Gβγ-dependent and Gβγ-independent Basal Activity of G Protein-activated K⁺ Channels

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Cardiac and neuronal G protein-activated K⁺ channels (GIRK; Kir3) open following the binding of Gβγ subunits, released from Gαi/o proteins activated by neurotransmitters. GIRKs also possess basal activity contributing to the resting potential in neurons. It appears to depend largely on free Gβγ, but a Gβγ-independent component has also been envisaged. We investigated Gβγ dependence of the basal GIRK activity (A_{GIRK,basal}) quantitatively, by titrated expression of Gβγ scavengers, in Xenopus oocytes expressing GIRK1/2 channels and muscarinic m2 receptors. The widely used Gβγ scavenger, myristoylated C terminus of β-adrenergic kinase (m-cβARK), reduced A_{GIRK,basal} by 70–80% and eliminated the acetylcholine-evoked current (I_{ACH}). However, we found that m-cβARK directly binds to GIRK, complicating the interpretation of physiological data. Among several newly constructed Gβγ scavengers, phosducin with an added myristoylation signal (m-phosducin) was most efficient in reducing GIRK currents. m-phosducin relocated to the membrane fraction and did not bind GIRK. Titrated expression of m-phosducin caused a reduction of A_{GIRK,basal} by up to 90%. Expression of Gβγ was accompanied by an increase in the level of Gβγ and Gα in the plasma membrane, supporting the existence of preformed complexes of GIRK with G protein subunits. Increased expression of Gβγ and its constitutive association with GIRK may underlie the excessively high A_{GIRK,basal} observed at high expression levels of GIRK. Only 10–15% of A_{GIRK,basal} persisted upon expression of both m-phosducin and cβARK. These results demonstrate that a major part of I_{basal} is Gβγ-dependent at all levels of channel expression, and only a small fraction (<10%) may be Gβγ-independent.

G protein-activated, inwardly rectifying K⁺ channels (GIRK, Kir3) mediate postsynaptic inhibitory effects of various neurotransmitters in the brain and atrium via seven-helix, G protein-coupled receptors (GPCRs) linked to pertussis toxin-sensitive G proteins of the G_{i/o} family. Opening of the channels is the result of a direct binding of Gβγ subunits released from the G_{i/o}βγ heterotrimers (1–4). The channel can also be activated by cytosolic Na⁺ and membranal phosphatidylinositol 4,5-bisphosphate (PIP2); the latter is essential for proper GIRK gating by both Na⁺ and Gβγ (3, 5, 6).

Whereas the physiological role of neurotransmitter-induced GIRK activity is well established, the basal activity of these channels (A_{GIRK,basal}) is often regarded as negligible and physiologically unimportant. This feature distinguishes GIRK from many other K⁺ channels of the Kir family, such as Kir1 and Kir2, which show high intrinsic activity under physiological conditions and are often referred to as “constitutively active.” Low A_{GIRK,basal} is supposed to ensure high signal-to-noise ratio for GIRK-related neurotransmitter signaling and to minimize participation of GIRK in resting membrane K⁺ conductance (see Ref. 2). However, some classical and many recent studies challenge this concept. In sinoatrial node cells, GIRK channels may contribute a major part of basal K⁺ conductance (7). Recent studies in intact neurons indicate that basal activity of GIRK may substantially contribute to resting K⁺ conductance, shunting the excitatory postsynaptic potentials and reducing the responses to glutamate (8). Substantial A_{GIRK,basal} has been reported in hippocampal pyramidal cells (9, 10) and in rat locus coeruleus slices (11). In murine locus coeruleus, where GIRKs mediate most of the inhibitory effects of opioids, knock-out of GIRK subunit genes depolarizes the cell’s resting potential by as much as 20 mV (8). In many cases, it was not clear whether the GIRK activity observed in resting cells under a variety of experimental conditions was a truly agonist-independent one or depended on the presence of “ambient” neurotransmitters or local hormones. For instance, in hippocampal pyramidal cells, one report assigned to ambient adenosine a major role in sustaining most of A_{GIRK,basal} (9), whereas another one contended that A_{GIRK,basal} was independent of adenosine or other neurotransmitters (10). In any case, it appears that GIRK plays a substantial role in determining the resting membrane potential at least in some neurons and in sinoatrial pacemaker cells. It is therefore conceivable that not only activation of GIRK by G_{i/o} coupled neurotransmitters, but also its inhibition by neurotransmitters that activate G_{i/o} (12–16) may serve as an important mechanism of regulation of neuronal excitability. These considerations urge for a better understanding of the mechanisms of regulation of A_{GIRK,basal}.

An important contributor to A_{GIRK,basal} is ambient free Gβγ. This has been shown in heterologous expression systems; co-

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Footnotes:

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The abbreviations used are: GIRK, G protein-activated K⁺ channel; ACh, acetylcholine; A_{GIRK,basal}, the basal activity of the GIRK channel; I_{basal}, the basal activity of GIRK measured in whole oocytes in a high K⁺ solution; I_{ACH}, the ACh-evoked GIRK response; 5HT, 5-hydroxytryptamine (serotonin); aa, amino acid(s); GST, glutathione S-transferase; GPCR, G protein-coupled receptor; m2R, muscarinic receptor type 2; PM, plasma membrane; PIP2, phosphatidylinositol 4,5-bisphosphate; m-cβARK, myristoylated C terminus of β-adrenergic receptor; ambient, in accordance with 18 U.S.C. Section 1734.

