Gα_{i1} and Gα_{i3} Differentially Interact with, and Regulate, the G Protein-activated K⁺ Channel*

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G protein-activated K⁺ channels (GIRKs; Kir3) are activated by direct binding of Gβγ subunits released from heterotrimeric G proteins. In native tissues, only pertussis toxin-sensitive G proteins of the G_{iα} family, preferably Gα_{i3} and Gα_{i2}, are donors of Gβγ for GIRK. How this specificity is achieved is not known. Here, using a pull-down method, we confirmed the presence of Gα_{iα}-GTP binding site in the N terminus of GIRK1 and identified novel binding sites in the N terminus of GIRK2 and in the C termini of GIRK1 and GIRK2. The non-hydrolyzable GTP analog, guanosine 5'-3-O-(thio)triphosphate, reduced the binding of Gα_{i3} by a factor of 2–4. Gα_{i3}-GTP bound to GIRK1 and GIRK2 much weaker than Gα_{i3}-GDP-Titrated expression of components of signaling pathway in Xenopus oocytes and their activation by m2 muscarinic receptors revealed that G_{i3} activates GIRK more efficiently than G_{iα}, as indicated by larger and faster agonist-evoked currents. Activation of GIRK by purified Gβγ in excised membrane patches was strongly augmented by coexpression of Gα_{iα} and less by Gα_{i2}. Differences in physical interactions of GIRK with GDP-bound Gα subunits, or Gβγ heterotrimer, may dictate different extents of Gβγ anchoring, influence the efficiency of GIRK activation by Gβγ, and play a role in determining signaling specificity.

The importance of G protein-activated K⁺ channels (GIRK; Kir3) is 2-fold: as abundant mediators of inhibitory neuronal signaling and as the first known and one of the best studied direct effectors of Gβγ (1, 2). GIRK channels convey inhibitory signals of many neurotransmitters acting via G protein-coupled receptors (GPCRs). They mediate many of the negative inotropic effect of acetylcholine (ACh) acting via the m2 muscarinic receptor (m2R) in the heart (3, 4) and many of the inhibitory actions of opioids, serotonin, γ-aminobutyric acid, dopamine, and other transmitters in the brain (2, 5). They also play important roles in neuronal regulations of behavior and in mediation of GPCR- and alcohol-induced analgesia (6, 7). GIRKs are inwardly rectifying, tetrameric K⁺ channels. GIRK1/2 heterotetramers, abundant in the brain, and GIRK1/4, prevailing in the heart, display a GIRK1/GIRK2 stoichiometry. Each subunit consists of a core transmembrane domain with two membrane-spanning α-helices M1 and M2, flanked by cytoplasmic N and C termini, and a re-entrant helix-P-loop between M1 and M2. Recently resolved crystal structures of the cytoplasmic domain of GIRK1 and of an entire bacterial inward rectifier KirBac1.1 (8, 9) show that the cytosolic domains of Kir channels tetramerize to form a cytoplasmic water-filled channel that presents continuation of the transmembrane pore (see Fig. 1A).

It is now unequivocally established that Gβγ, but not Gα, activates GIRK (10). Gβγ binds to sites located in the N and C termini of each GIRK subunit and triggers channel opening by an unknown mechanism (11). The role of Gα subunits in GIRK signaling has been controversial for many years (reviewed in Ref. 10), but it is now becoming clear that Gα subunits are not mere donors of Gβγ for GIRK activation. They physically interact with GIRK, and it has been proposed that they contribute to channel function in several ways: determining the specificity of activation by neurotransmitters, anchoring the Gβγ subunits to GIRK to ensure fast and specific activation (12, 13), and regulating GIRK basal activity and Gβγ-dependent gating (14). The latter seems to involve Gα GDP rather than Gα GTP. In Xenopus oocytes, expression of Gα_{i3} reduces an excessively high basal activity of overexpressed GIRK channels and enhances agonist-induced activation. In excised patches, coexpressed Gα_{i3} enhanced GIRK activation caused by added Gβγ protein. This suggested that Gα_{i3} (probably in its GDP-bound form) not only sequesters free Gβγ and donates it upon agonist activation, but also predisposes (primes) the channels to Gβγ activation (14). It is not known whether the priming effect is also produced by Gα subunits other than Gα_{i3}.

