Dynamic Integration of α-Adrenergic and Cholinergic Signals in the Atria

ROLE OF G PROTEIN-REGULATED INWARDLY RECTIFYING K⁺ CHANNELS

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Numerous heptahelical receptors use activation of heterotrimeric G proteins to convey a multitude of extracellular signals to appropriate effector molecules in the cell. Both high specificity and correct integration of these signals are required for reliable cell function. Yet the molecular machineries that allow each cell to merge information flowing across different receptors are not well understood. Here we demonstrate that G protein-regulated inwardly rectifying K⁺ (GIRK) channels can operate as dynamic integrators of α-adrenergic and cholinergic signals in atrial myocytes. Acting at the last step of the cholinergic signaling cascade, these channels are activated by direct interactions with βy subunits of the inhibitory G proteins (Gβy), and efficiently translate M₂ muscarinic acetylcholine receptor (M₂R) activation into membrane hyperpolarization. The parallel activation of α-adrenergic receptors imposed a distinctive “signature” on the function of M₂R-activated GIRK1/4 channels, affecting both the probability of Gβy binding to the channel and its desensitization. This modulation of channel function was correlated with a parallel depletion of Gβ and protein phosphatase 1 from the oligomeric GIRK1 complexes. Such plasticity of the immediate GIRK signaling environment suggests that multireceptor integration involves large protein networks undergoing dynamic changes upon receptor activation.

The heart functions in a continuously changing environment responding to excitatory and inhibitory signals from the sympathetic and parasympathetic branches of autonomic nervous system. Both branches use members of the large family of seven transmembrane G protein-coupled receptors (GPCRs) as membrane gateways for their opposing signals. At rest, parasympathetic signals reduce the heart rate through activation of Gᵦₒ-coupled M₂Rs. During exercise and stress, sympathetically derived catecholamines target coexisting ARs and increase the cardiac output by accelerating the heart rate and enhancing contractility. The β₁-, β₂-, and α₁-ARs are the most predominant AR subtypes in cardiac myocytes (1). These receptors activate different signaling cascades with α₁-ARs coupling to Gᵦₓ, β₁-ARs coupling to Gᵦₓ and β₂-ARs coupling to both Gᵦₓ and Gᵦᵣ pathways. Although the cellular processes triggered upon activation of individual adrenergic and M₂ receptors have been well delineated (1), how cardiac myocytes integrate the inhibitory and excitatory signals transmitted by these receptors remains less well understood.

Signal integration might arise from heterodimerization of members of distinct subfamilies of GPCRs. Such receptor heterodimerization expands the operational features of the receptors involved, modulating either receptor trafficking or function (2). Importantly, heterodimerization of β₁- and β₂-ARs in intact cardiac myocytes was recently reported to give rise to a new population of β-ARs with enhanced signaling properties (3).

Assembly of receptor heterodimers, however, is perhaps only one of the diverse mechanisms used by different GPCRs to achieve exquisite and dynamic control of membrane excitability in cardiac myocytes. Integration in vivo might also rely on highly organized signaling networks in which relevant proteins are physically maintained together. Recent studies indicate that atrial GIRK1/4 channels might play a role in a similar network responsible for the coordination of adrenergic and cholinergic signals in the heart (4). These channels are heterotetrameric membrane proteins consisting of two pore-forming subunits, GIRK1 (5, 6) and GIRK4 (7). Acting downstream from M₂Rs, GIRK channels are responsible for membrane hyperpolarization and ensuing reduction in heart rate following cholinergic stimulation. Channel activation is conferred by direct Gβy binding to multiple domains on the intracellular surface of the channel (reviewed in Refs. 8 and 9). Upon Gβy activation, atrial GIRK1/4 channels adopt four functional modes, characterized by different open probabilities and rendered active by putative binding of an increasing number of Gβy subunits to the channel (10, 11). As the Gβy concentration increases, the equilibrium among the four functional modes shifts gradually from low to high efficient gating. This operational feature enables GIRK1/4 channels to control membrane excitability with great...
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precission, which is a prerequisite for efficient integration of opposing signals. Furthermore, a fraction of atrial GIRK1/4 channels has recently been shown to form large protein complexes (12). Immunopurification of these macromolecular complexes identified GIRK1 association with Gβγ, G protein-coupled receptor kinase, cyclic adenosine monophosphate-dependent protein kinase, two protein phosphatases, PP1 and PP2A, receptor for activated C kinase 1, and actin, implicating these proteins as potential modulators of channel function (4). Oligomeric complexes containing GIRK channels, adenylyl cyclase, and GPCRs have also been identified (13, 14), suggesting the existence of large G protein-coupled signaling networks in the membrane.

To serve as integrators in similar networks, the GIRK channels must conform to two requirements. First, activation of different GPCRs should leave a unique mark on channel function at the single molecule level. Second, the signaling framework of the GIRK1 complexes should “memorize” previous events of GPCR activation. Here we examined whether the GIRK channels met these minimal requirements following concurrent stimulation of M2 and α-adrenergic receptors in atrial myocytes. First reported by Braun et al. (15), the inhibition of GIRK channel currents by GqPCRs is a well established phenomenon (reviewed in Ref. 16), which provides an optimal venue for addressing these questions. Remarkably, the Gq-coupled receptors recruit a large repertoire of parallel cellular processes to accomplish the inhibition of GIRK channel currents. Depletion of phosphatidylinositol 4,5-bisphosphate (PIP2), activation of protein kinase C (PKC), and direct binding of Gαq subunits to the channel have been all shown to contribute to this phenomenon (4, 16–25). How these processes modulate GIRK channel function at single molecule level remains less well understood. We therefore investigated GIRK1/4 channel function and the mechanisms underlying α-AR-mediated inhibition in intact atrial myocytes, using the single-channel experiments. These experiments record the stochastic time trace of repetitive transitions of an individual molecule undergoing conformational fluctuations triggered by receptor activation and allow measurements of molecular properties unattainable from ensemble data. Initially, we established the equivalence between the M2R and Gβγ activation of GIRK1/4 channels. Accordingly, the M2R-activated channels adopt four functional modes conferred by the apparent binding of an increasing number of Gβγ subunits to the channel protein. This finding validates the concept of Gβγ-driven control of four functional states, evolving from the same molecule, as a robust framework for understanding GIRK1/4 function and regulation. In this framework, the α-AR stimulation perturbs GIRK channel-Gβγ interactions, leading to a 2-fold reduction in the probability of Gβγ binding to the channel. In addition, the impaired channel function is associated with concerted changes in GIRK1/4 signaling environment, manifested by differences in the composition of the oligomeric GIRK1 complexes. Intriguingly, cholinergic and α-adrenergic signals differentially affect this environment, supporting the notion that the oligomeric GIRK1 assemblies provide a dynamic protein framework involved in the integration of these signals.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse monoclonal antibodies against Gβ, PP1, phosphoinositide-specific phospholipase C (PLC) β1, phosphoinositide 3-kinase (PI 3-kinase), β-arrestin, Gαq, and Gαq were purchased from BD Transduction Laboratories. Mouse monoclonal anti-M2R, rabbit polyclonal anti-K+ channel GIRK1 antibodies, and normal mouse IgG were from Oncogene Research Products (Boston, MA). Other antibodies used in this study were as follows: the rabbit polyclonal anti-M2R and rabbit polyclonal anti-Ca,1,2a (Alomone Labs, Jerusalem, Israel); mouse monoclonal 4A9 anti-phosphoserine (Calbiochem); rabbit polyclonal anti-actin, rabbit polyclonal anti-PLC phosphoinositide-specific phospholipase C (PLC) β1, phosphoinositide 3-kinase (PI 3-kinase), β-arrestin, Gαq, and Gαq were purchased from BD Transduction Laboratories. Mouse monoclonal anti-M2R, rabbit polyclonal anti-K+ channel GIRK1 antibodies, and normal mouse IgG were from Oncogene Research Products (Boston, MA). Other antibodies used in this study were as follows: the rabbit polyclonal anti-M2R and rabbit polyclonal anti-Ca,1,2a (Alomone Labs, Jerusalem, Israel); mouse monoclonal 4A9 anti-phosphoserine (Calbiochem); rabbit polyclonal anti-actin, rabbit polyclonal anti-PLC β2 (Q-15), and rabbit polyclonal anti-adenyl cyclase (R-32) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Horseradish peroxidase-linked mouse and rabbit whole antibodies, ECL Plus reagents, and Rainbow Molecular Weight Markers were purchased from Amersham Biosciences. Dynabeads M-280 sheep anti-mouse IgG, M-280 sheep anti-rabbit IgG, and M-450 rat anti-mouse IgM were from Dynal Biotech Inc. (Lake Success, NY). Immobilon-P transfer membrane was purchased from Millipore (Bedford, MA). Protease inhibitor mixture set III (PI) and phosphatases inhibitor mixture set 1 (Phl) were purchased from Calbiochem. N-Glycosidase F was purchased from Prozyme (San Leandro, CA). Electrophoretic and Western blotting reagents were provided by Bio-Rad. All other chemicals used in this study, including deoxycholic acid sodium salt and IGEPA CA-630, were of molecular biology grade and were purchased from Sigma.

