Identification of Critical Residues Controlling G Protein-gated Inwardly Rectifying K⁺ Channel Activity through Interactions with the βγ Subunits of G Proteins

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G protein-sensitive inwardly rectifying potassium (GIRK) channels are activated through direct interactions of their cytoplasmic N- and C-terminal domains with the βγ subunits of G proteins. By using a combination of biochemical and electrophysiological approaches, we identified minimal N- and C-terminal Gβγ-binding domains responsible for stimulation of GIRK4 channel activity. Within these domains one N-terminal residue, His-64, and one C-terminal residue, Leu-268, proved critical for Gβγ-mediated GIRK4 activity. Moreover, mutations at these GIRK4 sites reduced significantly binding of the channel domains to Gβγ. The corresponding residues in GIRK1 also showed a critical involvement in Gβγ sensitivity. In GIRK4/GIRK1 heteromers the GIRK4 His-64 and Leu-268 residues showed greater contributions to Gβγ sensitivity than did the corresponding GIRK1 His-57 and Leu-262 residues. These results identify functionally important channel interaction sites with the βγ subunits of G proteins, critical for channel activity.

Activation of atrial potassium (K⁺) channels underlies the acetylcholine (ACh) -induced reduction in heart rate during vagal activity (1). GTP-binding (G) proteins couple muscarinic receptors to K⁺ channel activation (2, 3). G protein activation of K⁺ channels is mediated through the βγ rather than the α subunits of G proteins (4).

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‡ The abbreviations used are: ACh, acetylcholine; G, GTP-binding; Gβγ, βγ subunits of G proteins; GIRK, G protein-gated inwardly rectifying K⁺; G1, GIRK channel 1; G4, GIRK channel 4; IRK1, inwardly rectifying K⁺ channel 1; PH, pleckstrin homology; βARK, β-adrenergic receptor kinase; GST, glutathione S-transferase; GFP, green fluorescence protein; PIP₂, phosphatidylinositol bisphosphate.

Four mammalian G protein-sensitive Inwardly Rectifying K⁺ (GIRK1–4 or Kir3.1–3.4) channels have been identified thus far (5–9). Both the N- (90 amino acids) and C-terminal (240 amino acids or more) ends of these channels are cytoplasmic (5). Two transmembrane domains (90 amino acids) surround the P-region that harbors the potassium ion “selectivity filter” (10). GIRK channels can function as highly active heteromultimers (pairing of GIRK1 with any of the other subfamily members) or low to moderately active homomultimers (‡‡GIRK2–4) reviewed in Refs. 11 and 12). Mutations at a specific position within the P-region of these channels (i.e. GIRK4-S143T or GIRK1-F137S) greatly enhance the activity of homomultimers (13, 14). Use of these highly active point mutants simplifies the experimental design of structure-function studies and allows assessment of the relative contributions of each of the two subunits in the heteromultimeric complex (14).

A number of studies have demonstrated direct binding of Gβγ subunits to the full-length GIRK proteins (15) or to segments of channel subunits (16–21). Although Gβγ subunits can interact directly with both the N and C termini of GIRK1, interactions with the C terminus of this channel were shown to be the strongest (16, 17). Moreover, the N terminus of GIRK1 but not the C terminus interacts with the Gaβγ heterotrimer (16, 20). Functionally, both native and recombinant hetero- or homomultimeric GIRK channels are activated by the Gβγ subunits (4, 8, 9, 22). No qualitative difference in the Gβγ sensitivity of active homomultimeric mutants versus heteromultimeric channels was observed (14). In contrast, the inwardly rectifying K⁺ channel IRK1 (5) is Gβγ-insensitive (23, 24), despite its high degree of similarity in protein sequence to the four members of the GIRK subfamily. By using a chimeric approach between GIRK4 and IRK1, a critical C-terminal Leu residue was identified (GIRK4-L339 or GIRK1-L333), mutation of which abolished the Gβγ-mediated agonist-induced GIRK channel activation but not the Gβγ-mediated agonist-independent (basal) GIRK channel activity (24). This result suggested that distinct channel sites may be involved in basal versus agonist-induced channel activation through the Gβγ subunits.

