

A Functional Model for G Protein Activation of the Muscarinic K⁺ Channel in Guinea Pig Atrial Myocytes

Spectral Analysis of the Effect of GTP on Single-channel Kinetics

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ABSTRACT To elucidate the functional interaction between the active G protein subunit (G_K^{*}) and the cardiac muscarinic K⁺ (K_{ACh}) channel, the effect of intracellular GTP on the channel current fluctuation in the presence of 0.5 μM extracellular acetylcholine was examined in inside-out patches from guinea pig atrial myocytes using spectral analysis technique. The power density spectra of current fluctuations induced at various concentrations of GTP ([GTP]) were well fitted by the sum of two Lorentzian functions. Because the channel has one open state, the open-close transitions of the channel gate represented by the spectra could be described as C₂ ↔ C₁ ↔ O. As [GTP] was raised, the channel activity increased in a positive cooperative manner. The powers of the two Lorentzian components concomitantly increased, while the corner frequencies and the ratio of the powers at 0 Hz remained almost constant. This indicates that G protein activation did not affect the gating of each channel but mainly increased the number of functionally active channels in the patch to enhance the channel activity. Regulation of the number of functionally active channels could be described by a slow transition of the channel states, U (unavailable) ↔ A (available), which is independent of the gating. The equilibrium of this slow transition was shifted by GTP from U to A. Monod-Wyman-Changeux's allosteric model for the channel state transition (U ↔ A) could well describe the positive cooperative increase in the channel availability by GTP, assuming that, in the presence of saturating concentrations of ACh, [G_K^{*}] linearly increased as [GTP] was raised in our experimental range. The model indicates that the cardiac K_{ACh} channel could be described as a multimer composed of four or more functionally identical subunits, to each of which one G_K^{*} binds.

KEY WORDS: potassium channels • kinetics • patch-clamp techniques

INTRODUCTION

In nodal and atrial myocytes of the heart, acetylcholine (ACh)¹ activates a specific class of inwardly rectifying K⁺ channels (the muscarinic K⁺ channel, K_{ACh}) via a pertussis toxin-sensitive GTP-binding protein (G protein, denoted as G_K after its function to regulate the K⁺ channel) in a membrane-delimited manner (Breitwieser and Szabo, 1985; Pfaffinger et al., 1985; Kurachi et al., 1986a; Kurachi et al., 1986b). The βγ subunit of G_K is responsible for the physiological activation of the K⁺ channel (Logothetis et al., 1987; Kurachi et al., 1989; Ito et al., 1992; Yamada et al., 1993; Yamada et al., 1994a; Wickman et al., 1994; Reuveny et al., 1994; for review see, Kurachi et al., 1992; Kurachi, 1994; Kurachi, 1995). Recently, a clone encoding the main subunit of the K_{ACh} channel, GIRK1/KGA, was isolated (Dascal et

al., 1993; Kubo et al., 1993). Furthermore, the K_{ACh} channel has been suggested to be a heteromultimer composed of GIRK1 and other subunits (Duprat et al., 1995; Kofuji et al., 1995; Krapivinsky et al., 1995). Among them, not only GIRK1 but CIR/GIRK4 and GIRK2 can be activated by G protein βγ subunits (G_{βγ}) when expressed alone, whereas GIRK3 cannot (Kofuji et al., 1995; Krapivinsky et al., 1995). Therefore, the functional interactions between G_{βγ} and the multimeric K_{ACh} channels could be different depending on the subunit compositions. To further elucidate the molecular mechanism of the K_{ACh} channel regulation, a functional analysis of the activation of the native channel by the active subunit of G_K (G_K^{*}, i.e., G_{Kβγ}) is mandatory, but not yet available.

Here, we present a functional model for G protein activation of the native cardiac K_{ACh} channel, which may be a heteromultimer composed of GIRK1 and CIR/GIRK4 (Krapivinsky et al., 1995). We previously reported that intracellular GTP activates the K_{ACh} channel in a positive cooperative manner, and suggested the possibility that several molecules of G_K^{*} may bind to a K_{ACh} channel (Kurachi et al., 1990; Ito et al. 1991; Yamada et al., 1993). In the present study, to clarify the

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¹Abbreviations used in this paper: ACh, acetylcholine; G_{βγ}, G protein βγ subunits; G_K^{*}, active G protein subunit; GTPγS, guanosine 5'-O-(3-thiotriphosphate); K_{ACh} channel, muscarinic K⁺ channel.

mechanism underlying the positive cooperative activation of the K_{ACh} channel by G_K^* , we analyzed the channel kinetics at different concentrations of GTP ([GTP]) using spectral analysis technique. To explain the cooperative interaction between G_K^* and the K_{ACh} channel, we simulated the experimental data according to Monod-Wyman-Changeux's (MWC) concerted allosteric model by assuming that, in the presence of saturating concentrations of ACh, $[G_K^*]$ in the membrane linearly increased as [GTP] applied to the intracellular side of the patch membrane was raised (Monod et al., 1965). Our analysis indicates that the cardiac K_{ACh} channel could be described as a multimer composed of four or more functionally identical subunits.