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expression of Ga subunits and a myristoylated C-terminal segment of β-adrenergic receptor kinase (m-βARK), used in the past as standard Gβγ scavengers in GIRK studies (17–22), has been reported to reduce A\textsubscript{GIRK,basal} to different extents, between 50 and 90%. It is not clear whether the remaining activity is Gβγ-independent or simply reflects insufficient Gβγ sequestration, leaving open the question of whether a Gβγ-independent A\textsubscript{GIRK,basal} exists. Such Gβγ-independent basal activity could be due to a direct activation by cytosolic free Na\textsuperscript{+} (23–25) and/or reflect an intrinsic Gβγ- and Na\textsuperscript{+}-independent channel activity. The variability of the reported results regarding the extent of inhibition of A\textsubscript{GIRK,basal} by Gβγ scavengers calls for a better quantitative assessment of the Gβγ-dependent component. Furthermore, the use of Ga as a Gβγ scavenger is problematic. Coexpressed Ga\textsubscript{i} subunits may affect the level of channel expression (20); they interact with GIRK itself and have been hypothesized to affect the gating directly (20, 26–28). This greatly complicates the interpretation of Gβγ-scavenging effects of Ga subunits. It is not known whether m-βARK, widely used to study modulation of ion channels by Gβγ (19, 20, 29–31), directly interacts with GIRKs or affects their expression. Therefore, further study is needed to reliably estimate the Gβγ-dependent part of A\textsubscript{GIRK,basal}.

An intriguing phenomenon related to A\textsubscript{GIRK,basal} has been discovered in Xenopus oocytes (20). At low expression levels (densities) of GIRK, A\textsubscript{GIRK,basal} is low, and activation by agonist and by purified Gβγ (in whole cells and in excised patches, respectively) is strong, as expected. However, when more GIRK channels are expressed, their basal activity becomes excessively large at the expense of agonist- or Gβγ-evoked activity. Coexpression of a Ga\textsubscript{i} subunit restores the normal gating pattern, with low A\textsubscript{GIRK,basal} and high agonist- or Gβγ-evoked activity. We proposed that A\textsubscript{GIRK,basal} is controlled by a number of factors, most prominently Gβγ and Ga, and that the balance between these factors collapses when the channel is expressed at high levels in Xenopus oocytes, partly because of a lack of Ga\textsubscript{i} (20, 28). The excessively high A\textsubscript{GIRK,basal} at high levels of GIRK expression remained incompletely understood. Theoretically, it seemed possible that at high densities GIRK behaves as a constitutively active channel.

To better understand the fundamental mechanisms of A\textsubscript{GIRK,basal}, we have initiated a systematic study of Gβγ dependence of A\textsubscript{GIRK,basal} in Xenopus oocytes at different levels of GIRK expression. This heterologous expression system is superior to others for quantitative studies, since it allows both a controlled expression of proteins in very wide ranges and an accurate measurement of expressed proteins in the whole cell and in the plasma membrane (PM). Titrated expression of m-βARK and a novel membrane-targeted Gβγ scavenger, myristoylated phosphocin (m-phosducin), revealed that at least 90% of A\textsubscript{GIRK,basal} is Gβγ-dependent at all levels of expression of GIRK. Gβγ-independent activity, if any, constitutes only a small fraction of A\textsubscript{GIRK,basal}. The excessively high basal activity observed at high levels of channel expression may be due to a constitutive association of GIRK with Gβγ, in the absence of sufficient Ga needed to preserve low A\textsubscript{GIRK,basal}.

**EXPERIMENTAL PROCEDURES**

**cDNA Constructs and RNA**—The coding sequences of all cDNAs used in this study for preparation of RNA were inserted into high expression oocyte vectors containing 5'- and 3'-untranslated sequences of Xenopus β-globin: pGEMHE or its derivative pGEMHJ or pBS-MXT, as described in our previous publications (12, 32), unless stated otherwise. All PCR-derived cDNAs were sequenced in full at the Tel Aviv University sequencing facility. The original cDNAs of human m2R, human 5HT-1A receptor, mouse GIRK2, rat m-βARK (aa 452–689), (12, 32), human βARK (GB X61157), and bovine phosphocin (GB M35529) were kindly provided by E. Peralta, P. Hartig, P. Kofuji, E. Reuveny, J. Lefkowitz, and M. Lohse, respectively.

PCR-derived full-length coding sequence of wild type phosphocin was inserted into the EcoRI restriction site of pGEMHJ, creating the template for RNA synthesis and expression of wild-type, nonmyristoylated phosphocin (n-phosducin). To create a membrane-attached derivative of phosphocin, we first constructed a new vector, pGEMHE-myr, on the basis of the pGEMHE vector (33) modified by E. Reuveny, using standard PCR techniques. A DNA sequence, atgggggtggaggaagcatatgctggagac, was inserted between Smal and EcoRI restriction sites of the pGEM-HE vector. On the protein level, this adds the first 15 N-terminal aa of Src, MGGSSKSPKPSQQR (34), followed by Glu-Phe (encoded by the nucleotide sequence of the EcoRI site), to any proteins whose coding sequence is inserted between EcoRI and any one of the following restriction sites of pGEMHE polylinker. PCR product corresponding to the entire coding sequence of bovine phosphocin, flanked by EcoRI and HindIII, was inserted into the respective restriction sites of pGEMHE-myr.

cDNA for nonmyristoylated C terminus of human βARK1, n-βARK, was created by inserting the cDNA sequence encoding aa 460–689\textsubscript{N} of βARK, preceded by a methionine, flanked by EcoRI and HindIII restriction sites, into the pGEMHJ vector. The comparison of physiological effects of m-βARK (rat) and n-βARK (human) is justified by high homology between these proteins (97% identity). Coexpressed proteins m2R-cβARK, 5HT1A-cβARK, and 5HT1A-1TM-cβARK, PCR-derived coding sequences of m2R, 5HT1A receptor (5HT1AR), and an 1–67 of 5HT1AR, respectively, flanked by EcoRI restriction sites, were inserted in frame just prior to the n-βARK sequence made as described above. Point mutations in m-βARK were done using the QuikChange site-directed mutagenesis kit (Stratagene).

