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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 $G_{i/o}$ 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 GIRK 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)[1] 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 $G\alpha_{i/o}\beta\gamma$ heterotrimers (1–4). The channel can also be activated by cytosolic Na^+ and membranal phosphatidylinositol 4,5-bisphosphate (PIP_2); 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 $G\alpha_q$ (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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[1] 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; PIP_2, phosphatidylinositol 4,5-bisphosphate; m-cβARK, myristoylated C terminus of β-adrenergic kinase; m-phosducin, phosducin with an added myristoylation signal; n-phosducin, nonmyristoylated phosducin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

expression of Gα subunits and a myristoylated C-terminal segment of β-adrenergic receptor kinase (m-cβARK), used in the past as standard Gβγ scavengers in GIRK studies (17–22), has been reported to reduce $A_{GIRK,basal}$ 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 $A_{GIRK,basal}$ exists. Such Gβγ-independent basal activity could be due to a direct activation by cytosolic free Na^+ (23–25) and/or reflect an intrinsic Gβγ- and Na^+-independent channel activity. The variability of the reported results regarding the extent of inhibition of $A_{GIRK,basal}$ by Gβγ scavengers calls for a better quantitative assessment of the Gβγ-dependent component. Furthermore, the use of Gα as a Gβγ scavenger is problematic. Coexpressed $G\alpha_i$ 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 Gα subunits. It is not known whether m-cβARK, 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 $A_{GIRK,basal}$.

An intriguing phenomenon related to $A_{GIRK,basal}$ has been discovered in *Xenopus* oocytes (20). At low expression levels (densities) of GIRK, $A_{GIRK,basal}$ 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 $G\alpha_i$ subunit restores the normal gating pattern, with low $A_{GIRK,basal}$ and high agonist- or Gβγ-evoked activity. We proposed that $A_{GIRK,basal}$ is controlled by a number of factors, most prominently Gβγ and Gα, 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 Gα (20, 28). The excessively high $A_{GIRK,basal}$ 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 $A_{GIRK,basal}$, we have initiated a systematic study of Gβγ dependence of $A_{GIRK,basal}$ 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-cβARK and a novel membrane-targeted Gβγ scavenger, myristoylated phosducin (m-phosducin), revealed that at least 90% of $A_{GIRK,basal}$ is Gβγ-dependent at all levels of expression of GIRK. Gβγ-independent activity, if any, constitutes only a small fraction of $A_{GIRK,basal}$. 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 Gα needed to preserve low $A_{GIRK,basal}$.

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-cβARK (aa 452–689_{end}) (12, 32), human βARK (GB X61157), and bovine phosducin (GB M33529) were kindly provided by E. Peralta, P. Hartig, P. Kofuji, E. Reuveny, R. J. Lefkowitz, and M. Lohse, respectively.

PCR-derived full-length coding sequence of wild type phosducin was inserted into the EcoRI restriction site of pGEMHJ, creating the template for RNA synthesis and expression of wild-type, nonmyristoylated phosducin (n-phosducin). To create a membrane-attached derivative of phosducin, 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, atggggagtagcaagagcaagcctaaggaccccagccagcgccgg, was inserted between SmaI and EcoRI restriction sites of the pGEM-HE vector. On the protein level, this adds the first 15 N-terminal aa of Src, MGSSKSKPKDPSQRR (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 phosducin, flanked by EcoRI and HindIII, was inserted into the respective restriction sites of pGEMHE-myr.

cDNA for nonmyristoylated C terminus of human βARK1, n-cβARK, was created by inserting the cDNA sequence encoding aa 460–689_{end} of βARK, preceded by a methionine, flanked by EcoRI and HindIII restriction sites, into the pGEMHJ vector. The comparison of physiological effects of m-cβARK (rat) and n-cβARK (human) is justified by high homology between these proteins (97% identity). To create the fusion proteins m2R-cβARK, 5HT1A-cβARK, and 5HT1A-1TMD-cβARK, PCR-derived coding sequences of m2R, 5HT1A receptor (5HT1AR), and aa 1–67 of 5HT1AR, respectively, flanked by EcoRI restriction sites, were inserted in frame just prior to the n-cβARK sequence made as described above. Point mutations in m-cβARK 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.[2] Unless indicated otherwise, RNAs were injected into the oocytes in the following amounts: m2R, 0.5 ng/oocyte; n-cβARK, m2R-cβARK, 5HTA1-cβARK, 5HTA1–1TMD-cβARK, and m-$c\beta ARK_{R587Q}$, 5 ng/oocyte; 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; $CaCl_2$, 1 mM; $MgCl_2$, 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 a two-electrode voltage clamp with OC-725B (Warner Instruments Corp.), using agarose cushion electrodes (36) filled with 3 M KCl, with resistances of 0.1–0.5 megaohms. Oocytes were held at −80 mV in the ND-96 solution, and GIRK currents were measured in high K^+ solutions, high K 96 or high K 24. High K 96 contained 96 mM KCl, 2 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes (pH 7.5). High K 24 contained 24 mM KCl, 72 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 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 phosducin and cβARK was studied as described (21). Briefly, GST-CT-GIRK1 fusion protein was purified, and its interaction with [^{35}S]methionine-labeled proteins (the various forms of cβARK, phosducin, and $G\beta_1\gamma_2$) synthesized in rabbit reticulocyte 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 [^{35}S]methionine-labeled proteins in 300 μl of a high K^+ buffer (150 mM KCl, 50 mM Tris, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.0) with the addition of 0.5% CHAPS. Then 30 μl of glutathione-Sepharose beads (Amersham Biosciences) 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 products 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 EGTA, 5 mM EDTA, 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.