MATERIALS AND METHODS

Solutions and Chemicals

The control bathing solution contained (in mM): 136.5 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.53 $MgCl_2$, 0.33 NaH_2PO_4 , 5.5 glucose, and 5.5 HEPES-NaOH buffer (pH 7.4). Nominally Ca^{2+} -free bathing solution was prepared by omitting $CaCl_2$. The composition of the high K^+ /low Cl^- solution was (in mM): 10 taurine, 10 oxalic acid, 70 glutamic acid, 25 KCl, 10 KH_2PO_4 , 11 glucose, 0.5 EGTA, and 10 HEPES-KOH (pH 7.3–7.4). In patch clamp experiments, the pipette solution contained (in mM): 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 HEPES-KOH (pH 7.4) and 0.5 μM acetylcholine chloride (ACh; Sigma Chemical Co., St. Louis, MO). During inside-out patch clamp recordings, the bath was perfused with the "internal solution" composed of (in mM): 140 KCl, 2 $MgCl_2$, 5 EGTA, and 5 HEPES-KOH (pH 7.3). Various concentrations of guanosine 5'-triphosphate (GTP, Na salt, Sigma Chemical Co.) and its nonhydrolyzable analogue, guanosine 5'-O-(3-thiotriphosphate) (GTP γ S, Li salt; Boehringer Mannheim, Mannheim, Germany) were added to the internal solution. $\beta\gamma$ Subunits of G proteins ($G_{\beta\gamma}$) were purified from bovine brain as described previously (Kobayashi et al., 1990).

Isolation of Single Atrial Myocytes

Single atrial myocytes of the guinea pig heart were obtained by the enzymatic dissociation method described previously (Kurachi et al., 1986b; Yamada et al., 1994b). The heart was retrogradely perfused through the coronary arteries on a Langendorff apparatus with nominally Ca^{2+} -free bathing solution containing collagenase (0.04% wt/vol, Sigma type I) for 20 min (37°C). The heart was then stored in the high K^+ /low Cl^- solution at 4°C. To isolate single myocytes, a small piece of atrial tissue was dissected and agitated in the recording chamber filled with the control bathing solution. Myocytes with clear striations and smooth surface were used for experiments.

Electrophysiological Recordings

Single K^+ channel currents were recorded in the inside-out configuration of the patch clamp technique (Hamill et al., 1981). The tips of patch electrodes were coated with Sylgard and fire-polished. The tip resistance was 5–8 $M\Omega$ when filled with the pipette solution. All recordings from inside-out patches were made at a holding potential of –60 mV. All experiments were performed at room temperature (~25°C).

The channel current was recorded using a patch clamp amplifier (EPC-7; List, Darmstadt, Germany) and stored on a video tape through a PCM converter system (VR-10B; Instrutech Corp.,

Great Neck, NY). For analysis, data were reproduced, low-pass filtered (Programmable Filter 3625 in Bessel or Butterworth response as noted below; NF Electronic Instruments, Yokohama, Japan), digitized by an AD converter (ITC-16; Instrutech Corp.), continuously acquired on a computer (Macintosh Quadra, Apple Computer, Inc., Cupertino, CA) with Pulse program (HEKA Elektronik, Lambrecht, Germany) and analyzed with Patch Analyst Pro program (MT Corporation, Hyogo, Japan).

Single-channel Analysis

Records were low-pass filtered at 4 kHz (–3 dB) by an eight-pole Bessel filter and digitized at 20 kHz. Current amplitude histograms were constructed, and $N \cdot P_o$ values (N is the number of functional channels in a patch; P_o is the time-averaged open probability of each channel) were determined. For open and closed time distributions, the threshold for event detection was set at half the amplitude of the single-channel currents (Colquhoun and Sigworth, 1983).

An over-all estimate of mean channel open time was calculated as described by Fenwick et al. (1982). The analysis program located transitions between current levels as crossings of the mid-line between adjacent levels and calculated the time interval spent in each current level (t_j , where j indicates the current level). An over-all estimate of mean channel open time (τ_o) was calculated as:

$$\tau_o = \frac{\sum_j j \cdot t_j}{N_o},$$

where N_o is the number of channel openings (transitions from a given level j to a subsequent open level $j + 1$). By this method, a mean channel open time can be estimated in patches with more than one channel.

