Stable complexes among G proteins and effectors are an emerging concept in cell signaling. The prototypical Gβγ effector, G protein-activated K+ channel (GIRK; Kir3) physically interacts with Gβγ but also with Giα. Whether and how Giα/o subunits regulate GIRK in vivo is unclear. We studied triple interactions among GIRK subunits 1 and 2, Giα3 and Gβγ. We used in vitro protein interaction assays and in vivo intramolecular Förster resonance energy transfer (i-FRET) between fluorophores attached to N and C termini of either GIRK1 or GIRK2 subunits. We demonstrate, for the first time, that Giα/o subunit to Gβγ interaction occurs within multiprotein complexes that include the GIRK-Gβγ signaling complex and the partner proteins alter each other’s conformation and gating with low Ibasal and high signal-to-background ratio upon Gβγ activation (11, 12). Thus, Giα and Gβγ may regulate the conformation of the channel both before and after activation. We explored this hypothesis in vitro and in vivo using biochemical, functional, and intramolecular Förster resonance energy transfer (i-FRET) methods. Our data reveal interdependent triple interactions among Giα3, Gβγ, and GIRK subunits, which correlate with distinct conformational and gating states of the GIRK channel.

**Stable complexes among G proteins and effectors are an emerging concept in cell signaling.**

Gαi and Gβγ Jointly Regulate the Conformations of a Gβγ Effector, the Neuronal G Protein-activated K+ Channel (GIRK)*

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*This work was supported, in whole or in part, by National Institutes of Health Grants GM68493 (to N. D.) and GM60419 (to C. W. D.). This work was also supported by the United States Israel Binational Science Foundation Grant 01-122 (to N. D. and C. W. D.), Israel Science Foundation Grants 49/08 and 1396/05 (to N. D.), and a Segol Fellowship (to M. R.).

† The on-line version of this article (available at http://www.jbc.org) contains supplemental methods and supplemental Figs. S1–S5.

§ The abbreviations used are: GPCR, G protein-coupled receptor; GIRK, G protein-activated K+ channel; ivt, in vitro translated; CT, C terminus; NT, N terminus; GST, glutathione S-transferase; i-FRET, intramolecular Förster resonance energy transfer; CFP, cerulean fluorescent protein; YFP, yellow fluorescent protein; GTPγS, guanosine 5’-3-(thio)triphosphate; ACh, acetylcholine; m2R, m2 receptor; DL, doubly labeled; PM, plasma membrane.

**Experimental Procedures**

Additional details on all methods are available in the supplemental methods.

**cDNA Constructs and Electrophysiology**—The cDNAs used in this study were obtained or prepared using standard PCR-based procedures. GINC was constructed as described (12), with an 8-amino acid linker GSTAGST replacing the transmembrane segment (amino acids 85–184). Doubly labeled (DL)-GIRK1 and DL-GIRK2 were created by fusing CFP to the NT and YFP to the CT of the GIRK subunit, via linkers (see supplemental methods). Other cDNA constructs were as in Ref.
**Goα, and Gβγ Control Conformation of a Gβγ Effector**

**FIGURE 1. Gβγ enhances the interaction between Goα3 and GIRK1.** A schematic depiction of the ivt synthesized GIRK1 fragments. B, pulldown of 35S-labeled ivt proteins by GST-Goα3, in the presence of GDP or GTPγS, with or without Gβγ. Autoradiograms of one-sixtieth of the total loaded protein (upper panel, Input) and of the bound protein (lower panel, Binding) are shown. C, summary of binding of the ivt synthesized GIRK1 proteins (normalized to that of G1NC to Goα3QL) to G1C, in the presence of either GDP or GTPγS, with or without the addition of 3 μg of purified Gβγ. D, the interaction of Goα3GA or G1C, with purified Gβγ, measured by Western blots as in B, in the presence of GDP or GTPγS. E, the interaction of G1NC with Goα3GA or Goα3QL, in the presence of either GDP or GTPγS, with or without Gβγ. The upper panel shows the summary of all experiments. Below the summary is a representative autoradiogram of pulldown G1NC, and the bottommost panel shows Western blot of bound G1C. In D and E, in each experiment, binding of Gβγ or G1NC was normalized to that measured with Goα3QL. Results are shown as mean ± S.E. *p < 0.05; **p < 0.01, as compared with the control group.