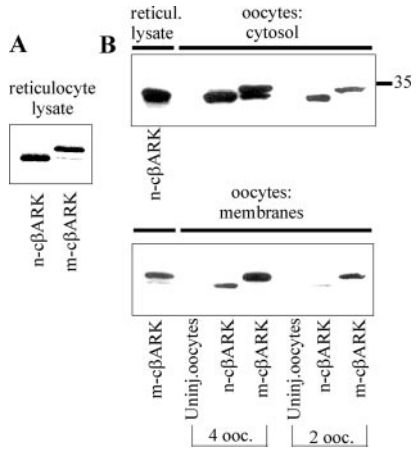

FIG. 1. **Myristoylation helps cβARK to associate with membranes.** *A*, autoradiogram of n-cβARK and m-cβARK expressed in reticulocyte lysate. *B*, Western blot of n-cβARK and m-cβARK expressed in oocytes (*ooc.*) and in reticulocyte lysate. Cytosolic (*top panel*) and membrane (*bottom panel*) fractions of oocytes were separated by differential centrifugation (100,000 × *g*). m-cβARK 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 Pierce. The intensity of labeling was quantified using TINA software (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 $G\alpha_{i1}$-GDP or purified $G\beta_1\gamma_2$, respectively, was added in 20-fold excess (w/v) over the antibodies. The purified $G\alpha_{i1}$ and $G\beta_1\gamma_2$ 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: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

RESULTS

m-cβARK Reduces I_{basal}, but It Also Binds to the C Terminus of the GIRK1 Subunit—To separate the Gβγ-dependent component of $A_{GIRK,basal}$, we have initially utilized the widely used Gβγ scavenger, m-cβARK. GIRK activation by Gβγ is membrane-delimited (1), so that only PM-attached Gβγ can activate the channel. Targeting of m-cβARK to the membranes is provided by the myristoylation signal (the first 15 aa of Src added to the N terminus of m-cβARK) (29, 34). For comparison, we have also constructed and tested a nonmyristoylated cβARK (n-cβARK), which should not be targeted to the membrane and thus is supposed to be less efficient than m-cβARK in inhibiting GIRK. As shown in Fig. 1, both m-cβARK and n-cβARK were well expressed in reticulocyte lysate (Fig. 1*A*) and in oocytes (Fig. 1*B*). m-cβARK showed two characteristic bands on SDS gels (Fig. 1, *A* and *B*). The lower band probably corresponded to the presence of a fraction of protein that remained nonmyristoylated, since only the upper band was present in the total membrane fraction (Fig. 1*B*, *bottom*). Only a small proportion of n-cβARK was found in the membrane fraction. Myristoylation of m-cβARK appeared to be more efficient in reticulocyte lysate than in the oocytes (Fig. 1, compare *A* and *B*). m-cβARK ran on the gel shown in this figure below the 35-kDa molecular size marker, but its actual molecular size was closer to 35 kDa, as deduced from its comparison with $G\beta_1$ (~37 kDa) and phosducin (~33 kDa; see Figs. 3 and 4). In summary, in *Xenopus* oocytes, m-cβARK is synthesized, myristoylated, and preferentially targeted to the membranes, as expected.

Next, we examined the effects of m-cβARK and additional Gβγ scavengers based on c-βARK on GIRK channels composed of subunits GIRK1 and GIRK2 (GIRK1/2). Oocytes were injected with either 0.2 ng/oocyte of RNA of each channel subunit (low/intermediate density of GIRK) or 1 ng/oocyte (high density of GIRK) (20) and 0.5 ng of RNA of the muscarinic m2 receptor, m2R. Fig. 2*A* shows typical recordings of K^+ currents without the coexpression of m-βARK ("control") or with m-cβARK (5 ng of RNA/oocyte). In these whole-cell records, $A_{GIRK,basal}$ is mirrored in the basal GIRK current, I_{basal} (*i.e.* the inward current that develops when the high Na^+ extracellular solution ND96 (96 mM Na^+, 2 mM K^+) is exchanged to a high K^+ solution (hK) with either 96 mM K^+ (Fig. 2*A*) or 24 mM K^+ (not shown). The agonist (acetylcholine; ACh)-induced current, I_{ACh}, was evoked by 10 μM ACh. The total GIRK current, I_{total}, is $I_{basal} + I_{ACh}$. Oocytes that expressed cβARK showed greatly reduced I_{basal} and I_{ACh}. The inhibitory effect of m-cβARK was qualitatively and quantitatively similar in both 96 and 24 mM K^+ solutions (data not shown).

We examined the dose dependence of the effect of m-cβARK, titrating its expression level by injecting various amounts of RNA (RNA of GIRK subunits was 0.2 ng/oocyte in these experiments). As shown in Fig. 2*B*, the effect of m-cβARK on I_{basal} was dose-dependent and did not fully saturate at the highest dose of RNA used, 10 ng/oocyte. In contrast, I_{ACh} was completely inhibited at 5 ng of RNA/oocyte of m-cβARK. The data were fitted to a Michaelis-Menten-like equation, assuming that, at saturating doses, m-cβARK would fully block the GIRK currents. The fit yielded values of ED_{50} (effective doses of RNA that cause 50% inhibition) of 3.04 and 0.1 ng of RNA/oocyte for I_{basal} and I_{ACh}, respectively. The estimate of ED_{50} for I_{ACh} was intrinsically less accurate than for I_{basal}, due to very strong inhibition of I_{ACh} observed already at 2 ng of RNA/oocyte. Nevertheless, it reflects a much higher sensitivity of the evoked current to m-cβARK than of the basal one. Unfortunately, at 10 ng/oocyte, the expressed m-cβARK appeared to be toxic to oocytes, which often did not survive the 3–4-day incubation period. Therefore, in the following experiments, 5 ng/oocyte of m-cβARK RNA were routinely injected into the oocytes. We note that, in our hands, 5 or 10 ng of RNA/oocyte usually yielded very high levels of expressed proteins, because RNAs used in this study were designed to give maximal protein yield upon expression in the oocytes (see "Experimental Procedures").

To make sure that the decrease in GIRK currents by m-cβARK 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 immunoimaging method (Fig. 2*C*). The channels were visualized in very large plasma membrane patches (usually several thousand μm^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. 2*C*, *b*, coexpression of m-cβARK did not affect the amount of channel protein in the PM.

Myristoylated m-cβARK seems to be suitable for the purpose of studying the Gβγ dependence of $A_{GIRK,basal}$, since it

[3] N. Dascal and E. Artzy, unpublished observations.

