 strains in which Lambert et al. "50 α subunits and mone sensitivity (i.e., EC C351G. To measure G trans inhibition (Fig. 1 point mutation within switch I of G activation, such as by GTP binding or addition of insensitive to RGS domain GAP activity, proteins. Coupling to =1 4 ; S. cerevisiae subunit for the G orient key catalytic residues poised for GTP hydrolysis.

Conversely, GDP-bound Gα(G202A) assumes a divergent conformation from that of wild-type Gα(GDP. In the GDP-bound state, switch II and switch III are normally unstructured and highly disordered when observed in crystal structures (26, 27); it is Ga activation, such as by GTP binding or addition of AlF4−, that normally elicits structural order in the switch regions, including switch II adopting a rigid, α-helical conformation and switch III being suitably stabilized for visualization (23, 28). In contrast, GDP-bound Gα(G202A) displayed sufficient structural order and suitable electron density to model all three switch regions (e.g., Fig. 1C and Fig. SL4). Switch regions I and II of Gα(G202A)-GDP are strikingly similar to their conformation in the GDP-AlF4−-bound state of both wild-type and G202A Gα(GDP, although minor alterations can be noted. Switch I does not approach the nucleotide-binding pocket as dramatically as in wild-type Gα-AlF4− GTP structure (Fig. S2); as previously stated, this may be in part due to the methyl side-chain of Ala-202 sterically hindering such a conformation. The catalytic R178 and T181 residues within switch I are, however, positioned similar to the wild-type Gα-AlF4− structure (cf. Fig. 1B vs. C; Fig. S2B).

Whereas the switch II [β3/2 loop] adopts a conformation somewhat distinct from wild-type Gα-AlF4− the catalytic Q204 residue is oriented toward nucleotide similar to the orientation seen in wild-type Gα(GDP-AlF4− (Fig. 1B). Together with the orientations of R178 and T181, this observation suggests that the G202A mutation causes allosteric conformational changes within Gα that orient key catalytic residues poised for GTP hydrolysis even when GDP-bound, confirming the hypothesis (22) that the G202A mutant is in a “pretransition” state resembling the changes brought about by RGS domain binding (11, 29). Notwithstanding these structural changes to key catalytic residues, the G202A mutation did not appreciably affect the affinity of the GDP-loaded Gαi subunit for the Gβγ dimer (Fig. S3).

Glycine to Alanine Substitution in Switch II Accelerates Yeast Gpa1 GT-Pase Activity and Restores Wild-Type Pheromone Sensitivity to RGS-Inensitive Yeast. Matting pheromone signaling in S. cerevisiae was one of the first signaling pathways shown to be regulated by an RGS protein (1, 30), with the yeast RGS protein Sst2 now well established as the principal trans-regulator of the pheromone response (31). Only one Gα subunit, Gpa1, is coupled to the yeast pheromone receptor, and it is highly similar to mammalian Gαi, including a nearly identical switch II (Fig. S4A). A “RGS-insensitivity” point mutation within switch I of Gα was first identified in S. cerevisiae (namely, Gpa1^{G302S} (32); this mutation renders Gα insensitive to RGS domain GAP activity, but leaves unaffected its capacity for intrinsic GTP hydrolysis and coupling to Gβγ, receptors, and effectors (32, 33).

We first established that the fast-hydrolysis switch II mutation functions similarly in yeast Gpa1 (i.e., G321A). Single-turnover GT-Pase assays with recombinant Gpa1 demonstrated enhanced intrinsic GT-Pase activity for Gpa1(G321A) of at least 16-fold over wild-type (Fig. S4B); this rate enhancement was similar to that provided to wild-type Gpa1 by the RGS domain of Sst2. To assess the effect of enhanced intrinsic GTP hydrolysis on receptor-mediated signaling, pheromone response assays were carried out in a-haploid S. cerevisiae strains in which the wild-type GPA1 locus was replaced (via homologous recombination) with Gpa1 point mutants: either Gpa1^{G321A} to provide faster-than-wild-type GTP hydrolysis, Gpa1^{G302S} to render the protein RGS-insensitive, or Gpa1^{G302S/G321A} to provide both activities. A pheromone pathway-specific lacZ reporter gene was used to assess dose-dependent changes in α-factor pheromone-induced transcriptional up-regulation (Fig. 2); pheromone signaling was independently measured using a “halo” assay (Fig. S5) indicative of pheromone-dependent growth inhibition (34, 35). Expression of RGS-insensitive Gpa1[G302S] led to a leftward-shifted pheromone dose–response curve, as well as larger halos and greater basal activity (Fig. 2 Inset; Fig. S5A), consistent with its original discovery as a “supersensitive allele” of Gpa1 (32) and demonstrating that endogenous Sst2 acts to decrease the sensitivity of pheromone receptor signaling through Gpa1. In contrast, expression of the fast-GTPase Gpa1[G321A] resulted in no change in basal activity or pheromone sensitivity, although a statistically significant, ~20% decrease in maximal efficacy was noted (as also observed with the G302S allele) (Fig. S5C). Addition of faster-than-wild-type hydrolysis to the RGS-insensitive Gpa1 mutant (i.e., Gpa1^{G302S/G321A}) was observed to supersede the effect of RGS-insensitivity in restoring wild-type basal activity and pheromone sensitivity (i.e., EC50), as well as the ultrasensitive nature of the response (i.e., Hill slope >1) (Fig. 2 and Fig. S5). Thus, the glycine-to-alanine fast hydrolysis mutation phenocopied the effects of the RGS protein Sst2 on agonist sensitivity.