Spectral Analysis of Single-channel Current Fluctuations

To avoid the ambiguities in analyzing open-close kinetics in multi-channel patches, we applied the spectral analysis technique to multi-channel current fluctuations. Multi-channel records were low-pass filtered at 5 kHz (–3 dB) by a Butterworth filter and digitized at 12.5 kHz. The data were divided into short segments of 8,192 points and were multiplied point by point by a Hanning window function and then Fourier-transformed following an algorithm for the fast Fourier transform. Power density spectra were calculated and averaged over 20–30 segments. Background noise was determined when the channel was completely closed in GTP-free solution and the power derived from background noise was subtracted. The averaged power density spectrum of current fluctuations was fitted by the sum of Lorentzian functions as follows:

$$S(x) = \sum_i \frac{S_i}{1 + \left(\frac{x}{F_i}\right)^2},$$

where $S(x)$ is the power spectral density at the frequency of x , S_i is the zero-frequency asymptote, and F_i is the corner frequency of "i"th Lorentzian component. To calculate rate constants from F_i , S_i , and τ_o , equations 11, 14, 15, 46, and 89–94 in Colquhoun and Hawkes (1977) were used.

Modeling

We used the Monod-Wyman-Changeux's (MWC) allosteric model (Monod et al., 1965) to simulate the positive cooperative activation of the K_{ACh} channels by intracellular GTP. The basic assumptions of the model are: (a) an oligomeric protein consists of the

finite number of functionally identical protomers, (b) each protomer interconverts between relaxed (R) and tense (T) states, (c) the R state has a higher affinity for the ligand than T state, and (d) all the protomers in a particular protein must be in the same state (all Rs or all Ts) and change the conformation together (concerted transition). As corollary, the binding of ligand increases the probability that all protomers in the protein are in the R state. Cooperativity is quantitatively dependent on the position of the equilibrium between the R and T states.

For simulation, we assumed that (a) the K_{ACh} channel is composed of m functionally identical subunits and (b) each subunit binds one G_K^* (see Fig. 4 A). The fraction of the channel molecules in the all-Rs form (Eq. 1 of Monod et al. [1965]) is:

$$Y = \frac{(1 + \alpha)^m}{L(1 + C\alpha)^m + (1 + \alpha)^m}, \quad (1)$$

where L is the allosteric constant for the concerted transition in the absence of ligand ($L = [T_0]/[R_0]$); $C = K_R/K_T$; and α is a normalization for the substrate concentration ($\alpha = [S]/K_R$). K_R and K_T are the microscopic dissociation constants of a ligand bound to the stereo-specific sites in R and T states, respectively.

Eq. 1 is divided by $1/(1 + LC^m)$ and normalized so that maximum value of Y is equal to 1.

$$Y = \frac{(1 + LC^m)(1 + \alpha)^m}{L(1 + C\alpha)^m + (1 + \alpha)^m}. \quad (2)$$

The relationship between the steady state availability of the functionally active K_{ACh} channel and [GTP] was fitted by the Eq. 2 by the least-squares method.

Statistical Analysis

Statistical inferences were made by Statistical Analysis System (SAS) software (SAS Institute Inc., Cary, NC). Comparisons among data at various [GTP] were made by the analysis of variance with Scheffe's test. A value of $P < 0.05$ was considered significant. The data were expressed as means \pm SEM. All curve fittings were made by the least-squares method.

RESULTS

Spectral Analysis of the K_{ACh} Channel Current Fluctuations Induced by Intracellular GTP

The concentration-dependent activation of the K_{ACh} channel by GTP was analyzed in inside-out patches (Figs. 1 and 2). We previously reported that the maximum activity of K_{ACh} channel induced by GTP applied to the cytosolic surface of inside-out patch membranes was increased as the concentration of ACh in the pipette was raised (Ito et al., 1991). ACh exerted its maximum effect at more than 0.1 μ M (Ito et al., 1991). When the pipette contained the maximum effective concentrations of ACh, more than 10 μ M GTP activated the K_{ACh} channel to the same extent as GTP γ S (10 μ M) (Ito et al., 1991).

In the present study, the pipette solution contained a maximum effective concentration of ACh (0.5 μ M). The intracellular side of the patch membrane was perfused with internal solution (~ 150 mM K^+) containing various concentrations of GTP. In a cell-attached mode,

brisk channel activity was observed (not shown). When the patch was excised in GTP-free internal solution, the activity completely disappeared (Fig. 1 A). GTP reactivated the channel slightly at 0.1 μ M ($N \cdot P_o = 0.159$), and to the same extent as 10 μ M GTP γ S at 10 μ M ($N \cdot P_o = 1.12$ in the presence of either 10 μ M of GTP or GTP γ S). The amplitude histograms of the recordings clearly indicated that the single-channel current amplitude did not change in the presence of different concentrations of [GTP] (not shown).

Fig. 1 B shows the power density spectra of current fluctuations constructed from the recordings shown in Fig. 1 A. Contribution of the background noise was removed by subtracting the power spectrum determined in the absence of GTP. All the spectra obtained in the presence of 0.1 or 10 μ M GTP or 10 μ M GTP γ S could be well fitted by the sum of two Lorentzian curves with similar values of corner frequencies. Corner frequencies of the slow (F_1) and the fast (F_2) components were respectively 39.31 and 292.48 Hz, in the presence of 0.1 μ M GTP; 32.13 and 305.99 Hz in 10 μ M GTP; and 31.65 and 294.12 Hz in 10 μ M GTP γ S. In contrast, the powers at 0 Hz of the two Lorentzians (S_1 and S_2 for slow and fast ones, respectively) significantly increased when [GTP] was raised: S_1 and S_2 were, respectively, 0.0063 and 0.00284 $pA^2 \cdot s$, in the presence of 0.1 μ M GTP; 0.0178 and 0.00672 $pA^2 \cdot s$ in 10 μ M GTP; and 0.0159 and 0.00576 $pA^2 \cdot s$ in 10 μ M GTP γ S. The S_1/S_2 ratio, however, remained almost constant at ~ 2 .

