Gβγ Inhibits Gα GTPase-activating Proteins by Inhibition of Gα-GTP Binding during Stimulation by Receptor*

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Gβγ subunits modulate several distinct molecular events involved with G protein signaling. In addition to regulating several effector proteins, Gβγ subunits help anchor Gα subunits to the plasma membrane, promote interaction of Gα with receptors, stabilize the binding of GDP to Gα to suppress spurious activation, and provide membrane contact points for G protein-coupled receptor kinases. Gβγ subunits have also been shown to inhibit the activities of GTPase-activating proteins (GAPs), both phospholipase C (PLC)-βs and RGS proteins, when assayed in solution under single turnover conditions. We show here that Gβγ subunits inhibit G protein GAP activity during receptor-stimulated, steady-state GTPase turnover. GDP/GTP exchange catalyzed by receptor requires Gβγ in amounts approximately equimolar to Gα, but GAP inhibition was observed with superstoichiometric Gβγ. The potency of inhibition varied with the GAP and the Gα subunit, but half-maximal inhibition of the GAP activity of PLC-β1 was observed with 5–10 nm Gβγ, which is at or below the concentrations of Gβγ needed for regulation of physiologically relevant effector proteins. The kinetics of GAP inhibition of both receptor-stimulated GTPase activity and single turnover, solution-based GAP assays suggested a competitive mechanism in which Gβγ competes with GAPs for binding to the activated, GTP-bound Gα subunit. An N-terminal truncation mutant of PLC-β1 that cannot be directly regulated by Gβγ remained sensitive to inhibition of its GAP activity, suggesting that the Gβγ-binding site relevant for GAP inhibition is on the Gα subunit rather than on the GAP. Using fluorescence resonance energy transfer between cyan or yellow fluorescent protein-labeled G protein subunits and Alexa532-labeled RGS4, we found that Gβγ directly competes with RGS4 for high-affinity binding to Gα-GDP-AIF4.

Gβγ subunits perform diverse roles in G protein-mediated signaling, almost all of which are based on their regulated binding to Gα. Gβγ binds most tightly to the GDP-bound form of Gα, usually considered the inactive conformation. Because Gβγ and GDP bind cooperatively to Gα, Gβγ stabilizes GDP binding and thus suppresses spontaneous Gα activation. Conversely, GDP stabilizes Gβγ binding and suppresses its ability to spontaneously regulate effector proteins. In contrast, Gβγ binds least tightly to the GTP-bound, activated conformation of Gα. Such negative cooperative binding permits Gβγ to regulate effectors during G protein activation. In solution, activation of Gα by a non-hydrolyzable GTP analog can drive physical dissociation of Gβγ (1–4) (see Refs. 5 and 6 for reviews). Complete dissociation of Gβγ from Gα-GTP also occurs to some extent in biological membranes during receptor-initiated activation of Gα (7, 8). However, recent data suggest that Gβγ bound to Gα-GTP can also interact productively with effector proteins without dissociating from the heterotrimer (8–11). This implies that the Gα-GTP-Gβγ complex somehow exposes the sites on Gα and Gβγ necessary for productive interaction with effector proteins while they remain bound to each other.

Gβγ-regulated effectors include K+ and Ca2+ channels, adenylyl cyclase, phospholipase C-β (PLC-β), PI 3-kinase, and some protein kinases (6). Effector interaction sites on the Gβγ molecule that exert these various functions have been mapped by a variety of chemical and mutagenic strategies. Together, they appear to cover a large fraction of the Gβγ surface, despite significant overlap that makes several effectors bind competitively with respect to each other (6, 12–16). Gβγ also binds a diverse collection of other proteins: G protein-coupled receptor kinases, phosducin, AGS1, and receptors (6).

In addition to its other functions, Gβγ subunits have been shown to inhibit the activity of G protein GAPs in solution-phase, single turnover assays (17–19). GAPs accelerate hydrolysis of GTP bound to Gα and thus promote Gα deactivation. GAPs can thereby inhibit steady-state signaling, accelerate signal termination when receptor agonist is removed, alter receptor selectivity among G proteins, and/or suppress basal (receptor-independent) signal output (see Ref. 20 for review). Gβγ inhibits the GAP activity of both RGS proteins and phospholipase C-βs and inhibits GAP activity toward all Gα subunits tested. The potency and extent of inhibition vary considerably among GAPs and Gα targets.