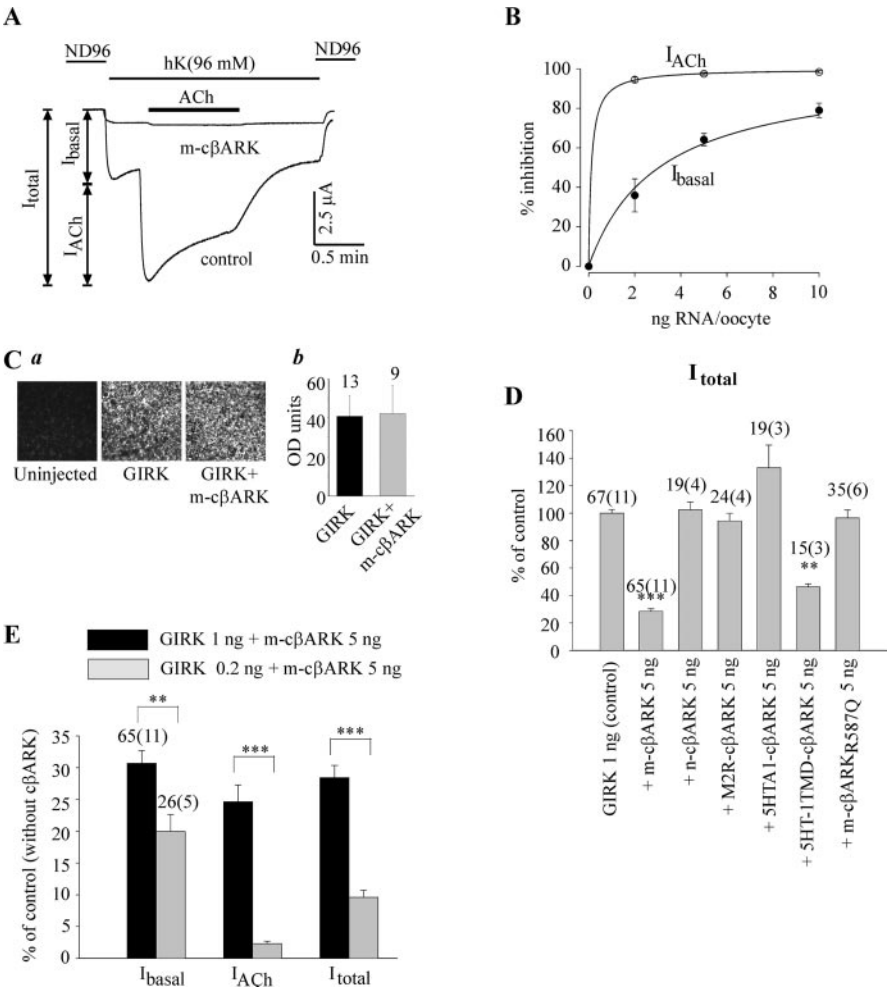

FIG. 2. **Effects of cβARK congeners on GIRK current.** *A*, examples of whole-cell GIRK currents measured by the two-electrode voltage clamp method. Oocytes were injected with 0.2 ng of RNA of each channel subunit and 0.5 ng of RNA of m2R with or without 5 ng of RNA of m-cβARK. *B*, extent of inhibition of I_{basal} and I_{ACh} is a dose-dependent function of the amount of injected RNA of m-cβARK. The *solid lines* show fits to the standard Michaelis-Menten equation for I_{basal}. *C*, coexpression of m-cβARK 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 cβARK on GIRK channel. The values of I_{total} are shown; changes in I_{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-cβARK 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 m2R, together or without 5 ng of RNA of m-cβARK. 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.

does not block the direct Na^+-induced activation (19). However, "side effects" are possible. One potential pitfall is that the Gβγ-binding segment is within a plekstrin homology domain, which also binds PIP_2 (38); thus, heavily overexpressed cβARK 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 Gβγ scavengers based on c-βARK. First, we made a point mutation, R587Q, in m-cβARK, which greatly impairs the ability of cβARK to bind Gβγ, but not PIP_2 (39). Expression of this construct in the oocytes did not have any significant effect on GIRK currents (Fig. 2*D*), suggesting that PIP_2 chelation is not involved in the effect of m-cβARK. To check for possible "side effects" of the myristoyl moiety, we expressed the nonmyristoylated n-cβARK and found that it did not significantly affect GIRK currents at 5 ng of RNA/oocyte (Fig. 2*D*). Although n-cβARK (aa 460–689 of cβARK) is 8 aa shorter than m-cβARK (aa 452–689), both constructs contain the Gβγ-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-βARK 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 m2R, the serotonin receptor 5HT1AR, and the first transmembrane domain of 5HT1AR, 5HT-1TMD. The first two constructs did not inhibit GIRK currents (Fig. 2*D*) after 3 days of incubation. Mild inhibitory effects of m2R-cβARK and 5HT1A-cβARK 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-cβARK significantly reduced I_{total} though less than m-cβARK (by ~55%, *versus* ~72% inhibition by cβARK; Fig. 2*D*). This result suggests that the presence of the myristoyl moiety is not necessary for c-βARK to inhibit GIRK.

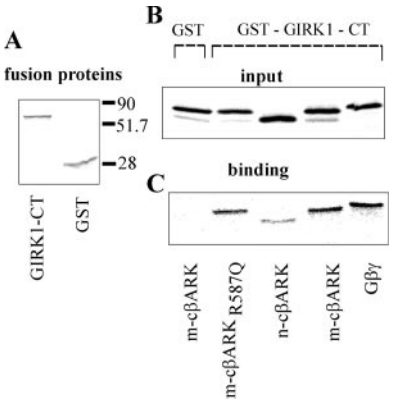

FIG. 3. **cβARK binds to C terminus of GIRK1.** Binding of different cβARK congeners to the GST-fused C terminus of GIRK1 (GST-GIRK1-CT) is shown in comparison with Gβγ synthesized *in vitro* in rabbit reticulocyte lysate. Representative of two experiments. *A*, Coomassie Blue staining of GST (used as control) and GST-GIRK1-CT. *B* and *C*, PhosphorImager autoradiogram of *input* (*B*) (*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).

Next, we compared the effect of m-cβARK on GIRK channels expressed at two different densities. Coexpression of m-cβARK (5 ng RNA/oocyte) was less effective in reducing I_{basal} and I_{ACh} when more channels were expressed (Fig. 2*E*). At high channel density (1 ng RNA/oocyte), I_{basal} was decreased by ~70%, and I_{ACh} 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 I_{total}. 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) under study, as well as on the cellular level of Gβγ (see Fig. 8). Another possibility is that the scavenger interacts with the 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 [^{35}S]methionine-labeled m-cβARK and m-cβARK$_{R587Q}$ synthesized *in vitro* in reticulocyte lysate almost as well as it bound Gβγ (Figs. 3 and 4*B*). 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 or of the first 8 aa in n-cβARK. Because m-cβARK$_{R587Q}$, 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 $A_{GIRK,basal}$ 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 Ca^{2+} 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 Gα, 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 I_{basal} by ~70% and I_{ACh} by only ~42% (see Fig. 5*B*). 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. 4*A*). Pull-down experiments showed that neither n-phosducin nor m-phosducin bound GST-GIRK1-CT (Fig. 4*B*). 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. 4*D* 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. 4*D*). 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. 5*A*). 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 I_{basal} to about the same extent, by 85–90%, at both channel densities. However, it was less effective than m-cβARK in reducing peak I_{ACh} in comparison with m-cβARK. The inhibition of I_{ACh} by m-phosducin was stronger at lower channel densities, suggesting competition among Gα, 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% I_{basal} 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 I_{basal}, supporting the possibility of the presence of measurable Gβγ-independent basal activity in GIRK channels (Fig. 5*B*). However, the main outcome of these experiments is that up to 90% of $A_{GIRK,basal}$ is Gβγ-dependent.