12. Xenopus oocytes were injected with RNAs, and whole-cell currents were measured using standard two-electrode voltage clamp procedures at 20–22 °C, in the ND96 (low K+) solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 5 mM HEPES, pH 7.5) or in a high K+ solution (24 mM K+, isotonically replacing NaCl in ND96) as described (12) (see supplemental methods). Differences in current amplitude, resulting from variations in GIRK expression, were corrected to channel expression, determined by the quantitation of YFP fluorescence as described (12). All currents were normalized to the control group.

**Pulldown Assay—**GST-fused Goα3 and its mutants were purified as described (13). [35S]Methionine-labeled segments of GIRK1 (G1NC (G1N1–84, C183–501)); G1NYP (YFP-N1–84); G1C (C184–501); G1C363–501 (C363–501)) were synthesized in rabbit reticulocyte lysate. Interaction among GST-fused Goα3, or its mutants with the in vitro translated (ivt), [35S]methionine-labeled segments of GIRK1 was studied by pulldown on glutathione-afﬁnity beads with either GDP or GTPγS (see supplemental methods). The eluted proteins were separated on 12% polyacrylamide–SDS gels. The radioactive signals from protein bands of the gels were imaged and quantitated using PhosphorImager and the software ImageQuant (GE Healthcare). Western blots were performed using Gβ antibody (Santa Cruz Biotechnology) and ECL reagents from Pierce.

**Imaging and Spectral FRET Analysis—**Fluorescent signals were collected with the Zeiss 510 META confocal microscope and analyzed as described (14), with modifications (see supplemental methods). Briefly, two spectra were collected from the animal hemisphere of each oocyte, with 405-nm (CFP excitation) and 514-nm (YFP excitation) laser lines. Net FRET signal of the CFP/YFP-labeled channels was calculated in the YFP emission range (with the 405-nm excitation) by consecutive subtraction of a scaled CFP-only spectrum (giving the A ratio parameter) and then of the ratio Aγ, which reports the direct excitation of YFP by the 405-nm laser. Therefore,

\[
\text{Ratio } A = \frac{F_{514}}{F_{514}} = \frac{F_{\text{direct}}}{F_{514}} + \frac{F_{\text{FRET}}}{F_{514}}
\]

(Eq. 1)

where

\[
F_{\text{direct}} = \frac{\text{Ratio } A_0}{A}
\]

(Eq. 2)

Then

\[
(A - A_0) = \frac{F_{\text{FRET}}}{F_{514}}
\]

(Eq. 3)

**Dynamic i-FRET—**Ionic currents were recorded in ND96 solution at −80 mV (see supplemental methods). Oocytes were repetitively excited with the 405-nm laser every 40 s. An ~150 × 100-μm region of the membrane area was imaged. The CFP and YFP fluorescence was collected using 470–500- and 505–550-nm band pass filters, respectively, and background signals were subtracted. YFP and CFP intensities at each point were normalized to an initial four measurements in each cell. This configuration allows leakage of CFP into the YFP recording window of ~0.30. Because this leakage is purely optical and constant irrespective of the FRET changes, we did not correct for it (15). The ratio of normalized intensities was denoted F_{YFP}/F_{CFP} (16). Some photobleaching occurred during repetitive excitations, but it had negligible effects on the F_{YFP}/F_{CFP} (see Fig. 4). Cells showing >15% bleaching of YFP or CFP were discarded.

**Statistical Analysis—**Results are shown as mean ± S.E. (see also supplemental methods). Asterisks or pound signs in Figs. 1, 3, and 4 indicate statistically significant differences as follows: * or #, p < 0.05; ** or ##, p < 0.01; *** or ###, p < 0.001, as compared with the control group, unless specified differently.