The physiological function of GAP inhibition by Gβγ is intriguing and presently unclear. It may represent a way to shield Gα-GTP from deactivation until it encounters an effector protein, or it may support continued activation in cases where Gβγ does not physically dissociate from Gα-GTP. The mechanism of inhibition is also not known in detail. We initially speculated that Gβγ might inhibit GAP activity by binding Gα-GTP and thus blocking the GAP binding site, but the relatively low concentrations of Gβγ that effectively inhibited several GAPs seemed inconsistent with the presumed low affinity of Gβγ for Gα-GTP. Gβγ might also interact directly with GAPs. Binding of Gβγ to RGS proteins has not been observed, but Gβγ does bind PLC-β and thus stabilize its phospholipasic activity. It was unknown whether the sites that cause phospholipase activation and GAP inhibition are identical.

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4 The abbreviations used are: PLC, PIP2-selective phospholipase C; PI, phosphatidylinositol; GAP, GTPase-activating protein; FRET, fluorescence resonance energy transfer; GTPyS, guanosine 5′-O-(3-thiotriphosphate); RGS4-197, a mutant RGS4 that lacks all cysteine residues except 197 (G25, C485, C715, C3125, C1835, C2045/C1213, C33A, C95A/C148V); RGS4-197FI, RGS4-197FI covalently labeled with Alexa532; GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; AChR, acetylcholine receptor.
We have now examined the mechanism whereby Gβγ inhibits G protein GAP activity using both enzyme kinetic approaches and direct optical measurements of Ga-RGS binding and dissociation. We find that Gβγ inhibits GAPs by competitively binding to Ga-GTP. In the case of PLC-β, the Gβγ binding site that mediates PLC stimulation is not required for inhibition of GAP activity. Gβγ binds Ga-GTP with unexpectedly high affinity, with values of $K_d$ well within the range of concentrations of Gβγ associated with regulation of its effectors. Thus Gβγ may protect Ga subunits from GAP-accelerated deactivation, and Gβγ from one heterotrimer may exert this effect on a heterologous Ga.

**EXPERIMENTAL PROCEDURES**

**Proteins**—m1AChR and m2AChR, Ga$q_1$, Ga$p$, Gβ1γ2y, and PLC-βs were expressed in Sf9 cells and purified as described (21). Heterotrimeric G proteins were prepared by mixing stoichiometric amounts of purified Ga and Gβ1γ2y. Gβ1γ2isofroms were used in all experiments. RGS-Z1 was expressed in Escherichia coli and purified as described (22). cDNAs for fusions of Ga$q_1$ with enhanced GFPs (Clontech) were prepared in pQE60 (Qiagen). GFP was inserted between residues 118 and 119 of Ga$q_1$, with the linker peptides GTSSGGGGS and SGGGGGTAG-­GHSHHHHHGGG. The citrine YFP mutant, which is minimally sensitive to quenching by F$^-$ (23), was prepared using the QuikChange protocol (Stratagene). Wild-type and mutant Ga$q_1$ were co-expressed in E. coli with yeast protein N-myristoyltransferase and purified as described (24). The PLC-β1Δ141 mutation, in which amino acid residues 2–141 are removed, was generated by PCR. The N-terminal hexahistidine tag was retained. Recombinant baculoviruses were produced as described previously (25). PLC-β1Δ141 was purified essentially as described for wild-type PLC-β1 (21). PLC-β2 and PLC-β3, both hexahistidine-tagged, were purified as described (21) after expression in Sf9 cells using baculovirus vectors that were a gift from Paul Sternweis (University of Texas Southwestern Medical Center). Purified phosducin was a gift from Barry Willardson ( Brigham Young University).

RGS4-197, a mutant RGS4 in which all cysteine residues except Cys379 were removed, was constructed by several rounds of QuikChange mutagenesis of the cDNA in the pGEX-2 expression vector. RGS4-197 displays essentially wild-type GAP activity both before and after its reaction with maleimides at the sole remaining cysteine residue. Mutant and wild-type RGS4 were expressed as glutathione S-transferase fusions using the pGEX-2 vector. For production of wild-type and mutant RGS4, transformed BL21DE3/pREP4 cells were grown in T7 medium (containing ampicillin, kanamycin, and isopropyl 1-thio­-β-D-galactopyranoside) for 6 h. Cell pellets were frozen in liquid N$_2$. Bacteria were thawed on ice and incubated with 0.5 mg/ml lysozyme in Buffer A (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol, 1 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride) for 30 min on ice. After sonication (three 1-min bursts), the suspension was centrifuged at 4 °C at 15,000 × g for 40 min. The supernatant was diluted 1:2 with Buffer A and mixed with glutathione-agarose for 2 h at 4 °C. The resin was washed with Buffer A and Buffer A plus 0.1% Lubrol plus 90 mM NaCl. Finally, the resin was equilibrated in 1 mM CaCl$_2$, removed from the column, and mixed with 4 μl/ml thrombin for 17–18 h at 4 °C. The resin was returned to the column and eluted with another 2 volumes of Buffer A plus 0.1% Lubrol, concentrated by ultrafiltration, frozen in liquid N$_2$, and stored at −80 °C.