I_{ACh} 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 I_{ACh} was greatly diminished compared with control (see Fig. 5*A*). The kinetics of decay of I_{ACh} were specifically studied in a separate experiment (n = 6 oocytes). Fig. 6*A* shows representative records from two oocytes, with and without coexpressed m-phosducin, after scaling the peak I_{ACh} currents to facilitate the comparison of decay kinetics. Fig. 6*B* summarizes the effects of m-phosducin on the amplitudes of I_{basal} and I_{ACh} measured at the peak and 1.5 min after the addition of ACh. On the average, within 1.5 min, I_{ACh} decayed to a mere 2.7 ± 1.3% of I_{ACh} measured at the same time point in control oocytes. The time constant of decay of I_{ACh} in the continuous presence of ACh, τ_{decay}, decreased from 34 s in control to less than 5 s in the

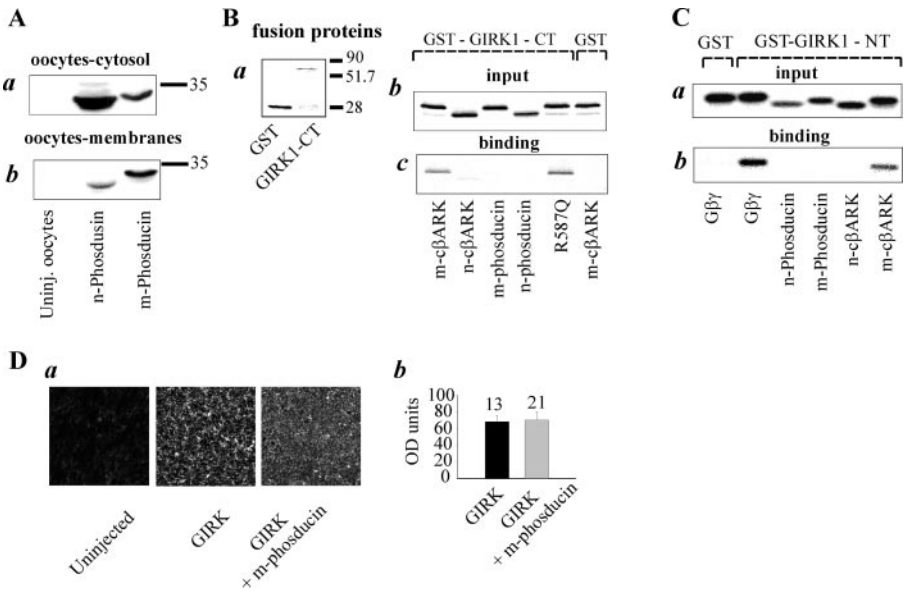

FIG. 4. **Characterization of the properties of n-phosducin and m-phosducin.** *A*, myristoylation promotes the association of m-phosducin with membranes. Oocytes expressing phosducin were separated into cytosol and membrane fractions by differential centrifugation, and phosducin protein was visualized using Western blot. Both n-phosducin and m-phosducin were present in the cytosol (*a*), but m-phosducin was enriched in the membrane fraction (*b*) compared with n-phosducin. *B*, test of binding of phosducin to GST-GIRK1-CT, in comparison with different cβARK congeners (two experiments). c-βARK and phosducin derivatives were synthesized *in vitro* in rabbit reticulocyte lysate. The *left panel* (*a*) shows Coomassie Blue staining of GST-GIRK1-CT and GST. The *right panels* show PhosphorImager autoradiograms of *input* (*b*) and *binding* of *in vitro* synthesized proteins to GST-GIRK1-CT or GST (*c*). For explanation of the terms *input* and *binding*, see the legend to Fig. 3. *C*, test of binding of phosducin to GST-GIRK1-NT, in comparison with cβARK and Gβγ. PhosphorImager autoradiogram of *input* (*a*) and *binding* (*b*) of *in vitro* synthesized proteins to GST-GIRK1-NT or GST are shown. *D*, m-phosducin does not affect the expression of GIRK in the PM. *a*, representative confocal images of channel protein staining in large membrane patches of oocytes. *b*, summary of the measurements of GIRK protein expression (1 ng RNA/oocyte) in the PM, after subtraction of the average intensity of signal measured in uninjected oocytes, in three separate experiments (oocyte batches).

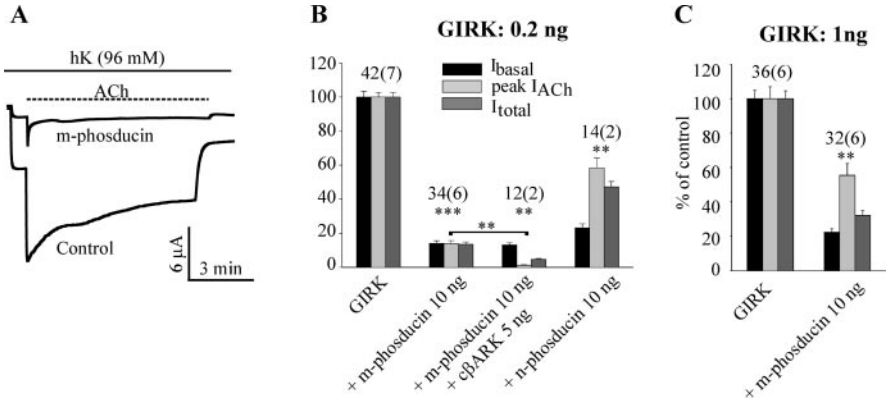

FIG. 5. **Gβγ scavengers cannot completely inhibit the basal activity of GIRK.** *A*, representative current records in oocytes expressing the channel alone or with m-phosducin. Oocytes were injected with 0.2 ng of RNA of each channel subunit and 0.5 ng of RNA of m2R with or without 10 ng RNA of m-phosducin. *B*, summary of effects of coexpression of m-phosducin, n-phosducin, and m-phosducin together with m-cβARK, at low/intermediate density of the GIRK channel. All parameters are shown as percentage of the same parameter recorded in the control group. Note that coexpression of cβARK on top of m-phosducin further reduces I_{ACh} ($p < 0.01$) but does not further reduce I_{basal}. *C*, summary of the effect of coexpression of m-phosducin at high density of the GIRK channel.