Fig. 2 summarizes the data from similar experiments ($n = 4-10$ for each [GTP]). Internal solution containing 10 nM to 100 μ M GTP was applied to inside-out patches. In each patch, the effect of three or four different concentrations of GTP was sequentially tested. At the end of each experiment, 10 μ M GTP γ S was added. The channel activity was measured after a steady state response was established at each [GTP], normalized to the maximum activity induced by 10 μ M GTP γ S and expressed as relative $N \cdot P_o$ (Fig. 2 A). The activation started at 0.1 μ M GTP (relative $N \cdot P_o = 0.028 \pm 0.012$, $n = 7$) and was enhanced as [GTP] was raised. At 10 μ M GTP, the maximum activation was attained (relative $N \cdot P_o = 0.99 \pm 0.012$, $n = 10$). The data were fitted by the Hill equation:

$$Y = \frac{1}{1 + \left(\frac{K_d}{[GTP]} \right)^H},$$

where Y is the relative $N \cdot P_o$; K_d is the [GTP] at the half-maximum channel activation; and H is the Hill coefficient. The fitted curve gave K_d of 0.301 μ M and H of 2.4, indicating that intracellular GTP activates the K_{ACh} channel in a positive cooperative manner (Dixon and Webb, 1979). Because the stoichiometry of GTP-binding to a G protein is 1:1 (Gilman, 1987), this positive