**Electrophysiological Recordings**—Viable atrial myocytes were obtained from 1- to 2-day-old Sprague-Dawley rats by a trypsin/chymotrypsin/elastase dissociation procedure and cultured as described previously (10). Unitary currents through KACH channels were recorded from cell-attached and inside-out patches using standard high resolution patch clamp methods (26) as described previously (10, 11). The composition of the bath solution was as follows (in mM): 150 KCl, 5 EGTA, 5 glucose, 1.6 MgCl2, 5 HEPES, pH 7.4; the composition of the pipette solution was as follows (in mM): 150 KCl, 1 CaCl2, 1.6 MgCl2, 5 HEPES, pH 7.4. Only recordings from a single GIRK1/4 channel or a pair of GIRK1/4 and scGIRK channels (11), residing in the same patch, were included in the analysis of GIRK1/4 channel gating behavior. In the latter case, the two types of GIRK channels were investigated as individual entities, and the gating equilibrium of GIRK1/4 channel was analyzed independently of the presence of a scGIRK channel in the same patch.

The modal behavior of M2R-activated GIRK1/4 channels was analyzed in the context of a model that assumes four functional modes of a single GIRK channel arising from different occupancy of four equivalent and independent Gβγ sensors in the channel (10, 11). Briefly, single channel recordings were divided into 400-ms consecutive segments, and the frequency of apparent openings, f, was calculated for each data segment. Initially, the data recordings were examined for homogeneity, divided into 400-ms consecutive segments, and the frequency, F, of the resulting F − t plots accurately identified the...
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apparently homogeneous sections of each recording (11). A data recording was judged as homogeneous if the mean F values calculated from the first and the second halves of this recording were statistically indistinguishable. The F values calculated from the individual 400-ms data segments in a homogeneous section of the recording were further used to generate a frequency histogram. The F histograms were subsequently fitted by the sum of several gamma distributions, \( \Gamma(f) \) as shown in Equation 1, and as described previously (11),

\[
\Gamma(f) = \sum N_k (f/f_k)^\gamma \exp(-f/f_k) \tag{Eq. 1}
\]

where \( \Gamma(f) \) is the distribution expected for the sum of two variables, when each variable has an exponential distribution, \( f_k^{-1} \exp(-f/f_k) \) with mean \( f_k \) (27). Previously, we have shown that F histograms generated from Gβγ-activated GIRK channels were fitted rather well by a sum of gamma terms over a wide range of Gβγ concentrations (11). The F histograms were further used to determine the number of gating modes accessible to the GIRK channels, the characteristic \( f_k \) values, and the relative occupancy of each mode, \( F_k = N_k/\Sigma N_k \). For each F histogram, the probability of Gβγ binding, \( P \), was computed from the relative occupancy of mode 1, \( F_{1} \), according to Equation 2,

\[
F_{1} = 4P(1-P)^3/(1-(1-P)^4) \tag{Eq. 2}
\]

The relative occupancy of the remaining modes, \( F_{4} \), was then examined as a function of the parameter \( P \) to verify that experimental \( F_k \) estimates were consistent with the theoretically derived probability of observing mode \( k \) as shown in Equation 3,

\[
F_{4} = (N!/(k!(N-k)!)) P^k(1-P)^{N-k} \tag{Eq. 3}
\]

where \( P \) is the probability of Gβγ binding to one of four putative G protein sensors in GIRK1/4 channel structure, and \( N = 4 \).

For each experimental condition, the fraction of blank data segments caused by GIRK1/4 channel desensitization, \( F_{4b} \), was calculated as the difference, \( (F_{4b} - F_{4}) \), where \( F_{4b} \) is the experimentally determined fraction of blank data segments, and \( F_{4} \) is the predicted fraction of time of the channel spends with no Gβγ bound to it. The \( F_{4b} \) fraction, \( F_{4b} = (1-P)^6 \), was calculated from the probability of Gβγ binding, \( P \), according to Equation 3.

Immunoprecipitation and Immunoblotting—The composition of GIRK1 signaling complexes was determined as described previously (4), with some modifications. Briefly, rat atrial tissue was homogenized in HME buffer containing 20 mM HEPES-KOH, pH 8.0, 2 mM MgCl\(_2\), 1 mM EDTA, supplemented with 0.32 M sucrose, PI and Phl. The homogenate was centrifuged at 500 x g for 15 min at 4 °C to remove the nuclei and mitochondria, and plasma membranes were then obtained by centrifugation (100,000 x g for 65 min) of the resulting supernatant on a cushion of HME-PI-Phl buffer with 1.3 M sucrose (28). The membranes at the interface were collected, washed twice, resuspended in HME-PI-Phl buffer containing 0.32 M sucrose and frozen as above. This modification of membrane preparation yielded plasma membranes containing the glycosylated p65 GIRK1 form only.

For the experiments involving stimulation of M2Rs, excised atria were equilibrated in oxygenated solution containing (in mM) 140 NaCl, 5.4 KCl, 1 CaCl\(_2\), 1.6 MgCl\(_2\), 5 HEPES, 5 glucose, pH 7.4, stimulated with 1 µM acetylcholine (ACh) for 5 min at room temperature and frozen in liquid nitrogen. To determine the effect of concurrent stimulation of M3 and α-adrenergic receptors on the composition of the GIRK1 complexes, atria were preincubated with β-AR antagonist propranolol (1 µM) for 5 min at room temperature, then treated with phenylephrine at a concentration of 1 µM for 5 min, followed by stimulation with 1 µM ACh for an additional 5 min in the presence of 1 µM phenylephrine. After a brief rinse with ice-cold physiologi- cal solution, the tissue was frozen in liquid nitrogen. Unstimulated tissue processed in parallel was used as a control.

