Muscarinic K⁺ Channel in the Heart

Modal Regulation by G Protein βγ Subunits

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ABSTRACT The membrane-delimited activation of muscarinic K⁺ channels by G protein βγ subunits plays a prominent role in the inhibitory synaptic transmission in the heart. These channels are thought to be heterotetramers comprised of two homologous subunits, GIRK1 and CIR, both members of the family of inwardly rectifying K⁺ channels. Here, we demonstrate that muscarinic K⁺ channels in neonatal rat atrial myocytes exhibit four distinct gating modes. In intact myocytes, after muscarinic receptor activation, the different gating modes were distinguished by differences in both the frequency of channel opening and the mean open time of the channel, which accounted for a 76-fold increase in channel open probability from mode 1 to mode 4. Because of the tetrameric architecture of the channel, the hypothesis that each of the four gating modes reflects binding of a different number of Gβγ subunits to the channel was tested, using recombinant Gβ1γ5. Gβ1γ5 was able to control the equilibrium between the four gating modes of the channel in a manner consistent with binding of Gβγ to four equivalent and independent sites in the protein complex. Surprisingly, however, Gβ1γ5 lacked the ability to stabilize the long open state of the channel that is responsible for the augmentation of the mean open time in modes 3 and 4 after muscarinic receptor stimulation. The modal regulation of muscarinic K⁺ channel gating by Gβγ provides the atrial cells with at least two major advantages: the ability to filter out small inputs from multiple membrane receptors and yet the ability to control the mean open time of the channel with great precision.

KEY WORDS: signal transduction • guanine triphosphate binding proteins • muscarinic receptor • atrial myocytes

INTRODUCTION

The cardiac muscarinic inward rectifier potassium channels (KAC₃ channels) are responsible for the acetylcholine- (ACh)¹ and adenosine-induced deceleration of the heart rate and atrioventricular conduction. When either mACh₄-muscarinic cholinergic or A₁-purinergic receptors are activated in cardiac atrial myocytes, they interact with heterotrimeric regulatory GTP-binding proteins (G proteins), promoting dissociation of the G proteins into a Ga-GTP subunit and a Gβγ complex. Once dissociated, both the Ga-GTP and Gβγ subunits proceed to regulate several effectors in the myocytes (Clapham and Neer, 1993). It is now well recognized that the Gβγ dimers confer the activation of the KAC₃ channels (Reuveny et al., 1994; Wickman et al., 1994). At the same time, the Ga subunits are thought to interact with additional signaling constituents, the regulators of G protein signaling (RGS proteins), that stimulate the intrinsic GTPase activity of the Ga subunit, leading to rapid reassociation of the G protein heterotrimer and termination of the signal (Doupnik et al., 1997; Saitoh et al., 1997).

Several members of an expanding family of structurally related G protein–activated inward rectifiers (GIRKs) have been recently identified (Dascal et al., 1993; Kubo et al., 1993; Lesage et al., 1994; Doupnik et al., 1995). The cardiac KAC₃ channels consist of two homologous subunits, GIRK1 and GIRK4 (also known as CIR) (Krapivinsky et al., 1995; Wickman et al., 1998), that are thought to form tetrameric structures with a (GIRK1)₂(GIRK4)₂ stoichiometry (Silverman et al., 1996; Tucker et al., 1996). Both GIRK1 and GIRK4 subunits are composed of two putative transmembrane domains, flanked by large hydrophilic NH₂- and COOH-terminal domains residing within the cell. These NH₂- and COOH-terminal regions of GIRK1 and GIRK4 are thought to be involved in Gβγ interactions to mediate channel activation (Takao et al., 1994; Dascal et al., 1995; Huang et al., 1995; Kunkel and Peralta, 1995; Huang et al., 1997).

While the structural domains involved in the Gβγ binding have been identified, the molecular mechanisms directing KAC₃ channel gating upon Gβγ binding remain enigmatic. Previous electrophysiological studies

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¹Abbreviations used in this paper: ACh, acetylcholine; GIRK, G protein–activated inward rectifiers.
have shown that the native cardiac \( K_{AC} \) channels exhibit sigmoidal stimulus–response curves, suggesting cooperative binding of three or four G\( \beta \gamma \) subunits to a single channel molecule (Ito et al., 1991, 1992). It has been further postulated that the function of the \( K_{AC} \) channels is controlled by two independent mechanisms: a G protein–independent fast gating of the channel and a G protein–dependent slow transition from an unavailable to an available \( K_{AC} \) channel state (Hosoya et al., 1996). Another intriguing possibility, however, is that G\( \beta \gamma \) subunits may control the function of the \( K_{AC} \) channel by governing the equilibrium between several functional modes of the channel. In fact, we have recently shown that multiple gating modes of the \( K_{AC} \) channel do exist in the atrial myocytes of the bullfrog \( Rana catesbeiana \) (Ivanova-Nikolova and Breitwieser, 1997). Yet it remains to be determined whether the modal regulation of the \( K_{AC} \) channel is evolutionarily conserved and, if so, how G\( \beta \gamma \) subunits control the balance between the different functional states of the channel in mammalian systems.

To address these questions, we studied the interactions of G\( \beta \gamma \) with the native \( K_{AC} \) channels in atrial myocytes of the neonatal rat heart. First, we examined the \( K_{AC} \) channel gating upon activation of muscarinic receptors in the atrial myocytes and identified the presence of four distinct patterns of \( K_{AC} \) channel gating. The different gating modes were characterized by differences in both the frequency of channel opening and the mean open time of the channel, which accounted for a 76-fold increase in channel open probability from mode 1 to mode 4. Further, to reveal the mechanisms underlying modal behavior of the \( K_{AC} \) channel, we reconstructed channel activation in excised membrane patches using purified recombinant G\( \beta \gamma \). Surprisingly, in the presence of G\( \beta \gamma \) alone, the four gating modes of the \( K_{AC} \) channel differed only in the frequency of channel openings, while the mean open time of the channel remained the same. Finally, the analysis of the equilibrium among the functional modes of the channel at different G\( \beta \gamma \) concentrations demonstrated that the four gating modes reflected binding of a different number of G\( \beta \gamma \) subunits to four equivalent and independent sites in the channel protein complex.

**Materials and Methods**

**Isolation and Cell Culture of Neonatal Cardiac Myocytes**

Viable atrial myocytes were obtained from 1–2-d-old Sprague Dawley rats by a trypsin/chymotrypsin/elastase dissociation procedure as described previously (Foster et al., 1990). The dissociated cells were centrifuged through Percoll step gradients to obtain cell preparations consisting of >94% myocytes. The myocytes were suspended in Modified Eagle’s Medium containing 5% newborn calf serum and 0.1 mM 5-bromo-2′-deoxyuridine and were plated at a density of \( 10^5 \) cells/cm\(^2\) on cover slips pre-coated with 0.1% gelatin. After overnight incubation, the cells were washed to remove nonadherent cells and cultured in a defined serum-free media for four additional days (Hansen et al., 1994).

