Identification of a Potassium Channel Site That Interacts with G Protein βγ Subunits to Mediate Agonist-induced Signaling*

(Received for publication, December 31, 1998, and in revised form, January 19, 1999)

Cheng He, Hailin Zhang‡, Tooraj Mirshahi‡, and Diomedes E. Logothetis§

From the Department of Physiology and Biophysics, Mount Sinai School of Medicine, City University of New York, New York, New York 10029

Activation of heterotrimeric GTP-binding (G) proteins by their coupled receptors, causes dissociation of the G protein α and βγ subunits. Gβγ subunits interact directly with G protein-gated inwardly rectifying K+ (GIRK) channels to stimulate their activity. In addition, free Gβγ subunits, resulting from agonist-independent dissociation of G protein subunits, can account for a major component of the basal channel activity.

Using a series of chimeric constructs between GIRK4 and a Gβγ-insensitive K+ channel, IRK1, we have identified a critical site of interaction of GIRK with Gβγ. Mutation of Leu339 to Glu within this site impaired agonist-induced sensitivity and decreased binding to Gβγ, without removing the Gβγ contribution to basal currents. Mutation of the corresponding residue in GIRK1 (Leu339) resulted in a similar phenotype. Both the GIRK1 and GIRK4 subunits contributed equally to the agonist-induced sensitivity of the heteromultimeric channel. Thus, we have identified a channel site that interacts specifically with Gβγ subunits released through receptor stimulation.

Signaling through GTP-binding (G) proteins depends on dissociation of the heterotrimer Gαβγ into the Gα-GTP and Gβγ subunits. Direct interactions of Gα or Gβγ (or both) with effector proteins transduces the external signal into an intracellular response. Atrial potassium (K+)-channels, the first example of a Gβγ-effector (1), are responsible for the acetylcholine (ACh)-induced reduction in heart rate during vagal activity (2).

Five members of the G protein-gated inwardly rectifying K+ (GIRK1–5) channel subfamily have been reported thus far (3–8). The presumed topology of these channels includes a cytoplasmic N terminus (≈90 amino acids), followed by two transmembrane domains with the “ion selectivity” P region in between (≈100 amino acids) and ending with a long cytoplasmic C terminus (over 200 amino acids) (3, 9). GIRK channels can function as highly active heteromultimers (pairing of GIRK1 with any other subtype) or low to moderately active homomultimers (GIRK2–5) (for review, see Ref. 10). Mutations at a specific position within the P-region of these channels (“P-region mutants,” e.g. GIRK4-S143T) greatly enhance the activity of homomultimers (11, 12). 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 (12).

Several studies have demonstrated direct binding of Gβγ subunits to entire GIRK proteins (13) or to segments of channel subunits (14–19). Although Gα subunits can interact directly with both N and C termini, interactions with the C terminus of the channel were shown to be the strongest (14, 15). In addition, the N terminus also binds to Gα subunits alone (14) or to the Gβγ heterotrimer (14, 18).

The βγ subunits of G proteins activate not only native GIRK heteromultimers (1, 6), but also recombinant hetero- or homomultimeric GIRK channels (7, 20). There is no qualitative difference in the Gβγ sensitivity of P-region homomultimeric mutants versus heteromultimeric channels (12). In contrast, the inwardly rectifying K+ channel IRK1 (21) is Gβγ-insensitive (22), despite its high degree of similarity to the five members of the GIRK subfamily.

We sought to identify those residues of GIRK critical for transducing effects of the Gβγ subunits. Our strategy was to generate chimeras between the GIRK4-S143T (referred to as GIRK4*) and IRK1 channels, and screen for differences in Gβγ-dependent function and binding to Gβγ. Mutagenesis at a single site, namely GIRK4(L339E), reduced binding to Gβγ and impaired agonist-induced activity, but left intact the Gβγ dependence of the basal activity. Thus, we have identified a site on an effector protein that interacts specifically with Gβγ released through receptor stimulation.