The role of Gα in the specificity of signaling from GPCR to GIRK is widely accepted (15), but the mechanisms are unclear. GIRK is similarly activated by all Gβγ tested (16, 17) except Gβα which, in combination with Gγ_{2α}, inhibits GIRK (17). However, in native tissues, Gβα is associated mainly with certain RGS (regulators of G protein signaling) proteins rather than Gγ (18, 19). Therefore, it is unlikely that selectivity of coupling is determined by Gγ; Gα subunits must play a role. In cardiac and neuronal cells only pertussis toxin-sensitive G proteins, Gα_{iα}, activate GIRK (10). Selectivity is observed even within the Gα family: in atrial and certain neurons in neuronal cells, upon activation of a variety of GPCRs, Gα_{i3} and Gα_{i2} rather than Gα_{i1} and Gα_{i4} appear to be the preferred donors of Gβγ for GIRK (see “Discussion”). The molecular mechanisms determining the specific activation of GIRK only by certain G proteins remain unknown.

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¶ The abbreviations used are: GIRK, G protein-activated K⁺ channel; aa, amino acid(s); GST, glutathione S-transferase; GPCR, G protein-coupled receptor; m2R, muscarinic receptor type 2; GTP-S, guanosine 5'-3-O-(thio)triphosphate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; c.a., cell-attached; ACh, acetylcholine.
In contrast to the normal physiological situation, in heterologous expression systems (Xenopus oocytes, Chinese hamster ovary and human embryonic kidney cells) GIRK can be activated by Giβγ released from a variety of G protein heterotrimeric. Any Goi3, (14, 20, 21), including the pertussis toxin-insensitive Goi3 activates GIRK (22, 23). Even Goi can donate Giβγ to activate GIRK in heterologous expression systems or in native cardiomyocytes if various components of the signaling pathway (β-adrenergic receptors, Goi or Giβγ) are overexpressed (24–26). Of all Goi tested, only Goi is unable to donate Giβγ in expression systems; activation of Goi by the relevant GPCRs inhibits GIRK, apparently via indirect pathways that involve second messengers (27, 28).

How, then, is the selective activation of GIRK via Goi in native cardiac and neuronal cells ensured? Evidently, selective coupling between GPCRs and Giβγ heterotrimers contributes to the specificity of signaling to GIRK by neurotransmitters (20, 29), but this cannot explain the selectivity at the G protein-GIRK interface (e.g., why Goi does not activate GIRK). Thus, factors such as colocalization or scaffolding may be involved in assigning the relevant Go to GIRK. It has been proposed that pre-formed complexes of GIRK with certain Giβγ heterotrimers may exist in native cells, assuring fast and selective activation of GIRK by Giβγ derived from the GIRK-anchored heterotrimer (12, 13, 30). Supportive evidence is that Goi and Goi bind to the N terminus and mutations in N terminus reduce the speed of activation of GIRK by agonists of GIRK1 (12, 14, 31). However, this attractive hypothesis has not been conclusively confirmed.

Fundamental questions regarding the proposed complexes of GIRK with regulatory proteins remain open: whether GIRK subunits besides GIRK1 bind Goi; whether additional Gi-binding segments besides N terminus exist in GIRK; whether other Goi subunits besides Goi3 and Goi bind to GIRK; and whether differences in binding of various Goi subunits to GIRK contribute to signaling specificity and gating. To start to address these problems, we compared Goi3 and Goi3 subunits, which showed striking differences in their binding to GIRK in a preliminary comparative assay, for their ability to directly interact with the channel molecule and for their ability to regulate the physiological parameters of channel function. We identified and compared biochemical determinations are shown in Figs. 1 and 2 were performed with GDP-bound Goi as well as its distal part, Goi, (data not shown), and the results obtained with these two proteins were pooled. For trypsin digestion, 1 μl of 0.25% stock of trypsin was diluted ×100 and incubated for 20 min at 30 °C with 30 μl of 150 mM-K+ buffer with 0.5% CHAPS containing 2 μl of Goi lysate, treated with GTPγS or GDP as described above. Western blots were done using standard procedures, using a common Goi antibody (Calbiochem) and ECL reagents from Pierce Inc. Intensity of Coomassie Blue labeling was quantified using TINA software (Raytest, Straubing-Hardt, Germany).

Results are shown as mean ± S.E.; numbers of cells, patches, or separate biochemical determinations are shown above the bars in the figures. Multiple group comparison was done using one-way analysis of variance by Student-Newman-Keuls or Tukey's tests. One or two asterisks indicate p < 0.05 and p < 0.01, respectively, as compared with control group. Values of % of parameters in Goi3- and Goi-expressing groups, which report measurements independent of the control group, were compared by t test.