RNA was synthesized in vitro using a protocol that ensures incorporation of the GTP cap mainly into the 5′ portion of the RNA (35) rather than along the whole length as with commercially available kits. Accordingly, the resulting RNAs are usually more efficient than those obtained by other standard protocols. Unless indicated otherwise, RNAs were injected into the oocytes in the following amounts: m2R, 0.5 ng/oocyte; n-βARK, m2R-cβARK, 5HT1A-cβARK, 5HT1A-1TM-cβARK, and m-cβARK, n-phosducin, 10 ng/oocyte. Xenopus Oocytes Preparation and Electrophysiology—Xenopus oocytes were prepared as described (12, 32), injected with RNA, and incubated for 3–4 days in ND-96 solution (NaCl, 96 mM; KCl, 2 mM; CaCl\textsubscript{2}, 1 mM; MgCl\textsubscript{2}, 1 mM; Hepes/NaOH, 5 mM; pH 7.6) supplemented with gentamycin (50 μg/ml) and sodium pyruvate (2.5 mM). All experiments were done at 20–22 °C. Whole-cell GIRK currents were measured using two-electrode voltage clamp with OC-725B (Warner Instruments Corp.), using agarose tissue electrodes (36) filled with 3 M KCl, with resistances of 0.1–0.5 megohms. Oocytes were held at −80 mV in the ND-96 solution, and GIRK currents were measured in high K\textsuperscript{+} solutions, high K 96 or high K 24. High K 96 contained 96 mM KCl, 2 mM NaCl, 1 mM MgCl\textsubscript{2}, 5 mM Hepes/NaOH, 5 mM; pH 7.5. High K 24 contained 24 mM KCl, 72 mM NaCl, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 5 mM Hepes (pH 7.5). Data acquisition and analysis were done using Axotape and pCLAMP software (Axon Instruments Inc., Foster City, CA).

Interaction between GST fusion proteins and phosphocin and cβARK was studied as described (21). Briefly, GST-CT-GIRK1 fusion protein was purified, and its interaction with [\textsuperscript{35}S]methionine-labeled proteins (the various forms of cβARK, phosphocin, and Gβ\textsubscript{γ}) synthesized in rabbit reticuloocyte lysate (Promega) was monitored using a standard pull-down procedure. GST-GIRK1-CT or GST-GIRK1-NT (5–10 μg) was incubated for 1 h with 5 μl of reticulocyte lysate containing the designated [\textsuperscript{35}S]methionine-labeled proteins in 300 μl of a high K\textsuperscript{+} buffer (150 mM KCl, 50 mM Tris, 5 mM MgCl\textsubscript{2}, 1 mM EDTA, pH 7.0) with the addition of 0.5% CHAPS. Then 30 μl of glutathione-Sepharose beads (Pharmacia Biosiences) were added, and the mixture was incubated for 30 min at 4 °C and washed in 1 ml of the same buffer (once with 0.5% and twice with 0.1% CHAPS). Bound proteins were eluted with 30 μl of 15 mM reduced glutathione and analyzed on 12% SDS-polyacrylamide gels. The labeled proteins were imaged and quantified by autoradiography using a PhosphorImager (Amersham Biosciences).

**Western Blot Analysis**—The oocytes were homogenized on ice in homogenization buffer (20 mM Tris, pH 7.4, 5 mM EDTA, 5 mM DTNB, and 100 mM NaCl) containing the Roche Applied Science protease inhibitor mixture. Debris was removed by centrifugation twice at 1000 × g for 15 min at 4 °C. Total cellular membrane fraction from 5–17 oocytes was obtained by 1 h of ultracentrifugation at 100,000 × g. For

2. N. Dascal, unpublished observations.
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RESULTS

m-βARK Reduces \( I_{\text{basal}} \), but It Also Binds to the C Terminus of the GIRK Subunit—To separate the \( G\beta\gamma \)-dependent component of \( A_{\text{GIRK,basal}} \), we have initially utilized the widely used \( G\beta\gamma \) scavenger, m-βARK. GIRK activation by \( G\beta\gamma \) is membrane-delimited (1), so that only PM-attached \( G\beta\gamma \) can activate the channel. Targeting of m-βARK to the membranes is provided by the myristoylation signal (the first 15 aa of Src added by the myristoylation technology), or 2.5 ng/oocyte of n-βARK expressed in oocytes (occ.) and in reticulocyte lysate. Cytosolic (top panel) and membrane (bottom panel) fractions of oocytes were separated by differential centrifugation (100,000 × g). m-βARK was preferentially targeted to the membrane fraction.

Western blots, protein samples were separated on SDS-12% polyacrylamide gels. Antibodies against cβARK, phosducin, and Gβ common (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used. Visualization of protein bands was performed using ECL reagents obtained from Raytest, Straubenhardt, Germany).

Confocal Imaging of GIRK, Gα, and Gβ in Plasma Membrane—The levels of expressed proteins in PM were measured as described (20, 37). Briefly, large oocyte membrane patches were attached to coverslips with their cytoplasmic leaflet facing the external solution; fixed, stained by 0.75 ng/μl of a specific GIRK1 antibody (Alomone Laboratories, Jerusalem), 0.5 ng/μl of a common Gβ antibody (Santa Cruz Biotechnology), or 2.5 ng/μl of a common Gα antibody (Calbiochem); and visualized using a Cy3-conjugated rabbit IgG (Jackson Immunoresearch Laboratories) using a Zeiss LSM 410 or a Leica TCS SP2 confocal microscope. Intensity of labeling (OD units) was measured with TINA software. In control experiments (Fig. 8, A and B), to estimate the specificity of labeling by the Gα and Gβ antibodies, purified Gα1,GDP or purified Gβ1,γ2, respectively, was used in 20-fold excess (w/v) over the antibodies. The purified Gα1 and Gβ1,γ2 were a generous gift from C. W. Dessauer (University of Texas, Houston).

Data Presentation and Statistics—Data are presented as mean ± S.E. Comparison between two groups of treatment was done using the two-tailed Student’s \( t \) test. Comparisons among several groups of data have been performed using one-way analysis of variance followed by Dunnet’s test. The level of statistical significance is indicated in the figures as follows: *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \).