presence of m-phosducin; thus, in the latter case, by 1.5 min a steady state was reached (Fig. 6*C*). No change in speed of activation could be detected, but the speed limit of the perfusion system used (~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, $G\alpha_{GDP}$, and m-phosducin for available Gβγ, as follows. The extent of inhibition of I_{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_{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 Gαβγ 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_{ACh} in the presence of coexpressed m-cβARK have been attempted but proved less reliable, since m-cβARK reduced peak I_{ACh} more potently than m-phosducin. However, in those oocytes where I_{ACh} could be reliably measured in the presence of m-cβARK, I_{ACh} did not show any fast decay (Fig. 6*D*; representative of at least 20 cells).

The dependence of the effect of m-phosducin on level of its

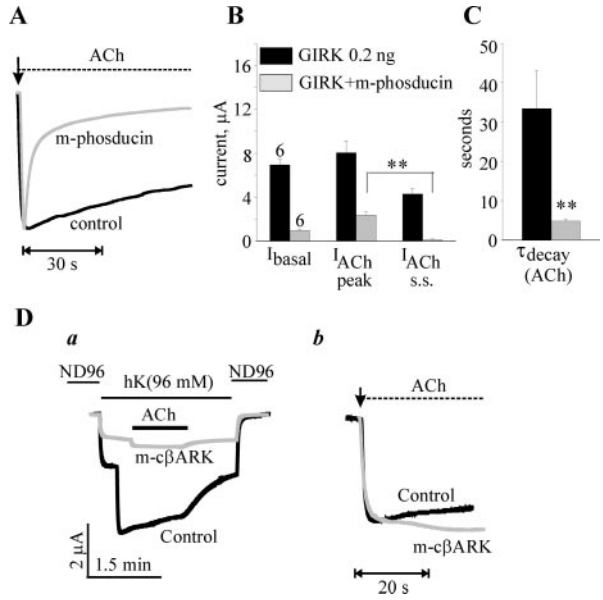

FIG. 6. **Kinetic aspects of the effect of m-phosducin on I_{ACh}.** *A*, comparison of kinetics of inactivation of I_{ACh}, in the same oocytes shown in Fig. 5*A*. I_{ACh} in the m-phosducin-expressing oocyte was scaled to I_{ACh} in the control oocyte to illustrate the differences in current kinetics. *B*, summary of amplitudes of I_{ACh} at the peak and in steady state (measured 1.5 min after the peak) in six oocytes of the same batch. *C*, m-phosducin accelerates the inactivation of I_{ACh}. *D*, c-βARK (5 ng of RNA/oocyte) does not accelerate the inactivation of I_{ACh}. GIRK was expressed at 1 ng of RNA/oocyte. Original current records (*a*) were scaled up (*b*) to illustrate the differences in current kinetics.

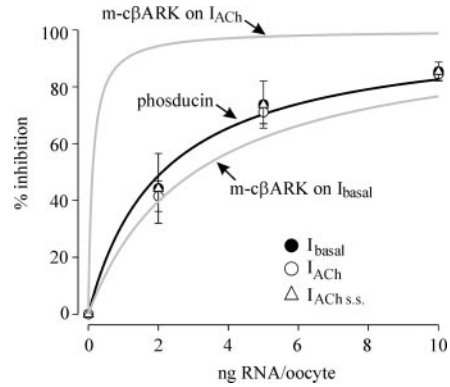

FIG. 7. **Dose dependence of m-phosducin inhibition of I_{basal} and I_{ACh}: A comparison with m-cβARK.** Oocytes were injected with 0.2 ng of RNA of each channel subunits, 0.5 ng of RNA of m2R, and different amounts of RNA of m-phosducin. Results from two batches of oocytes are summarized; each point represents mean ± S.E. of 6–9 cells. The *solid black line* is a fit to the standard Michaelis-Menten equation for I_{basal}; the *fitted lines* for I_{ACh} (peak and steady state) were very similar. The *gray lines* show the dose response for m-cβARK from Fig. 2*B*.

expression was studied in two separate experiments. The results are shown in Fig. 7, where they are compared with the dose dependence of m-cβARK from Fig. 2 (*gray lines*). m-phosducin reduced I_{basal} similarly to m-cβARK, with an ED_{50} of 2.1 ng of RNA/oocyte. Remarkably, the effect of m-phosducin on I_{ACh} was strikingly different from that of cβARK. The dose dependence of inhibition of peak I_{ACh} (*open circles*) was very similar to that for I_{basal} (*closed circles*), practically falling on the same Michaelis-Menten curve. Because ED_{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_{ACh} (Fig. 7, *triangles*). Again, it was very similar to that of I_{basal} and peak I_{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 $A_{GIRK,basal}$ 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 Gα in large patches of PM, using the imaging method described above (see Fig. 2*C*). The proteins were visualized by a GIRK1 antibody, a common Gβ antibody that recognizes Gβ subunits 1–4 but not $Gβ_5$, and a common Gα antibody that recognizes all Gα subunits. Fig. 8 demonstrates 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 Gα 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 $Gα_{i1}$ or $Gβ_1γ_2$). With both Gα 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 uninjected (native) oocytes, the endogenous Gα label was clearly above the background (Fig. 8*A*), whereas the endogenous Gβγ could not be clearly detected in this experiment (Fig. 8*B*). In the presence of coexpressed GIRK (2.5 ng of RNA/oocyte), both Gβ and Gα rose significantly above the control level. After subtraction of the background fluorescence, net increase in Gβ label was from an undetectable level to ~25 OD units ($p < 0.001$), whereas the level of Gα 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 Gα *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 Gα.