RESULTS

We explored the binding of Goi3 to GST-fused fragments of cytoplasmic N and C termini of GIRK1, using a standard pull-down method (Fig. 1B). The GST fusion proteins were immobilized of glutathione-agarose affinity beads. [35S]Methionine-labeled Goi3 was synthesized in vitro in rabbit reticulocyte lysate, as it was done previously to study the interaction of Goi3 with the N terminus of GIRK1 (14). In vitro synthesized Giβγ has also been successfully utilized to identify Giβγ binding sites of voltage-gated Ca2+ and GIRK channels (32, 33). The binding experiments were routinely conducted in buffers containing 0.5% CHAPS, which minimized nonspecific binding of Goi3 to GST. All initial screening and mapping experiments reported in Figs. 1 and 2 were performed with GDP-bound Goi3 (see “Experimental Procedures”). In each experiment the amount of bound Go protein was measured from autoradiograms using a PhosphorImager and normalized to the amount of the bound G1–C3 segment, which was present in all experiments (Fig. 1C). We stress that this methodology gives a highly sensitive and accurate quantitation of bound protein, because the radioactive signal from labeled Go is measured from dried gels by using the PhosphorImager directly, in an almost unlimited linear range, and with high precision. In comparison, in Western blotting, several additional intermediate steps are involved, which reduce the accuracy of measurement.

In initial experiments, we found that not only the N terminus of GIRK1, but also the full-length C terminus of GIRK1 (G1310) and of GIRK2 bound Goi3 (Fig. 1C and data not shown). We then examined the binding of Goi3-GDP, to the whole C terminus and to six non-overlapping GST-fused fragments of C terminus, G1–C1 through G1–C6 (Fig. 1B), previously used to map the
**Fig. 1.** GST-fused fragments of GIRK1 bind Go_{i3,GDP}. A, schematic presentation of a GIRK channel. The image was made using the program Swiss PDB Viewer. The transmembrane part is shown as for KcsA (60). The dashed lines represent the approximate boundaries of the lipid phase of the membrane. Cytoplasmic domains of two out of four subunits are shown according to Nishida and MacKinnon (8). The folding of the C terminus of GIRK1 between residues 372 and 501 is not known and is shown arbitrarily. Linkers between cytoplasmic and transmembrane parts have not been resolved in the crystal structure of GIRK. Parts of the C terminus of GIRK1 tinted black, gray, and light gray correspond to G1–C1, G1–C2, and G1–C3, respectively. B, linear presentation of GIRK is shown at the top, and the GST-fused segments, with numbers of first and last amino acids, are shown below: NT, N terminus; CT, C terminus; TM, transmembrane domain. C, a representative pull-down experiment showing the binding of Go_{i3,GTP} to GST fusion proteins of GIRK1. The upper panel shows Coomassie Blue-stained proteins eluted from the beads and subjected to SDS-PAGE, and the lower panel shows a PhosphorImager autoradiogram of the bound [35S]methionine-labeled Go_{i3}. Note that the Go-binding GST fusion proteins in this experiment (G1–C1 through G1–C3, and G1_{183–501}) have been added in lower amounts than GST fusion proteins of the second half of C terminus (G1–C4 through G1–C6); despite this, the latter did not bind Go_{i3}. D, Go_{i3} binding to GST fusion proteins of GIRK1, normalized to G1–C3.

Go_{i3} binding sites (32). Fig. 1C shows that three fragments covering all of the proximal part of C terminus of GIRK1, G1–C1 through G1–C3, bound Go_{i3,GDP}; G1–C3 showed the strongest binding (summarized in Fig. 1D). Interestingly, the whole C terminus (G1_{183–501}) bound Go_{i3} far weaker than G1–C3. It is possible that the complete C terminus is folded in such a way that the distal part beyond an 380 (which was proposed to play a role of a "switch" that regulates the activation of the channel in protein kinase A-dependent manner (34–36)) impedes the binding of Go_{i3} to the main C-terminal binding segments.