To make sure that the decrease in GIRK currents by m-βARK is not linked to changes in channel expression, we monitored the relative amounts of expressed GIRK channels in the PM using a confocal microscope-assisted immunomapping method (Fig. 2C). The channels were visualized in very large plasma membrane patches (usually several thousand \( \mu m^2 \)), adhered to a coverslip, with their cytoplasmic surface facing the external medium (37). The patches were extensively washed and nominally free from endoplasmic reticulum.3 As summarized in Fig. 2C, b, coexpression of m-βARK did not affect the amount of channel protein in the PM.

Myristoylated m-βARK seems to be suitable for the purpose of studying the \( G\beta\gamma \) dependence of \( A_{\text{GIRK,basal}} \), since it

3 N. Dascal and E. Artzy, unpublished observations.
does not block the direct Na\(^{+}\)-induced activation (19). However, “side effects” are possible. One potential pitfall is that the G\(\beta\)γ-binding segment is within a plekstrin homology domain, which also binds PIP\(_2\) (38); thus, heavily overexpressed c\(\beta\)ARK may in principle reduce the amount of PIP\(_2\) in the PM. Second, it is not known whether the myristoyl moiety affects the function of GIRK. To control for these possibilities, we constructed several membrane-targeted G\(\beta\)γ-scavengers based on c\(\beta\)ARK. First, we made a point mutation, R587Q, in m-c\(\beta\)ARK, which greatly impairs the ability of c\(\beta\)ARK to bind G\(\beta\)γ, but not PIP\(_2\) (39). Expression of this construct in the oocytes did not have any significant effect on GIRK currents (Fig. 2D), suggesting that PIP\(_2\) chelation is not involved in the effect of m-c\(\beta\)ARK. To check for possible “side effects” of the myristoyl moiety, we expressed the nonmyristoylated n-c\(\beta\)ARK and found that it did not significantly affect GIRK currents at 5 ng of RNA/oocyte (Fig. 2D). Although n-c\(\beta\)ARK (aa 460–689 of c\(\beta\)ARK) is 8 aa shorter than m-c\(\beta\)ARK (aa 452–689), both constructs contain the G\(\beta\)γ-binding domain (aa 643–670) as well as the full plekstrin homology domain (aa 561–655) (39). Therefore, the difference in length probably does not determine the difference in the effect on GIRK currents. Next, we constructed fusion proteins in which c\(\beta\)ARK was connected to C-terminal ends of several membrane proteins, which were expected to anchor these tandems to the PM, instead of the myristoyl moiety. The proteins chosen were m\(_2\)R, the serotonin receptor 5HT1AR, and the first transmembrane domain of 5HT1AR, 5HT-1TMD. The first two constructs did not inhibit GIRK currents (Fig. 2D) after 3 days of incubation. Mild inhibitory effects of m\(_2\)R-c\(\beta\)ARK and 5HT1A-c\(\beta\)ARK were seen after long periods of oocyte incubation, 4–5 days (data not shown), suggesting that these very large fusion proteins may be poorly expressed in the oocytes. However, 5HT-1TMD-c\(\beta\)ARK significantly reduced \(I_{\text{total}}\) though less than m-c\(\beta\)ARK (by 72% inhibition by c\(\beta\)ARK; Fig. 2D). This result suggests that the presence of the myristoyl moiety is not necessary for c\(\beta\)ARK to inhibit GIRK.

**FIG. 2. Effects of c\(\beta\)ARK congeners on GIRK current.** A, examples of whole-cell GIRK currents measured by the two-electrode voltage clamp method. Oocytes were injected with 0.2 ng of RNA of each channel subunit and 0.5 ng of RNA of m\(_2\)R with or without 5 ng of RNA of m-c\(\beta\)ARK. B, extent of inhibition of \(I_{\text{basal}}\) and \(I_{\text{ACh}}\) is a dose-dependent function of the amount of injected RNA of m-c\(\beta\)ARK. The solid lines show fits to the standard Michaelis-Menten equation for \(I_{\text{basal}}\). C, coexpression of m-c\(\beta\)ARK does not affect the level of expressed GIRK in the PM. a, representative confocal images of channel protein staining in large membrane patches. b, summary of the measurements of GIRK protein expression in the plasma membrane in oocytes of two donors. The net signal, in OD units, in each image was calculated by subtracting the average intensity of signal measured in uninjected oocytes. D, summary of effects of coexpression of different constructs containing c\(\beta\)ARK on GIRK channel. The values of \(I_{\text{total}}\) are shown; changes in \(I_{\text{basal}}\) were similar. All parameters are shown as percentages of the same parameter recorded in the control group in the same experiment. E, the effect of coexpression of m-c\(\beta\)ARK at high and low densities of the GIRK channel. Oocytes were injected with either 0.2 or 1 ng of RNA of each channel subunit and 0.5 ng of RNA of m\(_2\)R, together or without 5 ng of RNA of m-c\(\beta\)ARK. In D and E, the numbers above the bars denote the number of oocytes, with the number of experiments (oocyte batches) given in parentheses. Here and in the following figures, the statistical significance is denoted as follows: **, \(p < 0.01\); ***, \(p \leq 0.001\). The brackets below the asterisks show groups subjected to pairwise comparisons. The asterisks above the bars not denoted by brackets indicate statistically significant differences compared with the control group only.
Unexpectedly, we found that GST-GIRK1-CT bound in vitro in reticulocyte lysate almost as well as it bound Gβγ in the PM, when more channels were expressed (Fig. 2E). At high channel density (1 ng RNA/oocyte), Ibasal was decreased by ~70%, and IACH was decreased by ~75%, in comparison with ~80 and ~95% decrease, respectively, at lower channel density, 0.2 ng of RNA/oocyte. The same held true for Itotal: These differences are not unexpected for a pure Gβγ scavenger whose efficiency depends on the ratio of its own concentration to that of the Gβγ-binding effector (GIRK in our case) and is itself sequestered by high doses of the channel, so that the efficiency of Gβγ sequestration is reduced. Indeed, GIRK and βARK co-immunoprecipitate, along with a number of other components of a multiprotein signaling complex, from atrial cells (40).