Fig. 8*C* 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. 8*D* summarizes a separate series of experiments in which the levels of Gα 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, $A_{GIRK,basal}$, is observed in the absence of agonist and, in heterologous expression systems, of any coexpressed receptor (2). To understand the mechanisms underlying $A_{GIRK,basal}$, 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 cβARK 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 βARK (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 cβARK fused to the first transmembrane domain of the 5HT1A recep-

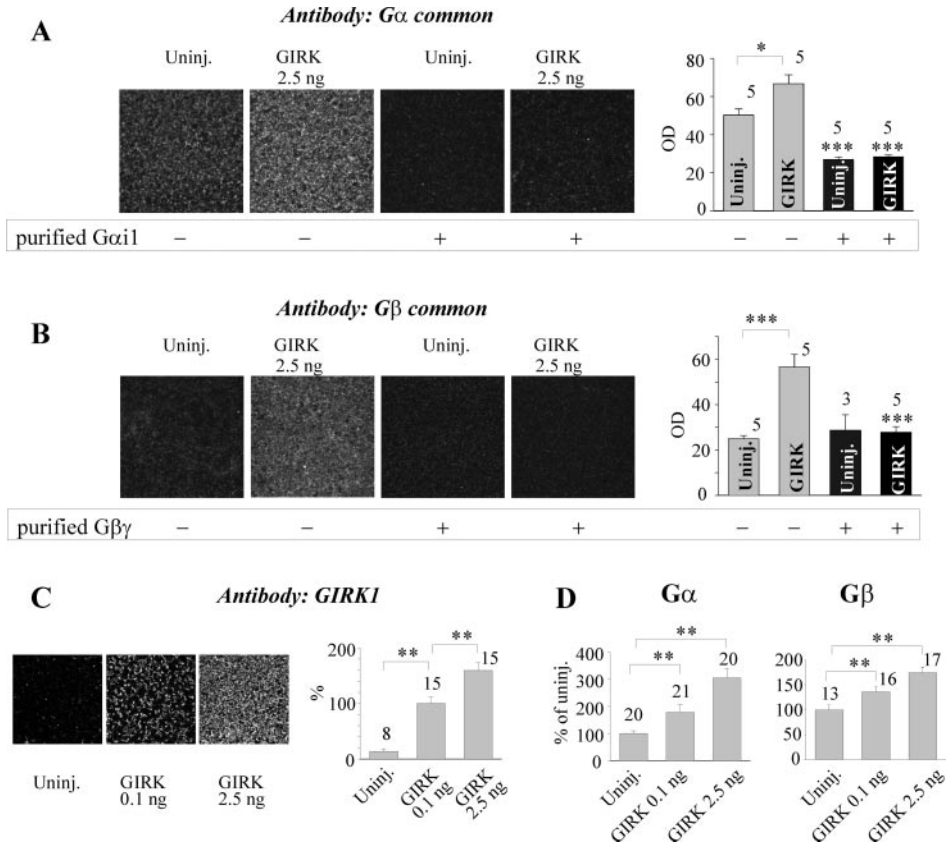

FIG. 8. **Expression of GIRK is accompanied by an increase in the amount of endogenous G*α* and G*β* in the plasma membrane.** In *A* and *B*, the levels of G*α* (*A*) and G*β* (*B*) were measured by immunocytochemistry in large PM patches. The *left panels* show representative confocal microscope images 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*; $G\alpha_{i1}$-GDP in *A* and $G\beta_1\gamma_2$ 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 GIRK1 was normalized to that observed in oocytes injected with 0.1 ng of RNA. *D*, summary of data from two or three oocyte batches showing the increase in the level of endogenous G*α* and G*β* upon coexpression of GIRK1/2 at two RNA doses, 0.1 and 2.5 ng/oocyte. Data were normalized to control levels of G*β* or G*α* detected in uninjected (*Uninj.*) oocytes. In these experiments, nonspecific labeling was not estimated, and the measured values represent total fluorescence signal (background + specific).

tor suggests that the myristoyl moiety itself is not essential; rather, membrane anchoring appears to be the important factor that ensures efficient competition between a G$\beta\gamma$ scavenger and GIRK for membrane-attached G$\beta\gamma$. Unfortunately, we find that, in addition to sequestering G$\beta\gamma$, m-cβARK directly binds to GIRK1. This interaction does not seem to interfere with the activation of the channel by G$\beta\gamma$, because m-cβARK$_{R587Q}$, which does not bind G$\beta\gamma$ but still binds GIRK1, has no effect on GIRK currents. Despite this consideration, the binding of m-cβARK to GIRK1 is a serious disadvantage that perplexes the interpretation of the experimental results.

Both m-phosducin and m-cβARK reduced I_{basal} in a dose-dependent manner. RNA titration experiments showed a slightly lower ED_{50} for m-phosducin, possibly due to better protein expression or PM accumulation of m-phosducin. Titrated expression of these two potent G$\beta\gamma$ scavengers, performed in a rigorous quantitative manner for the first time here, led us to the main empirical conclusion of this work; at least 90% of $A_{GIRK,basal}$ in intact oocytes is G$\beta\gamma$-dependent at all densities of GIRK channels.

The source of G$\beta\gamma$ that determines $A_{GIRK,basal}$ in the oocytes and other cells is not clear. It is widely believed that the G$\beta\gamma$-dependent component of $A_{GIRK,basal}$ reflects the concentration of free G$\beta\gamma$ in the membrane (2). The most obvious and best studied sources of free G$\beta\gamma$ in the PM are the G$\alpha\beta\gamma$ heterotrimers. The latter dissociate and supply free G$\beta\gamma$ by several mechanisms: basal dissociation of $G\alpha_{GDP}$ from G$\beta\gamma$ in the absence of GDP-GTP exchange (49); basal GDP-GTP exchange that results in great reduction of the affinity of Gα to G$\beta\gamma$ (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 G$\beta\gamma$—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 $G\alpha_{i/o}\beta\gamma$ complex, which guarantees both low $A_{GIRK,basal}$ and rapid and efficient channel activation by G$\beta\gamma$ released upon an encounter with an agonist-bound GPCR (20, 26–28, 40, 53). The observation that the levels of endogenous G$\beta\gamma$ and Gα 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 G$\beta\gamma$ and Gα in the endoplasmic reticulum or the Golgi complex before being relocated to the PM. Joint trafficking of

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 Gα such as $G\alpha_s$ and $G\alpha_z$ to replace the endogenous $G\alpha_{i/o}$ (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 Gα, 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; Gα-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 $A_{GIRK,basal}$ at high GIRK densities. Although the level of endogenous Gα also rises upon coexpression of GIRK, it appears insufficient to prevent the disproportional increase in $A_{GIRK,basal}$, possibly because the increase in the level of Gβγ in the PM is greater than that of Gα. Comparison of the relative increases in Gα and Gβ caused by the expression of GIRK (Fig. 8, *A* and *B*) seems to support this possibility. However, at present this interpretation remains presumptive, because the immunoimaging 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 Gα levels includes that of "irrelevant" Gα (not $G\alpha_{i/o}$) available in the oocytes, which cannot efficiently reduce $A_{GIRK,basal}$ and/or donate Gβγ upon activation of m2R.