We also examined Go_{i3} binding to GIRK2 using GST-fused GIRK2 segments shown in Fig. 2A (sequence alignments of N termini and parts of the C termini of GIRK1 and GIRK2 are shown in Fig. 2B). The strongest binding of Go_{i3,GDP} was observed in the large segment G2–C2, aa 310–414, and in its smaller subdivision G2–C1–2 (aa 310–380) (Fig. 2C; summarized in Fig. 2D). This segment mostly overlaps G1–C3 of GIRK1 except the first 20 aa (Fig. 2B, part b). The first half of the N terminus did not bind Go_{i3}, whereas the second half bound Go_{i3,GDP}, although apparently somewhat weaker than G2–C2–1. The absence of Go_{i3} binding to G2–C2–1, which lacks 20 C-terminal aa compared with the homologous G1–C2 that binds Go_{i3} well, hints that this 20-aa stretch contains an important binding determinant for Go_{i3}.

Incubation of in vitro synthesized Go_{i3} with GTP\_S protected Go_{i3} from trypsin digestion, as expected for a GTP\_S-bound Go_{i3} (37) (Fig. 3A). This result confirms GTP\_S binding and the consequent change in Go_{i3} conformation and attests to functional integrity of in vitro synthesized Go_{i3}. Under mild non-ionic detergent conditions (0.05% Tween 20), the extents of binding of Go_{i3,GTR} and Go_{i3,GTP\_S} to the N terminus of GIRK1 were comparable (Ref. 14 and data not shown). However, under the standard conditions used in most experiments, with 0.5% CHAPS, the binding of Go_{i3,GTP\_S} was weaker than that of Go_{i3,GDP} (Fig. 3C). The same was observed with the C-terminal G1–C5 segment (Fig. 3C). The summary in Fig. 3C shows that, in 0.5% CHAPS, the binding of Go_{i3,GTP\_S} to all Go-binding fusion segments of GIRK1 and GIRK2 was only about a third of that of Go_{i3,GDP} (Fig. 3C).

The Go_{i3} binding segments identified by pull-down also bind G\beta{gamma} (32). To rule out the possibility that Go_{i3,GDP} binds to GIRK indirectly, via G\beta{gamma} present in the reticulocyte lysate, we performed two types of control experiments. First, we verified that binding of Go_{i3} to different segments was not increased by the addition to the reaction mixture of various amounts of untreated reticulocyte lysate, suggesting that the binding of Go_{i3,GDP} to GIRK was not via an endogenous protein present in the lysate (data not shown). Second, addition of lysate containing in vitro synthesized G\beta{gamma}_2 did not increase the binding of Go_{i3,GDP} to G1_{1–84} and G1–C1 (data not shown). Two additional facts argue against involvement of G\beta{gamma} in mediating Go_{i3} binding to GIRK: 1) the binding preference for G\beta{gamma} in the C terminus of GIRK1 (G1–C1 > G1–C2 > G1–C3; see Ref. 32) is opposite to that of Go_{i3} and 2) Go_{i3,GDP} does not bind to G1–C3 (see below), despite the very high affinity of binding of Go_{i3,GDP} to G\beta{gamma} (38). We conclude that, under our experimental conditions, Go_{i3} binds directly to GST-fused segments of GIRK1.

Preliminary screening of several Go subunits indicated weak binding of Go_{i3,GDP} to fusion proteins of GIRK1. We thus focused on the comparison of Go_{i3} and Go_{i1}. Functional integrity of Go_{i1} was verified by the trypsin digestion test (data not shown). For each GST fusion protein, the binding reaction was performed with equal volumes of reticulocyte lysate containing...
the labeled Ga subunits, and the amount of bound Ga was normalized to the amount of Ga added to the reaction in the beginning (“input”). Figs. 4 (A and B) show examples of individual experiments, and Fig. 4C summarizes the results of all experiments with all GST fusion segments, showing the binding of Ga relative to Ga3. In all segments of GIRK1 tested, the binding of Ga3-GDP was consistently weaker than that of Ga3-GTP-s (Fig. 4, A and C), and the binding of Ga3-GTP-s was too weak to be reliably quantitated (data not shown). Some Ga3 binding was observed in the N terminus, confirming a previous finding (12), but it amounted only to about 20% of Ga3-GDP. G1–C3 did not bind Ga3-GDP at all. In GIRK2, both Ga3-binding segments (G2–N2 and G2–C2–1) gave very weak signal with Ga3-GDP (Fig. 4, B and C).