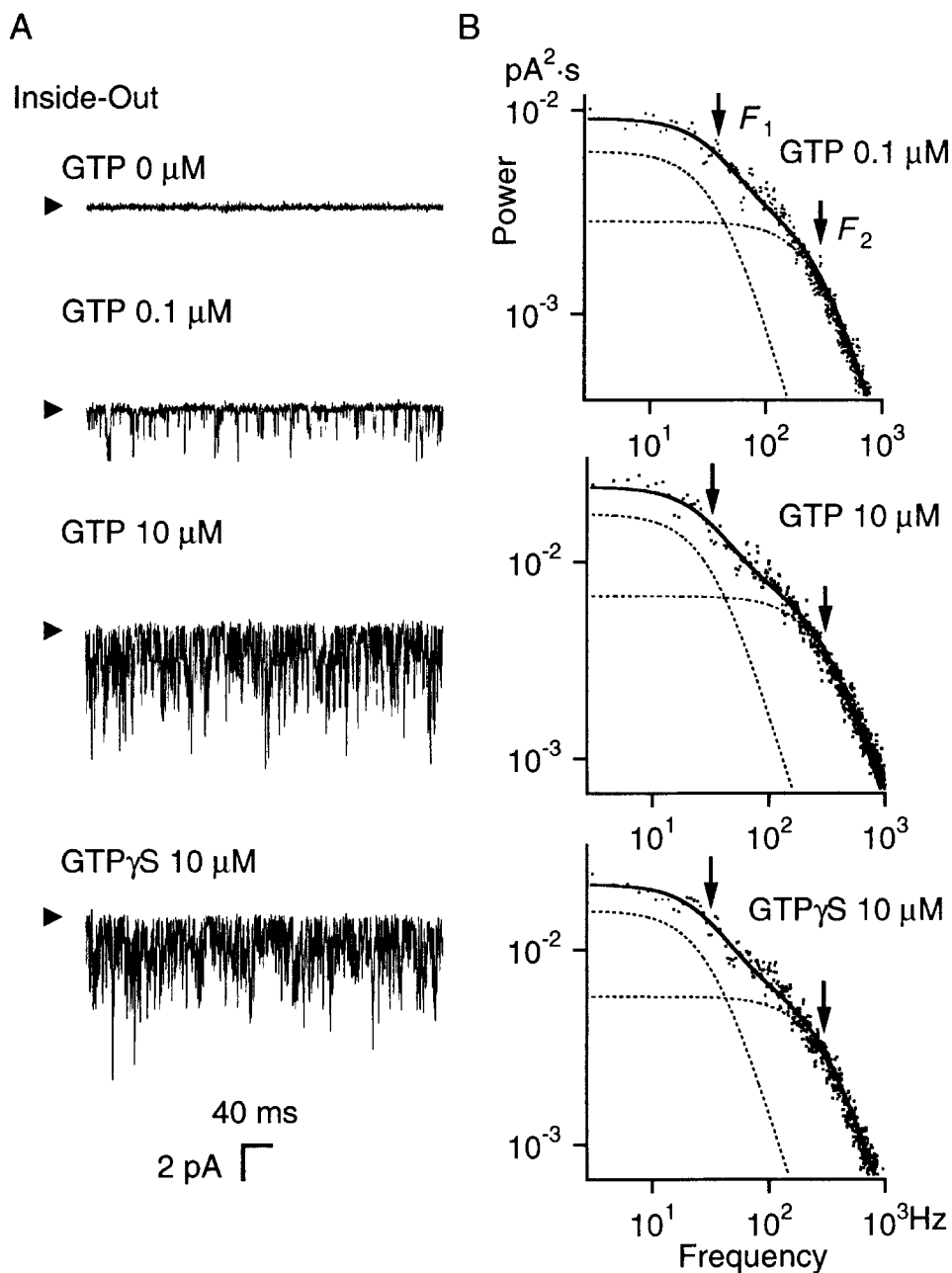


FIGURE 1. Effect of intracellular GTP on the power density spectra of current fluctuations of the K_{ACh} channel. (A) Inside-out patch recording at -60 mV. The pipette solution contained 0.5 μ M ACh. Increasing concentrations of GTP ($[GTP]$) were applied to the intracellular side of the patch membrane. GTP γ S was finally applied to measure the maximum channel activity of the patch. (B) Power density spectra calculated from the inside-out patch records in A. Each spectrum could be well fitted by the sum of two Lorentzian functions. F_1 and F_2 (arrows) indicate corner frequencies of the slow and fast Lorentzian components, respectively.

cooperativity could be attributed to the property of the interaction between G_K^* and the K_{ACh} channel (Kurauchi et al., 1990; Ito et al., 1991).

Spectral analyses of current fluctuations were performed at $[GTP]$ of 0.06 , 0.1 , 0.2 , 0.3 , 0.6 , 1.0 , 3.0 , and 10 μ M. From pooled data ($n = 4-10$), the corner frequencies (F_1 and F_2), the powers at 0 Hz (normalized to the S_1 value at 10 μ M GTP and expressed as relative (r) S_1 and rS_2) and the S_1/S_2 ratio were plotted against $[GTP]$ (Fig. 2, B and C). Neither corner frequencies nor the ratio of the powers at 0 Hz of the two Lorentzian components was affected by $[GTP]$ although $N \cdot P_o$ was increased by ~ 40 -fold when $[GTP]$ was increased in the same range (Fig. 2 A). Thus, the gating reaction

of the K_{ACh} channel represented by F_1 , F_2 or the S_1/S_2 ratio of the two Lorentzian components was independent of the level of G protein activation. On the other hand, the powers of both components increased as $[GTP]$ was raised (Fig. 2 C). Because the single-channel current amplitude did not change with $[GTP]$ (Fig. 1 A), this finding indicates that G protein activation increases the number of functional channels in the patch.

Kinetics

The power density spectra of the current fluctuations induced at different $[GTP]$ could be well fitted by the sum of two Lorentzian curves. This indicates that the channel has three functional states (Colquhoun and