RGS4-197 was labeled with the thiol-reactive probe, Alexa532-C$_7$-maleimide, which does not change its activity as a Ga$q_1$ GAP. Prior to labeling, RGS4-197 (800 μg) was dialyzed with 3000 volumes of buffer (25 mM Hepes, pH 7.5, 100 mM NaCl, 0.05% Lubrol) to remove dithiothreitol. A 10-fold molar excess of probe was added and incubated for 45 min at 0 °C, and the reaction was quenched with β-mercaptoethanol.

The reaction mixture was centrifuged at 4 °C at 13,000 rpm for 10 min, and the supernatant was applied to a G-25 gel filtration column (30 ml). Peak fractions were pooled, concentrated, frozen in liquid N$_2$, and stored at −80 °C. Labeling efficiency was determined according to the protein concentration (26) and the absorption of Alexa532 at 528 nm (ε = 78,000 cm$^{-1}$ M$^{-1}$).

Unilamellar receptor-G protein vesicles were reconstituted as described previously (21). Vesicles were incubated with 5 mM dithiothreitol at 0 °C for 1 h to activate receptors (21). The concentration of receptors in the vesicles was assayed by $[^3]$Hquinuclidinylbenzilate binding (27). Receptor-coupled Ga was measured according to carba­chol-stimulated $[^35]$GTPγS binding (21).

**Functional Assays**—Single turnover GAP assays, which measure acceleration of the hydrolysis of a preformed complex of Ga–γ-3P-GTP in detergent solution, were performed as described previously (28). Temperature, which was varied for some experiments, and concentrations used for specific experiments are given in the figure legends. Steady-state GAP activity was determined according to the increase in agonist-stimulated GTPase activity in phospholipid vesicles that contained trimeric Geβγγ and receptor (28).

To measure the rate of hydrolysis of Gs-bound GTP under the conditions of steady-state turnover, m1AChR-Gs vesicles and agonist were first mixed in assay buffer (21) for 25 min at 10 °C to allow association of receptor and Gs. An aliquot (20 μl) was then mixed with 5 μl of assay buffer that contained carbachol and [γ-32P]GTP (300 nM final concentration). The mixture was incubated for 1 min to initiate steady-state hydrolysis and allow accumulation of Gs–[γ-32P]GTP without excessive production of background $[^32]$P$_i$. At this point, defined as t = 0, further [γ-32P]GTP binding was quenched by a 1:2 dilution with 2 mM nonra­
Dissociation of Gαq-CFP and RGS4-197Fl was assayed in the same buffer used for equilibrium experiments. Fluorescence stopped flow measurements were performed using a Bio-Logic SFM3 stopped flow mixer in which syringe, mixing chambers, and delay lines are all under thermostat control. Gαq-CFP (20 nM) was incubated with 200 nM RG54-197Fl in one syringe of the mixer for at least 2 min at 25 °C. An aliquot of this mixture (50 μl) was mixed with an equal volume of 500 nM nonfluorescent Gαq in the presence or absence of 1 μM Gβγ2. Dissociation was measured by the loss of transferred acceptor fluorescence at 10-ms intervals in a 30-μl cuvette. Excitation was set at 431 nm using a monochromator, and emission was measured using a 520 nm cut-off filter.

**RESULTS**

Gβγ inhibits the GAP activities of RGS proteins and PLC-β (17–19), modulates nucleotide binding to Gαq, and is required essentially for G protein regulation by receptors (6). We therefore asked how Gβγ alters interactive regulation by receptors and GAPs during steady-state GTPase turnover, which includes all three processes. As shown in Fig. 1A, excess Gβγ2 markedly and potently inhibited steady-state GTPase activity in a coupled system composed of a receptor, a trimeric G protein, and a GAP. In the case of Gαq, m1AChR, and PLC-β1, inhibition was nearly complete, to about the level observed without a GAP (compare with Fig. 1B). Inhibition was maximal at 50 nM Gβγ and displayed an IC50 of about 5 nM above the amount of Gβγ already included in the vesicles as part of the Gαq heterotrimer (~2 nm). Gβγ inhibition of GAP activity during steady-state GTPase turnover is thus more potent than during a single catalytic cycle (19). Onset of inhibition was rapid upon the addition of Gβγ (5 min in Fig. 1) and displayed no obvious lag. Qualitatively similar fast and complete inhibition was observed when PLC-β3 was used as the GAP (not shown) or when the test system was

dioactive GTP, 2 mM ATP, and the indicated concentration of PLC-β1. Hydrolysis of bound [γ-32P]GTP was quenched at the appropriate times as described (28). Release of [32P]Pi was fitted to a single exponential equation to yield the rate constant k hydroly.