EXPERIMENTAL PROCEDURES

Human homologs of GIRK1 and GIRK4 (GenBank™ accession numbers U39195 and U39196) (7) or their point mutated active counterpart (GIRK1-F137S or GIRK1* and GIRK4-S143T or GIRK4*), subcloned in the pGEMHE plasmid vector (23), were used as described previously (11, 12). The chimeric cDNA constructs were produced by splicing by overlapping extension polymerase chain reaction (24). Polymerase chain reactions, 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 University, Ithaca, NY). The βARK-PH construct (amino acids 452–689) was altered to incorporate the 15 N-terminal residues of Src for membrane targeting. This construct, generously provided by Dr. Eitan Reuvens, was altered and subcloned into pGEMHE.

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 an RNA marker (Life Technologies, Inc.) as a standard.
**Identification of a G_{\beta\gamma} Potassium Channel Site**

*Xenopus* oocytes were surgically extracted, dissociated, 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/superscript; IRK1 channel, 0.25 ng; m2 receptor, 1.0 ng; \(\beta_{2}\)-adrenergic receptor, 2.0 ng; GIRK1 subunits, 1.0 ng; GIRK subunits, 1.0 ng; Ca\(_{\text{II}}\), 1.0 mM; Mg\(_{\text{II}}\), and 5 mM HEPES, pH 7.4. The bath solution \(\text{IRK}\) and amino acids 546–670 for \(\text{IRK}\) and amino acids 184–419 for \(\text{GIRK}\), and the PH domain of \(\text{βARK}\) that can act as “sinks” for endogenous G\(_{\beta\gamma}\), subunits, and (c) coexpression of channels with exogenous G\(_{\beta\gamma}\), subunits.

Expression of GIRK4* in oocytes led to large basal and ACh-induced currents (Fig. 1, A and C). Coexpression of either βARK-PH or Gia1 led to a significant reduction in basal currents. However, oocytes coexpressing Gia1, rather than βARK-PH, displayed ACh-induced currents. This result is consistent with the interpretation that Gia1, and not βARK-PH, bound to endogenous G\(_{\beta\gamma}\), may be available for receptor-mediated activation. Coexpression of GIRK4 with exogenous G\(_{\beta\gamma}\), enhanced agonist-independent K\(_{\text{out}}\) currents while preserving the ACh-induced response (as in Ref. 12). These results indicate that both the GIRK4 basal and agonist-induced currents are largely mediated by the G\(_{\beta\gamma}\), subunits. In contrast, oocytes injected with IRK1 exhibited no ACh-induced currents and did not respond to coexpression with Gia1, βARK-PH, or G\(_{\gamma}\) (Fig. 1, B and C).

**A Minimal Chimera between GIRK4 and IRK1 with a Defect in Agonist-induced Responses**—We constructed chimeras between GIRK4 and IRK1 (Fig. 2, left). We screened for minimal segments of GIRK4 that when replaced by the corresponding IRK1 regions impaired sensitivity to G\(_{\beta\gamma}\). Chimeras were named for the IRK1 segment replacing the corresponding GIRK4 region. We first replaced the full C terminus of GIRK4* with that of IRK1 (GIRK4*IRK\(_{179-1423}\)). This chimera showed intact basal but impaired agonist-induced currents, consistent with a previous report (22). Huang et al. (15) found the GIRK1(Glu\textsuperscript{181}-Pro\textsuperscript{162}) segment to be a minimal G\(_{\beta\gamma}\), binding region. From an alignment of the GIRK1 and GIRK4 primary amino acid sequences, residue GIRK1(Glu\textsuperscript{181}) corresponds to GIRK4(Asp\textsuperscript{124}). Thus, we tested the response of the chimera GIRK4*IRK\(_{1316-1423}\) that replaced the GIRK4(Met\textsuperscript{229}-Val\textsuperscript{415}) region with the corresponding IRK1 segment. Again this chimera exhibited intact basal but impaired agonist-induced currents. To narrow the region responsible for the alternation of the GIRK4 agonist-induced currents, we constructed and tested three additional chimeras GIRK4*IRK\(_{3116-1343}\), GIRK4*IRK\(_{3142-1430}\), and GIRK4*IRK\(_{3136-1624}\). All three chimeras displayed intact basal currents. However, the response of GIRK4*IRK\(_{3116-1431}\) was impaired to agonist. These results suggest that differences between the two channels in this region, GIRK4(Met\textsuperscript{229}-Tyr\textsuperscript{348}) and IRK1(Leu\textsuperscript{161}-Tyr\textsuperscript{341}), may be important in their differential sensitivity to G\(_{\beta\gamma}\). This is unlike the downstream regions where differences were without effects on G\(_{\beta\gamma}\), sensitivity.