In the present study we sought to identify additional specific residues of the GIRK subunits, critical for Gβγ-mediated activity. Two amino acid residues, the N-terminal GIRK4(H64) and the C-terminal GIRK4(L268), are shown to be critically involved in Gβγ-mediated currents. Moreover, mutation of each of these GIRK4 residues is shown to reduce binding to the Gβγ subunits. The corresponding GIRK1 residues are also found to be involved in the Gβγ sensitivity of GIRK1 subunits (GIRK1(H57) and GIRK1(L262)). In contrast to the mutation of GIRK4(L339) or GIRK1(L333), which selectively controlled ag-
onist-induced but not agonist-independent Gβγ-mediated currents (24), the two sites reported here are found to abolish all Gβγ-mediated currents.

EXPERIMENTAL PROCEDURES

Human homologs of GIRK1 and GIRK4 (GenBank accession numbers U39195 and U39196 (9)) or their point-mutated active counterparts (GIRK1-F137S or GIRK1* and GIRK4-S143T or GIRK4*), subcloned in the pGEMHE plasmid vector (25), were used as described previously (9, 14, 24). The chimeric cDNAs were constructed by splicing together overlapping extension PCR (ePCRs, using Vent DNA polymerase, were performed for only 15 cycles to avoid errors. Point mutations were generated using the Quickchange site-directed mutagenesis kit (Stratagene). The sequence of all constructs was confirmed by automated DNA sequencing (Sequencing facility, Cornell, Ithaca, NY). The products were stained with ethidium bromide and visualized under UV light for correct insertion of the ePCR products and correct reading frames. Presence of constructs was confirmed by automated DNA sequencing (Sequencing facility, Cornell, Ithaca, NY). The βARK-construct (amino acids 452–689) (referred to as βARK-Go) was used to sink Gβγ subunits away from the channel. For confocal studies GIRK1-GFP was used as described previously (27).

All constructs were linearized with NheI, and cRNAs were transcribed in vitro using the “message machine” kit (Ambion). RNAs were electrophoresed on formaldehyde gels, and concentrations were estimated from two dilutions using RNA marker (Invitrogen) as a standard. Xenopus oocytes were surgically extracted, dissociated, and defolliculated by collagenase treatment and microinjected with 50 nl of a water solution containing the desired cRNA. Unless otherwise indicated, we used the following approximate quantities: GIRK channel subunits, 1.0 ng per species; IRK1 channel, 0.25 ng; M2 receptor, 1.0 ng; Gβγ subunits, 1.0 ng; Gγ subunit, 1.0 ng; βARK-PH, 1.0 ng. Gαo, or Gβγ with Gγ2 were used in all Gβγ coexpression experiments.

Oocytes were incubated for 3 days at 19 °C. Whole oocyte currents were then measured by conventional two-microelectrode voltage clamp with a GeneClamp 500 amplifier (Axon Instruments). Agarose cushion microelectrodes were used with resistances between 0.1 to 1.0 megohms (28). Oocytes were constantly superfused with a high potassium solution containing (in mM) 91 KCl, 1 NaCl, 1 MgCl2, 5 KOH/HEPES (pH 7.4). To block or activate currents, the oocyte chamber was perfused with 100 mM composition of the same composition with 3 mM BaCl2 or 5 mM Ba2 + ionophores were used for open time histogram fitting. Recordings were made using Clampfit8 with solutions of the same composition with 3 mM BaCl2 or 5 mM Ba2 +. Opening time and closed state amplitudes were measured at the end of the 200-ms pulse at each concentration. Data were then fitted with a multiexponential function using the Clampfit8 software. Reversal potentials were measured at the peak of the response to ACh, and ACh-induced incremental openings were used for open time histogram fitting. Recordings were performed under control conditions and in the presence and absence of blockers (e.g. 10 μM apamin, 200 μM tetrodotoxin) or agonists (29, 31). Single channel recordings were performed, as described previously (29–31). Single channel currents were filtered at 1 kHz with a 6-pole low-pass Bessel filter, sampled at 5 kHz. Single channel data were analyzed with pCLAMP8 software supplemented with some of our own programs. Base-line drifts were carefully adjusted with Clampfit8 before idealization. Only those records containing a few or no double openings were used for open time histogram fitting. Recordings were performed on 2–5 min after impaling the oocytes just before application of ACh. ACh-activated currents were evaluated at the peak of the response to ACh, and Ba2+-insensitive currents were evaluated once steady-state inhibition was achieved (1–3 min after application of 3 mM BaCl2). Basal current is the difference between control and Ba2+-insensitive currents, and ACh-induced (or ACh-sensitive) current is the difference between ACh-activated and control currents. Error bars in the figures represent S.E. Each experiment shown or described was performed on 3–5 oocytes of the same batch. A minimum of 2–3 batches of oocytes were tested for each experiment shown.