In summary, our observations so far conform to the proposal (20, 28) that $A_{GIRK,basal}$ is controlled by both Gβγ and Gα. 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 $G\alpha_{i/o}$ subunits, thus causing an excessively high $A_{GIRK,basal}$. Taking together the previously accumulated evidence regarding the existence of GIRK-Gαβγ complexes and the new data presented here, we propose that GIRK is persistently, although reversibly, associated with $G\alpha_{GDP}\beta\gamma$ throughout its biosynthesis; Gα is a nonobligatory part of the complex, whereas Gβγ is bound persistently.

Using m-phosducin and m-cβARK 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 $A_{GIRK,basal}$ 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 $A_{GIRK,basal}$ 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 $A_{GIRK,basal}$ via the high affinity site of GIRK would be lower than that required to induce I_{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 $A_{GIRK,basal}$. Alas, the results with the two potent Gβγ scavengers used here were not unequivocal. m-phosducin and m-cβARK showed striking differences in the apparent potency of inhibition of peak I_{ACh} and in ED_{50}. The data obtained with cβARK appear to support the two-site model of He *et al.* (57); I_{ACh} was inhibited by much lower doses of m-cβARK than I_{basal}. However, m-phosducin, which showed an even greater apparent affinity in inhibiting $A_{GIRK,basal}$ than m-cβARK (Fig. 7), exhibited an identical apparent affinity for I_{ACh}.

A clue for the understanding of these differences may be provided by invoking the fact that m-cβARK binds GIRK. Phosducin reduced I_{basal} stronger than I_{ACh} but accelerated the decay of I_{ACh}. These effects of m-phosducin are compatible with those of a simple Gβγ sink (see "Results"). In contrast, m-cβARK inhibited I_{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 cβARK, such that it captures Gβγ released from the heterotrimeric Gαβγ 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-cβARK and Gβγ for binding to GIRK may also explain the effects of m-cβARK 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-cβARK 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_{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 $A_{GIRK,basal}$ 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_{basal}) on top of phosducin did not further reduce I_{basal} supports the existence of a Gβγ-independent component. This putative Gβγ-independent component of $A_{GIRK,basal}$, 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 PIP_2, which is an obligatory gating factor in these channels (60). Also, it has been proposed that intracellular Na^+ is an important regulatory factor that may substantially regulate $A_{GIRK,basal}$ (23, 46). Na^+ 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_{50} of the direct Na^+ effect is 20–30 mM (62), and the extent of direct activation is probably minor in resting cells, at physiological Na^+ concentrations of 5–10 mM. Further studies will be necessary to clarify whether a Gβγ-independent component of $A_{GIRK,basal}$ exists and what is the underlying mechanism.