An Agonist-insensitive Chimera with Intact G\(_{\beta\gamma}\)-mediated Basal Currents—The current resulting from expression of the chimera GIRK4*IRK\(_{3116-1431}\) had intact basal currents but impaired agonist-induced responses. To test its sensitivity to G\(_{\beta\gamma}\), the GIRK4*IRK\(_{3116-1431}\) chimera was coexpressed with Gia1 or βARK-PH. A significant reduction of basal currents was obtained, similar to the GIRK4 control (Fig. 3A). Yet, this chimera differed from GIRK4* (see Fig. 1) in that its expression alone or with Gia or G\(_{\beta\gamma}\) resulted in impaired ACh-induced currents. This result further supports the conclusion that this chimeric channel is defective in producing agonist-induced currents. Coexpression with G\(_{\beta\gamma}\) did not stimulate basal levels of activity. Because the G\(_{\beta\gamma}\), dependence of the basal currents was intact in the GIRK4*IRK\(_{3116-1431}\) chimera, it is likely that the highly active GIRK4* and IRK1 channels. All experiments were carried out by expression in *Xenopus* oocytes. In whole-cell experiments, G\(_{\beta\gamma}\), sensitivity, in the presence of coexpressed G protein-coupled receptor, was assessed by (a) K\(_{\text{out}}\) current responses to agonist stimulation, (b) coexpression of channels with proteins such as Gia subunits or the PH domain of βARK that can act as “sinks” for endogenous G\(_{\beta\gamma}\), subunits, and (c) coexpression of channels with exogenous G\(_{\beta\gamma}\), subunits.

**RESULTS**

**GIRK4* Unlike IRK1 Is G\(_{\beta\gamma}\)-sensitive**—We compared G\(_{\beta\gamma}\), sensitivities of basal and agonist-induced currents between the
other regions may be involved in $G_{\beta\gamma}$ mediation of basal currents.

A Point Mutation Sufficient to Specifically Impair Agonist-induced Currents without Affecting the $G_{\beta\gamma}$ Contribution to Basal Activity—We proceeded to test which of the distinct residues within the identified region of the GIRK4* and IRK1 channels were responsible for their differences in sensitivity to $G_{\beta\gamma}$. Eleven point mutations were made in which residues in the Met323-Tyr348 region of GIRK4* were mutated to the corresponding residues found in the Leu316-Tyr341 region of IRK1 (Fig. 3B). Mutant names refer to the position and amino acid of GIRK4 that was mutated to the corresponding IRK1 residue. Only GIRK4*(L339E) showed impaired agonist-induced responsiveness, mimicking the responses obtained with the GIRK4*(IRK L316-Y341) chimera.