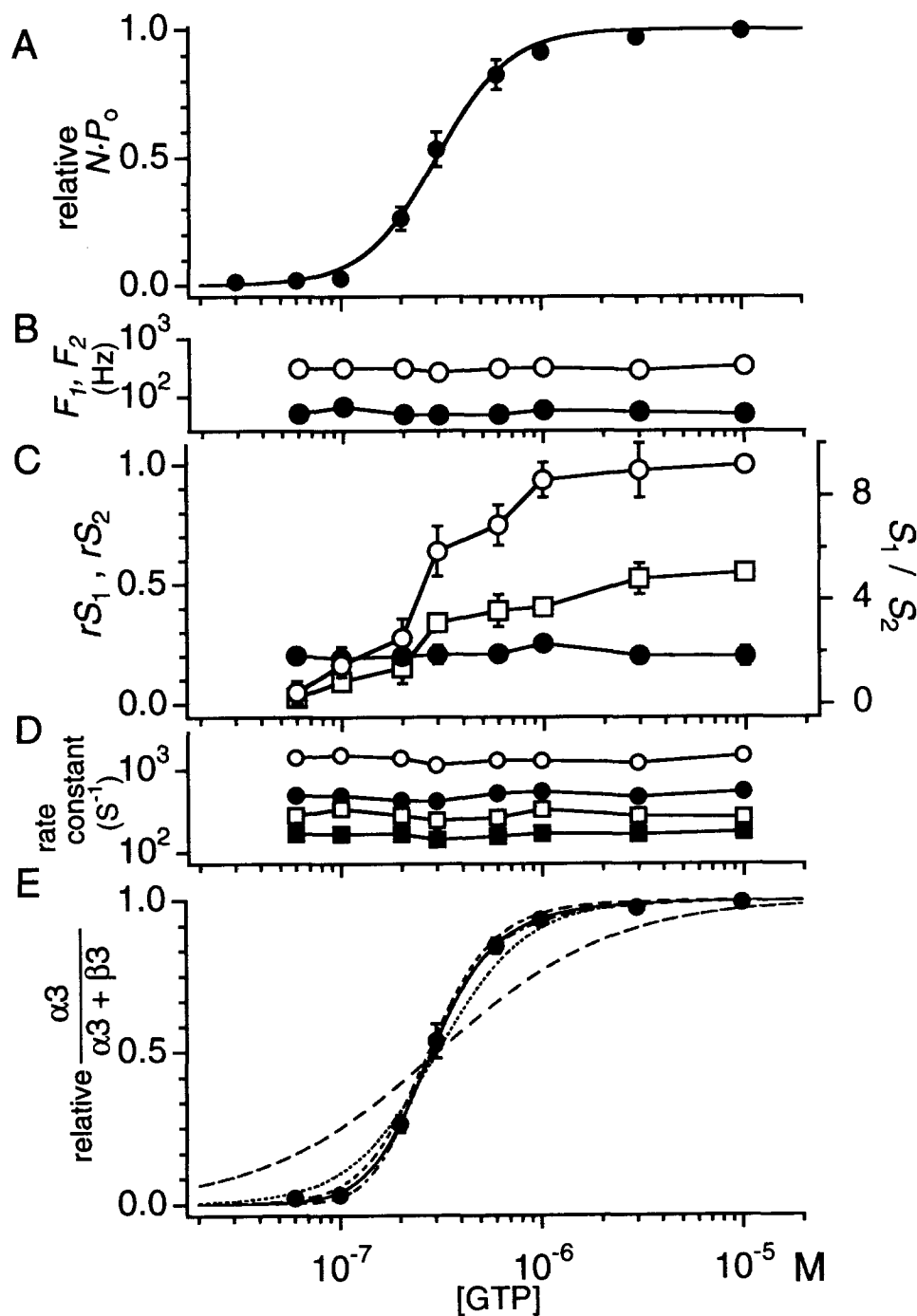
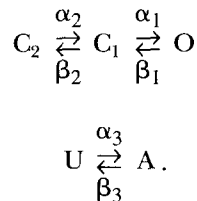


FIGURE 2. Concentration-dependent activation of the K_{ACh} channel by GTP. (A) Relative $N \cdot P_o$ was plotted against [GTP]. Symbols and bars represent means \pm SEM ($n = 4-10$). The continuous line is a fit by the Hill equation using the least-squares method. The Hill coefficient was 2.4. (B) F_1 (\bullet) and F_2 (\circ) were plotted against [GTP]. (C) S_1 , S_2 and S_1/S_2 ratios (\bullet) were plotted against [GTP]. S_1 or S_2 at each [GTP] was normalized to the S_1 value at $10 \mu\text{M}$ GTP and plotted as relative rS_1 (\circ) or rS_2 (\square). (D) The rate constants for the fast gating transitions, α_1 (\bullet), β_1 (\circ), α_2 (\blacksquare), and β_2 (\square), were calculated and plotted against [GTP] ($n = 4-10$). (E) Relative $\alpha_3/(\alpha_3 + \beta_3)$ values were plotted against [GTP]. Symbols and bars represent means \pm SEM ($n = 4-10$). Relative $\alpha_3/(\alpha_3 + \beta_3)$ was 0.0201 ± 0.000489 (at GTP $0.06 \mu\text{M}$), 0.0294 ± 0.0118 ($0.1 \mu\text{M}$), 0.261 ± 0.0277 ($0.2 \mu\text{M}$), $0.535 \pm 0.0562^*$ ($0.3 \mu\text{M}$), $0.849 \pm 0.0252^*$ ($0.6 \mu\text{M}$), $0.937 \pm 0.0138^*$ ($1.0 \mu\text{M}$), $0.974 \pm 0.0129^*$ ($3.0 \mu\text{M}$), and $0.991 \pm 0.0204^*$ ($10 \mu\text{M}$). *Indicates $P < 0.0001$ compared to the value at GTP $0.06 \mu\text{M}$. Fitting of the data by Eq. 2 was repeated with different presumed number of subunits ($m = 1-10$). In the panel, only 5 cases ($m = 1$ —, 2 ·····, 3 ---, 4 — — —, and 10 — — —) were shown.

Hawkes, 1977). Thus the open-close transitions of the channel gate could be described as $C_2 \leftrightarrow C_1 \leftrightarrow O$ or $C_2 \leftrightarrow O \leftrightarrow C_1$, because the single-channel analysis clearly indicated that the channel has a single open state (not shown; also see Sakmann et al., 1983; Kurachi et al., 1986b). For the simplicity, we chose the C-C-O model in this study, although both models can explain the experimental data. The corner frequencies of the two Lorentzian components did not change, when $N \cdot P_o$ increased when [GTP] was raised (Fig. 2, A and B). This implies that the gating of the channel is GTP inde-

pendent. The increase in the powers of both components when [GTP] was raised indicates the presence of a GTP-dependent process which regulates the number of functionally active channels (Fig. 2, A and C). This could be described by a slow transition of the channel states, U (unavailable) \leftrightarrow A (available), which is independent of the fast open-close gating transitions. The corresponding component did not appear in the spectral analysis, possibly because the transition between the two channel states was slow and infrequent compared to the gating transitions.