**RESULTS**

**GIRK-Goα-Gβγ Triple Interactions in Vitro—**We constructed a cDNA encoding the complete cytosolic domain of GIRK1, G1NC, in which the NT and CT were fused whereas the transmembrane region was replaced by a short linker (Fig. 1A), to test its interaction with the G proteins. Similar constructs were previously shown to have a strong propensity to form stable tetramers in solution (17, 18). The ivt [35S]-labeled G1NC gave a single protein band on SDS gel, as expected (Fig. 1B). Moreover, G1NC bound purified Gβγ (12). We have recently reported (12) that Gβγ enhances the interaction of Goα3 with the
The addition of purified Gβγ had no effect on the binding of GST-Gαi3 to separate GIRK1 N and C termini, in the presence of either GDP or GTPγS (Fig. 1E, summary in panel E). In contrast, the binding of G1NC to GST-Gαi3 GDP was enhanced ~9-fold by the addition of Gβγ, corroborating the previous report (12). Unexpectedly, Gβγ also strongly enhanced the binding of G1NC to GST-Gαi3 in the presence of GTPγS (Fig. 1, B and C), a condition where GST-Gαi3 poorly binds G1N (supplemental Fig. S1). To alleviate the concern that a residual amount of Gαi3βγ heterotrimers remaining in GTPγS could bias the results, we constructed GST-fused versions of two well-characterized Gαi3 mutants that mimic Gαi3 GTP and Gαi3 GDP, respectively: constitutively active Gαi3Q204L (Gαi3QL) and constitutively inactive Gαi3G203A (Gαi3GA) (see Ref. 12). GST-Gαi3GA showed the expected strong interaction with G1N,γ2 in GDP and much less in GTPγS, as reported (19). GST-Gαi3QL showed some interaction with G1N,γ2 in GDP (see also Ref. 20) but not in GTPγS (Fig. 1D, example in panel E).

Both GST-Gαi3GA and GST-Gαi3QL bound G1NC in the absence of Gβγ (Fig. 1E). Gβγ enhanced the binding of both Gαi3GA and Gαi3QL to G1NC, in GDP as well as in GTPγS, despite the great differences in their interaction with Gβγ (Fig. 1E). Thus, GIRK1 likely binds the active Gαi3 (Gαi3 GTPγS and Gαi3QL) directly and not through Gβγ; by binding to GIRK1, Gβγ enhances this interaction.

**G Protein Subunits Induce Non-identical Conformational Rearrangements in GIRK1 and GIRK2—iFRET was previously used to detect conformational changes in membrane proteins (16). We created a doubly labeled GIRK1 subunit (DL-G1) by fusing CFP (the donor) and YFP (the acceptor) to the extremities of NT and CT of GIRK1, respectively. DL-G1 was coexpressed with the neuronal GIRK2 subunit in Xenopus oocytes to give DL-G1/G2 channels, and visualized in the plasma membrane (PM) using a confocal microscope (Fig. 2). A linear relation of YFP to CFP fluorescence in DL-G1 confirmed the expected CFP/YFP molar ratio of 1:1 (supplemental Fig. S2C). We tested the functionality of all our tagged clones (Fig. 3) and found that DL-G1/G2 displayed similar function to wild-type G1/G2. The DL-G1/G2 channel had the characteristic Basal activity (Ibasal), was readily activated by acetylcholine (ACh) via a coexpressed muscarinic m2 receptor (m2R) and blocked by Ba2+ (Fig. 3Aa). Moreover, DL-G1/G2 exhibited the typical inward rectification (Fig. 3Ab). Importantly, the regulation of the channel by coexpressed Gαi3GA, Gαi3QL and the Gβγ-scavenging protein m-phosphoducin, was also identical to that of the wild-type G1/G2 channel (Fig. 3B) (12). We found that Gαi3GA strongly reduced Ibasal and when coexpressed with Gβγ, Gαi3GA did not reduce the total Gβγ-dependent current, Iγ (as also observed for the wild-type channel). Thus, Gαi3GA enhanced the relative activation by coexpressed Gβγ (Rγ) (supplemental Fig. S3A). Gαi3QL affected neither Ibasal, Iγ, nor Rγ, m-Phosphoducin reduced Ibasal but, in sharp contrast to Gαi3GA, also substantially diminished Iγ, acting as a typical Gβγ scavenger (Fig. 3B and supplemental Fig. S3A) (11, 12).