To measure the rate of GDP dissociation, [α-32P]GDP was first bound to m1AChR-Gαq vesicles by incubation with 2 μM [α-32P]GDP, 1 mM carbchol, and 10 nM PLC-β1, with or without Gβγ2, for 15 min at 15 °C. Aliquots of this mixture were diluted with antagonist and 5 mM unlabeled GTP, dissociation was allowed to proceed for various times, and bound [α-32P]GDP was measured as described (21).

Phospholipase activity was determined according to hydrolysis of [3H]PIP2 added as mixed micelles (27). Reactions were initiated by the addition of enzyme, and the reaction was allowed to proceed at 30 °C for 10 min. Gβγ-stimulated activity was determined in the presence of 1 μM free Ca2+. Gαq-stimulated activity was measured in the presence of Gαq that has been activated previously by incubation with GTPγS (29) plus 200 nM free Ca2+.

**Fluorescence Measurements**—Equilibrium fluorescence measurements were performed on a SPEX Fluorolog 211 spectrophotometer using a 3-mm cuvette with both excitation and emission slits set at 1.25 mm. Gαq-CFP was diluted in 200 μl of 20 mM NaHepes, pH 8.0, 3 μM GDP, 10 μM AlCl3, 10 mM MgCl2, and 5 mM NaF at 25 °C. After the addition of RG54-197Fl, the mixture was incubated for 2 min, and fluorescence was recorded between 460 and 580 nm with excitation at 341 nm. FRET between Gαq-CFP and RG54-197Fl was measured as the enhancement of RG54-197Fl emission at 552 nm or the decrease of Gαq-CFP emission at 475 nm over the sum of both components at the same concentrations. The individual emission intensities of both Gαq-CFP and RG54-197Fl (both excited at 431 nm) were scaled for concentration and subtracted from total observed fluorescence to give a measure of FRET intensity.
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FIGURE 3. Effect of Gβγ on GAP potency. A, carbachol-stimulated GTPase activity in m2AChR-Gz vesicles was measured in the presence of the indicated concentrations of PLC-β1 (○) or RGS4 (●) either with no addition (open symbols) or with 100 nm excess Gβγ (closed symbols). EC50 values obtained from fits to single-site saturation functions for PLC-β1 and RGS4 were 1.7 and 29 nm without Gβγ and 6.3 and 106 nm with Gβγ, respectively. B, carbachol-stimulated GTPase activity in m2AChR-Gz vesicles was measured in the presence of the indicated concentrations of RGS4 either with no addition (○) or with 100 nm Gβγ and 500 nm phosducin (●). EC50 values were 7 nm without added Gβγ, 33 nm with Gβγ, and 12 nm with Gβγ plus phosducin. Each experiment was repeated three times with similar results. C, steady-state GTPase activity was measured in the presence of 200 nM PLC-β1 and the indicated concentrations of carbachol with either no addition (○) or with 100 nm Gβγ (●). After fitting to a single-site activation model (solid lines), EC50 values for carbachol were 32 µM with or without Gβγ.

Inhibition of steady-state GTPase by Gβγ predominantly reflects inhibition of the GAP-accelerated GTP hydrolysis reaction itself rather than of other steps in the GTPase catalytic cycle. Gβγ significantly inhibited hydrolysis of Gαq-GTP in the vesicles (Fig. 2). Excess Gβγ did not alter the receptor-promoted dissociation of GDP from G protein (Fig. 2, inset), the other relatively slow step in the GTPase cycle (30). Thus, the modest effect of Gβγ in the single turnover assay (Fig. 2) is magnified when GAP activity is measured at steady-state (Fig. 1).

For both RGS proteins and PLC-β, Gβγ added to the receptor-G protein vesicles increased the amount of GAP needed to accelerate GTPase hydrolysis but had little if any effect on the maximal hydrolytic rate that could be attained. In a single turnover assay of GTP hydrolysis in vesicles, Gβγ increased the EC50 for PLC-β1 by more than 4-fold (Fig. 2). Gβγ also shifted the concentration dependence of GAP activity during steady-state hydrolysis stimulated by agonist-bound receptor. Similar effects were observed with RGS4 or PLC-β1 (Fig. 3A). Vesicles that contained m2AChR and Gz showed similar shifts in RGS4 potency in the presence of Gβγ (Fig. 3B). In each case, the maximal GTPase activity at high GAP concentration was about the same with or without Gβγ, but the EC50 for GAP was increased 4–6-fold. In contrast to its effects on GAP potency, excess Gβγ had no effect on the potency of agonist-bound receptor to accelerate steady-state GDP/GTP exchange (Fig. 3C), again indicating that Gβγ acts primarily at the GAP-promoted hydrolytic step of the GTPase cycle rather than at GDP/GTP exchange.