Glycine to Alanine Substitution in Switch II Restores Neurotransmitter Signaling Kinetics and Sensitivity to RGS-Inensitive Gα

To determine the effect of accelerating hydrolysis on GPCR signaling in mammalian cells, we used HEK 293 cells transiently expressing the D2 dopamine receptor (D2R) that couples to pertussis toxin (PTX)–sensitive Gi/o proteins. Coupling to endogenous Gi/o heterotrimers was blocked by PTX pretreatment, and D2R signaling was reconstituted by expressing PTX-insensitive Gi/oA C351G. To measure Gi/oA activation, we used a recently developed bioluminescence resonance energy transfer (BRET) assay (36), wherein Gβγ dimers labeled with the YFP variant Venus (Gβγ−Y++) are liberated by active Gi subunits and bind to a Renilla luciferase-labeled, membrane-associated C-terminal fragment of GRK3 (masGRKct-Rluc8). Energy transfer

Fig. 2. The glycine-to-alanine switch II mutation Gpa1^{G321A} restores wild-type pheromone sensitivity to RGS-insensitive yeast. Strain BY4741 of S. cerevisiae was transformed with integrating plasmids (pRS406 containing Gpa1^{WT}, Gpa1^{G302S}, Gpa1^{G321A}, or Gpa1^{G302S/G321A} as well as a plasmid (pRS423 FUS1-lacZ) containing the pheromone-inducible FUS1 promoter and lacZ reporter. Yeast grown to midlog growth phase were treated with the indicated concentrations of α-factor, and the resulting β-galactosidase activity was measured by fluorescence spectrophotometry. In the time frame of α-factor treatment and subsequent transcriptional readout (90 min), the yeast RGS protein Sst2 is induced (1). Data shown are the results of three independent experiments using multiple colonies (WT, n = 14; G302S, n = 14; G321A, n = 13; G302S/G321A, n = 13) performed in triplicate. Error bars, ±SE. (Inset) A similar transcriptional reporter experiment was conducted, but yeast were left untreated to measure basal β-galactosidase activity.
between masGRKct-Rluc8 and Gβ1γ2-V provides a biosensor of G-protein heterotrimer activation amenable to both steady-state measurements and time-resolved kinetic experiments (36).

D2R activation with the agonist quinpirole (10⁻¹⁰–10⁻⁵ M) produced a graded increase in BRET between masGRKct-Rluc8 and Gβ1γ2-V (Fig. 3A). In cells expressing PTX-insensitive Gα16, referred to here as wild-type (wt), the EC₅₀ for this response was 405 ± 49 nM (n = 12). Agonist sensitivity was increased for responses mediated by RGS-insensitive Gα16 G184S (EC₅₀ = 90 ± 12 nM; n = 12; Fig. 3A), suggesting that endogenous RGS proteins decrease agonist sensitivity in HEK 293 cells. Conversely, overexpression of RGS8 decreased agonist sensitivity even further (EC₅₀ = 2.4 ± 0.4 μM; n = 8; Fig. 3A), suggesting that wt Gα subunits are not saturated by endogenous RGS proteins. When the RGS insensitivity and fast hydrolysis mutations were combined (Gα16 G184S/G203A), RGS insensitivity was preserved (Fig. S6), and yet the fast hydrolysis phenotype prevailed. Increased agonist sensitivity observed with the RGS insensitivity mutation alone was reversed by adding the fast hydrolysis mutation (EC₅₀ = 1.2 ± 0.1 μM; Fig. 3A). Responses to saturating concentrations of quinpirole were increased further by addition of hydrolysis-resistant GTPγS (Fig. 3C), indicating that the reporter of heterotrimer activation (masGRKct-Rluc8) was not saturated and, thus, the maximum response should be sensitive to changes in Gα GTase rate. Indeed, when compared with responses mediated by either wt Gα16 or RGS-insensitive Gα16 subunits, maximum responses after RGS8 overexpression and for RGS-insensitive/fast hydrolysis double mutants were significantly decreased (P < 0.01; repeated-measures ANOVA; Fig. 3C). Importantly, maximal responses were equally blunted when GTP hydrolysis was accelerated by saturating RGS8 or by the fast hydrolysis mutation (P > 0.05; Fig. 3C). These results are thus consistent with those observed for yeast pheromone signaling, and indicate that changes in Gα GTase rate in both systems produce shifts in agonist sensitivity and response amplitude.