We quantified the iFRET signal using the spectral FRET technique (14) (Fig. 2C and supplemental Fig. S2E). Each oocyte was excited at two wavelengths, 405 and 514 nm, emission spectra were collected (Fig. 2, B and C, and supplemental Fig. S2E), and parameters characterizing bleed-through and energy transfer (ratios A0 and A, respectively) were calculated. The extent of FRET is proportional to (A − A0). Ratios A and A0 were linear over a broad range of wavelengths, a testimony to the absence of optical and calculation artifacts (14) (Fig. 2D).

We found a strong basal iFRET signal (A − A0) = 0.28 ± 0.02) in DL-GIRK1/2 (Figs. 2D and 4A), indicating proximity between the CFP and YFP moieties. Coexpression of Gβγ significantly increased iFRET (by ~17%; Fig. 4A) concomitantly with the increase in channel activity (Fig. 3B). Inversely, coexpression of Gαi3GA or m-phosphoducin markedly decreased both iFRET (by up to 20%) and Ibasal. Thus, low iFRET in DL-G1/G2 correlates with non-conductive conformation(s), and high iFRET corresponds to Gβγ-activated, open state(s) of the channel.
Coexpression of Gβγ with Gα13-GA restored the active state with large Iρβ and with high i-FRET. In contrast, coexpression of Gβγ did not reverse the m-phosducin-induced reduction in i-FRET and in Iργ (Figs. 3B and 4A), supporting the differentiation of the actions of Gα13-GA from those of a simple Gβγ scavenger (11). Expression of Gα13-QL did not significantly alter the basal i-FRET and currents, but further coexpression of Gβγ led to a strong increase in i-FRET (~30%), significantly greater than under all other conditions (Fig. 4A; see supplemental Fig. S4 for complete statistical analysis).

Unlike GIRK1, Gβγ does not enhance Gα13-GIRK2 interaction, and Gα3 does not regulate Iρbasal of homotetrameric GIRK2 (12). To assess whether Gα3 and Gβγ confer conformational changes upon GIRK2 within the GIRK1/2 heterotetramer, we constructed a doubly labeled GIRK2 (DL-G2) as in GIRK1 (see “Experimental Procedures”). DL-G2 was expressed with wild-type GIRK1 to produce G1/DL-G2 heterotetramers, which showed adequate regulation by Gα13, Gα3, and Gβγ (supplemental Fig. S3B) and showed basal i-FRET of 0.25 ± 0.02 (n = 44). Similarly to DL-G1, i-FRET of DL-G2 was reduced by Gα13-GA (by ~20%), an effect reversed by Gβγ (Fig. 4B). However, other parameters of i-FRET in the G1/DL-G2 heterotetramer were differently affected by the coexpressed G proteins. Coexpression of Gβγ did not increase i-FRET above basal level either in the presence or in the absence of Gα13. Most interestingly, concomitant expression of Gα13-QL with Gβγ significantly reduced the basal FRET signal (by ~26%), opposite to its effect on DL-G1.

As a control, we engineered a doubly labeled IRK1 (Kir2.1) channel, DL-IRK1 (Fig. 3C and supplemental Fig. S5). This inwardly rectifying K⁺ channel does not directly interact with, and is not regulated by, G proteins (21). DL-IRK1 exhibited PM localization, constitutively active gating, the typical strong inward rectification (Fig. 3, C and D) and strong basal i-FRET ((A - A₀) = 0.35 ± 0.01). Coexpression of Gβγ, Gα13-GA, Gα13-QL, or m-phosducin caused no significant changes in i-FRET (Fig. 4C) in channel currents. These results confirm the specificity of G protein regulation of conformational states of GIRK1/2 by G protein subunits, revealed by i-FRET.