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Detection of GFP-tagged channels in oocytes has been described previously in detail (33). Briefly, 2–4 days after injections, Xenopus oocytes were fixed in 4% parafomaldehyde overnight at room temperature. Fixed oocytes were embedded in 3% agarose, and 50-μm sections were cut using a Vibratome. The cut sections were mounted on cover slips and imaged using a Leica TCS confocal microscope. To compare fluorescence intensities between different oocyte sections, image acquisition parameters such as pinhole size, intensity, and offset were kept constant.

RESULTS

Minimal Gβγ-binding Domains in the N and C Termi

n of GIRK4 Channels—To identify minimal Gβγ-binding domains within the N and C termini of the GIRK4 channels, we constructed and purified GST fusion proteins and tested their binding to purified Gβγ subunits. We tested Gβγ-binding of the full N and C termini of GIRK4 and of fragments harboring incremental deletions of ~25 amino acids, until Gβγ binding was no longer detected. Gβγ binding of GIRK4 channel fragments was compared with that of the following: (a) the PH domain of the β-adrenergic receptor kinase (βARK) (e.g. Ref. 24); and (b) the corresponding full N- and C-terminal fragments of the IRK1 (or Kir2.1) channel, which is related to GIRK4 but is Gβγ-insensitive (e.g. Ref. 24); and (c) the GST protein alone, which is common to all purified fusion proteins. Fig. 1A shows Gβγ binding for the full N terminus, GIRK4 (1–92) (denoted as G4-(1–92)), and for a minimal N-terminal Gβγ-binding fragment G4-(41–92). Fig. 1B shows Gβγ binding for the full C terminus G4-(184–419), and no binding for fragments smaller than G4-(253–322). In fact, maximal Gβγ binding was obtained

FIG. 1. Identification of minimal Gβγ-binding regions on the N and C termini of GIRK4. A, GST fusion constructs were made with fragments of the N terminus of GIRK4. The full GST and GST-IRK1-(1–89) were used as negative controls, whereas GST-βARK-PH was used as a positive control for Gβγ-binding. Proteins were purified using glutathione 4B-Sepharose beads and were detected by Coomassie staining (top). Purified GST fusion proteins were incubated with Gβγ and glutathione-Sepharose beads. Following wash, the bound proteins were released from the beads by heating in protein sample buffer and were separated by SDS-PAGE. Gβ was detected by immunoblotting with anti-Gβ antibody. The position of Gβ is indicated by the arrow. The representative gel shown is one of two experiments performed. B, GST fusion proteins were made with fragments of the C terminus of GIRK4. The proteins were purified and detected, and Gβγ interactions were performed as described in A. The representative gel shown is one of three experiments performed.

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with fragment G4-(253–348), whereas deletion to position 322, G4-(253–322), caused a reduction in G/H9252/H9253 binding, consistent with our previous findings (24) implicating the 322–348 region as an important determinant of G/H9252/H9253 sensitivity. On the other hand, the binding of the full N-terminal segment G4-(41–92) was consistently weaker than that of the smaller N-terminal segment G4-(41–61). Overall these results suggest that G4-(41–92) and G4-(253–348) are, respectively, minimal domains of the GIRK4 channel N and C termini capable of maximal binding to G/H9252/H9253.