Gβγ Inhibits GAP Activity by a Km-based Mechanism—According to x-ray crystallographic structures, the two surfaces of Gα that interact with Gβγ and RGS proteins overlap substantially. These surfaces include both the switch regions and the N-terminal helix (22, 31–33). Inhibition might thus reflect competition between Gβγ and GAP for binding to Gα-GTP, although affinity of Gβγ for Gα-GTP is low. Alternatively, inhibition could result from an allosteric decrease of GAP-Gα affinity caused by the binding of Gβγ to the GAP-Gα-GTP complex.

An increase in Km without a change in Vmax is the hallmark of competitive inhibition, but it is also characteristic of a negative cooperative interaction between inhibitor and substrate. We therefore examined the effect of Gβγ on the Km of RGS-Z1 and RGS4, using GαL-GTP as substrate because the Km for these reactions is substantially lower than for other GAP-Gα pairs. For both RGS-Z1 and RGS4, the major effect of Gβγ was to increase the Km with a much smaller or negligible effect on Vmax (Fig. 4). This pattern was observed when Km and Vmax were determined by varying the concentration of either GαL-GTP or the GAP. The increase in Km by added Gβγ was mirrored by an increase in the concentration of Gβγ needed to inhibit the reaction at higher concentrations of GAP (Fig. 5). The half-maximal inhibitory concentration of Gβγ (IC50) increased nearly linearly over a 30-fold range of RGS4 concentration. The linear relationship suggests competitive inhibition, in this case caused by Gβγ binding to the GαL GTP substrate. If Gβγ bound to RGS with negative cooperativity with respect to Gα-GTP, or if it bound Gα while in a GAP-Gα-GTP ternary complex with similar effect, the curve would be expected to saturate at concentrations consistent with the affinity of Gβγ for the target. However, such a deviation from linearity is only predicted near or above the Km for the inhibitor, and the lowest observed IC50 for Gβγ was about 10 nM. Thus, although these data clearly show that Gβγ exerts its inhibition via an effect on Km, they do not distinguish the precise molecular mechanism.

Phosducin binds Gβγ competitively with respect to Gaq (34–36), and phosducin blocks inhibition of GAP activity by Gβγ (Figs. 3B and 6). Phosducin decreased the inhibitory potency of Gβγ and at high concentrations completely blocked its effect. The potency of phosducin is similar to its affinity for Gβγ (34, 35).

Inhibition of the GAP Activity of PLC-β Does Not Require the Known Gβγ Binding Site—PLC-β family members are stimulated both by activated Gαq and by Gβγ (37). The principal Gαq-binding domain of PLC-β is a coiled coil region that is C-terminal to the catalytic and C2 domains. This domain displays Gαq GAP activity when expressed separately, and its deletion eliminates both the GAP activity and the Gαq responsiveness of phospholipase activity (38–41). An N-terminal region that includes the PH domain is required for response to Gβγ, although other regions have also been implicated as important (42–45). To determine whether Gβγ binding to PLC-β at the PH domain is required for its inhibition of GAP activity, we compared the ability of Gβγ to inhibit the GAP activity of intact PLC-β1 and an N-terminally truncated mutant (Δ141). As shown in Fig. 7A, the GAP activity of the truncation mutant was inhib-
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FIGURE 4. Effect of Gβγ on GAP $K_m$. The GAP activity of RGS1 with the substrate $G_{\alpha_z}$-GTP was determined at varying concentrations of both substrate (A) and GAP (B) either in the presence (▲) or absence (●) of 300 nM Gβγ. The inset in B is expanded to show data near the origin. The fixed concentration of RGS1 was 0.25 nM (A), and that of $G_{\alpha_z}$-GTP was 2.8 nM (B). For each protocol, data from three experiments were combined and fit to the Michaelis-Menten equation to derive values of $K_m$ of 17 ± 4 nM or 15 ± 3 nM (control) and 62 ± 26 nM or 66 ± 5 nM (plus Gβγ) (± S.E. of fits). For the experiments shown, values of $V_{max}$ were 56 ± 7 and 52 ± 18 fmol/min without or with Gβγ (A) and 610 ± 35 and 670 ± 25 fmol/min (B). Control values of $K_m$ are higher than reported previously (22) because these assays were performed at 5 °C to decrease background hydrolysis. $K_m$ is temperature-dependent (data not shown).