We next tested the $G_{\beta\gamma}$ sensitivity of the basal currents of GIRK4*(L339E), and compared them with that of the GIRK4* control in the same batch of oocytes. Oocytes coexpressing GIRK4*(L339E), $G_{\beta\gamma}$, or $\beta$ARK-PH behaved similar to the chimera GIRK4*(IRK L316-Y341), demonstrating an intact $G_{\beta\gamma}$-mediated basal current component. (Fig. 4A). Inside-out patch recordings from oocytes expressing the mutant and control channels were performed to test their responses to $G_{\beta\gamma}$ subunits. Fig. 4B (left) compares activity from one batch of oocytes expressing GIRK4* and GIRK4*(L339E) channels. Perfusion of inside-out patches with purified $G_{\beta\gamma}$ was ineffective in stimulating GIRK4*(L339E) activity compared with control GIRK4*. Stimulation of currents by endogenous G proteins through GTP$_S$ application gave similar results as the application of purified $G_{\beta\gamma}$(data not shown, n > 3). Regardless of their sensitivity to $G_{\beta\gamma}$, control or mutant channels responded to a similar degree to intracellular Na$^+$ ions (27), thus providing a positive control for gating by Na$^+$ ions. These inside-out patch responses were consistent with the whole-cell data for GIRK4*(L339E). Perhaps the lack of stimulation of whole-cell currents by $G_{\beta\gamma}$ coexpressed with GIRK4*(L339E) reflects maximal basal currents for this mutant. To examine this possibility, we coexpressed $\beta$ARK-PH in oocytes (same batch as the experiments in Fig. 4B, left) with GIRK4* or GIRK4*(L339E) channels. We perfused inside-out patches from such oocytes with GTP$_S$ purified from bovine brain (Fig. 4B, right). Inside-out patches of oocytes coexpressing GIRK4*(L339E) and $\beta$ARK-PH convincingly responded to perfusion with exogenous $G_{\beta\gamma}$, presumably recovering the $\beta$ARK-PH inhibition of the basal currents seen in the whole-cell experiments. However, these responses were significantly smaller than those of the control GIRK4*. In all cases, GTP$_S$ application failed to stimulate channel activity by activating endogenous G proteins, serving as a positive control for $\beta$ARK-PH effectiveness (data not shown, n > 3). Through these ex-
experiments we conclude that the GIRK4(L339E) mutation selectively impairs agonist-induced Gbg-mediated responses.

The C Terminus of GIRK4(L339E) Shows Decreased Binding to the Gbg Subunit—To determine the effects of the GIRK4-(L339E) mutation of the C terminus on Gbg binding, we constructed and purified GST fusion proteins containing the C termini of GIRK4 (GIRK4C), GIRK4(L339E), (GIRK4-(L339E)C) and IRK1 (IRK1C), or βARK-PH. GST fusion proteins were expressed in bacteria and purified (Fig. 5, top). In vitro binding assays were performed with the recombinant bovine Gbg1g2 subunits purified from Sf9 cells.

As shown in Fig. 5 (middle and bottom panels), the C termini of GIRK4 and GIRK4(L339E) were able to bind Gbg as compared with negative controls (GST and IRK1C) and a positive control (βARK-PH). GIRK4(L339E)C binding to Gbg was significantly reduced. These results suggest that the critical Leu of GIRK channels, and perhaps neighboring residues, directly interacted with Gbg subunits. Additionally, because GIRK4-(L339E)C has reduced but measurable binding to Gbg, it is likely that additional C-terminal Gbg binding sites exist, which contribute to the Gbg dependence of basal currents.

Wild-type GIRK1/GIRK4 Subunits Contribute Equally to the Agonist-induced Activity of Heteromultimeric Channels—To determine whether the effect seen with the L339E mutant was specific to the GIRK4* subunit, we mutated the corresponding amino acid residue in GIRK1, L333E. We tested for Gbg sensitivity of basal currents of the agonist-insensitive chimera and point mutants. A, effects on the basal currents (at 280 mV) of the agonist-insensitive chimera GIRK4*(IRKL316-Y341) coexpressed with Gbg, βARK-PH, or Gila. ACh responses were impaired in all groups (p < 0.005, n = 3–5). The basal currents of GIRK4*(IRKL316-Y341) coexpressed with βARK-PH or Gila were also significantly reduced (p < 0.005, n = 3–5). B, currents (at –80 mV) from mutants resulting from substitution of amino acids within GIRK4*(IRKL316-Y341) (corresponding to GIRK4*(IRKL316-Y341)), which differ between GIRK4 and IRK1. Each of eleven mutations were introduced into the context of the GIRK4* backbone. Asterisks denote significant reduction in ACh-induced currents (p < 0.005, n = 3).
Identification of a $G_{b\gamma}$ Potassium Channel Site