We then took advantage of the ability of the BRET assay to indicate rapid changes in heterotrimeric G-protein activity to examine rates of response onset and recovery mediated by these Gα16 mutants. BRET between Gβ1γ2-V and masGRKct-Rluc8 was monitored during sequential addition of saturating concentrations of quinpirole (30 μM) and the antagonist haloperidol (10 μM). Both the onset and recovery of responses mediated by RGS-insensitive Gα16 G184S subunits were slowed when compared with wild-type (P < 0.01; n = 3–12 performed in quadruplicate; Fig. 3B), as seen previously in numerous cell types (37, 38). In contrast, overexpression of RGS8 accelerated both onset and recovery of responses mediated by wt Gα16, again suggesting that endogenous RGS proteins do not saturate these subunits (Fig. 3 B, D, and E). Once again, when the RGS insensitivity and fast hydrolysis mutations were combined, fast hydrolysis compensated for RGS insensitivity. Both response onset and recovery mediated by G184S/G203A double mutant subunits were significantly faster than those observed with wild-type Gα16 or RGS-insensitive Gα16 (P < 0.01; Fig. 3 B, D, and E), and were indistinguishable from those observed with RGS8 overexpression (P > 0.05; Fig. 3 D and E). Similar effects were observed using analogous PTX-insensitive Gα16 mutants in reconstituted signaling by the M4 muscarinic acetylcholine receptor, i.e., the fast hydrolysis mutation compensated for the loss of RGS sensitivity (Fig. S7 and Table S2). In this case, agonist (carbachol) sensitivity was less affected by changes in hydrolysis, similar to previous reports with native PTX-sensitive Gα subunits (14, 15). Taken together, these results indicate that accelerating GTP hydrolysis with a mutation that promotes a pretransition state for GTP hydrolysis reproduces the effects of RGS protein binding on agonist sensitivity and kinetics in mammalian cells.

Discussion

The discovery of RGS proteins resolved a longstanding discrepancy between the slow intrinsic GTase rate of Gα subunits seen in vitro and rapid termination of heterotrimeric G-protein signals seen in vivo (39, 40). However, early studies demon-
sthatting this important role of RGS proteins for timely response termination (14, 15) described new puzzling observations. By increasing the rate of GTP hydrolysis, RGS proteins were expected to speed response recovery, and this expectation was fulfilled. It was also expected that accelerating GTP hydrolysis would decrease steady-state agonist sensitivity, but this was not observed (14, 15). It appeared that the reason for the maintained agonist sensitivity in these studies was an unexpectedly large increase in the rate of response onset. The authors of these studies concluded that RGS proteins increased not only the rate of G-protein deactivation, but also the rate of G-protein activation, the latter so much so that agonist sensitivity and response amplitude were left unchanged (14, 15).

Two primary models have been proposed to account for RGS protein–mediated acceleration of response onset. The first of these stems from the observation that RGS proteins can interact directly or indirectly with GPCRs in addition to G proteins, as reviewed elsewhere (16, 18, 41). Thus RGS proteins might facilitate GPCR coupling to G proteins by directly promoting their physical interaction, i.e., by serving as physical scaffolds. The second model is similar to that originally proposed for activation of the Gq–effector and -GAP protein PLCβ (42), and suggests that rapid hydrolysis allows for rapid reactivation of Go by active receptor (9, 19, 20, 43). Such a mechanism could promote coupling by reducing the need for GPCRs and G proteins to associate by slow diffusion and collision events. The positive effect of RGS proteins because of this kinetic scaffolding mechanism would depend entirely on acceleration of GTP hydrolysis. A third potential mechanism, GAP-mediated enhancement of receptor-stimulated GDP release, was proposed recently based on modeling studies incorporating steady-state GTPase activity (19). Experimental data consistent with these models have been reported (17, 20, 44, 45). However, little experimental evidence was previously available to directly support or exclude any mechanism.

In the present study, we show that a mutation which imposes a pretransition state and accelerates GTP hydrolysis mimics the influence of RGS proteins on response kinetics and agonist sensitivity. Therefore, the GAP function of RGS proteins is sufficient to explain both the increase in the rate of deactivation and (independently) the rate of response onset. Our study leaves the underlying mechanism for this independent acceleration of receptor deactivation unexplained and thus our finding that the GAP function of RGS proteins remains incomplete. Our findings are generally consistent with, but do not definitively establish, a kinetic scaffolding model and/or other positive regulatory mechanisms that depend only on structural changes in Go. Furthermore, our results do not exclude the possibility that RGS proteins may also serve as physical scaffolds, perhaps as a means of achieving receptor-selective regulation (18, 46). However, our results do imply that physical scaffolding or other non-GAP functions of RGS proteins are not necessary to explain the effects of RGS proteins on response kinetics or agonist sensitivity.