**Dynamic Structural Rearrangements in GIRK1/2 upon GPCR Activation**—The changes in i-FRET caused by the presence of G protein subunits probably reflect the conformations of the channel at different activation steps. To verify that these static measurements reflect the conformations adopted by the channel during its physiological activation process, we monitored dynamic changes in i-FRET caused by activation of a GPCR (m2R). In these experiments, the electrophysiological response (K⁺ current) and fluorescence signals were collected simultaneously from individual oocytes (Fig. 5A).

Oocytes expressing DL-GIRK1/2, m2R, and the wild-type Gα13 were voltage-clamped at ~80 mV and continuously perfused with ND96 solution. Concomitantly, a region of
control experiments, where oocytes were exposed to 30 μM atropine, which completely inhibited the ACh-evoked K⁺ current (Fig. 5C). Importantly, pretreatment with pertussis toxin (PTX) completely inhibited both ACh-evoked currents and the ACh-induced change in i-FRET (Fig. 5D). The use of atropine rules out the involvement of the receptor in the induction of the change in conformation of the GIRK, and the latter is most probably caused by the direct interaction of the G proteins themselves. Finally, ACh did not induce any ionic currents or changes in $F_{YFP}/F_{CFP}$ in DL-IRK1 channel (Fig. 5E).

**DISCUSSION**

**Gβγ Is Crucial for Strong Interaction between GIRK1 and Goi—** Our biochemical results strongly support the idea of a preformed signaling complex of heterotrimeric Goiβγ with GIRK1, initially based upon the finding of a strong interaction of Goiβγ with the NT of GIRK1 in vitro (5). Indeed, Gβγ greatly enhanced the interaction of GIRK1 with Goiβγ under conditions favoring the formation of Goiβγ heterotrimers (Fig. 1) (12). Somewhat at odds with Huang et al. (5), our data suggest that both NT and CT are necessary for the formation of the strong GIRK1-Goiβγ complex as the effect of Gβγ was not present in separate N and C termini. Further quantitative binding studies may be needed to understand the reason for this discrepancy.

A novel and unexpected finding was that Gβγ also enhanced the interaction of GIRK1 with Goi3 and the constitutively active Goi-QQL in GTPγS, a condition when Goi-QQL-Gβγ interaction was detected by pulldown (supplemental Fig. S1). Therefore, we suggest that the conformational change in GIRK1 induced by the binding of Gβγ allosterically improves the direct binding of Goi3-GTP to GIRK1. Alternatively, GIRK1 may still bind Goi3-GTP via Gβγ, utilizing a second Gβγ-binding site on Goi3, distinct from the classical high affinity interaction site (22). In all, the biochemical data support the persistence of GIRK-Goi-i–Gβγ signaling complexes, both before and after activation by agonist-bound GPCR. The continuous attachment of Goi to GIRK1/2 would ensure a high local concentration of Goi and a diffusion-independent reassociation with Gβγ, which allows for fast termination of the physiological response upon removal of the agonist because of the fast kinetics of Goi-GDPP, Gβγ binding (23).

**i-FRET and GIRK Conformation**—The available partial crystal structures of GIRK tetramers indicate proximity between the NT of one subunit to the mid-CT of an adjacent subunit (17, 18, 24). In a tetrameric DL-G1/G2 channel, i-FRET could potentially arise from proximity of CFP-NT of one DL-G1 subunit to the CT-YFP of an adjacent DL-G1. This would require a 1–1–2–2 arrangement of subunits in the tetramer, which is probably viable, by analogy with 1–1–4–4 in GIRK1/GIRK4, although 1–4–1–4 is preferable (25, 26). However, it is important to note that the proximal NTs (~40 amino acids) and the distal CT (~130 amino acids in GIRK1, amino acids 370–501; ~45 amino acids in GIRK2) are omitted from the above crystal structures. The unique distal CT segment of GIRK1 is essential for the strong triple GIRK-Goi-i–Gβγ interaction and for the differential regulation of GIRK1 and GIRK2 by Goi and Gβγ (12). The flexible proximal NT and distal CT are probably long...
enough to allow for intrasubunit interactions. Such intrasubunit contacts were proposed to play an essential role in GIRK gating by Gβγ (27). Therefore, we interpret an increase in i-FRET as nearing of the NT and CT of either the same or adjacent GIRK subunits. The exact structural rearrangements remain to be determined. However, it is certain that changes in i-FRET reflect G protein-induced changes in conformation of the channel (Figs. 3C and 5B).