The atrial membranes were solubilized in MEB buffer (20 mM HEPES, pH 8.0, 2 mM MgCl\(_2\), 1 mM EDTA, 100 mM NaCl, 10 mM β-mercaptoethanol, 1% sodium deoxycholate, and 0.45% IGEPAL CA-630) with PI and Phl as described previously (4, 10). The detergent-insoluble material was removed by centrifugation at 100,000 x g for 1 h, and the supernatant, containing the solubilized membrane-associated proteins, was immediately used for immunoprecipitation. Protein concentration was determined by Bio-Rad DC protein assay against a standard curve of bovine serum albumin. Equal amounts of membrane proteins were used in each reaction.

Immunoprecipitations were performed with Dynal magnetic beads according to the manufacturer’s protocols using the MPC magnetic device (Dynal, Lake Success, NY) as described previously (4). The magnetic immunopurification eliminates all centrifugation steps from the classical immunoprecipitation process. As a result, any possible contamination of the immunoprecipitation product by large protein complexes that might be trapped within antibody-protein A/G beads latticework during centrifugation is also eliminated. Briefly, equal amounts of beads were incubated with the anti-phosphoserine (4A9 clone) or anti-Gβγ antibodies (3 µg per 1.2 x 10\(^7\) M-450 rat anti-mouse IgM beads) or anti-M2R antibody (Oncogene) (3 µg per 1.8 x 10\(^7\) M-280 sheep anti-mouse IgG beads) for 2 h with rotation at 4 °C. In each experiment, equal amounts of beads coated with normal mouse IgG (3 µg per 1.8 x 10\(^7\) M-280 sheep anti-mouse IgG beads) were used as a negative control. In addition, the specificity of the antibodies used for immunoprecipitation was tested in each experiment to ensure that these antibodies recognized only the protein of interest in the atrial membranes. Coated beads were washed and incubated with the solubilized membrane proteins for 2 h at 4 °C with rotation. The complexed beads were collected by magnetic precipitation, washed, resuspended in equal volumes of Laemmli sample buffer, and heated for 5 min at 95 °C (70 °C for M2R detection). Equal sample volumes were loaded on an 11% gel; the proteins were sep-
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arated by Tris-glycine SDS-PAGE and electrophoretically transferred to Immobilon-PSQ membranes. The proteins of interest were detected by Western blotting, and the immunoreactive complexes were visualized by enhanced chemiluminescence detection system. The immunoreactive protein bands were quantified using VersaDoc Imaging System and Quantity One (version 4.5.2) software (Bio-Rad). The changes in the composition of GIRK1 complexes after receptor stimulation were assayed as follows. The pixel intensities (arbitrary units) of the immunoreactive bands for each protein were quantified and normalized to the intensity of the corresponding protein band in the control sample. The ratio between the normalized amount of a particular protein and the normalized amount of GIRK1 protein in the same complex was then determined and the mean ± S.E. of four independent experiments reported. Finally, to verify that the protein bands of interest were properly quantified, in each experiment, a range of known amounts of atrial membrane proteins was also loaded on the same gel, and the relative protein levels were examined to confirm that pixel intensity of the immunoreactive bands grows linearly with increasing protein amount.

RESULTS

Effects of $\alpha$-AR Stimulation on GIRK1/4 Channel Function—Numerous studies, involving different cell types, have indicated that activation of $\alpha_2$-adrenergic or other $G_{q}\gamma$-coupled receptors leads to inhibition of whole-cell GIRK channel currents (15–25). To investigate the molecular mechanisms underlying such inhibition, we focused on GIRK1/4 channel function at the single molecule level and examined this function in cell-attached configuration following concurrent stimulation of $M_2$ and $\alpha$-adrenergic receptors in atrial myocytes. M2Rs activate two populations of GIRK channels in atrial myocytes, canonical 35-picosiemens GIRK1/4 channels and small conductance (sc) GIRK channels with a conductance of ~16 pico siemens (11). This report focuses on $\alpha$-AR regulation of the classical GIRK1/4 channels, because they are the major contributors to the inhibitory synaptic transmission in the heart.

Unitary currents, recorded from two different atrial myocytes in the presence of 1 $\mu$M acetylcholine, added to the pipette solution, are shown in Fig. 1. The M2R-activated channels exhibited inward rectification and direct dependence on $G_{q}\gamma$ for their activation. Patch excision in GTP-free solution abruptly and completely eliminated channel activity, whereas subsequent perfusion of the cytoplasmic side of the patch with nanomolar concentration of purified recombinant $G_{q}\gamma$-7 brought the activity back (data not shown). For each cell-attached experiment, data acquisition began 30 s after formation of a gigaseal. This delay was necessary for adjustment of holding potential and the appropriate gain setting of the patch clamp amplifier. As illustrated in Fig. 1, the sensitivity of individual GIRK1/4 channels to M2R stimulation varied from one channel to another. Regardless of such variability, channel gating remained stable over the course of the cell-attached recordings (usually 7–10 min).

In contrast, activation of atrial $\alpha$-ARs changed the profile of GIRK channel response to M2R stimulation. In whole-cell assays, inactivation of GIRK channels by $G_{q}\gamma$-coupled receptors was quantified as the ratio of steady-state current, after application of specified $G_{q}$-agonist, to the control steady-state current evoked in the same cell (15–25). In our experiments, however, attempts to record M2R-activated single channels for several minutes and then perfuse the experimental chamber with an agonist frequently led to patch excision, because of the mechanical perturbations associated with the perfusion. For this reason we reversed the order of $M_2$ and $\alpha$-adrenergic receptor activation. The atrial myocytes were incubated with $\beta$-AR antagonist, propranolol (1 $\mu$M), for 5 min and then treated with $\alpha$-AR agonist phenylephrine (1 $\mu$M) for additional 5 min. At the end of the 5th min, the electrophysiological experiments were conducted under conditions identical to the conditions in the control experiments (1 $\mu$M ACh was added to the patch pipette). Propranolol and phenylephrine were present in the experimental chamber for the duration of the experiment. Fig. 2 illustrates the activity of a GIRK1/4 channel recorded in cell-attached configuration, when $\alpha$-ARs and M2Rs are concurrently activated. The initial response of GIRK1/4 channels to M2R stimulation in the phenylephrine-treated myocytes was similar to the response in the control group. Combined activation of $\alpha$-ARs and M2Rs, however, led to extensive inactivation of GIRK1/4 channels within less than 5 min.

Gating Mechanism of M2R-activated GIRK1/4 Channels—Understanding the principles of integration of opposing stimuli by GIRK1/4 channels required a quantitative analysis of chan-
nel gating. We therefore analyzed the kinetic behavior of GIRK1/4 channels in control and phenylephrine-treated myocytes. We have previously shown that upon Gβγ activation, atrial GIRK1/4 channels undergo fast transitions between four functional modes rendered active by the binding of an increasing number of Gβγ subunits to the channel (10, 11). This Gβγ-dependent GIRK channel behavior is further complicated by slow modal transitions, which are independent of Gβγ concentration (11). The slow modal transitions, however, occur on a time scale ~1000-fold slower than the transitions between the four functional modes of GIRK1/4 channel. Such heterogeneity of channel behavior significantly hinders the interpretation of the single-channel recordings. We therefore developed quantitative approaches for detecting homogeneous sections of recordings and classification of heterogeneous channel gating into functionally distinct modes (10, 11).