The possibility of a direct interaction between cβARK and GIRK was probed using the standard pull-down methodology. We used a purified GST fusion protein containing the full-length cytosolic C terminus of GIRK1, GST-GIRK1-CT (aa 183–501), which was previously shown to bind Gβγ(21, 27, 41). Unexpectedly, we found that GST-GIRK1-CT bound [35S]methionine-labeled m-cβARK and m-cβARK translocated synthesized in vitro in reticulocyte lysate almost as well as it bound Gβγ (Figs. 3 and 4B). n-cβARK also bound to GST-GIRK1-CT, but distinctly more weakly than m-cβARK. At present, we do not know whether this difference was due to the absence of myristoylation of or of the first 8 aa in n-cβARK. Because m-cβARKsG8TQ, which binds GIRK1 but not Gβγ, does not affect GIRK currents (Fig. 2), it is plausible that most of the inhibitory effect of m-cβARK is independent of its binding to GIRK and represents a genuine Gβγ scavenging effect. However, the direct interaction between m-cβARK and GIRK1 is bound to complicate the interpretation of the results of this and previous studies.

A Novel Gβγ Scavenger, m-Phosducin, Reveals That Most GIRK Channel Activity Is Gβγ-dependent at All Channel Densities—In a search for a Gβγ scavenger that does not interact with GIRK, we constructed a myristoylated first intracellular loop of the N-type voltage-dependent Ca2+ channel, which is known to bind Gβγ (42). However, expression of this construct in the oocytes led to a rather weak inhibition of GIRK currents (data not shown). Next, we turned to phosducin, a soluble cytosolic Gβγ-binding protein originally identified in retinal photoreceptor cells, which has already been used as a Gβγ scavenger in heterologous expression systems (43). Phosducin also binds Go, but this interaction is of much lower affinity than that with Gβγ (44). In this paper, we designate the original cytosolic phosducin as n-phosducin to distinguish it from the myristoylated construct made by us.

Coexpression of n-phosducin (10 ng of RNA/oocyte) reduced Ibasal by ~70% and IACH by only ~42% (see Fig. 5B). To increase the likelihood that phosducin will access Gβγ in the PM, we constructed a DNA for myristoylated m-phosducin. The resulting protein has an added 15-aa myristoylation signal, the same as in m-cβARK, at the beginning of its N terminus. n-Phosducin expressed in the oocytes was preferentially associated with the cytosolic fraction, whereas m-phosducin was enriched in the membrane fraction (Fig. 4A). Pull-down experiments showed that neither n-phosducin nor m-phosducin bound GST-GIRK1-CT (Fig. 4B). Since the N terminus of GIRK1 also binds Gβγ (27), we examined whether the GST-fused N terminus of GIRK1 (GST-GIRK1-NT) binds cβARK and phosducin. Fig. 4D shows that GST-GIRK1-NT bound Gβγ and, to a lesser extent, m-cβARK, but neither n-phosducin nor m-phosducin interacted with the N terminus of GIRK1. Coexpression of m-phosducin did not affect the amount of expressed GIRK channels in the PM of *Xenopus* oocytes (Fig. 4D). Thus, m-phosducin has the desired properties of a PM-enriched Gβγ scavenger that does not interact with GIRK.

Coexpression of m-phosducin with GIRK at 10 ng RNA/oocyte efficiently decreased GIRK currents (Fig. 5A). The effect of phosducin was dose-dependent (see below) and did not saturate at the maximal RNA dose used, 10 ng/oocyte (this dose was not toxic and was routinely used in all experiments, unless stated otherwise). We compared the effects of m-phosducin (10 ng of RNA/oocyte) on GIRK currents at low/intermediate (0.2 ng/oocyte) and high (1 ng of RNA/oocyte) channel densities (Fig. 5, B and C). m-Phosducin reduced Ibasal to about the same extent, by ~85–90%, at both channel densities. However, it was less effective than m-cβARK in reducing peak IACH in comparison with m-cβARK. The inhibition of IACH by m-phosducin was stronger at lower channel densities, suggesting competition among Go, GIRK, and m-phosducin for available Gβγ. n-Phosducin was significantly less effective than m-phosducin in reducing GIRK currents. To examine whether the 10–15% IACH remaining in the presence of m-phosducin are Gβγ-dependent, we coexpressed, in addition, m-cβARK (5 ng/oocyte). This treatment did not further reduce Ibasal, supporting the possibility of the presence of measurable Gβγ-independent basal activity in GIRK channels (Fig. 5B). However, the main outcome of these experiments is that up to 90% of AACHbasal is Gβγ-dependent.

IACH of GIRK channels expressed in *Xenopus* oocytes slowly decays in the continuous presence of ACh, reaching a steady state after 3–5 min (45, 46). We noticed that, in the presence of m-phosducin, the decay was strongly accelerated. The steady state level of IACH was greatly diminished compared with control (see Fig. 5A). The kinetics of decay of IACH were specifically studied in a separate experiment (n = 6 oocytes). Fig. 6A shows representative records from two oocytes, with and without coexpressed m-phosducin, after scaling the peak IACH currents to facilitate the comparison of decay kinetics. Fig. 6B summarizes the effects of m-phosducin on the amplitudes of Ibasal and IACH measured at the peak and 1.5 min after the addition of ACh. On the average, within 1.5 min, IACH decayed to a mere 2.7 ± 1.3% of IACH, measured at the same time point in control oocytes. The time constant of decay of IACH, in the continuous presence of ACh, τdecay, decreased from 34 s in control to less than 5 s in the