Therefore, we assumed the existence of two independent reactions in the K_{ACh} channel kinetics, i.e., a fast gating process and a slow process regulating the channel availability, as in the following diagram:



The channel is functionally active (available) in the A state and can open when the gate takes the O state. In contrast, in the U state, the channel is functionally inactive (unavailable) and cannot open, even when the gate reaches the O state.

Rate Constants of K_{ACh} Channel Kinetics

According to the above model, we calculated the rate constants for the fast gating transitions ($\alpha_1, \beta_1, \alpha_2, \beta_2$) from parameters obtained from spectral analysis ($F_1, F_2, S_1/S_2$ ratio) and single-channel analysis (over-all mean open time) using equations of Colquhoun and Hawkes (1977).

$$\left\{ \begin{array}{l} 1/\beta_1 = \tau_o \\ \lambda_2 + \lambda_3 = -(\alpha_1 + \beta_1 + \alpha_2 + \beta_2) \\ \lambda_2 \lambda_3 = \alpha_1 \alpha_2 + \beta_1 \beta_2 + \beta_1 \alpha_2 \\ S_1/S_2 = -\left(\frac{\lambda_3}{\lambda_2}\right)^2 \frac{(\beta_1 + \lambda_3)(\alpha_2 + \lambda_2)}{(\beta_1 + \lambda_2)(\alpha_2 + \lambda_3)}, \end{array} \right.$$

where $\lambda_2 = -2\pi F_1$; $\lambda_3 = -2\pi F_2$; and τ_o is over-all mean open time. τ_o was 0.71 ± 0.03 ms, 0.66 ± 0.05 , 0.72 ± 0.04 , 0.87 ± 0.04 , 0.76 ± 0.06 , 0.78 ± 0.05 , 0.82 ± 0.04 , and 0.67 ± 0.08 at 0.06, 0.1, 0.2, 0.3, 0.6, 1.0, 3.0, and 10 μ M of [GTP], respectively ($n = 4-10$).

In this model, open probability of the channel is expressed as follows:

$$P_o = \frac{\alpha_3}{\alpha_3 + \beta_3} \cdot \frac{\alpha_1 \alpha_2}{\alpha_1 \alpha_2 + \beta_1 \beta_2 + \beta_1 \alpha_2},$$

$\alpha_3/(\alpha_3 + \beta_3)$ was normalized to the maximum value induced by 10 μ M GTP γ S and expressed as relative $\alpha_3/(\alpha_3 + \beta_3)$.

The relative $\alpha_3/(\alpha_3 + \beta_3)$ was calculated from the following equation:

$$\begin{array}{l} \text{relative } \frac{\alpha_3}{\alpha_3 + \beta_3} = \\ \text{relative } N \cdot P_o \times \frac{\frac{\alpha'_1 \alpha'_2}{\alpha'_1 \alpha'_2 + \beta'_1 \beta'_2 + \beta'_1 \alpha'_2}}{\frac{\alpha_1 \alpha_2}{\alpha_1 \alpha_2 + \beta_1 \beta_2 + \beta_1 \alpha_2}}, \end{array}$$

where $\alpha'_1, \beta'_1, \alpha'_2$, and β'_2 are the rate constant at 10 μ M GTP γ S.

As shown in Fig. 2 D, the rate constants for the fast gating transitions ($\alpha_1, \beta_1, \alpha_2, \beta_2$) remained constant at different [GTP]. On the other hand, relative $\alpha_3/(\alpha_3 + \beta_3)$, i.e., the steady-state availability of the K_{ACh} channel, significantly increased as [GTP] was raised ($P < 0.0001$, Fig. 2 E). Therefore, only the slow transition between states U and A is GTP dependent. These data indicate that G_K^* enhances the channel activity through an increase in the number of functionally active channels by increasing the steady state probability of the A state.