**Expression and Purification of G Protein Subunits**

Procedures for construction and selection of recombinant Baculoviruses encoding various G subunits of the G proteins have been described previously by Iniguez-Lluhi et al. (1992). cDNA containing the entire coding region for a particular subunit was transferred to the baculovirus expression vectors pVL1392 and pVL1393. Recombinant viruses were generated by cotransfection of *Spodoptera frugiperda* (SF9) insect cells with the recombinant pVL1392 and pVL1393 transfer vectors along with mutant *Autographa californica* nuclear polyhedrosis virus, as described by the supplier (PharMingen, San Diego, CA). All recombinant viruses were plaque purified and were verified by their ability to direct the expression of the appropriate proteins, as detected by immunoblotting. G\( \alpha \), G\( \beta \gamma \) heterotrimers containing a hexahistidine (H\( \delta \)) tag inserted into the G\( \alpha \) were expressed in SF9 cells as described (Kozasa and Gilman, 1995). H\( \delta \)-tagged heterotrimers were solubilized by 0.5% Lubrol and purified by adsorption to an H\( \delta \)-affinity nickel column. The G\( \beta \gamma \) dimers were then selectively eluted from the column using a HEPES buffer (pH 8) containing 0.03 mM AlCl\( _3 \), 50 mM MgCl\( \_2 \), and 10 mM NaF. The elution of the G\( \beta \gamma \) complex was monitored by immunoblotting, using G\( \beta \) and G\( \gamma \)–specific antibodies. The G\( \beta \gamma \) dimers were further purified to homogeneity using Fast Protein Liquid Chromatography Mono Q column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Purity of the final product was determined by sodium dodecyl sulfate (SDS)-PAGE and silver staining before use.

**Electrophysiology**

Single-channel currents through \( K_{AC} \) channels were recorded from cell-attached and inside-out patches of atrial myocytes using standard high resolution patch-clamp method (Hamill et al., 1981). The membrane potential was zeroed with the following bath solution (mM): 150 KCl, 5 EGTA, 5 glucose, 1.6 MgCl\( \_2 \), 5 HEPES, pH 7.4. Pipette solution contained (mM): 150 KCl, 1 CaCl\( \_2 \), 1.6 MgCl\( \_2 \), 5 HEPES, pH 7.4. Patch pipettes were made from borosilicate glass (World Precision Instruments, Inc., Sarasota, FL) on a Flaming Brown micropipette puller (Sutter Instruments, Co., Novato, CA) and firepolished on a microforge (Narishige Scientific Instrument Lab., Tokyo, Japan). The resistance of the patch pipettes (when filled with pipette solution) was 10–20 MOhm. Either acetylcholine or adenosine was added to the pipette solution to activate m\(_2\)-muscarinic or A\(_1\)-purinergic receptors, respectively. Currents were recorded with a Patch Clamp List-Medical EPC-7 amplifier (ALC Scientific Instruments Inc., Westbury, NY), additionally filtered at 2 kHz with an eight-pole Bessel filter (Frequency Devices Inc., Haverhill, MA) and the acquired data was stored on the hard disk of a computer. Data acquisition and analysis were performed using Digidata 1200 (Axon Instruments, Foster City, CA) supported by version 6.0 of pCLAMP software (Axon Instruments). Channel opening and closing transitions were identified with the half-amplitude threshold-old-crossing algorithm. Idealized records of the amplitudes, and channel open and close times, were generated by the FETCHAN Events List function. To determine the residence time of the \( K_{AC} \) channel in the different gating modes, the continuous records were divided into consecutive segments, the frequency of apparent openings, \( f \), was calculated for each segment and \( f \) histograms were generated for further analysis.
RESULTS

Evidence for the Presence of Different Functional States of the $K_{ACh}$ Channels in the Neonatal Rat Cardiac Myocytes

We have previously demonstrated that upon activation of endogenous G proteins in frog atrial myocytes, the $K_{ACh}$ channels exhibit bursting behavior, shifting between three predominant patterns of gating. They were termed low, medium, and high frequency modes, according to the frequency of channel openings during individual bursts (Ivanova-Nikolova and Breitwieser, 1997). Under basal conditions, infrequent, agonist-independent channel openings display exclusively a low frequency behavior, while a maximal activation of G proteins by GTP$_{gS}$ favors the high frequency behavior of the $K_{ACh}$ channels. To test whether such a multistep activation of the $K_{ACh}$ channel has been evolutionarily conserved as a signal transduction mechanism, we examined the gating behavior of $K_{ACh}$ channels after m$_2$-receptor stimulation in rat neonatal atrial myocytes.

The individual $K_{ACh}$ channels, activated in cell-attached configuration by 1 $\mu$M ACh, differed considerably in their gating behavior. One gating pattern was characterized by infrequent channel openings separated by long silent intervals, while a second one was dominated by the apparent clustering of openings into long bursts of activity. Fig. 1 provides an illustration of the differences between these patterns of gating. First, a comparison of the all-points histograms unveiled an order of magnitude difference in channel open probability, $P_o$. Second, a comparison of the open time distributions revealed a correlation between the proportion of brief and long $K_{ACh}$ channel openings and the pattern of gating. In 10 cell-attached patches selected for analysis (no recordings with superimposing openings were accepted for this or the following types of analysis), a sum of two exponentials provided an adequate fit to the open time distributions. In addition, the time constants of both fast and slow exponential components were similar for different $K_{ACh}$ channels and had values of $\sim$1 and $\sim$7 ms, respectively. The fractions of the histograms fitted by each of the two components, however, were different for the channels exhibiting the patterns of activity illustrated in Fig. 1, A and B. Thus, the changes in the kinetic behavior of the $K_{ACh}$ channels were associated with both a change in the open probability and a shift from a brief open state to a long open state of the channel. These two observations provided a basis for classification of the $K_{ACh}$ channel gating into functionally distinct modes. In bullfrog atrial myocytes, such classification was entirely based on analysis of channel gating within well defined individual bursts, assuming that each burst reflected a single event of interaction between the ion channel and G$\beta$. In rat neonatal atrial myocytes, however, similar unambiguous division of the single channel records into bursts was not