**FIG. 4.** Comparison of $G_{b\gamma}$ sensitivity of GIRK4* and GIRK4*(L339E) in whole-cell and inside-out patch experiments. A, two-electrode voltage clamp experiments plotting currents (at $-80 \text{ mV}$) of GIRK4* and GIRK4*(L339E) channels coexpressed with $G_{b\gamma}$, bARK-PH, or Gia1. ACh responses were impaired in the GIRK4*(L339E) groups, $p < 0.005, n = 3–6$. The basal currents of GIRK4*(L339E) coexpressed with bARK-PH or Gia1 were also significantly reduced, $p < 0.005, n = 3–6$. B, (left) inside-out patches from oocytes expressing the control or point mutant GIRK4* channels. Responses to patch perfusion with $G_{b\gamma}$ or Na* are shown for a representative patch and a number of patches tested within this batch of oocytes. $G_{b\gamma}$ increased channel activity significantly compared with control for GIRK4* ($p < 0.005, n = 3$) but not for GIRK4*(L339E) ($n = 4$); (right) inside-out patches from oocytes coexpressing the control or point mutant GIRK4* channels and bARK-PH. Responses to patch perfusion with $G_{b\gamma}$ or Na* are shown for a representative patch and a number of patches tested within this batch of oocytes. $G_{b\gamma}$ increased channel activity significantly compared with control for GIRK4* and GIRK4*(L339E) ($p < 0.005, n = 4$). The increase in channel activity in response to $G_{b\gamma}$ was significantly less in GIRK4*(L339E) compared with GIRK4* ($p < 0.005, n = 3–4$).

Activity of this point mutant in the context of the highly active homomultimer GIRK1(F137S) (see Refs. 11 and 12; referred to as GIRK1*).

Similar results were obtained with the GIRK1*(L333E) mutant as with the GIRK4*(L339E) mutant. Again, although ACh-induced currents were impaired by the mutation, the basal currents were reduced by Gia1 and bARK-PH (Fig. 6A, right).

In Fig. 6B shows that GTPyS application to inside-out patches expressing GIRK1* or GIRK1*(L333E) resulted in ~42-fold increase in GIRK1* activity but caused no increase in the current of GIRK1*(L333E) ($n = 5$).

We next sought to determine the relative contribution of wild-type GIRK subunits to agonist-induced activation in heteromultimeric channels (Fig. 6C). We introduced the Leu to Glu mutations at the 333 and 339 positions of the wild-type GIRK1 and GIRK4 subunits, respectively. We compared basal and agonist-induced currents of GIRK1/GIRK4 heteromultimeric channels, composed of both wild-type, both Leu to Glu mutants, and each wild-type to mutant combination. Our results suggest that each of the wild-type subunits contribute equally to agonist-induced activity, because heteromultimeric channels containing either Leu to Glu mutants displayed reduced agonist-induced sensitivity. Moreover, heteromultimeric channels containing both the subunits contained the Leu to Glu substitution showed significantly impaired agonist-induced currents. Thus, these results confirmed the importance of the residue for receptor-stimulated currents in heteromultimeric channels.

**Mutation of the Critical Leu Residue Does Not Distinguish among Channel Interactions with Specific $G_{b\gamma}$ Subunits or Signaling through Specific Receptors—Yeast two-hybrid experiments have shown that $G_{b\gamma}$ and $G_{b\gamma}$ interact with the N terminus of GIRK1 more strongly than do $G_{b\gamma}$ (19). To determine whether C-terminal mutation of the critical Leu residue could have altered the ability of the channel to interact with specific $G_{b\gamma}$ subunits, we coexpressed $G_{b\gamma}$, $G_{b\gamma}$, or $G_{b\gamma}$ with $G_{b\gamma}$ and GIRK1/GIRK4 or GIRK1(L333E)/GIRK4(L339E) heteromultimers. All $G_{b\gamma}$ combinations stimulated wild-type basal currents (2–4-fold, $n = 3$). When tested with the mutated channel subunits, $G_{b\gamma}$ or $G_{b\gamma}$ subunits failed to stimulate basal currents ($n = 3$). These results suggest that mutation of the critical Leu residue does not exert its effects by altering the specificity of channel-$G_{b\gamma}$ interactions. However, possible changes in the specificity of Leu mutant channels with the $G_{b\gamma}$ or $G_{b\gamma}$ subunits that were not tested cannot be ruled out.