Materials and Methods

Protein Purification, Single Turnover GTPase Assays, and Structure Determinations. Recombinant Gpa1 (aa 39–472) and Sst2 RGS domain (aa 408–698) were each cloned with N-terminal His6- and TEV-cleavage sites in a modified pET expression vector (Novagen). His6/TEV-tagged human Gβi1, both wild-type and G202A mutant, and the two yeast proteins were separately purified from clarified Escherichia coli lysate using sequential Ni-NTA affinity, TEV cleavage, anion exchange, and size-exclusion chromatographies essentially as previously described (47). Single-turnover GTPase assays using recombinant Gs subunits were conducted essentially as described previously (48, 49). For crystallization experiments, purified Gβi2(G202A) protein was concentrated to 10 mg/mL and stored in crystallization buffer (50 mM Hepes pH 8, 5 mM DTT, 1 mM EDTA, 1 mM MgCl2, and 100 μM GDP) with or without added AlCl3 (30 μM) and NaF (10 mM). Details of crystal growth and structure determinations are presented in SI Materials and Methods.

Phoreme Transcriptional Reporter Assay and Halo Assay of Growth Inhibition. Standard methods for manipulating S. cerevisiae expression plasmid DNA and the growth, maintenance, and transformation of yeast were used throughout, as previously described (34, 35). Yeast strains bearing integrated gpa1 mutation(s), created by homologous recombination and validated by PCR as described in SI Materials and Methods, were transformed with the transcriptional reporter plasmid pRS423 FUS1-lacZ, and β-galactosidase activity was determined as described elsewhere (34, 35). The phoreme-induced growth inhibition assay has also been described previously (50).

BRET Measurements. Agonist-dependent cellular measurements of bioluminescence resonance energy transfer between masGRKt-RLuc and Giβ2γ V were performed as previously described (36). Details, including curve-fitting examples (Fig. 5B), are available in SI Materials and Methods.

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Supporting Information

Supporting Information Corrected January 26, 2012

SI Materials and Methods

Single Turnover GTPase Assays. Mutations of Gly-321 to alanine in the S. cerevisiae Gpa1 DNA and Gly-202 to alanine in the human Gαi1 cDNA were separately achieved using QuikChange site-directed mutagenesis (Stratagene). Mutant or wild-type Gα protein (100 nM) was incubated at 30 °C for 15 min in buffer C (50 mM Tris, pH 7.5, 0.05% C12E10, 1 mM DTT, 5 mM BSA, 10 mM EDTA, 100 mM NaCl) containing ~1 × 10^13 cpm of [γ-32P]GTP (6,000 Ci/mmol). Samples were then placed on ice for 5 min. GTPase reactions (on ice) were initiated by adding 10 mM MgCl2 (with 100 mM GTP) to ensure measuring a single round of GTP hydrolysis, and timed reaction aliquots were quenched by charcoal slurry (containing 20 mM H3PO4, pH 3) followed by centrifugation (~4,000 × g for 10 min at 4 °C). Supernatants containing free [32P] inorganic phosphate were analyzed by scintillation counting. Background counts (in the absence of Gα) were subtracted from all experimental conditions.

Crystal Growth and Structure Determinations. Crystals of Gαi1-(G202A) protein (in GDP form) were grown using the hanging drop method in which 4 μL of protein was mixed 1:1 with 4 μL of buffer (1.8–2.2 mM ammonium sulfate, 100 mM sodium acetate, pH 5.5–6.5). Crystals formed in 2–4 days at 18°C. The GDP-bound protein crystallized in the space group I4 (a = b = 120 Å, c = 68.2 Å; α = β = γ = 90°) with 1 molecule in the asymmetric unit. Crystals were cryoprotected in crystallization buffer supplemented with 20% glycerol for ~1 min and submerged in liquid N2. Native data sets of 2.34 Å (GDP form) were collected at the UNC-Chapel Hill Biomolecular x-ray Crystallography Facility using an R-Axis IV++ beamline. Data were scaled and indexed using HKL-21000 (1). The structure of wild-type Gαi1-GDP (PDB id 1GDD; ref (2)), excluding waters and GDP, was used for molecular replacement (3). Model building was completed using Coot (4) and O (5), with successive rounds of simulated annealing, minimization, B-group, and rigid body refinements being completed by CNS (6). All electron density map calculations were completed with CNS. All structural images were generated using PyMol (DeLano Scientific) unless otherwise denoted.