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REFERENCES

1. Clapham, D. E., and Neer, E. J. (1997) *Annu. Rev. Pharmacol. Toxicol.* **37,** 167–203
2. Dascal, N. (1997) *Cell Signal.* **9,** 551–573
3. Stanfield, P. R., Nakajima, S., and Nakajima, Y. (2003) *Rev. Physiol. Biochem. Pharmacol.* **145,** 47–179
4. Kurachi, Y., and Ishii, M. (2004) *J. Physiol.* (*Lond.*) **554,** 285–294
5. Logothetis, D. E., and Zhang, H. (1999) *J. Physiol.* (*Lond.*) **520,** 630
6. Hilgemann, D. W., Feng, S., and Nasuhoglu, C. (2001) *Science's STKE* http://www.stke.org/cgi/content/full/OC_sigtrans;2001/111/re19
7. Ito, H., Ono, K., and Noma, A. (1994) *J. Physiol.* (*Lond.*) **476,** 55–68
8. Torrecilla, M., Marker, C. L., Cintora, S. C., Stoffel, M., Williams, J. T., and Wickman, K. (2002) *J. Neurosci.* **22,** 4328–4334
9. Takigawa, T., and Alzheimer, C. (2002) *J. Physiol.* (*Lond.*) **539,** 67–75
10. Luscher, C., Jan, L. Y., Stoffel, M., Malenka, R. C., and Nicoll, R. A. (1997) *Neuron* **19,** 687–695
11. Blanchet, C., and Luscher, C. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 4674–4679
12. Sharon, D., Vorobiov, D., and Dascal, N. (1997) *J. Gen. Physiol.* **109,** 477–490
13. Kobrinsky, E., Mirshahi, T., Zhang, H., Jin, T., and Logothetis, D. E. (2000) *Nat. Cell Biol.* **2,** 507–514
14. Meyer, T., Wellner-Kienitz, M. C., Biewald, A., Bender, K., Eickel, A., and Pott, L. (2000) *J. Biol. Chem.* **276,** 5650–5658
15. Leaney, J., Dekker, L., and Tinker, A. (2001) *J. Physiol.* (*Lond.*) **534,** 367–379
16. Lei, Q., Talley, E. M., and Bayliss, D. A. (2001) *J. Biol. Chem.* **276,** 16720–16730
17. Reuveny, E., Slesinger, P. A., Inglese, J., Morales, J. M., Iniguez-Lluhi, J. A., Lefkowitz, R. J., Bourne, H. R., Jan, Y. N., and Jan, L. Y. (1994) *Nature* **370,** 143–146
18. Vivaudou, M., Chan, K. W., Sui, J. L., Jan, L. Y., Reuveny, E., and Logothetis, D. E. (1997) *J. Biol. Chem.* **272,** 31553–31560
19. Petit-Jacques, J., Sui, J. L., and Logothetis, D. E. (1999) *J. Gen. Physiol.* **114,** 673–684
20. Peleg, S., Varon, D., Ivanina, T., Dessauer, C. W., and Dascal, N. (2002) *Neuron* **33,** 87–99
21. Ivanina, T., Rishal, I., Varon, D., Mullner, C., Frohnwieser-Steinecke, B., Schreibmayer, W., Dessauer, C. W., and Dascal, N. (2003) *J. Biol. Chem.* **278,** 29174–29183
22. Zhang, Q., Pacheco, M. A., and Doupnik, C. A. (2002) *J. Physiol.* (*Lond.*) **545,** 355–373
23. Sui, J. L., Chan, K. W., and Logothetis, D. E. (1996) *J. Gen. Physiol.* **108,** 381–391
24. Ho, I. H., and Murrell-Lagnado, R. D. (1999) *J. Biol. Chem.* **274,** 8639–8648
25. Zhang, H., He, C., Yan, X., Mirshahi, T., and Logothetis, D. E. (1999) *Nat. Cell Biol.* **1,** 183–188
26. Slesinger, P. A., Reuveny, E., Jan, Y. N., and Jan, L. Y. (1995) *Neuron* **15,** 1145–1156
27. Huang, C. L., Slesinger, P. A., Casey, P. J., Jan, Y. N., and Jan, L. Y. (1995) *Neuron* **15,** 1133–1143
28. Ivanina, T., Varon, D., Peleg, S., Rishal, I., Porozov, Y., Dessauer, C. W., Keren-Raifman, T., and Dascal, N. (2004) *J. Biol. Chem.* **279,** 17260–17268
29. Jing, J., Chikvashvili, D., Singer-Lahat, D., Thornhill, W. B., Reuveny, E., and Lotan, I. (1999) *EMBO J.* **18,** 1245–1256
30. Blackmer, T., Larsen, E. C., Takahashi, M., Martin, T. F., Alford, S., and Hamm, H. E. (2001) *Science* **292,** 293–297
31. Yevenes, G. E., Peoples, R. W., Tapia, J. C., Parodi, J., Soto, X., Olate, J., and Aguayo, L. G. (2003) *Nat. Neurosci.* **6,** 819–824
32. Vorobiov, D., Bera, A. K., Keren-Raifman, T., Barzilai, R., and Dascal, N. (2000) *J. Biol. Chem.* **275,** 4166–4170
33. Liman, E. R., Tytgat, J., and Hess, P. (1992) *Neuron* **9,** 861–871
34. Dascal, N., Doupnik, C. A., Ivanina, T., Bausch, S., Wang, W., Lin, C., Garvey, J., Chavkin, C., Lester, H. A., and Davidson, N. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 6758–6762
35. Dascal, N., and Lotan, I. (1992) in *Protocols in Molecular Neurobiology* (Longstaff, A., and Revest, P., eds) Vol. 13, pp. 205–225, Humana Press, Totowa, NJ
36. Schreibmayer, W., Lester, H. A., and Dascal, N. (1994) *Pflugers Arch. Eur. J. Physiol.* **426,** 453–458
37. Singer-Lahat, D., Dascal, N., Mittelman, L., Peleg, S., and Lotan, I. (2000) *Pflugers Arch. Eur. J. Physiol.* **440,** 627–633
38. Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) *Annu. Rev. Biochem.* **67,** 653–692
39. Carman, C. V., Barak, L. S., Chen, C., Liu-Chen, L. Y., Onorato, J. J., Kennedy, S. P., Caron, M. G., and Benovic, J. L. (2000) *J. Biol. Chem.* **275,** 10443–10452
40. Nikolov, E. N., and Ivanova-Nikolova, T. T. (2004) *J. Biol. Chem.* **279,** 23630–23636
41. Kunkel, M. T., and Peralta, E. G. (1995) *Cell* **83,** 443–449
42. Dolphin, A. C. (1998) *J. Physiol.* (*Lond.*) **506,** 3–11
43. Schulz, R. (2001) *Pharmacol. Res.* **43,** 1–10
44. Bauer, P. H., Bluml, K., Schroder, S., Hegler, J., Dees, C., and Lohse, M. J. (1998) *J. Biol. Chem.* **273,** 9465–9471
45. Kovoor, A., Henry, D. J., and Chavkin, C. (1995) *J. Biol. Chem.* **270,** 589–595
46. Vorobiov, D., Levin, G., Lotan, I., and Dascal, N. (1998) *Pflugers Arch. Eur. J. Physiol.* **436,** 56–68
47. Savage, J. R., McLaughlin, J. N., Skiba, N. P., Hamm, H. E., and Willardson, B. M. (2000) *J. Biol. Chem.* **275,** 30399–30407
48. Kim, C. M., Dion, S. B., and Benovic, J. L. (1993) *J. Biol. Chem.* **268,** 15412–15418
49. Sarvazyan, N. A., Remmers, A. E., and Neubig, R. R. (1998) *J. Biol. Chem.* **273,** 7934–7940
50. Ross, E. M. (1995) *Recent Prog. Horm. Res.* **50,** 207–221
51. Kenakin, T. (2004) *Mol. Pharmacol.* **65,** 2–11
52. Rebois, R. V., and Hebert, T. E. (2003) *Receptors Channels* **9,** 169–194
53. Hille, B. (1992) *Neuron* **9,** 187–195
54. Prybylowski, K., and Wenthold, R. J. (2004) *J. Biol. Chem.* **279,** 9673–9676
55. Ruiz-Velasco, V., and Ikeda, S. R. (1998) *J. Physiol.* (*Lond.*) **513,** 761–773
56. Yakubovich, D., Rishal, I., and Dascal, N. (2005) *J. Mol. Neurosci.* **25,** 7–19
57. He, C., Zhang, H., Mirshahi, T., and Logothetis, D. E. (1999) *J. Biol. Chem.* **274,** 12517–12524
58. He, C., Yang, X., Zhang, H., Mirshahi, T., Jin, T., Huang, A., and Logothetis, D. E. (2002) *J. Biol. Chem.* **277,** 6088–6096
59. Finley, M., Arrabit, C., Fowler, C., Suen, K. F., and Slesinger, P. A. (2004) *J. Physiol.* (*Lond.*) **555,** 643–657
60. Mirshahi, T., Jin, T., and Logothetis, D. E. (2003) *Science's STKE* http://stke.sciencemag.org/cgi/content/full/OC_sigtrans;2003/194/pe32
61. Rishal, I., Keren-Raifman, T., Yakubovich, D., Ivanina, T., Dessauer, C. W., Slepak, V. Z., and Dascal, N. (2003) *J. Biol. Chem.* **278,** 3840–3845
62. Ho, I. H., and Murrell-Lagnado, R. D. (1999) *J. Physiol.* (*Lond.*) **520,** 645–651