$$\text{Figure 1. Heterogeneity of } \text{K}_{ACh} \text{ channel gating in } \text{neonatal rat atrial myocytes. Two examples of single-channel activity recorded from cell-attached patches in the presence of } 1 \text{ } \mu \text{M ACh are shown in A and B to illustrate the kinetic heterogeneity of channel gating encountered in our experiments. The membrane potential of the patch was held at } -90 \text{ mV in both cases. In this and all figures, the downward deflections correspond to inward currents. All-points and open-time histograms generated from the data shown in A and B are presented on the right. The all-points histograms were fitted by the sum of two Gaussian functions to determine the channel open probability, } P_o. \text{ The } P_o \text{ value obtained from the } 204 \text{ s record is } 0.015 \text{ in A and } 0.297 \text{ in B. The open time distributions were fitted by the sum of two exponential components. The time constants of the fast and slow components are } \tau_{a1} = 1.56 \text{ ms (83.6%)} \text{ and } \tau_{a2} = 7.50 \text{ ms (16.4%)} \text{ in A and } \tau_{a1} = 1.22 \text{ ms (52.1%)} \text{ and } \tau_{a2} = 7.54 \text{ ms (47.9%)} \text{ in B. The time constants of the fast and slow components showed little variations from one experiment to another; however, the relative areas under the individual exponents were different.}$$
always possible. Therefore, we used a different approach for the classification of heterogeneous channel behavior into discrete gating modes. The continuous recordings were divided into consecutive, equally spaced time intervals and the channel behavior was assessed within these intervals. A duration of 400 ms was selected for the time intervals in this analysis based on the burst duration determined for the K\textsubscript{\textalpha}{\textalpha}Ch channels exhibiting well-delineated bursting behavior. For each 400-ms data segment, the frequency of openings, \( f \), and the probability of the channel being open, \( P_\alpha \), were calculated from the events list files and plotted vs. time. Fig. 2 illustrates this approach, using the two segments of K\textsubscript{\textalpha}{\textalpha}Ch channel recordings shown in Fig. 1. Visual inspection of a large number of \( f \) and \( P_\alpha \) plots verified that the selected time interval provided an adequate representation of the fluctuations in the K\textsubscript{\textalpha}{\textalpha}Ch channel gating. Similar plots were generated from each of the 10 cell-attached recordings selected for analysis (total of 30 min of single-channel data). The \( f \) and \( P_\alpha \) values within each 400-ms data segment were used to calculate the mean open time of the channel, \( t_\text{open} \). The \( t_\text{open} \) values were further analyzed with regard to the frequency of openings to determine to what extent the changes in the frequency of gating could be correlated with changes in the mean open time of the K\textsubscript{\textalpha}{\textalpha}Ch channel. The resulting \( t_\text{open} - f \) plot, shown in Fig. 3A, revealed a multistep augmentation in the average \( t_\text{open} \) from 2.28 ± 0.10 ms at \( f = 2.5 \) Hz (\( n = 999 \) segments) to 6.24 ± 0.28 ms at frequencies above 47.5 Hz (\( n = 231 \) segments). Each statistically significant step in the \( t_\text{open} \) augmentation is indicated by an arrowhead in the \( t_\text{open} - f \) plot and presumably reflects a rather abrupt transition from one pattern of channel gating to another. The analysis of K\textsubscript{\textalpha}{\textalpha}Ch channel gating, outlined above, identified transitions between four functional modes accessible to the channel upon activation of muscarinic receptors. A histogram of the frequency of openings was also generated to estimate the relative occupancy of different modes (Fig. 3B).

Fig. 4 provides a direct comparison of several functional properties of the four K\textsubscript{\textalpha}{\textalpha}Ch channel gating modes in neonatal rat atrial myocytes. Taken together, the differences in the frequency of channel openings and in the average open times account for significant changes in the open probability of the channel from one mode to the next. Thus, the mean open probability was ~20- and ~76-fold higher in modes 3 and 4, respectively, when compared with the open probability in mode 1. Such differences in the open probability would certainly have profound effects on the contributions of individual modes to the total current. Accordingly, modes 3 and 4 together contributed ~80% of the total current, although the K\textsubscript{\textalpha}{\textalpha}Ch channels spent only 23% of their active time in these two modes.

These data indicate that the modal behavior of the K\textsubscript{\textalpha}{\textalpha}Ch channels in neonatal rat atrial myocytes is qualitatively similar to the one described by us in bullfrog atrial myocytes (Ivanova-Nikolova and Breitwieser, 1997). The different number of gating modes identified in the rat myocytes (four, vs. three in the bullfrog myocytes) can be attributed to the elimination of mode 1 from the data selected for analysis in the bullfrog myocytes. In that case, individual bursts were defined as series of openings separated by closed intervals shorter than

\[ \text{Figure 2. Approach for classification of the K\textsubscript{\textalpha}{\textalpha}Ch channel gating. Continuous single-channel recordings were divided into consecutive, 400-ms segments and the channel open probability and the frequency of openings were determined for each individual segment. Plots of open probability and frequency of gating vs. time, derived from the continuous K\textsubscript{\textalpha}{\textalpha}Ch channel records illustrated in Fig. 1, are shown for comparison in A and B. Expanded current traces from each of the two records (illustrating the first five 400-ms segments in which K\textsubscript{\textalpha}{\textalpha}Ch channel activity was present) are shown on the right to reveal the kinetic behavior of the channel in greater detail.} \]
some critical interval, usually between 120 and 140 ms. As a result, the singular events (separated by closed intervals longer than 140 ms) that delineate gating mode 1 were completely excluded from the analysis.

Regulation of Modal Behavior of the K<sub>ACH</sub> Channels by G Protein βγ Subunits

To understand the mechanisms underlying the modal behavior of the K<sub>ACH</sub> channel, we reconstructed channel activation in excised membrane patches using recombinant Gβγ. A major advantage of this approach over receptor-mediated activation of the K<sub>ACH</sub> channel is that it provides a strictly controlled environment, abolishing potential contributions of intermediate signaling molecules to the K<sub>ACH</sub> channel–Gβγ interactions. Since the composition of the Gβγ involved in the physiological regulation of the K<sub>ACH</sub> channel is unknown, we selected the Gβ<sub>1γ5</sub> isoform for our experiments, based on its relative abundance in the heart (Hansen et al., 1995). Gβ<sub>1γ5</sub> dimers, expressed and purified from Sf9 cells, as previously described by Kozasa and Gilman (1995), were applied to activate directly the K<sub>ACH</sub> channels in inside-out membrane patches excised from the rat atrial myocytes. Fig. 5 illustrates the activity recorded from the same K<sub>ACH</sub> channel in a cell-attached patch in the presence of 1 μM adenosine (A), upon patch excision in GTP-free solution (B), and after application of 0.6 and 1.5 nM Gβ<sub>1γ5</sub> (C and D, respectively). Nanomolar concentrations of Gβ<sub>1γ5</sub> activated the K<sub>ACH</sub> channels in a concentration-dependent manner similar to that reported by Wickman et al. (1994). The activation of the K<sub>ACH</sub> channels developed over the course of 2–11 min after Gβ<sub>1γ5</sub> application and was sustained during continued presence of Gβ<sub>1γ5</sub>. The gating behavior of the individual K<sub>ACH</sub> channels remained heterogeneous even under such strictly controlled conditions, suggesting that the heterogeneity in the channel gating is an intrinsic property of K<sub>ACH</sub> channel–Gβγ interactions.

To reveal the source of this heterogeneity, the K<sub>ACH</sub> channel gating in the presence of Gβ<sub>1γ5</sub> was subjected to the same analysis routine described for analysis of the data recorded upon muscarinic receptor stimulation. The unitary currents through K<sub>ACH</sub> channels activated by different concentrations of Gβ<sub>1γ5</sub> were recorded for 20–30 min after Gβ<sub>1γ5</sub> application and was sustained during continued presence of Gβ<sub>1γ5</sub>. The gating behavior of the individual K<sub>ACH</sub> channels remained heterogeneous even under such strictly controlled conditions, suggesting that the heterogeneity in the channel gating is an intrinsic property of K<sub>ACH</sub> channel–Gβγ interactions.