$G_{b\gamma}$ subunits released from $G_{b\gamma}$ subunits by $\beta 2$-adrenergic receptor stimulation activate GIRK channels expressed in *Xenopus* oocytes (28). To test whether the critical Leu residue is involved in $G_{b\gamma}$ signaling by receptors other than m2, we coexpressed $\beta 2$-adrenergic receptor and $G_{b\gamma}$ subunits with GIRK1/GIRK4 or GIRK1(L333E)/GIRK4(L339E) heteromultimers. Isoproterenol-induced currents were obtained after expression of wild-type heteromultimers (~7.65 ± 2.17 μA at $-80 \text{ mV}$, $n = 3$) but not with mutants (~0.17 ± 0.09 μA at $-80 \text{ mV}$, $n = 3$). These results suggest that the $G_{b\gamma}$ released after activation of these two different receptors interact in a similar fashion with the critical GIRK Leu residue.

**DISCUSSION**

Since Soejima and Noma (29) first reported the membrane-delimited nature of the atrial muscarinic K+ channel, the mechanism of G protein gating of ion channels has received...
great attention. Over a decade ago, G protein-gated inwardly rectifying K channels provided the first example of a Gβγ-controlled signaling pathway. Yet, despite intense efforts, many questions remain unanswered regarding specific sites of interaction between the channel and Gβγ.

Biochemical studies from several groups have pointed to interaction of Gβγ, with the C and N termini of these channels. Specifically, Huang et al. (14), using deletion mutagenesis, found that deletion of the GIRK1(Val1573-Pro1584) segment reduced Gβγ binding of the remaining C-terminal fragment. In subsequent studies, Huang et al. (15) determined the GIRK1(Glu318-Pro462) segment as a minimal Gβγ binding region. Kunkel and Peralta (16), using a combination of chimeras and deletion mutations, reported the GIRK1(Thr290-Tyr356) region to be important in interactions with Gβγ.

In this study, we screened the C terminus of GIRK4 for residues that control channel activity. We made chimeras that replaced specific sections of GIRK4 with those from the G protein-insensitive channel, IRK1. Expression of a minimal chimera, the GIRK4*(IRK-L336,Y341), resulted in normal basal currents that did not respond to ACh when coexpressed with hm2 receptors. Expression of exogenous Gβγ did not enhance basal currents in this chimera. Yet, basal currents were inhibited by coexpression of Gi1 and βARK-PH.

Of all the amino acid differences between IRK1 and GIRK4 contained in this chimera, only the mutant GIRK4*(L339E) retained all properties of the chimera. This mutant displayed Gβγ-sensitive basal currents that were not ACh-sensitive and did not respond to exogenous Gβγ. Inside-out patch currents from oocytes expressing GIRK4*(L339E) and hm2 receptors were significantly smaller in response to Gβγ, or GTPγS. Binding of the Gβγ subunits to the GIRK4 C terminus bearing the L339E mutation was significantly reduced. Within the broader boundaries suggested by others (14–16), the region surrounding Leu339 is a Gβγ binding site with critical functional consequences.