Surface Plasmon Resonance–Based Assay of the Gαi/GDP/Gβγ Interaction. Wild-type and G202A mutant His6-Gαi1 proteins were purified as described previously in the main text except that, after Ni-NTA chromatography, protein fractions were pooled and resolved on a calibrated Superdex S200 gel filtration column equilibrated with HEPES pH 7.5 (50 mM), NaCl (150 mM), GDP (10 μM), and glycerol (2.5% vol/vol). Surface plasmon resonance-based measurements of Gαi1-GDP binding were performed on a Biacore 3000 (GE Healthcare) at room temperature using separate streptavidin biosensors (Sensor Chip SA) containing either 500 or 800 response units (RU) of immobilized, biotinylated Gβγ1γ2 (ref. 7), or 800 RU of biotinylated Gαi1 (8), as a negative control surface (i.e., Gα subunits do not dimerize). Before injection over biosensor surfaces, Gαi1 proteins were diluted in Biacore running buffer of 10 mM Hepes pH 7.4, 150 mM NaCl, and 0.005% (vol/vol) Nonidet P-40. Injections were performed using the KINJECT command with an injection volume of 200 μL and a dissociation time of 300 seconds at a flow rate of 20 μL/min. Bulk changes in refractive index were subtracted in BiaEvaluation software (GE Healthcare) using sensorgram curves derived from the nonspecific binding surface. Dose–response curves were subsequently plotted from the normalized binding observed at equilibrium (590 s after injection start; n = 2 for each dose) vs. GDP concentration; nonlinear regression was performed to determine apparent affinities (Kd values) and their standard error using GraphPad Prism v.5.0c.

Yeast Strains and Plasmids. The yeast S. cerevisiae strain used in this study was BY4741 (MATα leu2Δ met15Δ his3-1 ura3Δ), in combination with yeast shuttle plasmids pRS406 (ampR, URA3) and pRS423 FUS1lacZ. Gpa1 point mutants were integrated into BY4741 cells using the pRS406 integrating vector; by inserting truncated gpa1 lacking the promoter region and the start codon, it was possible to simultaneously insert the mutant gpa1 and knock out the wild-type GPA1 gene. The pRS406 GPA115388pbl plasmid was constructed by PCR amplification of GPA1 from genomic DNA with forward primer 5′-TAT CTA GAC GAG CAA TTC TGG CAG CTG G-3′ (containing an XbaI site) and reverse primer 5′-CAT AGC ATG CAC GTA TCA TAT GCT ATG G-3′ (containing a ClaI site). The PCR product was cloned into the XbaI/ClaI sites of the pRS406 multicloning region. Genomic integration of Gpa1 was used linearization via an unique HindIII site in the gpa1 open-reading frame downstream of both the G302S and the G321A point mutations. These two point mutations were created using QuikChange site-directed mutagenesis (Stratagene): G302S by using mutagenic primer 5′-GGC GTA TAA AGA CTA CAA GCA TTA CAG AAA CCG-3′ and its reverse complement, and G321A using mutagenic primer 5′-GGT TCT CGA CCG TGC TGG GCA GCC TTC TGA ACG-3′ and its reverse complement. Correct genomic integration of mutants was assayed by PCR. The forward primer was designed to terminate on the signal base-pair substitution of the mutant gene: G302S mutant detection primer was 5′-GGG CCG TAT AAA GAC TAC AA-3′ and the G321A mutant detection primer was 5′-TTC AAG GTT CTC GAC GCT GC-3′. Amplification of a PCR product using the mutant detection primers, and a lack of a PCR product using wild-type primers, was indicative of integration of the mutant gpa1.

Pheromone Transcriptional Reporter Assays. Yeast cells were grown to an OD_{600} of ~0.8 and treated with α-factor pheromone for 90 min before addition of a fluorescein di-β-galactopyranoside (FDG) solution (final concentrations: 83 nM FDG, 0.04% Triton X-100, 21.6 mM Pipes, pH 7.2) and further incubation in the dark at 37 °C for 60 min. Reaction was stopped by the addition of Na2CO3 to a final concentration of 143 mM, and fluorescence at 485–530 nm was measured with a VersaMax optical plate reader (Molecular Devices). Data were analyzed with Prism (GraphPad Software) and dose–response curves fit via nonlinear regression analysis (sigmoidal dose–response, variable slope).

Halo Assay of Pheromone-Induced Growth Inhibition. Yeast cells were grown to saturation in liquid medium, diluted (1:400) into 4 mL of 0.5% (wt/vol) dissolved agar (50 °C), and then spread onto culture plates of the same medium. Sterile filter discs were spotted with recombinant α-factor peptide (3, 1, 0.3, and 0.1 μg) and placed onto the freshly spread lawn of yeast cells. The resulting zone of growth inhibition was measured after 2 days incubation at 30 °C.

Plasmid DNA Constructs for Mammalian Expression. The Gβγ BRET sensor masGRKct-RluC8 contained amino acids G495-L688 of bovine GRK3 (NP_776925; a.k.a. β-adrenergic receptor kinase 2.
or βARK2), preceded by a myristic acid attachment peptide (mas; MGSSKSKTSNS). The stop codon of GRK3 was replaced with a GG linker, which was followed by the Renilla reniformis luciferase variant Rlu8c (9). The original masGRK3 construct was a generous gift from Stephen R. Ikeda (National Institute on Alcohol Abuse and Alcoholism, Rockville, MD). Gβ1γδ2-V was expressed by cotransfecting plasmids encoding amino acids 1–155 of the fluorescent protein Venus fused to a GGSGGG linker and the N terminus of human Gγ3 (Venus1-155-Gγ3) and amino acids 156–239 of Venus fused to a GGSGGG linker and the N terminus of human Gβ1 (Venus155-239-Gβ1) (10). RGS insensitivity (G184S) and switch-II fast hydrolysis (G203A) mutations were introduced into PTX-insensitive (C351G) human Gα1a. All constructs were made using an adaptation of the QuikChange (Stratagene) mutagenesis protocol, were expressed from pcDNA3.1 (Invitrogen), and were verified by automated sequencing.