The comparison of the t<sub>open</sub>–f plots obtained at different Gβ<sub>1γ5</sub> concentrations (ranging from 0.15 to 12 nM) with the t<sub>open</sub>–f plot in Fig. 3 A revealed a key difference in the function of the K<sub>ACH</sub> channels activated by Gβ<sub>1γ5</sub> alone. While the mean open time of the receptor-activated channels underwent synchronized changes with the increase in the frequency of gating, the mean open time of Gβ<sub>1γ5</sub>-activated channels (t<sub>open</sub> = 1.86 ± 0.09

![Figure 3](image-url)

**Figure 3.** Segregation of K<sub>ACH</sub> channel behavior into distinct gating modes. (A) The mean open time of the channel, t<sub>open</sub>, is plotted as a function of the frequency of channel openings. f. The t<sub>open</sub> value within each 400-ms data segment was calculated from the f and P<sub>o</sub> values within the same segment. The t<sub>open</sub> estimates were compiled from a total of 30 min of single-channel data recorded from 10 different cell-attached patches in the presence of 1 μM ACh, and the mean t<sub>open</sub> value for a particular f population was determined. For the frequencies above 47.5 Hz, where the number of data segments was relatively small, the mean t<sub>open</sub> value for each frequency, f, was averaged over a 5-Hz interval (f ± 2.5 Hz). The arrowheads in the t<sub>open</sub>–f plot and arrowheads indicate the statistically significant augmentations in the mean t<sub>open</sub> value and presumably reflect the transitions from one pattern of K<sub>ACH</sub> channel gating to another. (B) Histogram of frequencies of openings generated from the same set of data shown in A.
ms, \( n = 10 \) was unaffected by the frequency of gating (Fig. 6A). Such a difference in the gating of receptor-activated and \( \mathrm{G}_{\beta_1 \gamma_5} \)-activated \( \mathrm{K}_{\mathrm{ACh}} \) channels suggests that a combination of molecular interactions might contribute to the phenomenon of modal behavior. Given the ability of GIRK1 subunits of the \( \mathrm{K}_{\mathrm{ACh}} \) channel to bind \( \mathrm{G}_\alpha \) to a binding site different from that for \( \mathrm{G}_{\beta\gamma} \) (Huang et al., 1995), one intriguing scenario is that binding of both \( \mathrm{G}_\alpha \) and \( \mathrm{G}_{\beta\gamma} \) to the \( \mathrm{K}_{\mathrm{ACh}} \) channel is necessary to achieve the augmentation of \( t_{\text{open}} \) found after muscarinic receptor activation. Alternatively, different subclasses of \( \mathrm{G}_{\beta\gamma} \) might exert different effects on the \( \mathrm{K}_{\mathrm{ACh}} \) channel regulation and the method outlined in the present work is sensitive enough to capture the distinctions between channel gating in the presence of \( \mathrm{G}_{\beta_1 \gamma_5} \) and in the presence of the unidentified, but hypothetically distinct, \( \mathrm{G}_{\beta\gamma} \) released upon muscarinic receptor stimulation. Further studies will be required to distinguish between these two possibilities.

At the same time, the comparison of the frequency distributions obtained at different \( \mathrm{G}_{\beta_1 \gamma_5} \) concentrations (Fig. 6B) revealed that the increases in \( \mathrm{G}_{\beta\gamma} \) concentrations are translated into increases in the frequency of \( \mathrm{K}_{\mathrm{ACh}} \) channel openings. These distributions should consist of a sum of geometric components, and the number of components should reflect the number of conducting conformations of the channel (Colquhoun and Hawkes, 1981, 1983). We therefore used the geometric components in the \( f \) histograms to classify the gating behavior of \( \mathrm{G}_{\beta_1 \gamma_5} \)-activated channels into distinct modes. In the presence of 0.15 nM \( \mathrm{G}_{\beta_1 \gamma_5} \), a single geometric component was sufficient to fit the \( f \) histograms, while a sum of up to four geometric components was required for the adequate fit of the data generated in the presence of higher \( \mathrm{G}_{\beta_1 \gamma_5} \) concentrations. The mean frequencies of the first through fourth components showed little variations from one \( \mathrm{K}_{\mathrm{ACh}} \) channel to another and averaged 2.2 ± 0.2 Hz (\( n = 24 \)), 12.7 ± 0.6 Hz (\( n = 20 \)), 33.6 ± 1.9 Hz (\( n = 18 \)), and 65.0 ± 4.2 Hz (\( n = 8 \)), respectively. These values are similar to the frequencies found for modes 1–4 of the receptor-activated \( \mathrm{K}_{\mathrm{ACh}} \) channels in the atrial myocytes (see Fig. 4 for comparison). Accordingly, with regard to the frequency of openings, the \( \mathrm{G}_{\beta_1 \gamma_5} \)-activated \( \mathrm{K}_{\mathrm{ACh}} \) channels behaved in a manner approaching that of the receptor-activated channels, converting between four functional modes.

On the basis of these results, we proposed that binding of a different number of \( \mathrm{G}_{\beta\gamma} \) subunits to four \( \mathrm{G}_{\beta\gamma} \)-binding sites in the tetrameric \( \mathrm{K}_{\mathrm{ACh}} \) channel structure gives rise to its four functional modes. For simplicity, we further assumed that the four \( \mathrm{G}_{\beta\gamma} \)-binding sites are functionally equivalent and independent. These assumptions imply that the four sites have the same binding affinity for \( \mathrm{G}_{\beta\gamma} \), and that \( \mathrm{G}_{\beta\gamma} \) binding to one site is not affected by the \( \mathrm{G}_{\beta\gamma} \) occupancy of the remaining sites. Such a simple model predicts that the probability of observing each gating mode is given by the binomial distribution \( \left[ \frac{N}{k}(N-k) \right] P^k(1-P)^{N-k} \), where \( N \) is the total number of \( \mathrm{G}_{\beta\gamma} \)-binding sites, \( k \) is the number of the occupied binding sites, and \( P \) is the probability that one of the four \( \mathrm{G}_{\beta\gamma} \)-binding sites is occupied. To test this prediction, we quantified the equilibrium among the four gating modes at different \( \mathrm{G}_{\beta\gamma} \) concentrations. The equilibrium probability (or relative occupancy) of different modes was estimated from the fraction of the total \( f \) histogram fit by the corresponding geometric component. In experiments using the same concentrations of \( \mathrm{G}_{\beta_1 \gamma_5} \), some variations in the relative occupancy of different modes were observed. These variations can be explained by differences in the probability of \( \mathrm{G}_{\beta\gamma} \) binding, \( P \), and can be attributed either to a different amount of \( \mathrm{G}_{\beta_1 \gamma_5} \) incorporated in the membrane or to a different binding affinity of the channel for \( \mathrm{G}_{\beta_1 \gamma_5} \). Therefore, in each experiment, the probability of \( \mathrm{G}_{\beta\gamma} \) binding was calculated from the fraction of the \( f \) histogram fit by the first geometric component and the relative occupancy of different gating modes.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Properties of the four gating modes of the m2 receptor-activated \( \mathrm{K}_{\mathrm{ACh}} \) channel. Three different parameters: mean open time (A), frequency of channel openings (B), and mean open probability (C) were evaluated with regard to modal behavior of the channel after muscarinic receptor stimulation. The values for the mean open time of each gating mode are: \( t_{\text{open}} = 2.28 \pm 0.10 \) ms (mode 1), \( t_{\text{open}} = 2.75 \pm 0.08 \) ms (mode 2), \( t_{\text{open}} = 3.53 \pm 0.19 \) ms (mode 3), and \( t_{\text{open}} = 6.24 \pm 0.38 \) ms (mode 4). The values for the frequency of channel openings are: \( f_1 = 2.5 \), \( f_2 = 10.3 \pm 0.1 \), \( f_3 = 31.7 \pm 0.3 \), and \( f_4 = 69.1 \pm 1.2 \) Hz. The values for the mean open probability are: \( P_1 = 0.0057 \pm 0.0003 \), \( P_2 = 0.0281 \pm 0.0010 \), \( P_3 = 0.1128 \pm 0.0065 \), and \( P_4 = 0.4341 \pm 0.0195 \). Data are mean ± SEM (\( n = 231–1,116 \)).
was examined as a function of Gβγ binding to the channel, rather than as a function of Gβγ concentration. The corresponding plot from this analysis is shown in Fig. 7 and verifies that the equilibrium probability of each mode is binomially distributed as predicted by the model. In this way, the analysis of the equilibrium among the four K_{ACh} channel functional states in the presence of Gβ_{1γs} predicts the existence of four equivalent and independent Gβγ binding sites in the channel structure.