We utilized Xenopus oocytes to examine how coexpressed Ga3 and Ga1 affect the function of GIRK and to see whether their effect on various parameters of channel function correlate with the observed differences in their binding. To avoid promiscuous interactions caused by excessive expression of signaling proteins, which may obscure specificity (39), it is important to avoid overexpression and to titrate protein levels (which do not necessarily correlate with the amount of injected RNA). Titration of protein levels was especially important when comparing the effects of two homologous Ga proteins, Ga3 and...
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under two-electrode voltage clamp. Exchanging the external solution from a low K\(^+\) (2 mM) ND96 solution to a high K\(^+\) (24 mM) solution revealed the basal GIRK current, I\(_{\text{basal}}\). Addition of 10 \(\mu\)M acetylcholine elicited the agonist-evoked current, I\(_{\text{ACh}}\) (Fig. 6A). When GIRK was expressed at low density (15–50 pg of RNA of each channel subunit per oocyte), coexpression of an excess of Go\(_{i3}\) or Go\(_{i3}\) (0.5 ng of RNA) reduced I\(_{\text{basal}}\) by about half (Fig. 6B, part a). I\(_{\text{ACh}}\) was not altered in Go\(_{i3}\)-expressing cells but augmented in Go\(_{i3}\)-expressing cells (p < 0.01, Fig. 6B, part e). The total GIRK current (I\(_{\text{total}}\)) was reduced, especially by Go\(_{i1}\) (Fig. 6B, part e). Go\(_{i3}\) appeared to cause a stronger enhancement than Go\(_{i1}\), but the difference was not statistically significant. The strong reduction in I\(_{\text{basal}}\) and a decrease in I\(_{\text{total}}\) suggest a substantial scavenging of free GB\(_{\gamma}\), and much of the enhancement of I\(_{\text{ACh}}\), with both Go\(_{i3}\) and Go\(_{i3}\), to avoid a situation in which the weakly interacting Go\(_{i1}\) present at high levels, “mimics” the effects of the strongly interacting but poorly expressed Go\(_{i3}\).

The protein levels of Go\(_{i3}\) and Go\(_{i1}\) were measured upon injection of 1 ng of RNA/oocyte, an amount widely used in functional experiments (see below). The amounts of Go\(_{i}\) were quantitated using Western blot methodology with a common Go\(_{i}\) antibody. Because it is possible that the antibody does not bind with the same affinity to Go\(_{i1}\) and Go\(_{i3}\), we performed a calibration procedure. [\(^{35}\)S]Met-labeled Go\(_{i1}\) and Go\(_{i3}\) were synthesized in reticulocyte lysate, run on gel, and transferred to a blot membrane. The radioactive signal from the membrane (Fig. 5A, “PhosphorImager”) was compared for the two proteins and corrected for the number of methionines in each molecule, giving the relative molar amounts of each protein on the blot membrane (normalized to Go\(_{i1}\)). The chemiluminescence signal obtained from Western blot of the same blot membrane (Fig. 5A) could then be calibrated to give a correction factor (equal to 1 if the antibody recognized the two proteins equally well), which can be used to correct the quantitative results from Western blots of Go in any cell. Go\(_{i3}\) and Go\(_{i1}\) (1 ng of RNA) were expressed in oocytes, and signals from Western blots of membrane fractions (Fig. 5B) were quantitated and corrected. The results indicated similar levels of expression of the two Go proteins, with a slight preference toward Go\(_{i1}\) (~40%; Fig. 5C).

In most cases the choice of amounts of RNAs injected for functional experiments was based on titrations made in our previous works and designed to avoid excessive protein levels. Heterotetrameric GIRK1/2 channels were expressed either at a low level (density), 0.025–0.05 ng of RNA/oocyte of each GIRK subunit, or at a high density (1 ng of RNA/oocyte). The amounts of RNAs of Go subunits were varied depending on the density of expressed channel (14). To activate GIRK, human m2 receptor (m2R) was expressed at 0.5 ng of RNA per oocyte, sufficient to activate all expressed GIRK channels (23). No coexpression of GB\(_{\gamma}\) is needed, because the oocytes usually have high levels of endogenous GB\(_{\gamma}\) sufficient for activation of all GIRK channels even when those are at high density (14).

Whole cell GIRK currents were studied in intact oocytes. Results of several representative experiments are shown. The terms input and binding have the same meaning as in Fig. 2C. B, comparison of binding of Go\(_{i1}\) and Go\(_{i3}\) to GST-fused fragments of GIRK2. A representative experiment is shown. C, summary of experiments like those in A and B for Go\(_{i1}\) and Go\(_{i3}\). For each GST fusion protein, the binding of Go\(_{i1}\) was presented as percent of binding of Go\(_{i3}\) after correction for differences in inputs of Go\(_{i1}\) and Go\(_{i3}\).