**Fig. 3. cβARK binds to C terminus of GIRK1.** Binding of different cβARK congeners to the GST-fused C terminus of GIRK1 (GST-GIRK1-CT) is shown in comparison with Gβγ synthesized in vitro in rabbit reticulocyte lysate. Representative of two experiments. A, Coomassie Blue staining of GST (used as control) and GST-GIRK1-CT. B and C, Phosphor-Imager autoradiogram of input (I) (i.e. the radioactive signal from a 5-μl sample taken of the 300-μl reaction mixture before the addition of glutathione affinity beads) and binding (C) of the proteins to the GST fusion protein (the bound protein eluted from the beads with glutathione).
The presence of m-phosducin; thus, in the latter case, by 1.5 min a steady state was reached (Fig. 6C). No change in speed of activation could be detected, but the speed limit of the perfusion system used (110 ± 1 s) did not enable a conclusive measurement. The effect of phosducin is readily understood in general kinetic terms and can be quantitatively described by a kinetic model (not shown), assuming a simple competition among GIRK, Gβγ/GDP, and m-phosducin for available Gβγ, as follows. The extent of inhibition of \( I_{\text{basal}} \) is determined by the concentrations of the proteins involved and by the actual \( K_D \) values, since the system has sufficient time to reach equilibrium. At equilibrium, a certain percentage of Gβγ is bound to Gα within heterotrimers and is liberated from Gα upon activation by agonist. This leads to an initial increase in free Gβγ concentration, followed by re-equilibration and capturing of much of the Gβγ by the scavenger. The initial large peak of \( I_{\text{ACH}} \) can be explained kinetically by assuming that Gβγ released upon transmitter activation first gets access to the channel and only later can be captured by the scavenger. This supports the view that a Gβγ/Gα complex is closely associated with GIRK (see “Discussion”), although a similar property can also be conferred by a kinetic characteristic (a “slow” scavenger with a low on-rate).

Measurements of the kinetics of \( I_{\text{ACH}} \) in the presence of coexpressed m-cARK have been attempted but proved less reliable, since m-cARK reduced peak \( I_{\text{ACH}} \), more potently than m-phosducin. However, in those oocytes where \( I_{\text{ACH}} \) could be reliably measured in the presence of m-cARK, \( I_{\text{ACH}} \) did not show any fast decay (Fig. 6D; representative of at least 20 cells).

The dependence of the effect of m-phosducin on level of its...
expression was studied in two separate experiments. The results are shown in Fig. 7, where they are compared with the dose dependence of m-cARK from Fig. 2 (gray lines). m-Phosducin reduced $I_{\text{basal}}$ similarly to m-cARK, with an $E_{D_{50}}$ of 2.1 ng of RNA/oocyte. Remarkably, the effect of m-Phosducin on $I_{\text{ACH}}$ was strikingly different from that of cARK. The dose dependence of inhibition of peak $I_{\text{ACH}}$ (open circles) was very similar to that for $I_{\text{basal}}$ (closed circles), practically falling on the same Michaelis-Menten curve. Because $E_{D_{50}}$ is conceptually similar to dissociation constant, $K_D$, which is a measure of the equilibrium state, we have also measured the dose dependence of inhibition of steady state $I_{\text{ACH}}$. Again, it was very similar to that of $I_{\text{basal}}$ and peak $I_{\text{ACH}}$.

Expression of GIRK Is Accompanied by an Increase in the Amount of Gβγ in the PM—We have previously shown that the amount of endogenous Gβγ measured by Western blot methodology in total cellular membrane fraction of *Xenopus* oocytes does not change appreciably when GIRK is overexpressed (20). How can $A_{\text{GIRK,basal}}$ remain 90% Gβγ-dependent when more and more channels are expressed if free Gβγ concentration stays constant and there is less and less Gβγ per channel? One possibility is that the expression of GIRK leads to an increase in the amount of Gβγ in the PM. To explore this possibility, we monitored changes in GIRK1/2, Gβγ, and Ga in large patches of PM, using the imaging method described above (see Fig. 2C). The proteins were visualized by a GIRK1 antibody, a common Gβγ antibody that recognizes Gβ subunits 1–4 but not Gβ2, and a common Ga antibody that recognizes all Ga subunits. Figure 8 shows that the increase in the amount of GIRK in the membrane, brought about by injection of RNA, was accompanied by an increase in the amounts of endogenous Ga and Gβ in the PM. Specificity of labeling was controlled for by incubating the PM patches with the antibodies in the presence of an excess of the respective antigens (purified Ga1 or Gβ1,γ2). With both Ga and Gβγ, the background labeling remaining in the presence of the antigen was the same in native and GIRK-expressing oocytes, suggesting a complete block of the specific labeling by both antigens. In un.injected (native) oocytes, the endogenous Ga label was clearly above the background (Fig. 8A), whereas the endogenous Gβγ could not be clearly detected in this experiment (Fig. 8B). In the presence of coexpressed GIRK (2.5 ng of RNA/oocyte), both Ga and Gβ rose significantly above the control level. After subtraction of the background fluorescence, net increase in Ga label was from an undetectable level to ~25 OD units ($p < 0.001$), whereas the level of Gβ increased from ~25 to ~38 OD units ($p < 0.05$) (i.e. less than 2-fold). Although this rough comparison does not provide true quantification of the increase in Ga versus Gβ levels, it appears to indicate that the relative increase in the level of Gβ, caused by the expression of GIRK, was greater than that of Ga.

Fig. 8C shows that the expression of the GIRK protein in the PM increased with an increase in the dose of injected RNA. The background labeling with the GIRK1 antibody in uninjected oocytes was very low, suggesting high specificity of the antibody and the expected absence of GIRK1 protein in native oocytes. Fig. 8D summarizes a separate series of experiments in which the levels of Ga and Gβ in the PM were measured upon the expression of GIRK at two different levels. The levels of both G protein subunits were increased already when a low dose of GIRK RNA, 0.1 ng/oocyte, was injected, and a further increase was observed upon injection of a high dose of GIRK RNA, 2.5 ng.