It is interesting to note that in a small subset of Gβ_{1γs} experiments (3 of 27), the mean frequencies of gating for each mode deviated by a factor of ~2 from the values estimated for m_{2} receptor-activated K_{ACh} channels. In that case, the mean frequencies of the four modes were f_{1} = 3.7 ± 0.3, f_{2} = 21.4 ± 1.1, f_{5} = 54.7 ± 1.9, and f_{5} = 104.5 ± 5.6 Hz, while the conductance and the mean open time of these channels were similar to those determined for the rest of the Gβ_{1γs}-activated channels. Such differences in the channel gating, although infrequent, point to certain structural variability among native K_{ACh} channels in the heart. Evidently, a combination of K^+ channels susceptible to Gβγ regulation shapes the responsiveness of the atrial myocytes to G protein activation and the basic approach for classification of the K_{ACh} channel gating outlined in the present work creates a sensitive tool for capturing the functional variety among these channels.

![Figure 5](image-url)  
**Figure 5.** Activation of the K_{ACh} channels by recombinant Gβ_{1γs}. (A1) Representative single-channel activity recorded in cell-attached configuration from an atrial myocyte in the presence of 1 μM adenosine. The membrane potential was clamped at ~90 mV. (A2) Part of the trace in A1 (arrowhead) has been expanded to show the transitions between the closed and open states of the channel with a higher resolution. (B) Channel activity disappeared after the patch excision in GTP-free solution. The membrane potential continued to be clamped at ~90 mV for the entire experiment. (C–D) Application of nanomolar concentrations of Gβ_{1γs} (0.6 nM in C1 and 1.5 nM in D1) restored the channel activity in a concentration-dependent manner. Extended current traces starting at the points indicated by the arrowheads in C1 and D1 are shown on the right in C2 and D2, respectively.

![Figure 6](image-url)  
**Figure 6.** Modal classification of K_{ACh} channel gating in the presence of Gβ_{1γs}. (A) The t_{open}−/− plots were generated from the analysis of the data illustrated in Fig. 5, C and D. In the presence of Gβ_{1γs}, the mean open time of the K_{ACh} channel was independent of the frequency of channel gating and approached the t_{open} value estimated for gating mode 1 of receptor-activated channels. (B) Histograms of frequencies of openings are shown for the experiments illustrated in A. The histogram shown at left corresponds to Gβ_{1γs} concentration of 0.6 nM, whereas the histogram shown at right corresponds to Gβ_{1γs} concentration of 1.5 nM. Both histograms were fitted by the sum of three geometrics (Colquhoun and Hawkes, 1981): P(f) = \( \mu_{1} f^{-1} (1 - \mu_{1})^{-1} + \mu_{2} f^{-1} (1 - \mu_{2})^{-1} + \mu_{3} f^{-1} (1 - \mu_{3})^{-1} \) (continuous line). The mean frequency values and the relative areas of different components are \( \mu_{1} = 1.4 \) Hz (73.2%), \( \mu_{2} = 6.9 \) Hz (23.5%), and \( \mu_{3} = 17.6 \) Hz (3.3%) at 0.6 nM Gβ_{1γs}; and \( \mu_{1} = 1.5 \) Hz (54.9%), \( \mu_{2} = 12.6 \) Hz (35.5%), and \( \mu_{3} = 37.0 \) Hz (9.6%) at 1.5 nM Gβ_{1γs}. The proportion of high frequency data segments consistently increased with Gβ_{1γs} concentration.
Discussion

The results outlined in the present work characterize the mechanism of membrane-delimited activation of muscarinic K⁺ channels by Gβγ subunits in neonatal rat atrial myocytes and render an important clue to understanding the principles underlying the specificity of Gβγ-mediated signaling in general. These two aspects of our findings will be discussed below.

Mechanism of Gβγ Regulation of KₐCh Channels

Activation of KₐCh channels by acetylcholine and adenosine in sinoatrial nodal cells and atrial myocytes modulates both heart rate and cardiac contractility. The channel activation is brought about by a pertussis toxin–sensitive G protein (Breitwieser and Szabo, 1985; Pfaffinger et al., 1985) in a membrane-delimited manner (Soejima and Noma, 1984), using the G protein βγ subunits as direct information carriers between the membrane receptors and the KₐCh channels (Reuveny et al., 1994; Wickman et al., 1994). Because of their significance in the regulation of the heart function, KₐCh channels have been extensively studied at both whole-cell and single-channel levels (for review see Kurachi, 1995), yet the molecular mechanism underlying Gβγ–KₐCh channel interactions remains obscure. The only available functional model for G protein activation of the KₐCh channel was based on data from spectral analysis of current fluctuations at different GTP concentrations (Hosoya et al., 1996). In that case, the spectral analysis identified transitions only between two closed (C₁ and C₂) and one open (O) state of the KₐCh channel. Accordingly, the proposed model was based on two independent processes: a GTP-independent fast gating (C₂ ↔ C₁ ↔ O) and a slow, GTP-dependent transition (not detected in the power spectra) between unavailable and available channel states. Our recent single-channel analysis of the KₐCh channel regulation, however, clearly indicated that the channel activity is much more complex than previously thought and this activity is distributed between several gating modes (Ivanova-Nikolova and Breitwieser, 1997). To understand the mechanisms underlying such modal behavior of the KₐCh channel, in the present study, we performed an extensive single-channel analysis of the activation of the native KₐCh channel by recombinant Gβγ subunits. On the basis of this analysis, we formulated an alternative model of Gβγ–KₐCh channel interactions in which binding of a different number of Gβγ subunits to four Gβγ-binding domains in the KₐCh channel structure gives rise to four conducting conformations of the protein complex, linked in a dynamic equilibrium by the Gβγ concentration.