Regions within Minimal G/H9252/H9253-binding Domains Responsible for Stimulation of GIRK4 Channel Activity—To identify regions within the two minimal G/H9252/H9253-binding domains of GIRK4 involved in stimulating channel activity, we constructed chimeras between the GIRK4 and IRK1 channels. GIRK4 regions of ~25 amino acids, within the minimal N- and C-terminal G/H9252/H9253-binding domains, were replaced by the corresponding IRK1 regions. These chimeras were constructed in the background of GIRK4(S143T) (referred to as G4*), which has been shown previously to form functional homomeric channels (14). Heterologous coexpression of control and mutant channels with muscarinic M2 receptors was carried out in Xenopus laevis oocytes. Measurement of K/H9254 currents was performed using two-electrode voltage clamp. Fig. 2A shows that the N-terminal chimera G4*(IRK1 S35-V56), which replaced the GIRK4 region between 40 and 61 with the corresponding IRK1 region between Ser-35 and Val-56, showed normal agonist-independent (basal) and agonist-induced G4* currents. Similarly, the C-terminal chimera G4*(IRK1V270-D291) replacing the GIRK4 region between 277 and 298 also showed normal basal currents but lacks ACh-induced currents. These results suggested that neither the 40–61 nor the 277–298 GIRK4 regions possessed any key residues responsible for the differences in G/H9252/H9253 sensitivity between GIRK4 and IRK1. Chimera G4*(IRK1L316-Y341) showed basal but not agonist-induced currents. This result agreed with previous work showing the corresponding GIRK4 region (e.g. G4-(323–348)), and in particular a Leu residue (G4 Leu339 or G1 Leu333) was critical in G/H9252/H9253-mediated agonist-induced activa-
tion as well as for maximal binding to Gβγ (Ref. 24; also compare in Fig. 1B Gβγ binding to GST-G4-(253–322) versus GST-G4-(253–348)). In contrast, chimeras G4*(IRK1Q57-V86), G4*(IRK1D246-I269), and G4*(IRK1F292-Y315) described in Fig. 2A, distinct amino acids in these regions of GIRK4* were replaced with the corresponding IRK1 residues. One representative batch of 3–5 oocytes is shown for each series of point mutants. A, three amino acids, His-64, Tyr-71, and Leu-84, in GIRK4* N terminus were found to be critical for GIRK4* function. Currents were recorded at −80 mV. B, in the GIRK4* C terminus two amino acids, Leu-268 and Ala-318, were also found to be critical for GIRK4* function. Currents were again recorded at −80 mV.

**Fig. 3. Identification of amino acids in GIRK4* that are critical for channel activity.** To identify amino acids critical for the non-functional phenotype of the chimeric channels GIRK4*/IRK1(Q57-V86), GIRK4*/IRK1(D246-I269), and GIRK4*/IRK1(F292-Y315) described in Fig. 2A, distinct amino acids in these regions of GIRK4* were replaced with the corresponding IRK1 residues. One representative batch of 3–5 oocytes is shown for each series of point mutants. A, three amino acids, His-64, Tyr-71, and Leu-84, in GIRK4* N terminus were found to be critical for GIRK4* function. Currents were recorded at −80 mV. B, in the GIRK4* C terminus two amino acids, Leu-268 and Ala-318, were also found to be critical for GIRK4* function. Currents were again recorded at −80 mV.

**Single Residue Differences between GIRK4 and IRK1 Critical for Channel Function**—Within the N-terminal GIRK4-(62–91) region that proved critical for the functional integrity of G4*, 13 residues are different from the corresponding amino acids in IRK1. Mutation of each G4* N-terminal residue within this region to the corresponding IRK1 amino acid revealed three differences critical for channel function. Each of the G4*(H64F), G4*(Y71Q), and G4*(L84I) mutants abolished completely channel activity (Fig. 3A). Similarly, within the C-terminal GIRK4-(253–276) and GIRK4-(299–322) regions that produced non-functional chimeras, there are 11 amino acid differences from IRK1. Two of the 11 mutants, G4*(L268I) and G4*(A318C), also abolished completely K+ currents. The effects of these mutations were more severe than those in the previously identified mutant G4*(L339E), which resulted in loss of agonist-induced currents but left intact Gβγ-mediated basal currents (24).