Initially, we examined the recordings from M2R-activated GIRK1/4 channels for the presence of slow modal transitions. Each recording was divided into 400-ms consecutive segments, and the frequency of openings, $f$, was calculated for individual data segments. The mean frequency, $F$, was then calculated for 30-s intervals of data recordings (by averaging the $f$ values from 75 consecutive data segments) and plotted against time. Analysis of GIRK1/4 channel activity recorded from seven control and seven phenylephrine-treated myocytes is presented in Fig. 3. The mean frequency of channel gating measured in cell-attached patches from control myocytes during the first 60 s of recording was 19.9 ± 2.5 Hz ($n = 7$). This value is statistically indistinguishable from the frequency estimate obtained for the GIRK1/4 channels in phenylephrine-treated myocytes for the first 60 s of recording (18.2 ± 2.3 Hz, $n = 7$). However, inactivation of M2R-activated GIRK1/4 channels in the control myocytes was virtually absent for the duration of our experiments; the mean frequency of channel gating measured 4 min after the onset of the cell-attached recording was 18.9 ± 2.5 Hz ($n = 7$).

In contrast, M2R-activated GIRK1/4 channels in phenylephrine-treated myocytes showed a prominent inactivation under identical recording conditions; the mean frequency was only 4.8 ± 1.6 Hz ($n = 7$) 4 min after the beginning of the recording. Thus, stimulation of α-ARs in atrial myocytes controlled differentially the amplitude of the initial M2R-activated response and GIRK1/4 channel inactivation. The initial amplitude of the response was unaffected (Fig. 3), whereas the channel inactivation was enhanced from 8.4% of the initial response in control myocytes to 74.4% in phenylephrine-treated myocytes.

Having generated the $F - t$ plots for the individual single-channel recordings, we compiled the $f$ values within the apparently homogeneous section of each recording to construct a frequency histogram. The $f$ histograms were then fitted with gamma functions to quantify the equilibrium among the gating modes of M2R-activated GIRK1/4 channels. Fig. 4 illustrates these two stages in the analysis of the M2R-activated GIRK1/4...
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The frequency of channel gating was averaged over 30-s data segments and plotted against time for seven individual GIRK1/4 channels activated by 1 μM ACh in cell-attached patches from control myocytes (left panel) and for seven GIRK1/4 channels activated in phenylephrine (Phe)-treated myocytes (right panel). Although the single-channel activity was recorded from different atrial myocytes, the data recorded under identical experimental conditions were combined on the same grid to illustrate the global effect of α-ARs on the activity of GIRK1/4 channels and membrane hyperpolarization. The initial response of the GIRK1/4 channels in the phenylephrine-treated group was undistinguishable from the response in the control group. However, six of seven GIRK channels in the phenylephrine-treated group exhibited a prominent inactivation within 5 min of concurrent α-AR and M2R stimulation. In contrast, the gating of the GIRK channels in the control group remained stable over the course of the recording. The frequency of channel gating for GIRK channels 1 and 2 in the control group (left panel) was derived from the initial 7 min of GIRK1/4 channel recordings illustrated in Fig. 1, A and B, respectively. The F − t plot for GIRK channel 2 in the phenylephrine-treated group (right panel) was derived from the initial 7 min of GIRK1/4 channel recording illustrated in Fig. 2.

The histograms (Fig. 4, A and B, bottom panels) were amenable to fitting with gamma functions, suggesting that M2R-activated GIRK1/4 channels have gating properties similar to those of Gβγ-activated channels. Fig. 5 illustrates the profile of GIRK1/4 channel activity in a phenylephrine-treated myocyte (the unprocessed data shown in Fig. 2), as revealed by analysis of mean frequency of gating F (top panel). The F − t plot accurately detected the transition from high to low efficient GIRK1/4 channel gating, triggered by the concurrent activation of α-adrenergic and M2 receptors in the membrane. To obtain quantitative estimates of channel gating, we generated separate f histograms (Fig. 5, middle and bottom panels) from the initial and late phase (denoted by the horizontal lines in the F − t plot) of the recording. A comparison of these histograms revealed that the inactivation of GIRK1/4 channel was associated with a shift in the modal equilibrium toward the less efficient gating modes. Previously we have shown that similar shifts in modal equilibrium of Gβγ-activated channels are predictive of reduction in the probability of Gβγ binding to the channel (11). Receptor-mediated activation of the GIRK channels, however, relies on conformational changes in heterotrimeric G proteins, triggered upon agonist binding to the M2Rs. Accordingly, the interactions between M2Rs and endogenous G proteins theoretically can modulate the gating equilibrium of GIRK channels. We therefore examined whether the gating of M2R-activated channels differs from that of Gβγ-activated channels. To address this question, we analyzed the gating equilibrium of GIRK1/4 channels activated at 1 μM ACh. The probability of Gβγ binding to the channel, P, was computed from the relative occupancy of gating mode 1, assuming this mode ensues from the binding of a single Gβγ to the channel (see “Experimental Procedures”). The relative occupancy of the remaining modes was then examined as a function of P and compared with the theoretically derived distributions for the probability of binding of increasing numbers of Gβγ subunits to the channel. Fig. 6 illustrates this analysis for the two sets of experimental data for M2R-activated GIRK channels from control (A) and phenylephrine-treated myocytes (B). In the latter case, the probability of Gβγ binding to the channel, P, was derived from the f histograms generated from the low efficient phase of GIRK1/4 channel gating. The curves labeled “mode 2” and “modes 3 and 4” show the probability of binding of 2, and 3 and 4 Gβγ subunits to the channel, respectively, as calculated from the binomial distribution (see Equation 3). The theoretical curves superimposed on the experimental data for the occupancy of mode 2 (Fig. 6, diamonds) and modes 3 and 4 (triangles) illustrate the compatibility between channels shown in Fig. 1. The F − t plots (Fig. 4, A and B, top panels) verified the homogeneity of the channel activity upon M2Rs activation. More importantly, our analysis revealed that
the model and the experimental observations. Altogether, the analysis of modal equilibrium confirmed that the M2R-activated GIRK1/4 channels follow the predictions of the model in which incremental binding of Gβγ to four equivalent and independent sensors in the protein complex confers the four functional modes of the channel.

In the framework of this model, we next examined the probability of Gβγ binding to GIRK1/4 channels after M2R activation and the effect of α-ARs on Gβγ binding. At 1 μM ACh, the probability of Gβγ binding for M2R-activated GIRK channels from control myocytes ranged from 0.36 to 0.63. In contrast, the P values for channels from phenylephrine-treated myocytes were clustered in the range of 0.09–0.27. The mean probability of Gβγ binding to the channel (at 1 μM ACh) was 0.55 ± 0.03 for the control (n = 7) and only 0.23 ± 0.07 for the phenylephrine-treated myocytes (n = 7). These results identified the reduction in the probability of Gβγ binding to GIRK1/4 channels as a major mechanism underlying the α-AR inhibition of these channels.