**DISCUSSION**

**Most of the Basal Activity of GIRK Is Gβγ-dependent at All Levels of GIRK Expression—**Basal activity of GIRK channels, $A_{\text{GIRK,basal}}$, is observed in the absence of agonist and, in heterologous expression systems, of any coexpressed receptor (2). To understand the mechanisms underlying $A_{\text{GIRK,basal}}$, we utilized *Xenopus* oocytes, unsurpassed for titrated expression of proteins for rigorous quantitative studies (which we term “expression pharmacology”). Of the several Gβγ scavenger constructs used here, myristoylated derivatives of cARK and phosducin proved most efficient. The two scavengers have similar high affinities of binding to Gβγ in vitro: $K_D$ of 42 nM in a detergent solution for phosducin (47) and 32 nM in phospholipid vesicles for 6ARK (48). Myristoylation ensured preferential incorporation of these proteins into membrane fractions and, in parallel, stronger inhibition of GIRK activity than by nonmyristoylated congeners. The strong reduction of GIRK currents by cARK fused to the first transmembrane domain of the 5HT1A recep-
**FIG. 8. Expression of GIRK is accompanied by an increase in the amount of endogenous Ga and Gβ in the plasma membrane.** In A and B, the levels of Ga (A) and Gβ (B) were measured by immunocytochemistry in large PM patches. The left panels show representative confocal microscope images of the membrane patches labeled with the respective antibodies. The right panels summarize the label intensities measured in patches from oocytes of the same donor frog (numbers of patches imaged are shown above the bars). The labeling remaining in the presence of excess antigens (black bars; Ga, GDP in A and Gβ, G i2 in B) represents nonspecific background fluorescence. The levels of nonspecific signal were compared with total signal observed in the absence of the antigen (asterisks above black bars) separately for native and for GIRK-expressing oocytes. C, the level of expressed GIRK1 in PM patches increases as a function of the amount of injected RNA. The right panel summarizes data from three oocyte batches. Nonspecific labeling in the absence of expressed channel was very low; therefore, the level of expressed GIRK1 in PM patches increases as a function of the amount of injected RNA. The right panel summarizes data from three oocyte batches showing the increase in the level of endogenous Ga and Gβ upon coexpression of GIRK1/2 at two RNA doses, 0.1 and 2.5 ng/oocyte. Data were normalized to control levels of Ga and Gβ detected in uninjected (Uninj.) oocytes. In these experiments, nonspecific labeling was not estimated, and the measured values represent total fluorescence signal (background + specific).

The source of Gβγ that determines A_{GIRK,basal} in the oocytes and other cells is not clear. It is widely believed that the Gβγ-dependent component of A_{GIRK,basal} reflects the concentration of free Gβγ in the membrane (2). The most obvious and best studied sources of free Gβγ in the PM are the Gαβγ heterotrimer. The latter dissociate and supply free Gβγ by several mechanisms: basal dissociation of Gα_GDP from Gβγ in the absence of GDP-GTP exchange (49); basal GDP-GTP exchange that results in great reduction of the affinity of Gα to Gβγ (50); and GPCR-catalyzed GDP-GTP exchange that occurs as a result of a constitutive activity of the GPCR in the absence of an agonist (51) or is induced by an ambient agonist present in the extracellular medium (the latter seems unlikely in isolated, follicle-free, constantly perfused oocyte preparations).

Estimation of the relative contribution of these processes to A_{GIRK,basal} in oocytes and other cells will require further study. **GIRK May Be Constitutively Associated with Gβγ—**The idea that many end effectors of GPCRs operate within multiprotein complexes that include the GPCRs, heterotrimeric G proteins, and effectors themselves has gained wide acceptance (52). It has been proposed that, under normal physiological conditions, GIRK is associated with a Gαβγ complex, which guarantees both low A_{GIRK,basal} and rapid and efficient channel activation by Gβγ released upon an encounter with an agonist-bound GPCR (20, 26–28, 40, 53). The observation that the levels of endogenous Gβγ and Ga in PM increase upon expression of GIRK (Fig. 8) provides new support to this hypothesis and further extends it, indicating that GIRK is preassociated with Gβγ and Ga in the endoplasmic reticulum or the Golgi complex before being relocated to the PM. Joint trafficking of

![Diagram](image-url)
ion channels and associated proteins is a well-documented phenomenon (e.g., see Ref. 54). The change in Gβγ levels was not previously detected in total membrane fraction (20), probably because of the very low surface/volume ratio in the oocyte, where the PM constitutes at best a few percent of total cellular membranes.

The protein complex comprising GIRK and G protein subunits may also contain other components, such as protein kinase A, phosphatases, and βARK (40). It is probable that the complex is a dynamic one and that the interactions within it are reversible, as indicated by the ability of exogenously expressed Ga such as Gaα and Gaα to replace the endogenous Gaα (32, 55) and by the finite affinities of protein-protein interactions in vitro. On the other hand, there are indications that the complex may be rather stable: the components are co-precipitated by a single antibody (40); pull-down experiments show strong binding between GIRK and Gaα, Gβγ, and βARK (see Refs. 21, 27, and 41 and Fig. 3); and known affinities of interactions among some of the protein components are in the nanomolar range (Gβγ-GIRK, <10 nM; Ga-Gβγ, 0.2–2 nM) (discussed in Ref. 56).

A stable association of GIRK with Gβγ, together with the increase in Gβγ levels in PM, provides the basis for an understanding of the excessively high A\textsubscript{GIRK,basal} at high GIRK densities. Although the level of endogenous Gaα also rises upon coexpression of GIRK, it appears insufficient to prevent the disproportional increase in A\textsubscript{GIRK,basal}, possibly because the increase in the level of Gβγ in the PM is greater than that of Gaα. Comparison of the relative increases in Gaα and Gβγ caused by the expression of GIRK (Fig. 8, A and B) seems to support this possibility. However, at present this interpretation remains prescriptive, because the immunolocalizing method used here, although sensitive, does not provide quantitative information on the absolute levels of proteins measured with different antibodies. It is also possible that the observed rise in Gaα levels includes that of “irrelevant” Gaα (not Gaα) available in the oocytes, which cannot efficiently reduce A\textsubscript{GIRK,basal} and/or donate Gβγ upon activation of m2R.