**Rescue of Non-functional GIRK4 Mutants Allows Identification of Gβγ-sensitive Residues**—Inwardly rectifying K+ channels, including GIRK subfamily members, depend on phosphatidylinositol-bisphosphate (PIP2) for their activity. In fact, gating molecules, such as the Gβγ subunits or Na+ ions, have been shown to strengthen channel-PIP2 interactions (34–37). It has been shown previously that amino acids in the GIRK4 region (214–252) and the corresponding IRK1 region (207–245) are critical determinants of interactions of these channels with PIP2. One mutant, G4*(I229L), exchanging a GIRK4 residue for the corresponding one in IRK1, strengthened channel-PIP2 interactions sufficiently enough to allow gating of the mutant channel by PIP2 alone in the absence of gating molecules, such as Gβγ or Na+ ions. Yet this mutation preserved Gβγ stimulation of K+ currents. Each of the three N-terminal and two C-terminal single point mutants that showed no K+ currents was paired with the I229L mutation, and each of the double
Identification of Residues Controlling GIRK Channel Activity

mutant channels was expressed in Xenopus oocytes and tested for activity. Fig. 4A shows that all three N-terminal mutants G4*(I229L,H64F), G4*(I229L,Y71Q), and G4*(I229L,L84I) showed convincingly inwardly rectifying basal K⁺ currents. In fact, all of these mutants that now displayed basal currents also showed intact agonist-induced currents (Fig. 4, A right, and C right). Because activity of each of the “functionally lethal” point mutants H64F, Y71Q, and L84I was rescued by the I229L mutation, we proceeded to test whether Gβγ subunits were contributors to the rescued basal K⁺ currents. We have shown previously that molecules that bind Gβγ can serve as a “sink” to sequester Gβγ subunits away from the channel, inhibiting basal channel activity (24). Fig. 4B shows βARK-PH coexpression with each of the rescued N-terminal double mutants and with the control single mutant, G4*(I229L). Of the three N-terminal mutants, only G4*(I229L, H64F) was insensitive to βARK, indicating that with the H64F mutation the Gβγ subunits were no longer contributing to the rescued basal currents. Fig. 4C shows a similar rescue approach for the two C-terminal “functionally lethal” mutants. Again, the rescued G4*(I229L,L268I) and G4*(I229L,A318C) currents were convincingly above background (−4 μA versus −0.5 μA) and clearly inwardly rectifying (Fig. 4C). Again, these rescued C-terminal mutants showed intact agonist-induced currents. Of these two C-terminal rescued mutants, the L268I currents were not significantly affected by βARK coexpression (Fig. 4D). Thus, of the five non-functional mutants, one in the N terminus, G4*(H64F), and one in the C terminus, G4*(L268I), did not show inhibition by the Gβγ sink, βARK-PH, suggesting that the His-64 and Leu-268 residues are critical for Gβγ sensitivity.

Effects of Mutations of the His-57 and Leu-262 Residues on the Gβγ Sensitivity of GIRK1—The two identified Gβγ-sensitive residues in GIRK4 are identical in GIRK1. We proceeded to test their importance in GIRK1 in the background of GIRK1(F137S) (referred to as G1*), which has been shown previously to yield functional homomers (13, 14). Fig. 5A shows that G1*(L262I), the mutant corresponding to G4*(L268I), greatly reduced but did not abolish basal G1* currents. βARK-PH inhibited the remaining basal currents, suggesting that in GIRK1 additional residues may contribute to Gβγ-mediated basal currents. In contrast, the N-terminal GIRK1* mutant G1*(H57F) behaved as the corresponding G4*(H64F), abolishing completely K⁺ currents. As expected, the double mutant G1*(H57F,L262I), containing the non-functional H57F mutation, abolished all G1* currents.