Basal currents from the GIRK4*(L339E) channel were inhibited by coexpression of Gβγ sinks, such as the βARK-PH. Because binding of the L339E mutant of the GIRK4 C terminus was not abolished and because basal currents of GIRK4*(L339E) could be inhibited by βARK-PH or Gi1, it is likely that additional Gβγ binding sites contribute to basal channel activity. Moreover, because Gβγ perfusion of inside-out patches activated GIRK4*(L339E) channels only when they were coexpressed with βARK-PH, it is likely that this activation reflected reversal of the βARK-PH inhibited basal currents. We hypothesize that the basal binding sites may be high affinity and saturated in both the whole-cell and inside-out patch experiments. Recent evidence has suggested another region of GIRK4 (Ser290-Arg325) capable of high affinity binding to Gβγ (30). It is possible that such a site accounts for part or all of the basal channel activity.

How does GIRK4*(L339E) impair specifically agonist-induced stimulation? In the simplest model, free Gβγ would be bound to high affinity basal sites. The GIRK4*(IRK-L336,Y341) chimera and the GIRK4*(L339E) mutant may impair a low affinity binding of this region to Gβγ subunits. Normally, agonist-induced liberation of Gβγ subunits would increase the local free Gβγ concentration, allowing interaction with a low affinity site, encompassing GIRK4*(Leu339), and leading to stimulation of channel activity. Further work will be required to test this hypothesis.

GIRK1*(L333E) channels displayed similar properties to GIRK4*(L339E). Again, basal currents from this mutant channel were sensitive to Gβγ, but no ACh-induced currents could be detected. Double mutations in heteromultimeric GIRK1*(L333E)/GIRK4*(L339E) channels expressed in oocytes showed similar properties to the highly active homomultimeric mutants discussed above. Furthermore, mutation of both channels in a heteromultimer was required for the ACh-insensitive phenotype, whereas reduced agonist-induced currents were obtained with one or the other of the two subunits mutated. These results suggest that there is an equivalent contribution of GIRK1 and GIRK4 to Gβγ-mediated ACh-induced activity. Additionally, co-expression of different Gβγ combinations or different receptors such as the β2-adrenergic receptor did not alter the unique properties of these mutant channels. This suggests that signaling through different receptors and by different Gβγ combinations activates the channel through conserved interactions.

Biochemical evidence has suggested multiple binding sites in the C- and N-terminal segments of GIRK channels (15, 19, 30). The multiplicity of Gβγ and other effector proteins is in agreement with the finding that the Gβγ-phosphducin co-crystals show multiple sites of interaction between the two proteins (31). Our data combine biochemical with functional evidence.
for more than one $G_{i2}$ binding site on the channel. Surprisingly, distinct functional roles could be assigned to multiple binding sites; one designed to interact with $G_{i2}$ released from receptor stimulation, whereas additional site(s) may interact with free $G_{i2}$ to yield basal activity. Thus, these results suggest that for the $K^+$ channel the multiplicity of interactions may subserve distinct functional roles.

Acknowledgments—We are grateful to Eitan Reuveny for generously sharing with us the modified version of the ARK-PH construct and to Dr. Maureen Linder for graciously guiding us with the $G_{i2}$ purification. We thank Drs. David Clapham and Robert Margolieske for critical review of the manuscript, Grigory Krapivinsky for advice with the binding studies, and Mariana Max, Eitan Reuveny, and Ming Ming Zhou for helpful discussions.