The heterogeneity of the KₐCh channel gating is a major obstacle in the interpretation of the single-channel recordings from both receptor- and Gβγγγ-activated channels. Therefore, in the present study, we initially developed a basic approach for classification of the KₐCh channel gating into functionally distinct modes. The method is sensitive enough to capture subtle changes in the KₐCh channel regulation and greatly simplifies the kinetic analysis of the data. The analysis routine is based on fragmentation of continuous single-channel recordings into consecutive, equally spaced segments and subsequent characterization of the channel gating within individual segments. The characterization of channel gating combines the analysis of three different parameters: the frequency of openings, the probability of the channel being open, and the mean open time of the channel. The results from this analysis were then compiled to generate the frequency histograms and t_open–f plots as final readouts that convey the information about the dynamics of the KₐCh channel–Gβγ interactions.

In the present work, this method was successfully applied to classify the KₐCh channel gating into functionally distinct modes either by the appearance of several kinetic components in the frequency distributions, or by the orchestrated augmentations of KₐCh channel open time within the t_open–f plots. Two different series of experiments, one of which conserved the integrity of transduction cascade from the activation of muscarinic...
receptors to the activation of the K\textsubscript{ACh} channels, and another in which K\textsubscript{ACh} channel activation was brought about solely by purified recombinant G\textsubscript{b}\textsubscript{2}G\textsubscript{\gamma}\textsubscript{5}, indicated the presence of four functional modes of the channel. Remarkably, the two series of experiments yielded similar values for the predominant frequencies of channel openings within different gating modes. The values for the m\textsubscript{2} receptor-activated channels were: \(f_\text{m2, ACh} = 2.5\), \(f_\text{m2, ENa} = 10.3 \pm 0.1\), \(f_\text{m2, ACh} = 31.7 \pm 0.3\), and \(f_\text{m2, ACh} = 69.1 \pm 1.2\) Hz, while the values for the G\textsubscript{b}\textsubscript{2}G\textsubscript{\gamma}\textsubscript{5}-activated channels were: \(f_\text{Gbg, ACh} = 2.2 \pm 0.2\), \(f_\text{Gbg, ACh} = 12.7 \pm 0.6\), \(f_\text{Gbg, ACh} = 33.6 \pm 1.9\), and \(f_\text{Gbg, ACh} = 65.0 \pm 4.2\) Hz. Thus, our analysis identified the frequency of channel openings as a principal parameter in the classification of the K\textsubscript{ACh} channel activity into distinct gating modes.

Intriguingly, G\textsubscript{b}\textsubscript{2}G\textsubscript{\gamma}\textsubscript{5} alone was not able to reproduce the augmentation in the mean open time of the K\textsubscript{ACh} channel in modes 3 and 4 associated with the channel activation through muscarinic receptors (Fig. 3 A). One possible reason for this could be that the unidentified G\textsubscript{\beta}\gamma subunits released upon muscarinic receptor stimulation are structurally different from G\textsubscript{b}\textsubscript{2}G\textsubscript{\gamma} and the active conformations of the channel vary depending on different G\textsubscript{\beta}\gamma combinations. Alternatively, the augmentation in the mean open time could be regulated by different structural domains of the channel molecule and might require the binding of the G\alpha subunit to the channel. Validation of these possibilities requires direct experiments, which are currently under way.

Nevertheless, G\textsubscript{b}\textsubscript{2}G\textsubscript{\gamma}\textsubscript{5} was able to govern the equilibrium between the four functional modes of the channel. Multiple biochemical mechanisms might underline the modal behavior of the K\textsubscript{ACh} channels. Any hypothetical mechanism, however, should take into consideration two important structural characteristics of the channel: its tetrameric structure (Silverman et al., 1996; Tucker et al., 1996) and the presence of numerous G\textsubscript{\beta}\gamma-binding domains in the channel complex. Because of its simplicity, we considered a paradigm in which binding of G\textsubscript{\beta}\gamma subunits to four equivalent G\textsubscript{\beta}\gamma sites and the active conformations of the channel complex would give rise to four distinct conformational states of the K\textsubscript{ACh} channel. As long as the four gating modes of the channel can be regarded as reporters of such different conformational states, this paradigm predicts the probability of observing each gating mode as a function solely of the probability that one of the four G\textsubscript{\beta}\gamma-binding sites is occupied, \(P\). In this case, the probability function of each gating mode should follow the binomial distribution,

\[
P_1 = \frac{N!}{k!(N-k)!} P^k (1-P)^{N-k},
\]

for binding of one, two, three, or four G\textsubscript{\beta}\gamma subunits to four G\textsubscript{\beta}\gamma-binding sites on the tetrameric channel. To test this prediction, the equilibrium among the four gating modes was quantified from the histograms generated at different G\textsubscript{\beta}\gamma concentrations, and the relative occupancy of each mode, \(\tilde{N}_i\), was examined as a function of the parameter \(P\). In each instance, this parameter was computed from the relative occupancy of mode 1, \(\tilde{N}_1\), according to the equation \(\tilde{N}_1 = 4P(1-P)^3/[1 - (1 - P)^4]\). This equilibrium analysis clearly indicated that the probability of observing each gating mode is binomially distributed as one would predict if the four G\textsubscript{\beta}\gamma-binding sites are identical and independent. The structural basis for this functional equivalence of the G\textsubscript{\beta}\gamma-binding sites in the heterotetrameric K\textsubscript{ACh} channel remains to be determined. The NH\textsubscript{2}- and COOH-terminal domains of both GIRK1 and GIRK4 bind G\textsubscript{\beta}\gamma; however, the affinity of the COOH-terminal regions is different for the two subunits (Huang et al., 1997). In view of this fact, our data suggest that the GIRK1 and GIRK4 subunits must be arranged in a precise pattern to form the four equivalent G\textsubscript{\beta}\gamma-binding sites, perhaps using G\beta\gamma-binding blocks from both GIRK1 and GIRK4 polypeptide chains.

The probability of one of the four G\textsubscript{\beta}\gamma-binding sites to be occupied, \(P\), was a hyperbolic function of the G\textsubscript{\beta}\gamma concentration (Fig. 8 A), and was well approximated by the equation:

\[
P = P_\text{max} [G\beta\gamma] / ( [G\beta\gamma] + K_\text{d} ),
\]

where \(K_\text{d}\) is the microscopic dissociation constant for G\textsubscript{\beta}\gamma binding to the channel. The least-squares fit to the data provided a value of 0.63 for the parameter \(P_\text{max}\) and a \(K_\text{d}\) value of 1.29 nM. These values were used in Eqs. 1 and 2 to calculate the probability of observing each gating mode, \(P_i\), as a function of the G\textsubscript{b}\textsubscript{2}G\textsubscript{\gamma}\textsubscript{5} concentration. Then from the \(P_i\) values and the experimentally determined open probability of the K\textsubscript{ACh} channel in each gating mode (\(P_{\text{o1Gbg}} = 0.0055 \pm 0.0006\), \(P_{\text{o2Gbg}} = 0.0242 \pm 0.0015\), \(P_{\text{o3Gbg}} = 0.0642 \pm 0.0048\), and \(P_{\text{o4Gbg}} = 0.1409 \pm 0.0127\)), we generated the theoretical stimulus–response curve for a K\textsubscript{ACh} channel with four gating modes arising from the binding of a different number of G\textsubscript{\beta}\gamma subunits to four equivalent and independent binding sites. The resulting curve (Fig. 8 B, dotted line) is sigmoidal, and its fit with the Hill equation,