Although the analysis of both Gβγ-activated and M2R-activated GIRK1/4 channels overwhelmingly supported the model of channel gating in four conformations rendered active by the binding of an increasing number of Gβγ subunits to the channel, this model failed to predict accurately the probability of observing the nonconducting conformations of the channel. Thus, upon Gβγ activation, the proportion of blank data segments in GIRK1/4 channel recordings significantly exceeded the probability of observing gating mode 0 (no Gβγ bound to the channel) calculated from the binomial distribution (11). This discrepancy strongly suggests that an additional Gβγ-dependent desensitization process might contribute to the efficiency of GIRK1/4 channel gating. Because this process could prove vital in channel modulation, we examined GIRK1/4 channel desensitization in control and phenylephrine-treated myocytes. The fraction of desensitized channels, FDes, was defined as the difference (FDes – F0) between the experimentally determined fraction of blank data segments, FDes, and the predicted fraction of time the channel being with no Gβγ bound, F0, calculated from the probability of Gβγ binding, P, according to Equation 3. At 1 μM ACh, FDes was 9.82 ± 2.88% for the control (n = 7) and 19.07 ± 3.82% for the phenylephrine-treated myocytes (n = 7). Altogether, these results establish that parallel activation of α-ARs in atrial myocytes imposes a unique modulatory “signature” on M2R-activated GIRK1/4 channels, affecting both the probability of Gβγ binding to the channel and its desensitization.

Effect of Receptor Stimulation on the Composition of GIRK1-Gβ-Signaling Complexes—Having established the effect of α-AR stimulation on GIRK1/4 channel function in vivo, we asked whether channel modulation could be correlated with concurrent changes in channel signaling environment in vitro. To address this question we determined the composition of immunopurified GIRK1-Gβ signaling complexes after coactivation of M2 and α-adrenergic receptors. Fresh atrial tissue was first preincubated with propranolol for 5 min, followed by stimulation with 1 μM phenylephrine for 5 min, and then stimulated with 1 μM ACh for an additional 5 min. This protocol accurately replicates the receptor stimulation protocol from our electro-
physiological experiments. Two additional samples, one incubated with vehicle for the ligand only (control) and another one stimulated with 1 μM ACh for 5 min, were processed in parallel. Following receptor stimulation, the GIRK1-Gβ signaling complexes were initially immunopurified using the phosphoserine-specific 4A9 antibody. Previously, we have shown that the 4A9 antibody selectively detected the core p54 form of GIRK1 protein in rat atrial membranes, and this antibody could be used for immunopurification of the oligomeric GIRK1-Gβ complexes (4). The resulting 4A9 protein complexes were analyzed by immunoblotting for the presence of GIRK1, Gβ, actin, and PP1. Fig. 7 (top left) illustrates the agonist-mediated changes in the composition of the 4A9 complexes. To quantify these changes, the amount of each protein was normalized to the amount of the same protein in the control 4A9 complexes. Thus, the relative amount of each component was set at a value of unity in the absence of receptor stimulation. Because both GIRK1 isoforms, the core p54 and the glycosylated p65 form, were present in the 4A9 complexes (Fig. 7, top left panel), we used the sum of the pixel intensities of the 54- and 65-kDa bands for quantification of the amount of GIRK1 protein in these complexes. To confirm that the sum of the intensities of these two bands accurately reflects the amount of total GIRK1 protein, we allowed N-glycosidase F to completely deglycosylate the p65 form of GIRK1, and then we quantified the intensity of a single GIRK1/p54 band. We observed that the total amount of GIRK1 protein in the 4A9 complexes was a rather stable parameter in all three experimental conditions (data not shown).

To examine the stoichiometry of the 4A9 complexes after receptor stimulation, the relative amount of each protein was normalized to the amount of total GIRK1 protein in the same complexes. The results from this analysis for Gβ, actin, and PP1 are illustrated in Fig. 7 (dark bars). A comparison of Gβ immunoreactive bands in the 4A9 complexes yielded data consistent with the results from the analysis of GIRK1/4 channel gating (Fig. 6C). The level of Gβ increased by 2.40 ± 0.30-fold (n = 4) following the M2R stimulation but only by 1.54 ± 0.15-fold after concurrent stimulation of M₂ and α-adrenergic receptors. Furthermore, the levels of actin and PP1 increased (1.77 ± 0.22-fold and 1.60 ± 0.09-fold, respectively, n = 4) after M2R stimulation; however, these protein levels were not affected by the simultaneous activation of M₂ and α-adrenergic receptors. These results suggest the intriguing possibility that the signaling environment of GIRK channels undergoes dynamic transformations upon receptor stimulation.

To confirm that the identified changes in the 4A9 complexes were a consequence of receptor stimulations, we immunopuri-

![Figure 6. Modal equilibrium of M2R-activated GIRK1/4 channels.](image-url)

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**FIGURE 6. Modal equilibrium of M2R-activated GIRK1/4 channels.** A, gating equilibrium of M2R-activated channels in control myocytes. For each channel the occupancy of different gating modes was estimated from the analysis of histograms. The sojourns of the channels in gating modes 3 and 4 are represented together. In each experiment, the probability of Gβγ binding to one of the four Gβγ-sensors, P, was computed from the occupancy of mode 1, F₁, according to Equation 2. The solid lines represent the probability of binding of 1, 2, and 3 and 4 Gβγ subunits to the channel. The symbols represent the experimentally determined occupancies of mode 1 (circles), mode 2 (diamonds), and modes 3 and 4 (triangles). B, gating equilibrium of M2R-activated channels in phenoxyphrine (Phe)-treated myocytes. The probability of Gβγ binding to the channel, P, was derived from the histograms generated from the low efficient phase of GIRK1/4 channel gating after the slow modal transition triggered by the α-ARs. Stimulation of α-ARs shifted the modal equilibrium toward less efficient gating modes 1 and 2. C, mean probability of Gβγ binding to GIRK1/4 channels. At 1 μM ACh, p = 0.55 ± 0.03 for the control (n = 7) and p = 0.23 ± 0.07 for the phenoxyphrine-treated myocytes (n = 7), p < 0.05.
fied the oligomeric GIRK1 complexes using Gβ-specific antibodies as an alternative “bait” and examined their composition. The selection of a Gβ antibody for these experiments was based on information about the immunogen used to generate the antibody and localization of this immunogen in the three-dimensional Gβγ structure (29, 30). According to the supplier (BD Transduction Laboratories), the monoclonal Gβ antibody was generated against Gβ residues 130–145. This immunogen includes a surface-localized residue, Thr-143, essential for the interaction between Gβγ and Gaα-GDP that is not accessible in heterotrimeric G proteins (29, 30). We therefore hypothesized that the Gβ antibody should be able to recognize specifically the native Gβγ subunits but not the heterotrimeric Gaαβγ proteins. Furthermore, the same Gβ residue (Thr-143) is required for Gβγ interaction with PLC β2 and G protein-coupled receptor kinase but not for Gβγ activation of GIRK1/4 channels and adenylyl cyclase (31). Accordingly, the Gβ antibodies should be able to bind Gβγ-GIRK1/4-channel complexes but not Gβγ-PLC β2 and Gβγ-G protein-coupled receptor kinase complexes. We have previously established that the Gβ-complexes, immunopurified from atrial membranes, indeed consist of the same set of proteins found in the 4A9 complexes (4). Additionally, comparison of the stoichiometry of Gβ and 4A9 complexes in the present study (Fig. 7, light and dark bars, respectively) revealed a remarkable consistency in the composition of these complexes.