In summary, our observations so far conform to the proposal (20, 28) that A\textsubscript{GIRK,basal} is controlled by both Gβγ and Gaα. Expression of GIRK is accompanied by a rise in ambient Gβγ available for activation of GIRK, which is not accompanied by a comparably strong rise in the level of Gaα, subunits, thus causing an excessively high A\textsubscript{GIRK,basal}. Taking together the previously accumulated evidence regarding the existence of GIRK-Gaβγ complexes and the new data presented here, we propose that GIRK is persistently, although reversibly, associated with Gaα-Gβγ throughout its biosynthesis; Gaα is a nonobligatory part of the complex, whereas Gβγ is bound persistently.

Using m-phosducin and m-βARK to Assess the Existence of Different Gβγ-binding Sites in GIRK—It has been proposed, on the basis of mutational analysis of GIRK1 and GIRK4, that agonist-evoked activity is mediated by binding of Gβγ to a low affinity site(s), and A\textsubscript{GIRK,basal} depends on the interaction of Gβγ with another, high affinity, site(s) in the GIRK protein (57). Later works suggested that an intact putative site underlying A\textsubscript{GIRK,basal} is also crucial for agonist-evoked activity (58) and that a residue implicated in low affinity Gβγ binding (57) may be involved in channel gating rather than Gβγ binding (21, 59). Thus, the issue of two types of Gβγ-binding site remains unresolved.

Gβγ scavengers can be used to probe the existence in GIRK of Gβγ-binding sites with different affinities to Gβγ. The concentration of Gβγ required to induce A\textsubscript{GIRK,basal} via the high affinity site of GIRK would be lower than that required to induce I\textsubscript{ACH} via the low affinity site. Consequently, less Gβγ scavenger would be needed to suppress activation of GIRK by Gβγ released following GPCR activation than by ambient Gβγ that determines A\textsubscript{GIRK,basal}. Alas, the results with the two potent Gβγ scavengers used here were not unequivocal. m-phosducin and m-βARK showed striking differences in the apparent potency of inhibition of peak I\textsubscript{ACH} and in ED\textsubscript{50}. The data obtained with c-βARK appear to support the two-site model of He et al. (57); I\textsubscript{ACH} was inhibited by much lower doses of m-βARK than I\textsubscript{basal}. However, m-phosducin, which showed an even greater apparent affinity in inhibiting A\textsubscript{GIRK,basal} than m-βARK (Fig. 7), exhibited an identical apparent affinity for I\textsubscript{ACH}.

A clue for the understanding of these differences may be provided by invoking the fact that m-βARK binds GIRK. Phosducin reduced I\textsubscript{basal} stronger than I\textsubscript{ACH} but accelerated the decay of I\textsubscript{ACH}. These effects of m-phosducin are compatible with those of a simple Gβγ sink (see “Results”). In contrast, m-βARK inhibited I\textsubscript{ACH} with a high apparent affinity but did not accelerate its decay. It can be shown that such properties can be conferred by very fast Gβγ scavenging by c-βARK, such that it captures Gβγ released from the heterotrimeric Gaβγ before Gβγ can reach the channel. Anchoring of c-βARK to the channel would enable such extra fast scavenging. More complicated schemes, which assume the formation of a high affinity triple cβARK-Gβγ-GIRK complex, or a competition between m-βARK and Gβγ for binding to GIRK may also explain the effects of m-βARK described here. Unfortunately, all interpretations are complicated by the fact that GIRK directly binds cβARK, and its use as a “straightforward” Gβγ scavenger in GIRK studies should be avoided.

To summarize, we contend that, as a Gβγ scavenger, m-βARK is inferior to m-phosducin in GIRK studies. The results obtained with m-phosducin are consistent with the existence of a single type of Gβγ binding site underlying both basal and agonist-evoked activation of GIRK or the existence of separate sites with very similar affinities for Gβγ.

Possible Mechanism of Gβγ-independent Basal Activity—Inhibition of the whole-cell basal GIRK current, I\textsubscript{basal}, by both scavengers used here was dose-dependent and did not fully saturate even at the highest dose of scavengers used. Therefore, we cannot state with certainty that the remaining ~10–15% of A\textsubscript{GIRK,basal} is Gβγ-independent. The fact that the expression of c-βARK at 5 ng of RNA/oocyte (a dose that normally inhibits 70–80% of I\textsubscript{basal}) on top of phosducin did not further reduce I\textsubscript{basal} supports the existence of a Gβγ-independent component. This putative Gβγ-independent component of A\textsubscript{GIRK,basal}, although small, is not negligible and may be physiologically relevant. It is also interesting from the biophysical point of view. What determines the open-closed equilibrium of GIRK in the absence of the main physiological gating factor, Gβγ? “Spontaneous” transitions from closed to open state are observed in many ion channels in their “resting” state, but it is seldom clear to what extent such transitions are controlled by an extrinsic modulatory factor. In GIRK channels, one such factor may be the ubiquitously present PiP2, which is an obligatory gating factor in these channels (60). Also, it has been proposed that intracellular Na\textsuperscript{+} is an important regulatory factor that may substantially regulate A\textsubscript{GIRK,basal} (23, 46). Na\textsuperscript{+} may activate GIRK in a Gβγ-dependent manner (61) (this component should be absent in oocytes expressing the Gβγ scavengers) and also via a direct binding (23). The EC\textsubscript{50} of the direct Na\textsuperscript{+} effect is 20–50 mM (62), and the extent of direct activation is probably minor in resting cells, at physiological Na\textsuperscript{+} concentrations of 5–10 mM. Further studies will be necessary to clarify whether a Gβγ-independent component of A\textsubscript{GIRK,basal} exists and what is the underlying mechanism.