G Protein-regulated Conformational States of the GIRK Channel—Measurements of single-channel or population currents in native and chimeric GIRK channels demonstrated that heterotrameric GIRK channels assume a number of closed and open conformations with a variable number of Gβγ molecules bound, from a closed, non-conducting state (interpreted as Gβγ-devoid), via intermediate states with one, two, or three Gβγ molecules bound and correspondingly increasing open probability (Pₒ), to a fully Gβγ-activated state with four Gβγ bound and the highest Pₒ (27–30). Analysis of i-FRET, when combined with biochemical and functional assays, extends our understanding of the rules and modes of G protein–GIRK interactions and of the regulation of channel conformation. A simplified schematic of some of the interactions and conformations is presented in Fig. 6.

Preactivated (resting) states of GIRK1/2 probably reflect a mixture of conformations of GIRK1/2 channels, associated with a variable number of Gaβγ heterotrimers and/or Gβγ that lack matching GaGa (Fig. 6, b and d), as suggested by functional data (11). Correspondingly, DL-G1/G2 and G1/DL-G2 expressed alone at high levels yield high Ibasal (10) and an intermediate i-FRET signal. A substantial basal GIRK activity has been reported in hippocampal and cortical neurons (31, 32); therefore, the preactivated states are physiologically relevant.

Overexpression of high doses of the G protein subunits or Gβγ scavengers shifts the channel population toward more homogenous states, some of which are detected by our i-FRET assay. 1) The first is Gβγ-free state, with low Ibasal (Fig. 6A). This state is promoted by m-phosducin, which removes Gβγ away from the channel (6, 11). It is characterized by low i-FRET and is depicted in Fig. 6 as having a large distance between NT and CT of GIRK1. 2) The second is Gβγ-activated state(s), with high i-FRET and GIRK currents, presumably with several (up to four in the full tetramer) Gβγ dimers bound at activation sites (Fig. 6, d and e). 3) Joint expression of Gβγ and GaαQL confers a conformation (Fig. 6C) characterized by the highest i-FRET in GIRK1 but the lowest i-FRET in GIRK2 (Fig. 3G). These opposite changes in i-FRET further point toward a difference in regulation of GIRK1 and GIRK2 subunits by Gaα and Gβγ (12). Neither GaαQL nor Gβγ alone confer such changes in i-FRET, indicating concomitant binding of Gaα and Gβγ to the heterotrameric GIRK1/2.

Cells overexpressing Gβγ, Gβγ + GaαGA, or Gβγ + GaαQL show high constitutive, non-desensitizing GIRK currents (11, 12), clearly reporting the open states of the channel. Despite the different relative positions of GIRK N and C termini, these various open state(s) give similar whole-cell currents (but it remains to be seen whether they have distinct single-channel properties). The overall conformation of the channel in the presence of ACh should resemble that seen in static FRET experiments upon coexpression of Gβγ or, even more closely, Gβγ + GaαQL. Accordingly, dynamic activation of GIRK by ACh via coexpressed m2R caused an increase in both GIRK current and i-FRET in DL-G1/G2. The increase in i-FRET most probably corresponds to the opening of the channel because GIRK channels expressed in Xenopus oocytes show little ACh-induced desensitization over time periods of several minutes (33). Control experiments showed no changes in i-FRET upon exposure to the muscarinic antagonist atropine and after treatment with pertussis toxin, which prevents the activation of Gaα by GPCRs. Finally, no G protein, or GPCR, changes in i-FRET were observed in the double-labeled Gβγ-insensitive IRK1 channel. These control experiments demonstrate the authenticity and specificity of i-FRET changes caused by Gaα and Gβγ in GIRK1/2.