Mammalian Cell Culture and Transfection. HEK 293 cells (ATCC) were propagated in plastic flasks according to the supplier’s protocol. Cells were transfected using linear polyethyleneimine (MW 25,000; Polysciences Inc) at an N/P ratio of 20; up to 3 μg of plasmid DNA was transfected per well of a six-well plate. Cells were propagated in plastic plates by rinsing with PBS-EDTA and triturating in PBS. Suspension cells were transferred to black 96-well microplates (Nunc, Thermo Scientific) and benzyl-coelenterazine (coelenterazine h; 5 μM; Nanolight Technologies) was added to all wells immediately before making measurements. Luminescence measurements were made using a photon-counting multimode plate reader (Mithras LB940; Berthold Technologies). The raw BRET signal (em535/480) was calculated as the emission intensity at 520–545 nm divided by emission intensity at 475–495 nm. Net BRET was this ratio minus the same ratio measured from cells expressing only the BRET donor (Rlu8c). Data points were generally collected every 0.6 s, although a subset of experiments was performed with data points collected every 0.1 s. Raw data points were fitted to a single exponential function using the method of least squares and the Levenberg-Marquardt algorithm. Fitted curves were judged as adequate when the coefficient of determination (R²) was >0.8. Curve fitting was performed to provide an objective estimate of response onset and recovery kinetics, even in cases when it was clear that more than a single process governed the time course (e.g., acute response desensitization with rapid hydrolysis; Fig. 3B).

BRET-Based Assessment of RGS Domain/Gα Mutant Interactions. Venus was fused to the C terminus of rat RGS8 with a GG linker. Rlu8c flanked by GGSGGG linker was fused to the various Gα1a mutants internally between amino acids 91 and 92. Cells expressing these constructs were exposed to either PBS or PBS with 10 mM MgCl₂, 60 μM AlCl₃, and 10 mM NaF (to form aluminum tetrafluoride).
**Fig. S1.** Electron density representation of the final model of Gαi1(G202A) bound to GDP, highlighting the bound nucleotide, the Gαi1 residues Arg-178 and Thr-181 of switch-I, and the mutant alanine (in italics) of switch-II at residue position 202. Experimental electron density is shown as a 2Fo-Fc simulated annealing composite omit map contoured at 1σ (electron density shown as blue mesh). Ordered waters are highlighted with purple asterisks. Image was generated in Coot.

**Fig. S2.** Comparisons of the structural models of wild-type Gαi1 in its inactive (GDP-bound; A) and transition-state mimetic (GDP·AlF4−-bound; B) forms, with the Gαi1 G202A mutant, highlighting dispositions of the three switch regions (SI, SII, and SIII) and the key catalytic residues Arg-178, Thr-181, and Gln-204. Structural models were derived from PDB records: 1GP2 (orange), wild-type Gαi1·GDP (within the Gαi1·Gαβγ heterotrimer complex which leads to ordered switch regions vs the disorder seen with isolated, wild-type Gαi1·GDP); 1GF1 (red), wild-type Gαi1·GDP·AlF4−; and, 3UMS (blue), Gαi1(G202A)·GDP.
Fig. S3. The G202A mutation does not dramatically alter the affinity of Gα·GDP for Gβγ. Wild-type Gαi1·GDP and Gαi1(G202A)·GDP (at indicated concentrations) were separately injected over immobilized, biotinylated Gβ1γ1 biosensor surfaces. Surface plasmon resonance (SPR) responses achieved at equilibrium binding were normalized to maximal resonance unit (RU) responses and plotted vs. Gα concentration. Resultant Bmax curves were fit by nonlinear regression to obtain dissociation constant (Kd) values which are presented with standard error (GraphPad Prism) (Inset).

Fig. S4. The fast-hydrolysis glycine-to-alanine switch II mutation is transferable to S. cerevisiae Gpa1. (A) Alignment of human Gαi1 (GenBank AAM12619) and S. cerevisiae Gpa1 (GenBank NP011868) highlighting conservation of switch II residues (green dots), including Gly-202 of Gαi1 (Gly-321 of Gpa1; red asterisk). Secondary structure and colored highlights of conserved nucleotide contacts (magenta), GTPase catalytic residues (orange), and Gβ contact residues (red dots) follow those of Johnston and Siderovski (1). (B) Single-turnover GTP hydrolysis data demonstrating enhanced intrinsic GTPase rate of Gpa1(G321A). Purified Gα protein (100 nM) was loaded with [γ-32P]GTP (∼1 × 106 cpm) for 15 min at 30 °C in the absence of Mg2+, followed by 5 min incubation on ice. Baseline aliquots were removed and MgCl2 (10 mM)−containing buffer, with or without the RGS domain of Sst2 (aa 420–689), was added to initiate reactions. Aliquots were taken at indicated timepoints and analyzed for [32P]Pi content as previously described (2). Calculated GTPase rates were as follows: Gpa1(wt): 0.28 min⁻¹; Gpa1(wt) + Sst2(RGS domain): 4.2 min⁻¹; Gpa1(G321A): 4.6 min⁻¹.