Finally, to rule out the possibility that other Gβγ-binding proteins were associated with the Gβ and 4A9 complexes, we analyzed these complexes by immunoblotting for the presence of Gaα, Gaα, adenylyl cyclase, PLC β1, PLC β2, PI 3-kinase, and β-arrestin. We have not been able to detect association of these proteins with both the Gβ and 4A9 complexes isolated from atrial membranes (data not shown). Notably, selective binding of Gaα (32) and adenylyl cyclase (13, 14) to neuronal GIRK channels has been well documented in heterologous expression systems and brain. The absence of Gaα, and adenylyl cyclase in the atrial GIRK1 oligomeric complexes most probably reflects the ability of different cell types to assemble divergent signaling networks.

Taken together, the analysis of the Gβ and 4A9 complexes indicates that both antibodies selectively recognize the native GIRK1-Gβ complexes in atrial membranes. Accordingly, we can accurately identify receptor-induced changes in the GIRK1 protein environment using either Gβ- or 4A9-protein complexes.

The presence of two GIRK1 isoforms in the complexes isolated from crude membrane preparations, however, introduces ambiguity in the interpretation of our data, because the two forms may localize to different membrane compartments and associate with different protein partners. To verify that the oligomeric GIRK1 complexes are relevant to the physiological GIRK1/4 channel function, we further purified the atrial membranes to yield a fraction enriched in plasma membranes. This fraction contained the p65 form of GIRK1 only (Fig. 8, top left panel). We directly confirmed the identity of the p65 GIRK1 form by deglycosylation of the membrane proteins with N-glycosidase F, prior to electrophoresis. This treatment converted the p65 form of GIRK1 (Fig. 8, Load) into the core p54 form (Fig. 8, Loadβ). Neither M2R stimulation nor coactivation of M2 and α-adrenergic receptors triggered changes in the membrane density of the GIRK1/p65 protein (Fig. 8, top left panel, Load, lanes 1–3).

Next, we sought to purify the GIRK1/p65-Gβ oligomeric complexes and to determine the effect of receptor stimulation on their composition. The 4A9 antibodies do not recognize the glycosylated form of GIRK1 on Western blots (4). Furthermore, these antibodies exhibit very low affinity for the native GIRK1/p65-Gβ complexes and immunoprecipitate less than 2% of the GIRK1/p65 protein from the plasma membranes (data not shown). In contrast, the products of parallel immunoprecipitations using Gβ antibodies contained ~25% of the GIRK1/p65 protein (Fig. 8, Load, lanes 1–3 versus lanes 7–9; IP, anti-Gβ). The specificity of Gβ association with GIRK1/p65 protein was further confirmed by the absence of other Gβγ binding proteins, Gaα, Gaα, adenylyl cyclase, PLC β1, PLC β2, PI 3-kinase, and β-arrestin, in the Gβ-protein complexes. We therefore used the Gβ antibodies for immunopurification of the oligomeric GIRK1/p65 complexes from the
FIGURE 8. Effect of receptor stimulation on the composition of GIRK1/p65 signaling complexes. Left panel, top, atrial tissue was treated with either a vehicle for the ligand (denoted C) or 1 μM ACh (denoted A) or with a combination of 1 μM ACh and 1 μM phenylephrine (denoted P). Equal amounts of plasma membrane proteins were incubated with equal amounts of Dynal beads coated with either Gβγ/H11005 or IgG as a negative control. The immunoprecipitation products were collected, and the components were separated by 11% SDS-PAGE. Plasma membrane proteins were run on the same gel (lanes 1–3, Load). The products from the immunoprecipitations (IP) using normal mouse IgG were loaded in lanes 4–6; and the Gβγ-immunocomplexes were loaded in lanes 7–9. The segments probed for Goα, actin, PP1, Gβγ, and IgG contain 5% of each membrane lysate used for immunoprecipitation, whereas the segments probed for GIRK1 contain 20% of the lysates before (Load) and after (Load*) treatment with endo-N-glycosidase F. Proteins were transferred to a polyvinylidene difluoride membrane, and sections were probed with the antibodies indicated to the left (Blot). Bar graphs, stoichiometry of GIRK1/p65 complexes. The Gβγ immunoprecipitation products of four independent experiments were analyzed; the protein levels of GIRK1, Gβγ, actin, and PP1 were quantified and normalized to the level of GIRK1 (p65) protein in the oligomeric complexes. The relative changes in the protein levels of Gβγ, actin and PP1 are shown: Goα/GIRK1 (left panel, bottom), [Gβγ/GIRK1]ACh = 1.67 ± 0.20 (p < 0.05), and [Gβγ/GIRK1]ACh+Phe = 1.48 ± 0.10 (p < 0.05); [Actin]/[GIRK1]ACh+Phe = 1.48 ± 0.10 (p < 0.05); [PP1]/[GIRK1]ACh = 1.94 ± 0.27 (p < 0.05), and [PP1]/[GIRK1]ACh+Phe = 1.01 ± 0.14.

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atrial plasma membranes. Once again, analysis by immunoblotting revealed receptor-dependent changes in the composition and stoichiometry of multimeric GIRK1 complexes (Fig. 8, bar graphs). We found a significant increase in Gβγ association with GIRK1/p65 protein 5 min after M2R stimulation (1.67 ± 0.20-fold, n = 4, p < 0.05; Fig. 8, bottom left). This increase, however, could be blocked 5 min after concurrent stimulation of M2 and α-adrenergic receptors (0.89 ± 0.14 of the control, n = 4; Fig. 8, bottom left). Additionally, we found a significant increase in actin association with the GIRK1/p65-Gβγ complexes after M2R stimulation (1.50 ± 0.12-fold, n = 4, p < 0.05), and this increase was not eliminated by the simultaneous activation of M2 and α-adrenergic receptors (1.48 ± 0.10-fold, n = 4, p < 0.05; Fig. 8, top right). Finally, the profile of PP1 association with the multimeric complexes (Fig. 8, bottom right) followed the pattern observed for the Gβγ protein. The level of PP1 was increased by 1.94 ± 0.27-fold (n = 4, p < 0.05) after M2R activation; however, it was unaffected (1.01 ± 0.14 of the control, n = 4) after concurrent stimulation of M2 and α-adrenergic receptors.

Because direct interactions between Goα and the neuronal GIRK channels, as well as between Goα and the heterologously expressed GIRK1 and GIRK4 subunits, have been recently shown (33, 34), we investigated whether Goα associates with the GIRK1-Gβγ complex following α-AR stimulation. Goα was detected as a single band of 42 kDa in the atrial membranes. Immunoblotting of the GIRK1-Gβγ complexes for Goα did not detect Goα association with the oligomeric complexes (Fig. 8, top left). The existence of Goα-GIRK complexes in the atrial membrane, however, cannot be entirely ruled out, because some protein interactions could have been disrupted by the detergent solubilization of the membrane proteins.