FIGURE 5. Increasing concentrations of GAP decrease the inhibitory potency of Gβγ. The GAP activity at 0.1 (●), 0.5 (▪), and 3.0 (▲) nM RGS4 in a single turnover assay was measured at increasing concentrations of Gβγ. The concentration of $G_{\alpha_z}$-GTP substrate was 2.7 nM. The inset shows the IC50 for Gβγ at each RGS4 concentration. Data shown are means ± S.E. from three separate experiments, each performed in duplicate.

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FIGURE 6. GAP inhibition by Gβγ is blocked by phosducin. The $G_{\alpha_z}$ GAP activity of RGS4 was measured at increasing concentrations of Gβγ in the presence (▲) or absence (●) of 100 nM phosducin. Phosducin shifted the IC50 for Gβγ from 31 to 150 nM, a difference about equal to the phosducin concentration. Inset, $G_{\alpha_z}$ GAP activity was measured in the presence of 50 nM Gβγ and the indicated concentrations of phosducin. Data in the main panel and the inset are expressed relative to the activity observed in the absence of Gβγ. Each point is the mean of data from two experiments each performed in duplicate with a range ≤ 10% of the mean.
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To differentiate competitive binding and negatively cooperative binding, we asked whether Gβγ increases the rate of dissociation of the Ga11-Gβγ complex. Negatively cooperative interactions, although defined at equilibrium, generally result from an increase in the dissociation rates of the two negatively interacting ligands (Gβγ and RGS) in this case) from a low-affinity ternary complex (Ga-Gβγ-RGS). In contrast, a competitive mechanism has no such kinetic effect, because competing ligands never bind simultaneously. Dissociation of the complex of Ga11-GDP-AIF1 and RGS4 was measured in the presence or absence of 1 μM Gβγ under the same conditions used for the experiment in Fig. 8A. Gβγ had no effect on the rate of dissociation of Ga11-RGS4 (Fig. 8B) even though it markedly inhibited Ga11-RGS4 binding (Fig. 8A). Thus, if Gβγ accelerates the dissociation of Ga11-RGS4, its affinity for the Ga11-RGS4 complex must be extremely low, such that concentrations much higher than 1 μM are required (see "Discussion").

We evaluated the affinity of Gβγ binding to Ga11-GDP-AIF1 to test the feasibility of the competitive inhibition mechanism because Gβγ binds to active Ga subunits with reduced affinity. We found that Ga11 binds Gβ1γ2 with KD ~ 150 nM under our standard assay conditions (Fig. 8C). The KD can also be calculated from the I50 obtained in the experiment of Fig. 8A, assuming a competitive mechanism and given the affinity with which Gβγ binds RGS4. From these data, the KD for Ga11-Gβγ binding is 114 nM, in good agreement with the value obtained in Fig. 8C. This affinity is also consistent with the potency with which Gβγ inhibited GAP activity given the likely assumption that Ki = KD for the GAP-catalyzed hydrolysis of Ga11-GTP (see Ref. 28 for calculations).

DISCUSSION

These data show that Gβγ subunits inhibit G protein GAP activity in a membrane environment during stimulation by receptor, thus providing a model for the regulation of GAPs in agonist-stimulated cells. Inhibition displayed rapid onset (Fig. 1) and occurred in the range of 20 to 200 nM Gβγ (Figs. 2 and 3), similar to the concentrations required for activation of known Gβγ-regulated effectors such as K+ or Ca2+ channels, P1 3-kinase, PLC-β, or adenylyl cyclase (6). Inhibition of GAPs provides a novel mechanism for cross-talk among G protein pathways, most simply by allowing Gβγ from one G protein to regulate a GAP that is modulating the activity or kinetics of another. For example, inhibition of Gi GAP activity by Gβγ provides a plausible mechanism for the Gi-mediated potentiation of Ca2+ signaling by Gαi an effect that is hard

See Ref. 28 for calculations.)

FIGURE 7. Functional interaction of Gβγ with wild-type PLC-β1 and the Δ141 mutant. The responses to Gβγ of wild type PLC-β1 (solid line) and the Δ141 mutant (dotted line) were compared in several assays. A, inhibition of steady-state Gαi GAP activity measured with MACHi-Gαi, vesicles in the presence of carb chol. Gβγ was added at the indicated concentrations. B, phospholipase C activity was assayed with the soluble enzyme and micellar substrate at increasing concentrations of Gβγ. C, phospholipase activity on the micellar substrate was measured at increasing concentrations of GTPγS-activated Gαi1. These data are representative of two or three replications of each experiment. In all cases, both the phospholipase and GAP-specific activities of the mutant are somewhat lower than that of wild-type PLC-β1, probably because of diminished stability.