Fig. 4. Go\(_{i3}\) binds to GIRK subunits better than Go\(_{i1}\). A, comparison of binding of Go\(_{i3}\) and Go\(_{i1}\) to GST-fused fragments of GIRK1. Results of several representative experiments are shown. The terms input and binding have the same meaning as in Fig. 2C. B, comparison of binding of Go\(_{i1}\) and Go\(_{i3}\) to GST-fused fragments of GIRK2. A representative experiment is shown. C, summary of experiments like those in A and B for Go\(_{i1}\) and Go\(_{i3}\). For each GST fusion protein, the binding of Go\(_{i1}\) was presented as percent of binding of Go\(_{i3}\) after correction for differences in inputs of Go\(_{i1}\) and Go\(_{i3}\).
are shown in Fig. 7A. On the average, levels of basal activity recorded in cell-attached (c.a.) configuration were reduced by each Gα compared with the control group where the channel was expressed alone (Fig. 7B, part a). The basal activity in these experiments corresponded to medium to high channel density (see Ref. 14).

After 1–2 min of c.a. recording, the patch was excised into a Na+- and GTP-free, high K+ bath solution that contained 2 mM MgATP (the latter is needed to sustain viable levels of phosphatidylinositol bisphosphate, necessary for proper GIRK function (41)). After an initial reduction, which was similar in all oocyte groups (not shown), the basal activity stabilized. 20 nM purified recombinant Gαi3 and Gαi1 was added 3 min after excision, and activation relative to basal activity in the same patch was measured. The results are presented as -fold activation relative to either basal c.a. activity (Fig. 7B, part b) or to basal activity
in excised configuration (Fig. 7B, part c). Both types of analysis showed that both Ga3 and Ga1 enhanced Gβγ-induced activation, but Ga3 was more efficient, causing −4-fold priming. Ga1 caused 2- to 3-fold priming. The absolute values of Gβγ-activated currents (which correspond to Itotal in whole cell recordings) did not differ significantly in the three groups, although a certain tendency to decrease was observed in the presence of Ga1 (Fig. 7B, part d).

**DISCUSSION**

We have identified Ga3-GDP binding sites in N- and C-terminal parts of the ubiquitous GIRK1 and the neuronal GIRK2 subunits and investigated the correlation between Ga binding and the effect on channel function. Coexpression of Ga3 improved the speed and extent of agonist- and Gβγ-induced activation of GIRK. A homologous member of the Ga family, Ga1, showed substantially weaker binding to GIRK than Ga3, and correspondingly served as a poorer donor of Gβγ and a weaker enhancer of Gβγ-dependent activation. Our results support the postulate (13, 14) that direct interactions of GDP-bound Ga subunits (or Gαβγ heterotrimers) with GIRK regulate the biological effect of Gβγ and take part in determining signaling specificity. Active modulation of a Gβγ effector by Ga3-GDP (or GaαGDPβγ) is a novel aspect of G protein signaling and warrants attention as a phenomenon that may exist in other protein-coupled signaling pathways.

**Ga3-GDP Binds to Strategically Important Parts of GIRK**—In addition to the previously identified N-terminal binding site in GIRK1 (12), we found Ga3 binding sites in the N terminus of GIRK2 and in the C terminus of both GIRK1 and GIRK2. The strongest binding was to the G1–C3 segment in GIRK1 (amino acids 320–370), and a homologous segment G2–C2–1 in GIRK2 (aa 310–380, correspond to aa 299–369 of GIRK1; see Fig. 3B). Ga3 binds much weaker than Ga3 to both GIRK subunits. The existence of multiple N- and C-terminal Ga3-binding segments implies large Ga-interacting surfaces, which probably contribute to three-dimensional Ga3 binding sites formed by one or more GIRK subunits.