The low i-FRET and small GIRK currents observed after the expression of GaαGA are indistinguishable from those seen in Gβγ-free channels in the presence of m-phosducin, as if GaαGA removed Gβγ away from the channel. However, given the compelling evidence for a strong triple association between GIRK1, Gaα, and Gβγ (Fig. 1) (5–7, 34), we hypothesize that the GaαGA-Gβγ heterotrimers, formed after expression of GaαGA, remain mostly associated with GIRK1/2. In support, we observe specific differences in the effects of GaαGA (but not the QL mutant) on i-FRET and the total GIRK current when coexpressed with Gβγ. This corroborates the hypothesis that it is the GDP-bound Gaα (or Gβγ heterotrimer) that regulates the basal activity of GIRK and “primes” the channel for activation by Gβγ (11, 12).

The new biochemical and i-FRET results, as well as previous functional observations, are compatible with a “two-site model” in which GIRK possesses an anchoring site for Gaα (and/or Gaαβγ) and a separate activation site, where the binding of Gβγ leads to channel opening (12) (Fig. 6). The assumption of separate binding sites for Gaα (or Gaαβγ) and Gβγ is supported, although not proved, by the absence of competition among Gaα and Gβγ for binding to G1NC and G2NC under conditions that favor either the formation or the dissociation of Gaαβγ heterotrimers and by the more-than-additive character of i-FRET

FIGURE 6. The two-site model of GIRK regulation by Gaα and Gβγ. For simplicity, the GIRK channel is shown as if composed of only one GIRK1 subunit. The locations of anchoring and activation sites are shown arbitrarily. Distances between NT and CT are depicted schematically, where high i-FRET is interpreted to indicate proximity between the N and C termini (of the same or adjacent subunits). Inversely, low i-FRET correlates with distancing of the termini. +phos., m-phosducin.
changes induced by GaQL and Gβγ. The two sites may lie in proximity (or even partly overlap). According to the two-site model, the high basal activity of overexpressed GIRK1/2 is due to an excess of Gβγ associated with the channel, over Ga (11, 12, 35). Under these conditions, some channels have Gβγ “uncompensated” by Gα and thus bound to the activation sites (Fig. 6d). The coexpressed Ga3GA associates with Gβγ subunits, obstructing their interaction with the activation sites (hence the decrease in tbasal and i-FRET). The newly formed Ga3GA heterotrimers are docked at the anchoring site (Fig. 6b). When more Gβγ is coexpressed, free Gβγ subunits bind at the activation sites, hence the high i-FRET and currents (Fig. 6e).

The two-site hypothesis provides an economical description of a normal physiological situation; upon activation of GPCR, Gβγ detaches from Gα and interacts with the activation site, whereas GaGGTP stays at the anchoring site (Fig. 6c). (Alternatively, Gβγ may stay at the same site, and it is Gα that shifts to another location. Also, Gα and Gβγ do not have to fully disengage (16, 36); a rearrangement followed by an exposure of a few crucial residues on Gβγ may be sufficient). Once GTP is hydrolyzed by Gα, Gβγ rebinds Gα and becomes docked again.

Conclusions—Coordinated biochemical, functional, and i-FRET data support the existence of a persistent, dynamic GIRK1/2-Gαi,Gβγ complex, both before and after activation by agonist-bound GPCRs. We propose that GIRK1 acts as the nucleator of the complex and that both active and inactive Ga3 may remain bound to the channel, ensuring fast and specific activation and termination of the signal. New in vitro and in vivo data reveal intricate triple interactions among GIRK1, Ga3, and Gβγ and show that the presence of Gβγ is crucial to ensure strong GIRK1-Gαi interactions. At least some of the effects of Gβγ on GIRK1-Gαi interaction appear to be allosteric. Gα and Gβγ induce mutually dependent conformational rearrangements in GIRK1/2, characterized by distinct changes in i-FRET in the GIRK subunits and often, but not always, by changes in channel activity.

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