Relative Contributions of the His-64/57 and Leu-268/262 Residues of the GIRK4/1 Heteromer—To examine the relative contributions of the GIRK1 and GIRK4 subunits to the heteromeric GIRK1/ GIRK4 channels, we expressed each double mutant subunit with the corresponding wild-type subunit and recorded basal currents. Fig. 5B shows that the G1/G4(H64F,L268I) heteromeric channels showed no activity, in contrast to the G1(H57F,L262I)/G4 channels that exhibited small inwardly rectifying K⁺ currents. Heteromeric assembly of mutant and wild-type subunits did contribute to these small currents (see below). When both mutant subunits were coexpressed, G1(H57F,L262I) and G4(H64F,L268I), no K⁺ currents could be measured. These results suggest a dominant role for the two identified residues in GIRK4 over GIRK1 subunits in a heteromeric channel. We further explored the relative contributions of each of the N- and C-terminal mutations to the heteromeric channels. Fig. 5C shows expression of each of the N- and C-terminal mutants alone or in combination with their wild-type counterpart. The G4(L268I)/G1 showed greatly reduced currents. Of the G1 mutants, the G1(H57F)/G4 heteromers also showed greatly attenuated K⁺ currents, whereas the G1(L262I,G4 showed attenuated but larger heteromeric currents than any of the other mutant/wild-type combinations. To test whether currents resulting from the G1(mutant)/G4(wild-type) combinations reflected activity from heteromeric channels or homomeric wild-type G4 channels, we examined the single channel characteristics of each of the
heteromeric combinations exhibiting basal currents. As shown in Fig. 6, G4 homomeric activity is characterized by short lived and not clearly resolved transitions at any particular level (see also Ref. 9). In contrast, G1/G4 heteromeric activity exhibits a clear peak in the single channel conductance amplitude histogram. Each of the G1 mutant combinations with G4 wild-type, G1(H57F)/G4, G1(L262I)/G4, and G1(H57F, L262I)/G4, showed clear peaks in their amplitude histograms, indicating that functional heteromeric channels were formed. Similarly, the only G4 mutant exhibiting channel activity when paired with the wild-type G1 (i.e. G1(G4(L268I)) also showed clear heteromeric channel amplitude transitions (not shown). Open time histograms could be fitted by two exponentials $\tau_1$ and $\tau_2$ yielding a mean open time $\tau$ (Fig. 6B). The predominant change in the open time kinetics due to heteromerization occurred on the second open time component, $\tau_2$. Thus, all heteromeric combinations that displayed activity indeed reflected contributions from heteromeric channel assemblies. These results, taken together with the results of Fig. 5C, suggest that the G4(H64F) mutant acted as a dominant negative in the heteromeric G1/G4(H64F) channel, whereas the G4(L268I) mutant greatly reduced heteromeric G1/G4(L268I) currents. Because the G4(L268I) mutant did not but the G4(H64F) mutant did abolish basal currents when coexpressed with wild-type GIRK1 channels, it seems that the role of His-64 is more critical than Leu-268 in controlling heteromeric channel activity. G1(H57F) also greatly reduced but did not abolish heteromeric G1(H57F)/G4 currents. The G1(L262I) caused even less inhibition of heteromeric G1(L262I)/G4 currents, consistent with its effects in G1$^*$ homomers (Fig. 5A). Overall, these results underscore the dominant role of GIRK4 in heteromeric G$\beta$Y sensitivity through the two identified N- and C-terminal residues.

N- and C-terminal Point Mutations Reduce GIRK4 Channel Binding to the G$\beta$Y Subunits—To test whether the H64F and L268I mutations affected channel binding to the G$\beta$Y subunits, we constructed and purified GST fusion proteins of the minimal G$\beta$Y-binding domains with or without the point mutations of interest, and then we tested their binding to purified G$\beta$Y subunits. Both the H64F and the L268I mutations significantly reduced binding to G$\beta$Y of the N- and C-terminal binding fragments, respectively (Fig. 7).