According to the crystal structure of the cytosolic domain of GIRK1 (8), the Ga-binding segments lie at the surface of this domain and are exposed to cytosol, which makes them available for protein-protein interactions (see Fig. 1A). These segments harbor (or lie close to) aa residues crucial in the regulation of GIRK gating, for instance glutamate 304 in GIRK1 (315 in GIRK2) and some adjacent residues (42, 43). The sequence VDY (340–342 in GIRK1, 346–348 in GIRK2), which is homologous to IDY (aa 298–300) in Kir Bac1.1, and adjacent segments in other Kir channels, are sites of interaction with the N terminus, which play an important role in gating processes in Kir channels (9, 44). The N-terminal Ga binding sites in GIRK subunits contain, at the boundary with the first transmembrane α-helix, a stretch (aa 64–77 in GIRK1) highly conserved among all Kir channels (9). This segment in KirBac1.1 forms a “slide” helix linking the transmembrane, pore-forming part of the channel with the cytosolic domain; it has been proposed to play a key role in Kir gating by linking movements of the cytosolic domain and of transmembrane helices M1 and M2 (9). Hence, the N- and C-terminal segments of GIRK that interact with Ga overlap, or are located close to, parts of channel molecule that are crucially involved in channel gating.

The binding sites for Ga3 are also spatially close to, and may partially overlap, the Gβγ binding sites found in the N and C termini (12, 32, 45, 46). This implies a complex pattern of interaction between G protein subunits and the channel, which may involve elements of competition and/or synergistic interactions. However, because the continued presence of coexpressed Ga3-GDP does not impair (actually enhances) activation by added Gβγ in excised patches, competition between Ga3-GDP and Gβγ for binding to GIRK under physiological conditions appears unlikely.

Ga3, as an Anchor and Donor of Gβγ—Anchoring of Gβγ (within a Gaβγ heterotrimer attached to the channel) and donating it upon addition of agonist is the most acknowledged potential function of Ga. Our working hypothesis, based on suggestions of Lily Jan and collaborators (12, 13) and later our own (14), is that physical interaction between Ga and GIRK allows fast coupling between the GPCR and GIRK. It enables the formation of a signaling complex that includes Ga, Gβγ, and the effector and enhances signaling specificity. Here, we utilized the differences in Ga3 and Ga3 binding to GIRK1 and GIRK2 to further explore this hypothesis in Xenopus oocytes. These cells are uniquely suited for quantitative titration of protein levels by exactly varying the amounts of injected RNA.
over a wide range. Here and previously (14), levels of expressed proteins have been also assessed by direct immunoochemical measurements. Uncertainties introduced by possible differences in GPCR-Gα coupling were avoided by using m2R, which couples equally well to all members of Gαi/o family (47).

Mass action law dictates that, for similar expression levels of two Gα species, more GIRK-Gαβγ complexes will be formed with Gα that binds GIRK with higher affinity, as long as the expression level of Gα is non-saturating. The existence of such a complex will ensure faster activation by agonist than in the case when Gβγ diffuses from a non-anchored heterotrimer (30). Therefore, kinetics of activation provide reliable information on the efficiency of Gα-GIRK coupling (29, 48). Since IACh shows desensitization (reviewed in Ref. 5), faster activation should also result in a greater agonist response amplitude (49). However, the kinetic parameter is more informative, because the difference in amplitudes of IACh may also stem from the less well understood phenomenon of priming (see below) or from an increase in surface expression of GIRK caused by coexpression of Gαi3 (14). We found both faster kinetics of activation and larger IACh with coexpressed Gαi3 than with Gαi1, supporting the hypothesis described above. The functional inferiority of Gαi1 was seen at all doses of Gα tested. However, it was most prominent at moderate expression levels, and became less pronounced at high levels of Gαi1. Such “promiscuous” coupling between poorly interacting proteins is common in heterologous expression systems upon overexpression of one of the components (39). This is in agreement with mass action law: when the concentration of Gαi3 becomes saturating (presumably in great excess over GIRK), it should be able to cause the same maximal effect as Gαi3 if the only difference between them is the affinity of interaction with GIRK. However, when expressed in excess, both Gαi3 and Gαi3 also act as general scavengers of Gβγ, forming Gαβγ heterotrimer not complexed with GIRK and reducing I total (14). If less Gαi3 is complexed with GIRK compared with Gαi3, more Gαi3 is available for general Gβγ scavenging. Our results support this notion: although Gαi3 accelerates the activation and increases I ACh at moderate and high expression levels, it never reaches the efficiency attained by Gαi3, and causes a greater reduction in I total than Gαi3 at 5 ng of RNA/oocyte (Fig. 6B). In all, our results suggest that Gαi3 is less efficient than Gαi3 as a Gβγ donor for GIRK under most conditions.