In Vitro Kinetic Assay for Monitoring Changes in GIRK1 Oligomeric Complexes—Altogether, our data indicate that receptor stimulation triggers changes in the signaling environment of GIRK channels, and these changes can be accurately monitored by analysis of GIRK1-Gβγ complexes. This analysis, however, provided only a static picture of the events following GPCR stimulation. In addition, electrophysiological recordings of the GIRK channel activity have shown that the channel open probability approaches zero in the absence of ACh in the pipette (data not shown). On the contrary, our biochemical experiments revealed a significant association between Gβγ and GIRK1, even when the oligomeric complexes were immunopurified from control atrial tissue in the absence of receptor stimulation. This apparent discrepancy between the control electrophysiological and biochemical experiments can be attributed to a detergent-induced release of Gβγβ from the heterotrimeric G proteins along the steps of membrane solubilization and immunoprecipitation, even in the absence of a receptor agonist. We therefore sought to develop a biochemical assay, which would allow us to monitor dynamic changes in the composition of GIRK1-Gβγ complexes regardless of receptor-independent generation of Gβγβ. We hypothesized that a population of GIRK1-Gβγ complexes, formed under equilibrium conditions, can be captured on magnetic beads and then modified by brief application of receptor agonists or antagonists for different GPCRs present in the reac-
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![Dynamic regulation of the composition of GIRK1-Gβ signaling complexes](image)

FIGURE 9. Dynamic regulation of the composition of GIRK1-Gβ signaling complexes. A, beads coated with Gβ-specific antibodies were incubated with solubilized membrane proteins from control atria for 2 h at 4 °C. At the end of the incubation, the solubilized atrial membranes were supplemented either with a vehicle for the agonists (1, Control) or a mixture of 40 μM Mg-ATP, 1 μM GTP, and 1 μM propranolol (2, Mg-ATP + GTP) for 20 min at 4 °C, or a combination of 1 μM ACh and 1 μM phenylephrine (Phe) for 10 min at 4 °C in the presence of Mg-ATP, GTP, and propranolol (3, ACh + Phe). The Dynal beads are depicted as brown spheres. By analogy with the model of GIRK channel gating (11), the GIRK1/4 channels are shown as gray tetramers with four identical Gβγ sensors (red ovals), which undergo hypothetical conformational changes upon Gβγ (black propellers) binding. For simplicity, each Dynal bead is depicted with a cargo of a single Gβ-specific antibody and a single GIRK1/4 channel-Gβγ complex bound to it. At the end of each reaction, the GIRK1-Gβ complexes were quickly separated from the rest of the membrane proteins and washed, and their composition was determined by immunoblotting (A, lanes 7–9). Lanes 1–3, control membrane lysate incubated with vehicle for the agonists (A, 1); a mixture of Mg-ATP, GTP and propranolol (A, 2); or ACh + phenylephrine (A, 3). The segment probed for actin, PP1, and Gβ contains 5% of the membrane lysates used for immunoprecipitation, whereas the segment probed for GIRK1 contains 20% of these lysates. The products from three parallel immunoprecipitations using Dynal beads coated with normal mouse IgG were treated in the same manner and loaded in lanes 4–6. B, GIRK1-Gβ complexes were bound under equilibrium conditions to Dynal beads coated with Gβ-specific antibodies in five identical and parallel reactions, and the solubilized membranes were then treated with a mixture of 1 μM ACh and 1 μM phenylephrine for different time intervals (1, 2, 5, 10, and 20 min, respectively) in the presence of Mg-ATP, GTP, and propranolol. At the end of each reaction, the GIRK1-Gβ complexes were separated by magnetic immunoprecipitation from the remaining membrane proteins. The resulting oligomeric complexes were analyzed by immunoblotting for the presence of GIRK1, Gβ, actin, and PP1 (top panel). Lanes 1–5 correspond to 1, 2, 5, 10, and 20 min of αAR and M2R stimulation. The intensities of the protein bands were quantified as before; however, the relative amount of each protein was normalized to the level of the same protein in the GIRK1-Gβ complexes obtained after 1 min of concurrent stimulation of M₂ and α-adrenergic receptors (instead of the control complexes) for consistency with the electrophysiological experiments illustrated in Fig. 3. After a 10-min stimulation of M₂ and α-adrenergic receptors, the relative amount of actin associated with GIRK1-Gβ complexes was 85.3 ± 4.5% of the amount associated after a 1-min stimulation (middle panel, n = 4), whereas the relative amount of PP1 was only 44.7 ± 3.6% (bottom panel, circles, n = 4), and the relative amount of Gβ was 50.0 ± 5.3% (bottom panel, gray bars, n = 4).

In our experiments confirmed the premise that the receptor activation modulates the composition of the preformed GIRK1-Gβ complexes. Furthermore, we revealed features of this modulation that are independent of the integrity of the cellular structures and can be studied in a preparation of isolated membrane proteins.

Having established the feasibility of the new assay, we used the same approach to monitor the temporal pattern of Gβ and PP1 dissociation from GIRK1 after concurrent stimulation of M₂ and α-adrenergic receptors (Fig. 9B). Once again, the
GIRK1-Gβ complexes were bound to the Dynal beads under equilibrium conditions in five identical and parallel reactions, and the solubilized membranes were then treated with a mixture of 1 μM ACh and 1 μM phenylephrine for different time intervals (1, 2, 5, 10, and 20 min, respectively) in the presence of Mg-ATP, GTP, and propranolol. At the end of each reaction, the GIRK1-Gβ complexes were segregated by magnetic immunoprecipitation from the remaining membrane proteins. The resulting oligomeric complexes were then analyzed by immunoblotting for the presence of GIRK1, Gβ, actin, and PP1 (Fig. 9B, top). The results from the analysis of four independent experiments are illustrated in the middle and bottom panels of Fig. 9B. In these series of experiments, the intensities of the protein bands were quantified as before; however, the relative amount of each protein was normalized to the level of the same protein bands were quantified as before; however, the relative amount of each protein was normalized to the level of the same protein from the atrial membrane lysates, and the resulting immunoprecipitates were subjected to SDS-PAGE. The relative amount of M2Rs in atria treated for 5 min with 1 μM ACh alone was 81.5 ± 1.7% of the control (n = 3). In contrast, the amount of M2Rs in atria incubated for 5 min with 1 μM phenylephrine and then treated for 5 additional min with a combination of 1 μM ACh and 1 μM phenylephrine (Phe) (lane 3), and membrane lysates were incubated with Dynal beads coated with antibodies against M2R (Oncogene). The immunoprecipitation products were probed with antibodies against M2R (Alomone Labs) and mouse IgG L-chains (Blot). The protein levels were quantified and normalized to the level of IgG L-chains in the immunoprecipitation product. The relative changes in M2R protein levels determined from three independent experiments are averaged and shown in the bar graph.

The relative amount of M2Rs in atria treated for 5 min with 1 μM ACh alone was 81.5 ± 1.7% of the control (n = 3). In contrast, the amount of M2Rs in atria incubated for 5 min with 1 μM phenylephrine and then treated for 5 additional min with a combination of 1 μM ACh and 1 μM phenylephrine (Phe) (lane 3), and membrane lysates were incubated with Dynal beads coated with antibodies against M2R (Oncogene). The immunoprecipitation products were probed with antibodies against M2R (Alomone Labs) and mouse IgG L-chains (Blot). The protein levels were quantified and normalized to the level of IgG L-chains in the immunoprecipitation product. The relative changes in M2R protein levels determined from three independent experiments are averaged and shown in the bar graph.

Finally, we examined the potential role of M2Rs in atrial myocytes. Channel inhibition, however, is not static. At a mechanistic level, channel inhibition is brought about by a

DISCUSSION

We have established the ability of GIRK1/4 channels to coordinate the processing of α-adrenergic and cholinergic signals in the membrane of atrial myocytes. Functionally, this coordination is manifested as inhibition of GIRK1/4 channel activity upon concurrent stimulation of α-adrenergic and M2 receptors in the myocytes. Channel inhibition, however, is not static. Although the initial GIRK response is comparable with the response evoked by activation of M2Rs alone, channel activity quickly declines when α-ARs and M2Rs are activated together. At a mechanistic level, channel inhibition is brought about by a
combination of diminished probability of Gβγ binding to the channel and increased channel desensitization. Furthermore, the dynamic modulation of channel function is accompanied by changes in the composition of GIRK1 oligomeric complexes. Such plasticity of the channel environment together with PIP<sub>2</sub> depletion and PKC activation might be paramount in the coordination of α-adrenergic and cholinergic signals in the membrane. These different aspects of our findings will be discussed below.