FIGURE 8. Competition by Gβγ for RGS4 binding to Ga11. A, equilibrium binding of 200 nM RGS4-1777F1 to 20 nM Ga11-CFP was measured by FRET at 25 °C in the presence of GDP, Al3+, F1, and increasing concentrations of unlabeled Gβγ. Data show the increase of RGS4-1777F1 fluorescence caused by energy transfer from Ga11-CFP. Similar results were obtained by observing Ga11-CFP quenching (data not shown). The solid line shows a fit to a single-site competition equation with IC50 ~ 290 ± 30 nM (standard error of fit). No improvement of the fit was obtained with a two-site model. Allowing the base line to float below zero improved the fit (dotted line) and gave an IC50 = 480 nM, with base line ~ -37 arbitrary fluorescent units (AFU). We believe that the lower value of IC50 (~ 300 nM) is more reliable than the higher value because base-line fluorescence is the difference between two relatively larger numbers and because any photobleaching artifact would drive error in that direction. This experiment was repeated three times with similar results. B, the dissociation of 20 nM Ga11-CFP from RGS4-1777F1 (200 nM total concentration) was monitored at 25 °C by stopped flow fluorescence measurement of FRET. The two proteins were allowed to bind in the syringe of the stopped flow mixer under the conditions described in A. Dissociation was initiated by mixing with an equal volume of 500 nM unlabeled Ga11, in the same buffer in either the presence (solid line) or absence (dotted line) of 1 μM Gβγ. Data points shown are from one shot each. Data from multiple shots were fit to a single exponential function to yield dissociation rate constants of 1.02 ± 0.05 s-1 (n = 12 shots) without Gβγ and 1.13 ± 0.08 s-1 (n = 14) with Gβγ. C, equilibrium binding of Ga11-ctitrine to 20 nM Ga11-CFP was measured in the presence of GDP, Al3+, F1, and increasing concentrations of Ga11-CFP.290 nM (standard error of fit). No improvement of the fit was obtained with a two-site model. Allowing the base line to float below zero improved the fit (dotted line) and gave an IC50 = 150 nM under our standard assay conditions (Fig. 8C). The solid line shows a fit to a one-site saturation equation with IC50 = 480 nM, with base line ~ -2 arbitrary fluorescent units (AFU). FRET between the two fluorophores. Data show the enhancement of Ga11-ctitrine fluorescence at 527 nm. The solid line shows a fit to a one-site saturation equation with KD ~ 146 nM and maximal fluorescence ~ 315 arbitrary fluorescent units.
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to rationalize by other known mechanisms of Gα action. Most Gα effects
reflect the action of Gβγ rather than of Gαi, at least in part because Gαi
(and Gαs) is expressed at high levels compared with other G proteins and
releases its Gβγ subunits more readily (5, 8, 47). Signal potentiation by
G/Go may frequently reflect inhibition of GAPs acting on the Gαi family,
Gαi or Gα12/13. In addition to potentiating other signals, GAP inhibition by
Gβγ may allow one G protein to prolong the duration of a signal con-
veyed by another. GAPs, particularly the RGS proteins, thus form a large
new group of candidate Gβγ-regulated effectors.

Whether free Gβγ actually is found in the plasma membrane upon G
protein stimulation and what its concentration might be are unknown
and currently not measurable. Recent studies by Bünemann et al. (9)
suggest that complete dissociation of Gβγ from Goi is not favored in
native plasma membranes during stimulation by agonist, although Gβγ
signaling was observed. Dissociation of Goi from Gβγ was observed
under similar conditions (8). Goi binds Gβγ less tightly than does Goi
(47). Goi-GTP and Gβγ apparently also remain associated during
receptor-promoted stimulation of K⁺ channels by Gβγ (10, 11), and
PLC-β can be stimulated by both Goi and Gβγ (37). It is then likely that
conformational isomerization in a Gαγ heterotrimer is sufficient to
allow signaling by Gβγ without complete dissociation. Because activa-
tion decreases affinity of Go for Gβγ, it is also likely that the rate of Gβγ
dissociation also increases, allowing Gβγ a kinetic window for Go exchange. The possibility that Gβγ interacts with multiple partners
simultaneously, or changes partners in a multiprotein complex, is thus
plausible, but its reality awaits direct physical measurement. Neverthe-
less, Gβγ inhibits GAP activity and stimulates known cellular effectors
over the same concentration range, which argues that GAP inhibition
may occur physiologically regardless of whether completely free Gβγ is
the active agent. More substantial evidence will depend on the develop-
ment of intracellular assays of GAP interactions with Gα-GTP
substrates.