\[
R = R_\text{max} / \{ 1 + (k_d / [G\beta\gamma])^n \},
\]

yields a Hill coefficient, \(N\), of 1.2 and an apparent dissociation constant, \(k_d\), of 2.71 nM. The theoretical and the experimental (Fig. 8 B, solid line) G\textsubscript{b}\textsubscript{2}G\textsubscript{\gamma}\textsubscript{5} concentration–response curves are identical in the concentration range 0.15–2.5 nM; however, at higher G\textsubscript{b}\textsubscript{2}G\textsubscript{\gamma}\textsubscript{5} concentrations, the theoretical curve approaches the saturation limit more gradually than the experimental curve (\(k_d\) of 1.09 nM and Hill coefficient of 1.73). This dis-
crepancy between the theoretical and the experimental curves can be explained by the desensitization of the KACh channel. We found that as Gβγ concentration was raised, the proportion of the blank data segments exceeded the probability of observing gating mode 0 (no Gβγ bound to the channel), P0, calculated from Eq. 1. This behavior is expected if the KACh channel enters a desensitized state that depends on the Gβγ concentration. An additional possibility is that as the Gβγ concentration increases, the Gβγ dimers aggregate in the membrane and, consequently, the membrane concentration of Gβγ deviates from the one in the experimental chamber. Consistent with this possibility, the probability of Gβγ binding, P, saturated at Pmax of 0.63 instead of reaching a value of 1. Perhaps a combination of these two factors suppresses the KACh channel responses at high Gβγ concentrations, and thus creates the steeper, switch-like stimulus–response curve observed in our experiments.

Physiological Relevance of Modal Regulation of the KACh Channel by Gβγ

The Gβγ-driven control of the modal prevalence is not only evolutionarily conserved in KACh channel regulation from frog to mammalian atrial myocytes, as demonstrated in the present study, but is also widespread and biologically versatile in other signaling systems. The same mechanism is encountered in the neurotransmitter-mediated downmodulation of neuronal N-type Ca2+ channels (Delcour and Tsien, 1993) and in the persistent activation of Na+ channels in mammalian central neurons (Alzheimer et al., 1993; Ma et al., 1997). In both systems, the G protein βγ subunits were implicated as the signal-relaying units that interact with these channels (Herlitze et al., 1996; Ikeda, 1996; Ma et al., 1997). The identity of the Gβγ subunits and the mechanism through which they accomplish the modal control may vary from one signaling cascade to another; still, in each case the existence of multiple functional states of the effector molecule helps to ensure the sensitivity and fidelity of Gβγ-mediated signaling. In the case of the KACh channel, the type of regulation described here can be exploited in at least two different ways. The cell could downregulate the microscopic affinity of the KACh channel for Gβγ, in which case the system would filter out small changes in the Gβγ concentration and yet allow the KACh channel to respond to sufficient changes in Gβγ concentration that occur upon stimulation of either m2-muscarinic or A1-purinergic receptors. Alternatively, the KACh channel affinity for Gβγ could be upregulated so that a small change in the Gβγ concentration could translate into a large response. The variability in the apparent affinity of the channel for Gβγ encountered in our experiments implies that the cell, indeed, regulates the sensitivity of the KACh channel to receptor stimulation. Recently, phosphatidylinositol 4,5-bisphosphate (PIP2) was identified as one of the potential factors contributing to such regulation (Huang et al., 1998; Sui et al., 1998).

Sensitivity is only one of several important aspects of the behavior of a signaling system. Another is its specificity. In this aspect, a signaling molecule with multiple functional states like the KACh channel has the potential to filter out the ”membrane noise” associated with the
G protein-mediated signaling. Activation of G proteins is a highly conserved signaling strategy used by a very large number of different G protein-coupled receptors colocalized in the cell membrane. Once the G proteins are activated and the Gβγ subunits are released, a heterogeneous population of Gβγ subunits is potentially available to act directly on the K_{AC} channels. Under such circumstances, the specificity might arise from a differential ability of different Gβγ subunit combinations to activate the K_{AC} channels. Studies with a limited number of purified recombinant Gβγ subunits, however, indicate that different Gβγ combinations activate the channel with comparable efficacies (Wickman et al., 1994). Thus, there might be additional mechanisms for ensuring the specificity of interactions between the expanding number of signaling partners. One attractive possibility is that the receptor, G protein, and the K_{AC} channel exist as a precoupled complex (Huang et al., 1995). Based on present study, an additional mechanism, the presence of functional states of the K_{AC} channel with very low open probabilities, is suggested. Such states would absorb some fraction of the Gβγ released in the membrane without producing substantial current through the channel, thus filtering small inputs from multiple membrane receptors.

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REFERENCES

Alzheimer, C., P.C. Schwindt, and W.E. Crill. 1993. Modal gating of Na⁺ channels as a mechanism of persistent Na⁺ current in pyramidal neurons from rat and cat sensorimotor cortex. J. Neurosci. 13:660–673.

Breitwieser, G.E., and G. Szabo. 1985. Uncoupling of cardiac muscarinic and β-adrenergic receptors from ion channels by a guanine nucleotide analogue. Nature. 317:558–560.

Clapham, D.E., and E.J. Neer. 1993. New roles for G-protein by-dimers in transmembrane signalling. Nature. 365:403–406.

Colquhoun, D., and A.G. Hawkes. 1981. On the stochastic properties of single ion channels. Proc. R. Soc. Ser. B 211:205–235.

Colquhoun, D., and A.G. Hawkes. 1983. The principles of the stochastic interpretation of ion-channel mechanisms. In Single Channel Recording. B. Sakmann and E. Neher, editors. Plenum Publishing Corp., New York. 135–175.

Dascal, N., W. Schreibmayer, N.F. Lim, W. Wang, C. Chavkin, L. Di-Magno, C. Labarca, B.L. Kieffer, C. Gaveriaux-Ruff, D. Trol linger, H.A. Lester, and N. Davidson. 1993. Atrial G protein-activated K⁺ channel: expression cloning and molecular properties. Proc. Natl. Acad. Sci. USA. 90:10235–10239.

Dascal, N., C.A. Doupnik, T. Ivanina, S. Bausch, W. Wang, C. Lin, J. Garvey, C. Chavkin, H.A. Lester, and N. Davidson. 1995. Inhibition of function in Xenopus oocytes of the inwardly rectifying G-protein-activated atrial K⁺ channel (GIRK1) by overexpression of a membrane-attached form of the C-terminal tail. Proc. Natl. Acad. Sci. USA. 92:6765–6762.

Delcour, A.H., and R.W. Tsien. 1993. Altered prevalence of gating modes in neurotransmitter inhibition of N-type calcium channels. Science. 259:980–984.

Doupnik, C.A., N. Davidson, and H.A. Lester. 1995. The inward rectifier potassium channel family. Curr. Opin. Neurobiol. 5:268–277.

Doupnik, C.A., N. Davidson, H.A. Lester, and P. Kofuji. 1997. RGS proteins reconstitute the rapid gating kinetics of Gβγ-activated inwardly rectifying K⁺ channels. Proc. Natl. Acad. Sci. USA. 94: 10461–10466.