**DISCUSSION**

A number of attempts have been made prior to this study to localize the N- and C-terminal channel regions responsible for direct binding with the G$\beta$Y subunits (16–18, 38). Specifically, Huang et al. (17), using a deletion mutagenesis approach, have localized the G$\beta$Y-binding region of GIRK1 to the N-terminal domain including amino acids 34–86 and the C terminus involving two separate fragments, 318–374 and 390–462. Fragments corresponding to GIRK1-(34–86) and GIRK1-(318–374) from GIRK-(2–4) also displayed G$\beta$Y binding. Kunkel and Peralta (18), with a combination of chimeras and deletion mutations, reported the GIRK1-(290–356) region to be important in interactions with G$\beta$Y. Krupivinsky et al. (38) used peptides derived from GIRK1 or GIRK4 amino acid sequences to compete direct G$\beta$Y binding to atrial purified K$^+$ channels. Effective peptides included the GIRK1-(364–383) region and the GIRK4-(209–225) and -(226–245) regions. It is difficult to discern commonly identified channel regions of interactions with G$\beta$Y from these studies.

Our results indicate that in GIRK4 minimal G$\beta$Y-binding domains involve the N-terminal 41–92 residues and the C-terminal 253–348 residues. The N-terminal region GIRK4-(41–92) indeed corresponds closely to the GIRK1(34–86) region, as identified by Huang et al. (17). Similarly, the C-terminal GIRK4-(253–348) region overlaps with the GIRK1 regions identified by Kunkel and Peralta (18) and by Huang et al. (16, 17). Moreover, comparisons of these studies together suggest
that GIRK1 may contain additional G\(\beta\)\(\gamma\)-binding sites in its unique C-terminal region, which are distinct from other subfamily members.

In our previous study (24) we determined that when the 323–348 region of GIRK4 was replaced with the corresponding region from IRK1, it supported G\(\beta\)\(\gamma\)-mediated basal currents but not agonist-induced currents. In our present study, deletion of this region from a C-terminal fragment showing maximal binding decreased binding to G\(\beta\)\(\gamma\) (compare the G\(\beta\)\(\gamma\) binding to the GST-G4-(253–348) versus the GST-G4-(253–322)). These results suggest that the difference in binding reflects channel-G\(\beta\)\(\gamma\) interactions occurring during agonist-induced stimulation. The difference in binding contributed by the 322–348 region was similar to that obtained upon mutation of a single residue within this region (i.e. L339E; see Ref. 24). In addition, GIRK4*(L339E) lacked ACh-sensitive currents but displayed G\(\beta\)\(\gamma\)-sensitive basal currents. Because G\(\beta\)\(\gamma\) binding and G\(\beta\)\(\gamma\)-mediated basal currents were intact in GIRK4*(L339E), it was concluded that additional G\(\beta\)\(\gamma\)-binding sites contribute to basal channel activity.

In the present study we have identified two channel sites critical for G\(\beta\)\(\gamma\) interactions and overall channel activity. Mutation of GIRK4(H64) and GIRK4(L268) reduced binding to G\(\beta\)\(\gamma\) and abolished K\(^{+}\) currents. Interestingly, mutation of the corresponding residue His-57 in GIRK1 also abolished K\(^{+}\) currents. This result is in contrast to that obtained from mutation of the corresponding C-terminal residue Leu-262, which significantly reduced K\(^{+}\) currents but did not abolish them. This result is consistent with the idea that the GIRK1 C terminus contains additional G\(\beta\)\(\gamma\) interaction sites that contribute to G\(\beta\)\(\gamma\)-mediated activity (17).

Mutations of G\(\beta\)\(\gamma\)-interacting residues that abolished basal currents also showed no agonist-induced currents (e.g. GIRK4*(H64F), GIRK4*(L268I), or GIRK1*(H57F)). On the other hand, mutations that attenuated but did not abolish basal currents also preserved agonist-induced responses (e.g. GIRK1*(L262I) or heteromeric channels of wild-type GIRK4 with mutant GIRK1(H57F), GIRK1(L262I), or GIRK1(H57F, L262I)). These results, as well as those from the GIRK4*(L339E) mutant that supports no agonist-induced currents but displays basal currents, suggest that the presence of basal