REFERENCES

1. Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J., and Clapham, D. E. (1987) Nature 325, 321–326
2. Trautwein, W., and Dudel, J. (1958) Pflügers Arch. 266, 324–334
3. Kubo, Y., Reuveny, E., Slesinger, P. A., Jan, Y. N., and Jan, L. Y. (1993) Nature 364, 802–806
4. Dascal, N., Schreibmayer, W., Lim, N. F., Wang, W., Chavkin, C., DiMaggio, L., Labarca, C., Kieffer, B. L., Caveriaux-Ruff, C., Trottier, D., Lester, H. A., and Davidson, N. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10235–10239
5. Lesage, F., Duprat, F., Fink, M., Guillaume, E., Coppola, T., Lazdunski, M., and Hugnot, M. J. P. (1994) FEBS Lett. 353, 37–42
6. Krapivinsky, G., Gordon, E. A., Wickman, K., Velimirovic, B., Krapivinsky, L., and Clapham, D. E. (1995) Nature 374, 135–141
7. Chan, K. W., Langan, M. N., Sui, J. L., Kozak, J. A., Pabon, A., Ladias, J. A. A., and Logothetis, D. E. (1996a) J. Gen. Physiol. 107, 381–397
8. Hedin, R. E., Lim, N. F., and Clapham, D. E. (1996) Neuron 16, 423–429
9. Doyle, D. A., Cabral, J. M., Pfuetzner, A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) Science 280, 69–77
10. Sui, J.-L., Chan, K., Langen, M.-N., Vivaudou, M., and Logothetis, D. E. (1999) in Advances in Second Messenger and Phosphoprotein Research (Armstrong, D., and Rossie, S., eds) Academic Press, Orlando, FL, in press
11. Chan, K. W., Sui, J. L., Vivaudou, M., and Logothetis, D. E. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 14195–14198
12. Vivaudou, M., Chan, K. W., Sui, J. L., Jan, L. Y., Reuveny, E., and Logothetis, D. E. (1996) J. Gen. Physiol. 107, 1238–1244
13. Krapivinsky, G., Krapivinsky, L., Wickman, K., and Clapham, D. E. (1995) J. Biol. Chem. 270, 29059–29062
14. Huang, C. L., Slesinger, P. A., Casey, P. J., Jan, Y. N., and Jan, L. Y. (1995) Neuron 15, 1133–1143
15. Huang, C. L., Jan, Y. N., and Jan, L. Y. (1997) FEBS Lett. 405, 291–298
16. Kunkel, M. T., and Peralta, E. G. (1995) Cell 83, 443–449
17. Imanobe, A., Ito, H., Ito, M., Hossaya, Y., and Kurachi, Y. (1995) Biochem. Biophys. Res. Commun. 217, 1238–1244
18. Cohen, N. A., Sha, Q., Makrina, E. N., Lopatin, A. N., Linder, M. E., Snyder, S. H., and Nichols, C. G. (1996) J. Biol. Chem. 271, 32301–32305
19. Yan, K., and Gautam, N. (1996) J. Biol. Chem. 271, 17597–17600
20. Reuveny, E., Slesinger, P. A., Inglese, J., Morales, J. M., Izquieta-Lubi, J. A., Leffkowitz, R. J., Bournne, H. R., Jan, Y. N., and Jan, L. Y. (1994) Nature 370, 143–146
21. Kubo, Y., Baldwin, T. J., Jan, Y. N., and Jan, L. Y. (1993) Nature 362, 127–133
22. Kubo, Y., and Izuka, M. (1996) Biochem. Biophys. Res. Commun. 227, 240–247
23. Liman, E. R., Tytgat, J., and Hess, P. (1992) Neuron 9, 861–871
24. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. R., and Pease, L. R. (1989)
Identification of a $G_{B_{\gamma}}$ Potassium Channel Site

25. Schreibmayer, W., Lester, H. A, and Dascal, N. (1994) *Pflügers Arch.* 426, 453–458

26. Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, B. F. J. (1981) *Pflügers Arch.* 391, 85–100

27. Sui, J. L., Chan, K. W., and Logothetis, D. E. (1996) *J. Gen. Physiol.* 108, 381–391

28. Lim, F. N., Dascal, N., Labarca, C., Davidson, N., and Lester, H. A. (1995) *J. Gen. Physiol.* 105, 421–439

29. Soejima, M., and Noma, A. (1984) *Pflügers Arch.* 400, 424–431

30. Krapivinsky, G., Kennedy, M. E., Nemec, J., Medina, I., Krapivinsky, L., and Clapham, D. E. (1998) *J. Biol. Chem.* 273, 16946–16952

31. Gaudet, R., Bohm, A., and Sigler, P. B. (1996) *Cell* 87, 577–588

32. Kozasa, T., and Gilman, A. G. (1995) *J. Biol. Chem.* 270, 1734–1741