Fig. S5. **Gpa1**<sub>G321A</sub> restores proper pheromone signaling to RGS-insensitive yeast. Strain BY4741 of *S. cerevisiae* was transformed with integrating plasmids (prS406) containing **Gpa1**<sub>WT</sub>, **Gpa1**<sub>G302S</sub>, **Gpa1**<sub>G321A</sub>, or **Gpa1**<sub>G302S/G321A</sub>. (A) Yeast were plated onto solid medium and exposed to paper discs containing α-factor (counterclockwise starting from bottom left: 0.1, 0.3, 1.0, and 3.0 μg) for 48 h. (B) To ensure equal expression of the integrated **Gpa1** gene locus in all four strains, yeast grown to midlog phase were collected and fractionated/resolved by 10% SDS/PAGE for immunoblotting using anti-**Gpa1** antibodies and anti-Sst2 antibodies. Diploid yeast that do not express **Gpa1** or Sst2 served as a negative control (first lane). The nonspecific protein band below **Gpa1** was used to determine equal loading. (C) Quantification of the halo formation and transcriptional response and assays on the indicated haploid yeast strains. Halo length represents the diameter of the halo for the highest concentration of pheromone tested (3.0 μg). A 50% effective dose (EC<sub>50</sub>) and Hill slope values were derived from measured transcriptional responses over 11 different pheromone concentrations subjected to nonlinear regression analysis (sigmoidal dose–response, variable slope).

<table>
<thead>
<tr>
<th><strong>Gpa1</strong></th>
<th>Halo length (mm)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>Hill slope</th>
<th>Max. response (%&lt;sub&gt;wt&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wildtype (wt)</td>
<td>21</td>
<td>2.07 ± 0.08</td>
<td>2.1 ± 0.1</td>
<td>100</td>
</tr>
<tr>
<td>G302S (RGS-i)</td>
<td>29</td>
<td>0.26 ± 0.02</td>
<td>1.1 ± 0.1</td>
<td>83.5 ± 1.4</td>
</tr>
<tr>
<td>G321A (fast GTPase)</td>
<td>21</td>
<td>1.98 ± 0.07</td>
<td>2.2 ± 0.1</td>
<td>80.5 ± 1.0</td>
</tr>
<tr>
<td>G302S/G321A</td>
<td>21</td>
<td>1.82 ± 0.06</td>
<td>2.2 ± 0.1</td>
<td>86.2 ± 1.1</td>
</tr>
</tbody>
</table>

Fig. S6. Aluminum tetrafluoride (AlF<sub>4</sub><sup>-</sup>)–induced BRET signal was observed to be significantly greater for wild-type **GαoA** ("wt") and the fast-hydrolysis G203A ("GA") mutant of **GαoA**, compared with the RGS-insensitive G184S-containing **GαoA** mutants (n = 5 in quadruplicate; P < 0.001, one-way ANOVA with Bonferroni means comparison).

**GαoA-Rluc8 + RGS8-V**

![Graph showing AlF<sub>4</sub><sup>-</sup>-induced BRET signal](image)

- wt
- GS
- GA
- GS/GA

* Indicates significantly greater BRET signal compared to the control.
Fig. S7.  The glycine-to-alanine switch II mutation G202A restores rapid onset and recovery kinetics to responses of the M4 muscarinic acetylcholine receptor as mediated by RGS-insensitive G\(_{\alpha i1}\) in HEK 293 cells.  (A) Normalized BRET between masGRKct-Rluc8 and G\(_{\beta 1\gamma 2}\)-V is plotted against time during sequential addition of carbachol (30 \(\mu\)M) and atropine (100 \(\mu\)M) for each of the four G\(_{\alpha i1}\) variants indicated (all pertussis-toxin insensitive).  Traces represent the mean of three experiments, each performed in quadruplicate.  (B) Average onset time constants plotted for each of the four G\(_{\alpha i1}\) variants (\(\pm\) SEM, \(n=5\)).  (C) Average recovery time constants plotted for each of the four G\(_{\alpha i1}\) variants (\(\pm\) SEM, \(n=3\), performed in quadruplicate); \(^*P<0.05\), \(^{**}P<0.01\) vs. wt.

Fig. S8.  Examples of single exponential curves fitted by the method of least squares. (A) Onset phases mediated by D2R and the indicated G\(_{\alpha oA}\) subunits were fitted to a single exponential function; the agonist quinpirole was added at time = 0; \((n=6)\).  In these experiments only a single wavelength (355 nm) was monitored to allow data points to be acquired every 0.1 s.  (B) Recovery phases; haloperidol was added at time = 0 to cells that had been pretreated with quinpirole \((n=6)\).  In these experiments, the BRET ratio was monitored every 0.6 s.  Derived mean onset and recovery time constants \((\tau)\) and \(R^2\) for these six experiments \((\pm SEM)\) are given.