Initially, we examined whether the gating properties of the M2R-activated GIRK1/4 channels differ from those of Gβγ-activated channels. The heterogeneity of GIRK channel gating requires new methods for interpretation of single-channel data. We therefore developed a quantitative approach for classification of channel behavior in functionally distinct modes (10, 11). We first asked, “How long a GIRK channel resides in a particular functional mode before switching to another?” To answer this question, we analyzed the bursting behavior of GIRK channels. Our analysis indicated that the mean burst duration is ~400 ms, and this duration is independent of the gating pattern (10). Accordingly, the recordings were divided into consecutive, 400-ms intervals, and the channel frequency of openings, f, and the open probability, p<sub>o</sub>, were calculated within these intervals. The f and p<sub>o</sub> values were then compiled to generate f and p<sub>o</sub> histograms. Because each gating mode contributes a single kinetic component to these histograms, the histogram analysis reveals the number of functional modes accessible to the channel. Additionally, the f and p<sub>o</sub> histograms provide the means to quantify the equilibrium among different modes (11). Upon Gβγ activation, our analysis identified the presence of four functional modes accessible to the GIRK1/4 channels. These four modes differ in their open probability and characteristic frequency of gating. More importantly, the concentration dependence of the modal equilibrium is consistent with a simple model of Gβγ binding to the channel. In this model, the incremental occupancy of four equivalent and independent Gβγ sensors in the tetrameric channel structure confers the four functional modes, corresponding to singly, doubly, triply and quadruply Gβγ-occupied GIRK1/4 channels (11). Accordingly, the probability of observing each gating mode would be a function only of the probability of Gβγ binding to one of the four Gβγ sensors, P, and this probability can be determined from analysis of modal equilibrium in each experiment. Here, we used the same approach to analyze the gating of M2R-activated GIRK1/4 channels and to probe the effects of α-ARs on the channel function. Our analysis revealed that the basic properties of Gβγ activation of GIRK1/4 channels are conserved after M2R stimulation. Thus, receptor activation partitions the channels among four functional modes with characteristic frequencies comparable with those of Gβγ-activated channels. Furthermore, the equilibrium probability (or relative occupancy) of different modes is binomially distributed as predicted by the model entertained previously for Gβγ activation of the channel. The equivalence between the M2R and Gβγ activation of GIRK channels yields an important insight into GIRK channel regulation; it indicates that M2R activation neither affects the equivalence of the four putative Gβγ sensors in the channel nor obstructs Gβγ binding to these sensors.

The molecular mechanisms underlying the heterogeneous GIRK behavior might be much more convoluted. Yet the manifestation of these mechanisms in the form of equilibrium between four functional modes of the channel creates a powerful framework for quantification and interpretation of heterogeneous single-channel behavior. Moreover, the analysis of the gating equilibrium allowed us to determine the probability of Gβγ binding to the channel, P, in the intact myocytes following M2R activation and to evaluate the impact of α-ARs on this probability. In phenylephrine-treated myocytes, the fraction of GIRK1/4 channels residing in the high efficient modes 3 and 4 was substantially lower than that in control myocytes. Thus, the mean probability of Gβγ binding to the channel declined ~2-fold in the phenylephrine-treated myocytes (from P ~ 0.55 to P ~ 0.23). Concordantly, the fraction of channels residing in nonconducting states increased ~2-fold beyond the predicted fraction of channels with no bound Gβγ. Such dual modulation of GIRK1/4 channels can be attributed to several parallel cellular processes triggered by stimulation of α-ARs in atrial myocytes. These receptors activate phospholipase C, which uses PIP<sub>2</sub> as a substrate to generate diacylglycerol and inositol 1,4,5-trisphosphate, thus depleting membrane PIP<sub>2</sub>. In parallel, accumulation of diacylglycerol promotes activation of PKC. The G<sub>q</sub>-mediated inhibition of GIRK channels arises essentially from the PIP<sub>2</sub> depletion (16–20, 24, 25) and PKC activation (4, 21–23), although G<i>α</i><sub>q</sub> subunits might also contribute directly to this phenomenon (16, 33, 34). Furthermore, Keselman et al. (36) have recently shown that PIP<sub>2</sub> depletion and PKC activation positively reinforced one another in their inhibition of GIRK channels. Numerous studies from different groups, however, have reported conflicting findings on the relative contributions of PIP<sub>2</sub> depletion and PKC activation to channel inhibition (reviewed in Refs. 23 and 36). Such diverse findings might be attributed to differences in the experimental conditions employed in the different studies. Alternatively, the mode of operation in G<sub>q</sub>,PCR-mediated inhibition of GIRK channels might depend on the highly organized GIRK1 complexes, which maintain cell-specific order among the diverse signaling components. To do so the GIRK1 complexes should have a dynamic nature and change their composition upon receptor stimulation. We therefore probed the composition of GIRK1 complexes in a series of biochemical experiments, which closely replicated the electrophysiological experiments. These experiments produced several interesting observations. First, the propensity of Gβ to associate with GIRK1 complexes (Fig. 9B) correlates remarkably well with the probability of Gβγ binding, derived from the analysis of channel gating (Fig. 6C). This observation independently confirms that modal behavior of GIRK channels reflects the interactions between the channels and Gβγ. Hence, the analysis of gating equilibrium can be reliably used for quantification of these interactions. More importantly, our biochemical experiments revealed receptor- and time-dependent differences in the association of actin and PIP1 with GIRK1-Gβ complexes. The increased association of actin with these complexes upon M2R activation is particularly interesting in the light of a recent study by Cho et al. (25), which implicates the integrity of actin cytoskeleton in the control of the membrane mobility of PIP<sub>2</sub>. Therefore, future
studies will investigate whether changes in the protein levels of actin in GIRK1 complexes would affect PIP$_2$ sensitivity of the GIRK channels. Even more importantly, our studies revealed that the temporal pattern of PP1 association with GIRK1-G$eta$ complexes closely resembles the association profile of G$eta$ with GIRK1/4 channels. Although the precise role of such protein choreography in the regulation of the channel function is not known, the studies presented here demonstrate for the first time a dynamic link between channel signaling environment and function. In addition, our studies launch several new areas of inquiry. What is the temporal sequence of association and dissociation of different protein kinases with the GIRK1 complexes? Which events orchestrate the dynamic modulation of GIRK1/4 channel function by PIP$_2$ and which events favor PKC-plexes? Which events orchestrate the dynamic modulation of the channel function? In addition, our studies launch several new areas of inquiry. What is the temporal sequence of association and dissociation of different protein kinases with the GIRK1 complexes? Which events orchestrate the dynamic modulation of GIRK1/4 channel function by PIP$_2$ and which events favor PKC-plexes? Which events orchestrate the dynamic modulation of the channel function? In addition, our studies launch several new areas of inquiry. What is the temporal sequence of association and dissociation of different protein kinases with the GIRK1 complexes? Which events orchestrate the dynamic modulation of GIRK1/4 channel function by PIP$_2$ and which events favor PKC-plexes? Which events orchestrate the dynamic modulation of the channel function?

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