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**Fig. 6.** Single channel kinetics reveal formation of heteromeric channels between GIRK4 wild-type and non-functional GIRK1 mutants. A, representative traces of cell-attached recordings from Xenopus oocytes expressing GIRK4/GIRK1(G4/G1), G4 alone, G4/G1(H57F), G4/G1(L262I), G4/G1-(H57F, L262I). All-point histograms for each trace are shown on the right. B, summary bar graph of the two time-constants and mean open time (\(\tau_1\), \(\tau_2\), and \(\tau\)) of the channels shown in A. Records were sampled at 5 kHz at a membrane potential of \(-80\) mV and were filtered at 1 kHz. Heteromeric expression of G4 wild-type and non-functional G1 mutants gave rise to channels with significantly longer mean open times (\(p < 0.005\)) and bursting behavior.
activity is a requirement for agonist-induced activity.

Biochemical and electrophysiological evidence from several studies, including ours, have suggested multiple binding sites in the C- and N-terminal segments of GIRK channels (16, 17, 21, 38). The multiplicity of Gβγ-binding sites with effector proteins is in agreement with a number of studies including the crystal structure of the Gβγ/phosducin complex, where multiple sites of interaction are seen between the two proteins (39). We have provided evidence suggesting distinct functional roles to specific binding sites. Thus, the Leu-339 site interacts with Gβγ released from receptor stimulation (24), whereas the His-64 and Leu-268 sites interact with Gβγ to yield basal activity and enable overall Gβγ-mediated stimulation. Our results suggest that for the K1 channel the multiplicity of interactions subserves distinct functional roles.

The N but not the C terminus of GIRK1 has been shown to support interactions with the heterotrimeric Gβγγ protein. It is intriguing to hypothesize that such interactions of the channel with the heterotrimeric G protein confer specificity to Gβγ signaling by hardwiring the signaling molecules into a receptor-channel-effector macromolecular complex. The N-terminal GIRK channel mutations (GIRK4-H64F and GIRK1-H57F) abolished all K+ currents. The mechanism by which the N-terminal mutation resulted in a complete loss of function is not known. The GIRK4(H64F) mutation decreases binding to Gβγ, and consequently it could result in a decrease in the binding of the heterotrimeric G protein to the N terminus and disruption of the presumed macromolecular complex.

In a recent study we identified three functionally important Gβ1 residues that interact with GIRK channels.2 One of these, Ser-98, is shared with Ga subunits and is thought to be involved in agonist-induced interactions. The other two Gβ1 residues, Ser-67 and Thr-128 that do not interact with Ga subunits, affect basal K+ currents when mutated. Possible interactions of GIRK4 residues His-64/His-57 and/or Leu-268/Leu-262 with the Gβ1 residues Ser-67 and Thr-128 remain to be tested.

Mutation of the two Gβγ-interacting residues, GIRK4* (H64F) and GIRK4*(L268I), abolished both basal and agonist-induced activities. The activity of each of these two mutants could be rescued by strengthening channel-PIP2 interactions with the mutation GIRK4*(I229L) (35). Thus, although agonist-induced activation requires basal activity, intact basal channel-Gβγ interactions do not seem to be a prerequisite for agonist stimulation of the channel. We have shown previously (24) that agonist stimulation of channel activity involves Gβγγ signaling with residues GIRK4(L339) and GIRK1(L333). In the present study we show that Gβγ-mediated basal activity depends on interactions with sites GIRK4(H64,L268) and GIRK1(H57,L262). Because Gβγγ stimulation has been shown to lead to stabilization of channel-PIP2 interactions (34, 25), we previously proposed that Gβγ gating of the channel may proceed through modulation of channel-PIP2 interactions (37).

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previous (24) and present work has implicated distinct channel sites in interactions with Gβγ to produce basal or agonist-induced currents. Thus, it is possible that each of the channel-Gβγ interactions (i.e. basal versus agonist-dependent) stabilize distinct channel-PIP₂ interactions. Precise identification of specific channel-PIP₂ interaction sites modulated by each of the channel-Gβγ interaction sites remains to be determined.

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Identification of Critical Residues Controlling G Protein-gated Inwardly Rectifying K⁺ Channel Activity through Interactions with the βγ Subunits of G Proteins
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