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Table S1. Data collection and refinement statistics for Gαi1(G202A) crystal structure

<table>
<thead>
<tr>
<th>Gαi1(G202A)·GDP PDB id 3UMS</th>
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<tbody>
<tr>
<td>Data collection</td>
<td></td>
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<tr>
<td>Space group</td>
<td>I4</td>
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<tr>
<td>Unit cell dimensions a, b, c (Å)</td>
<td>120.5, 120.5, 67.9</td>
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<tr>
<td>α, β, γ (°)</td>
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<tr>
<td>Resolution range (Å)</td>
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<tr>
<td>No. of unique reflections</td>
<td>19,799/344</td>
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<tr>
<td>Rmerge</td>
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<tr>
<td>Mean I/σ²</td>
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<tr>
<td>Completeness (%)</td>
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<tr>
<td>Redundancy</td>
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<td>Refinement</td>
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<tr>
<td>No. of reflections (working/test set)</td>
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<tr>
<td>Rwork/Rfree (%)</td>
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<tr>
<td>No. of atoms (non-hydrogen):</td>
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</tr>
<tr>
<td>Protein</td>
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</tr>
<tr>
<td>GDP/SO₄²⁻/Cl⁻</td>
<td>28/5/1</td>
</tr>
<tr>
<td>Waters</td>
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<tr>
<td>Average B-factor (Å²)</td>
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<td>Protein</td>
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<tr>
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<tr>
<td>Waters</td>
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<td>Ramachandran plot (% in region):</td>
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<td>97.0</td>
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<td>Generously allowed</td>
<td>3.0</td>
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<tr>
<td>Disallowed</td>
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Numbers in parentheses pertain to the highest-resolution shell.

Table S2. Signal onset and recovery data as measured in this study and those of Saitoh et al. (1) and Doupnik et al. (2), along with effective concentration at 50% maximum (EC₅₀) and the maximum agonist-induced response divided by the maximum response in the presence of GTPγS (Rmax; e.g., Fig. 3C)

<table>
<thead>
<tr>
<th>Receptor system</th>
<th>τ_{onset} (s)</th>
<th>τ_{recov} (s)</th>
<th>EC₅₀ (M)</th>
<th>Rmax</th>
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<tbody>
<tr>
<td>D2R GαoA wt</td>
<td>1.8</td>
<td>7.5</td>
<td>4.1 × 10⁻⁷</td>
<td>0.69</td>
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<tr>
<td>D2R GαoA G184S</td>
<td>3.0</td>
<td>31.9</td>
<td>9.0 × 10⁻⁸</td>
<td>0.75</td>
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<tr>
<td>D2R GαoA G203A</td>
<td>0.8</td>
<td>2.9</td>
<td>4.9 × 10⁻⁷</td>
<td>0.51</td>
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<tr>
<td>D2R GαoA wt + RGS8</td>
<td>0.9</td>
<td>1.9</td>
<td>2.4 × 10⁻⁶</td>
<td>0.35</td>
</tr>
<tr>
<td>D2R GαoA GS/GA</td>
<td>0.8</td>
<td>2.1</td>
<td>1.2 × 10⁻⁶</td>
<td>0.44</td>
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<tr>
<td>M4R Gκ1 wt</td>
<td>2.07</td>
<td>10.43</td>
<td>5.1 × 10⁻⁷</td>
<td>0.81</td>
</tr>
<tr>
<td>M4R Gκ1 G183S</td>
<td>3.00</td>
<td>41.13</td>
<td>2.0 × 10⁻⁷</td>
<td>0.89</td>
</tr>
<tr>
<td>M4R Gκ1 G202A</td>
<td>1.02</td>
<td>7.91</td>
<td>2.8 × 10⁻⁷</td>
<td>0.89</td>
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<tr>
<td>M4R Gκ1 GS/GA</td>
<td>0.86</td>
<td>7.56</td>
<td>1.3 × 10⁻⁷</td>
<td>0.86</td>
</tr>
</tbody>
</table>

(Saitoh) D2R -RGS8 | 2.91 | 12.76 | 6.7 × 10⁻⁷ |
(Saitoh) D2R +RGS8 | 1.10 | 4.71 | 1.0 × 10⁻⁶ |
(Saitoh) M2R -RGS8 | 6.02 | 11.36 | 2.1 × 10⁻⁷ |
(Saitoh) M2R +RGS8 | 1.19 | 3.91 | 2.5 × 10⁻⁷ |
(Doupnik) M2R -RGS4 | 1.40 | 21.00 | ~50 × 10⁻⁹ |
(Doupnik) M2R +RGS4 | 0.60 | 5.00 | ~50 × 10⁻⁹ |