The strength of Gα-GIRK interaction is not the only factor that determines the identity of Gαi/o that couples a GPCR to GIRK in native cells. Additional factors include coupling specificity at the GPCR-Gα interface (21) and Gα availability. Theoretically, a cell with excess Gαi1 may preferentially use it for agonist signaling to GIRK. Yet, published data imply a preferential use of Gαi3 (or Gαi3) over Gαi1 and Gαi3 to activate GIRK. An antibody- and antisense-based study suggested that somatostatin-induced GIRK current in AtT-20 cells critically depends only on Gαi3, whereas in locus coeruleus neurons it is mediated by Gαi2 and not by Gαi1, Gαi3, or Gαi5 (50). Notably, all Gαi/o subunits are present in the brain (51). In the atrium, despite the abundance of Gαi5 (52, 53), only Gαi2 and Gαi3, but not Gαi3, mediate the m2R-mediated GIRK current I ACh, as shown by targeted inactivation and re-expression of Gαi subunits (54–56). Importantly, m2R does not prefer Gαi2 or Gαi3 over Gαi5, because it specifically uses Gαi3 to inhibit ventricular Ca2+ channels (55). Unfortunately, the role of Gαi3 has not been specifically examined in these works, and its abundance in the heart is controversial (51, 53). A correlation between relative abundance of Gαi2 or Gαi3 and their use as the main donors of Gβγ cannot be ruled out at present.

Our results show that, in the zwitterionic detergent CHAPS, binding of Gαi3-GTPS to GIRK is significantly weaker than of Gαi3-GDP. Stronger binding of Gαi3GDP than Gαi3-GTPS to the N terminus of GIRK1 has also been reported (12). However, under mild non-ionic detergent conditions (0.05% Tween 20) the binding of Gαi3 to N terminus of GIRK1 was not reduced by GTPS (14). Even if measurements of binding in CHAPS accurately reflect the changes in Gα binding that take place in vivo, our results indicate that Gαi3-GTP remains bound to the channel following the GPCR-catalyzed exchange of GDP to GTP. When GTP is hydrolyzed, fast recapture of Gβγ by closely positioned Gαi would ensure fast deactivation upon washout of agonist. Indeed, for fast-dissociating agonist-receptor complexes, macroscopic deactivation is limited only by the speed of hydrolysis of GTP but not Gβγ dissociation form GIRK (57). The role of GPCR and of RGS proteins and the mode of their interaction with the G protein within such a multiprotein complex, remain to be elucidated.

Gαi as a Regulator of GIRK Gating and Function—Experiments in excised patches provide the opportunity to examine whether Gαi affects the function of the channel in separation from the donor function. Added Gβγ (not Gβγ released from an endogenous Gαβγ “donor” pool as in whole cell experiments) activates GIRK channels in the absence of coexpressed Gα. Coexpression of Gαi3 not only reduces I basal but also enhances activation of GIRK by Gβγ, thus Gαi3 serves as a primer for Gβγ effect (14). We proposed that this priming effect and part of the reduction in I basal are the consequences of a direct interaction of Gαi3 with the channel; we also envisaged a link between the two effects (14). The patch clamp experiments presented here showed that the extent of priming correlated with binding of Gαi3 and Gαi3, Gαi3 enhanced GIRK activation caused by Gβγ in the absence of agonist or GPCR, and under the conditions of little general scavenging of Gβγ (as witnessed by the preservation of the amplitude of total Gβγ-activated current). The weaker priming by Gαi3 supports the view that this phenomenon depends on direct interaction between Gαi3 and GIRK. Because the patches were excised into a GTP-free solution, it is most probable that the priming effect is caused by Gαi3-GDP or Gaβγ rather than Gαi3-GTP. On the other hand, Gαi1 and Gαi3 caused a similar reduction in I basal (both in patches and in whole cells). This weakens the possibility of a direct inhibitory effect of Gαi3 on basal activity. However, because the latter appears to be mostly Gβγ-dependent (14, 58), a direct scavenging of Gβγ may play a major role in the reduction of I basal by Gαi3, masking a minor contribution of a direct effect of Gαi3 to the channel, prevents channel activation via the high affinity site, reducing I basal, but changes the conformation of GIRK to enhance activation via the low affinity site once free Gβγ becomes available. Variants of this model may explain the known facts related to basal and evoked activities of GIRK, but a more specific formulation will require further experimentation.

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