Foster, K.A., P.J. McDermott, and J.D. Robishaw. 1990. Expression of G proteins in rat cardiac myocytes: effect of KCl depolarization. Am. J. Physiol. 288:H1432–H1441.

Hamill, O.P., A. Marty, E. Neher, B. Sakmann, and F.J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch. 391:85–100.

Hansen, C.A., S.K. Joseph, and J.D. Robishaw. 1994. Ins 1,4,5-P₃ and Ca²⁺ signaling in quiescent neonatal cardiac myocytes. Biochim. Biophys. Acta. 1224:517–526.

Hansen, C.A., A.G. Schroering, and J.D. Robishaw. 1995. Subunit expression of signal transducing G proteins in cardiac tissue: implications for phospholipase C-β regulation. J. Mol. Cell Cardiol. 27:471–484.

Herlitze, S., D.E. Garcia, K. Mackie, B. Hille, T. Scheuer, and W.A. Catterall. 1996. Modulation of Ca²⁺ channels by G-protein βγ subunits. Nature. 380:258–262.

Hosoya, Y., M. Yamada, H. Ito, and Y. Kurachi. 1996. A functional model for G protein activation of the muscarinic K⁺ channel in Guinea pig atrial myocytes. J. Gen. Physiol. 108:485–495.

Huang, C.-L., P.A. Slesinger, P.J. Casey, Y.N. Jan, and L.Y. Jan. 1995. Evidence that direct binding of Gβγ to the GIRK1 G-protein-gated inwardly rectifying K⁺ channels is important for channel activation. Neuron. 15:1133–1143.

Huang, C.-L., Y.N. Jan, and L.Y. Jan. 1997. Binding of the G protein βγ subunit to multiple regions of G protein-gated inward-rectifying K⁺ channels. FEBS Lett. 405:291–298.

Huang, C.-L., S. Feng, and D.W. Hilgemann. 1998. Direct activation of inward rectifier potassium channels by PIP₂ and its stabilization by Gβγ. Nature. 391:803–806.

Ikeda, S. 1996. Voltage-dependent modulation of N-type calcium channels by G-protein βγ subunits. Nature. 380:255–258.

Iniguez-Lluhi, J.A., M.I. Simon, J.D. Robishaw, and A.G. Gilman. 1992. G protein βγ subunits synthesized in SF9 cells. J. Biol. Chem. 267:23409–23417.

Ito, H., T. Sugimoto, I. Kobayashi, K. Takahashi, T. Katada, M. Ui,
and Y. Kurachi. 1991. On the mechanism of basal and agonist-induced activation of the G protein-gated muscarinic K+ channel in atrial myocytes of guinea pig heart. *J. Gen. Physiol.* 98:517–535.

Ito, H., R.T. Tung, T. Sugimoto, I. Kobayashi, K. Takahashi, T. Kata da, M. Ui, and Y. Kurachi. 1992. On the mechanism of G protein βγ subunit activation of the muscarinic K+ channel in guinea pig atrial cell membrane. *J. Gen. Physiol.* 99:961–983.

Ivanova-Nikolova, T.T., and G.E. Breitwieser. 1997. Effector contributions to Gβγ-mediated signaling as revealed by muscarinic potassium channel gating. *J. Gen. Physiol.* 109:245–253.

Kozasa, T., and A.G. Gilman. 1995. Purification of recombinant G proteins from Sf9 cells by hexahistidine tagging of associated subunits. *J. Biol. Chem.* 270:1734–1741.

Krapivinsky, G., E.A. Gordon, K. Wickman, B. Velimirovic, L. Krapivinsky, and D.E. Clapham. 1995. The G-protein-gated atrial K+ channel I_{KACH} is a heteromultimer of two inwardly rectifying K+ channel proteins. *Nature.* 374:125–141.

Kubo, Y., E. Reuveny, P.A. Slesinger, Y.N. Jan, and L.Y. Jan. 1993. Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel. *Nature.* 364:802–806.

Kunkel, M.T., and E.G. Peralta. 1995. Identification of domains conferring G protein regulation on inward rectifier potassium channels. *Cell.* 83:443–449.

Kurachi, Y. 1995. G protein regulation of cardiac muscarinic potassium channel. *Am. J. Physiol.* 269:C821–C830.

Lesage, F., F. Duprat, M. Fink, E. Guillemare, T. Coppola, M. Lazdunski, and J.P. Hugnot. 1994. Cloning provides evidence for a family of inward rectifier and G-protein coupled K+ channels in the brain. *FEBS Lett.* 353:37–42.

Ma, J.Y., W.A. Catterall, and T. Scheuer. 1997. Persistent sodium currents through brain sodium channels induced by G protein βγ subunits. *Neuron.* 19:443–452.

Pflaffinger, P.J., J.M. Martin, D.D. Hunter, N.M. Nathanson, and B. Hille. 1985. GTP-binding proteins couple cardiac muscarinic receptors to a K channel. *Nature.* 317:536–538.

Reuveny, E., P.A. Slesinger, J. Inglese, J.M. Morales, J.A. Inigues Lluhi, R.J. Lefkowitz, H.R. Bourne, Y.N. Jan, and L.Y. Jan. 1994. Activation of the cloned muscarinic potassium channel by G protein βγ subunits. *Nature.* 370:143–146.

Saitoh, O., Y. Kubo, Y. Miyatani, T. Asano, and H. Nakata. 1997. RGS8 accelerates G-protein-mediated modulation of K+ currents. *Nature.* 390:525–529.

Silverman, S.K., H.A. Lester, and D.A. Dougherty. 1996. Subunit stoichiometry of a heteromultimeric G protein-coupled inward-rectifier K+ channel. *J. Biol. Chem.* 266:19528–19535.

Soejima, M., and A. Noma. 1984. Mode of regulation of the ACh-sensitive K-channel by the muscarinic receptor in rabbit atrial cells. *Pflügers Arch.* 400:424–431.

Sui, J.L., J. Petit-Jacques, and D.E. Logothetis. 1998. Activation of the atrial K_{MACH} channel by the βγ subunits of G proteins or intracellular Na+ ions depends on the presence of phosphatidylinositol phosphates. *Proc. Natl. Acad. Sci. USA.* 95:1307–1312.

Takao, K., M. Yoshii, A. Kanda, S. Kokubun, and T. Nukada. 1994. A region of the muscarinic-gated atrial K+ channel critical for activation by G protein βγ subunits. *Neuron.* 13:747–755.

Tucker, S.J., M. Pessia, and J.P. Adelman. 1996. Muscarine-gated K+ channel: subunit stoichiometry and structural domains essential for G protein stimulation. *Am. J. Physiol.* 271:H379–H385.

Wickman, K.D., J.A. Inigues-Lluhi, P.A. Davenport, R. Taussing, G.B. Krapivinsky, M.E. Linder, A.G. Gilman, and D.E. Clapham. 1994. Recombinant G-protein βγ subunits activate the muscarinic-gated atrial potassium channel. *Nature.* 368:255–257.

Wickman, K., J. Nemec, S.J. Gendler, and D.E. Clapham. 1998. Abnormal heart rate regulation in GIRK4 knockout mice. *Neuron.* 20:103–114.