Competition between Gβγ and GAPs—Both steady-state kinetics and
protein–protein binding data indicate that Gβγ inhibits GAP activity
competitively, competing with GAP for binding to Goi-GTP and pro-
tecting it from GAP-accelerated deactivation. Initial indication for a
competitive mechanism came from the predominant increase in Km
caused by Gβγ with little if any effect on Vmax. This mechanism is
further supported by the ability of Gβγ to increase the EC50 of RGS
proteins in GAP assays (Fig. 4) and the ability of GAPs to increase the
IC50 of Gβγ (Fig. 5). Formally, such behavior would also be consistent
with negatively cooperative binding interactions; Gβγ might bind either
Gα-GTP or GAP (or both) and decrease its affinity for the other. The
dependence of Km on inhibitor is commonly used to differentiate these
mechanisms, but we were unable to measure Km at high enough concen-
trations to observe its saturation at high concentrations of Gβγ.
However, the absence of an effect of Gβγ on the rate of dissociation of
the RGS4-Goi complex (Fig. 8B) lends support to a competitive mecha-
nism. Finally, Gβγ inhibits the GAP activity of the Δ141 truncation
mutation of PLC-β1, which lacks the known Gβγ binding site. If inhibition
derived from negative cooperativity, Gβγ would have to bind to a
yet unknown site on the PLC-β molecule.

Competitive inhibition depends on the surprisingly high-affinity binding of Gβγ to the GTP-activated form of Goi. The data in Fig. 8
are the first direct measurement of the affinity of the active form of a Gα
subunit for Gβγ and indicate that affinity is much higher than was
previously supposed. The affinities of Gβγ for Goi-GTPγS and Goi-
GDP-AlF4 are very similar and therefore probably reflect the affinity
for the GTP-bound form as well. High-affinity binding of Goi-GTP and
Gβγ is also supported by the observations that Goi and Gβγ may remain
bound during the receptor-stimulated GTPase cycle (48, 49). The life-
time of the Goi-GTP-Gβγ complex (τ = 1/kd) is thus significantly longer
than the lifetime of the GTP-activated state of the Gα subunit (τ = 1/k

Structural Basis of Competition between Gβγ and GAPs—At one
level, competition between GAPs and Gβγ for binding to Goi-GTP is
predictable because both RGS proteins and Gβγ bind to the same sur-
face of the Gα subunit, which includes the switch regions (31, 32, 50).
Binding here is thought to mediate both the GAP activity of RGS pro-
teins and the GDP dissociation inhibitor activity of Gβγ. The second
surface in Goi to make contact with both Gβγ and RGS proteins is the
N-terminal helix (22, 32, 51). However, the structure of this part of the
Gα-RGS interface is unknown. The only available crystallographic
structure of a Ga-RGS protein complex shows the Ga N-terminal helix
making crystal contact with an RGS protein in the adjacent unit cell (31),
most likely as a crystallization artifact. Little structural information is
available about the binding of Gaγ-GTP to PLC-β. The C-terminal
region of PLC-β is required both for stimulation of phospholipase ac-
ivity by Goi and for Goi GAP action, but the precise interface remains
 speculative (38–40). Again, more than one binding site may be
involved.

A complication in interpreting competition between Gβγ and GAPs
for Goi-GTP is that crystallographic data on these interfaces were
obtained with the GDP/AlF4-bound form of Goi for the Ga-RGS com-
plex and with the GDP-bound form for the Gαγ trimer. Although the
structure of the Ga globular domain is fundamentally the same in both
cases, the orientations of the N-terminal helix are markedly different.
Indeed, the N-terminal region of Ga subunits is markedly flexible,
although mostly helical when determined by x-ray crystallography.
During the GTPase catalytic cycle, the conformation of Ga presumably
oscillates between the active and inactive conformation several times
per second. It is unclear in this case that Ga ever assumes either canonical
conformation. It is plausible to propose an oscillating, or pivoting,
binding mode in which Ga may remain bound to both GAP and Gβγ
during the GTPase cycle. Binding might be maintained via the Ga
N-terminal helix, with the switch regions on the globular domain alter-
nately interacting with GAP and Gβγ. This mechanism would remove
protein association/dissociation reactions from the rapid GTPase cycle
and thus accelerate cycle transit. It might also permit quasi-continuous
activity of Gβγ as long as receptor-catalyzed GTP binding was suffi-
ciently rapid. How it would impact on the rate of GAP-accelerated GTP
hydrolysis is uncertain. Mutation of Ga that differentially alters its affi-
nity for either GAP or Gβγ may help address these questions, as well
dynamic studies of the conformations and orientations of Ga during the
GTPase cycle.

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