Allosteric Model of the K_{ACh} Channel Activation by G Protein Subunits

The maximum effect of GTP on channel activity was increased as the concentration of ACh was raised (Ito et al., 1991). The maximum GTP effect may be determined through distinct mechanisms in the presence of different concentrations of ACh. In the presence of relatively low concentrations of ACh (e.g., 0.01 μ M), the channel activity induced by the maximum effective concentration of GTP is smaller than that induced by GTP γ S (10 μ M) (Ito et al., 1991). Under these conditions, either GTP γ S or exogenous $G_{\beta\gamma}$ applied to the patch on top of GTP further increased the channel activity to the same extent as GTP γ S (10 μ M) alone. These results indicate that the maximum GTP effect in the presence of the relatively low concentrations of ACh is determined by the availability of the receptor-activated G proteins for GTP but not the availability of K_{ACh} channels for G_K^* . On the other hand, the maximum GTP effect in the presence of more than 0.1 μ M ACh (always obtained at [GTP] > 10 μ M) was equivalent to that induced by GTP γ S (10 μ M) (Figs. 1 and 2 A in this study; see also Ito et al., 1991). The K_{ACh} channel activity induced by ACh (0.5 μ M) plus GTP (10 μ M) was not further enhanced by $G_{\beta\gamma}$ (10 nM) (Fig. 3 A). The $N \cdot P_o$ value in the presence of both GTP and $G_{\beta\gamma}$ was 0.976 ± 0.0478 (mean \pm SEM, $n = 6$) of that in the presence of GTP alone. Similarly, exogenous $G_{\beta\gamma}$ (10 nM) also did not further increase the channel activity after the channel was preactivated by GTP γ S (10 μ M) (Fig. 3 B). The $N \cdot P_o$ value with GTP γ S and $G_{\beta\gamma}$ was 0.983 ± 0.0342 (mean \pm SEM, $n = 6$) of that with GTP γ S alone. Therefore, in the presence of saturating concentrations of ACh, the maximum GTP effect is determined by the availability of K_{ACh} channels for G_K^* but not the availability of the receptor-activated G proteins for GTP. This is consistent with previous studies (Ito et al., 1992; Wickman et al., 1994; Yamada et al., 1994a), which showed that, at high concentrations of either purified or recombinant exogenous $G_{\beta\gamma}$, K_{ACh} channel activity reaches the maximum plateau level, probably because channels are saturated with $G_{\beta\gamma}$. Therefore, it

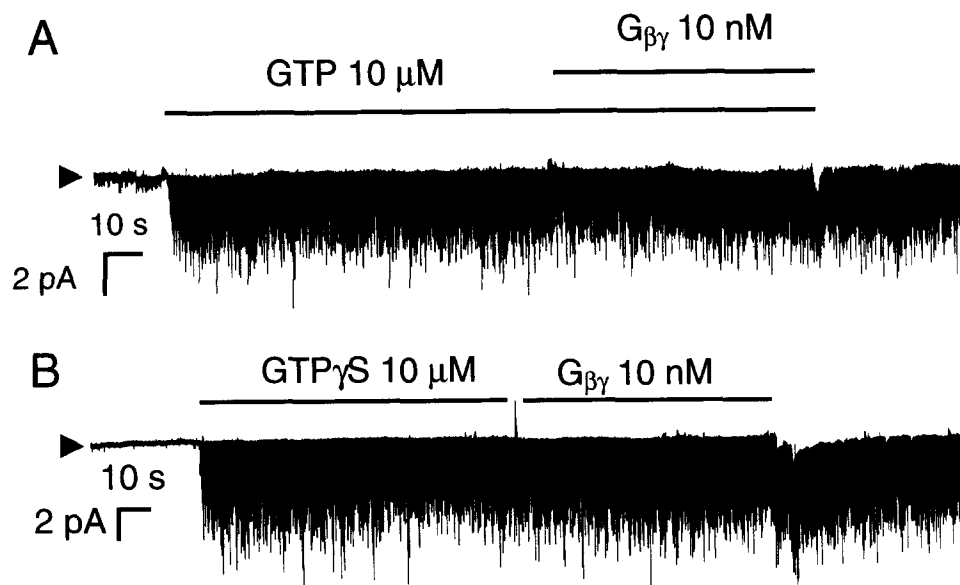


FIGURE 3. Effect of exogenous G protein $\beta\gamma$ subunit on the channel activity. (A) The pipette solution contained $0.5 \mu\text{M}$ ACh. In the inside-out patch condition, $G_{\beta\gamma}$ (10 nM) was added in the presence of $10 \mu\text{M}$ GTP. Exogenous $G_{\beta\gamma}$ did not further activate the channel. (B) In the inside-out patch configuration, 10 nM $G_{\beta\gamma}$ was perfused after the channel was activated by $10 \mu\text{M}$ $\text{GTP}\gamma\text{S}$. Exogenous $G_{\beta\gamma}$ did not further increase the channel activity.

may be possible to assume that in the presence of a saturating concentration ($0.5 \mu\text{M}$) of ACh, $[G_K^*]$ in the membrane linearly increases as $[GTP]$ is raised in our experimental range. Based on this assumption, we analyzed the positive cooperative activation of the K_{ACh} channel by G_K^* with the MWC concerted allosteric model (Monod et al., 1965), although other allosteric models could also be used.

Fig. 4 A shows the schematic illustration of this model. For formulation of the model, we assumed; (a) the K_{ACh} channel consists of m functionally identical subunits, (b) each of the subunits binds one G_K^* , and (c) the channel is available (i.e., in the A state) when all subunits are in the R state and unavailable (in the U state) when all in the T state. The relationship between $[GTP]$ and relative $\alpha_3/(\alpha_3 + \beta_3)$, the steady-state avail-

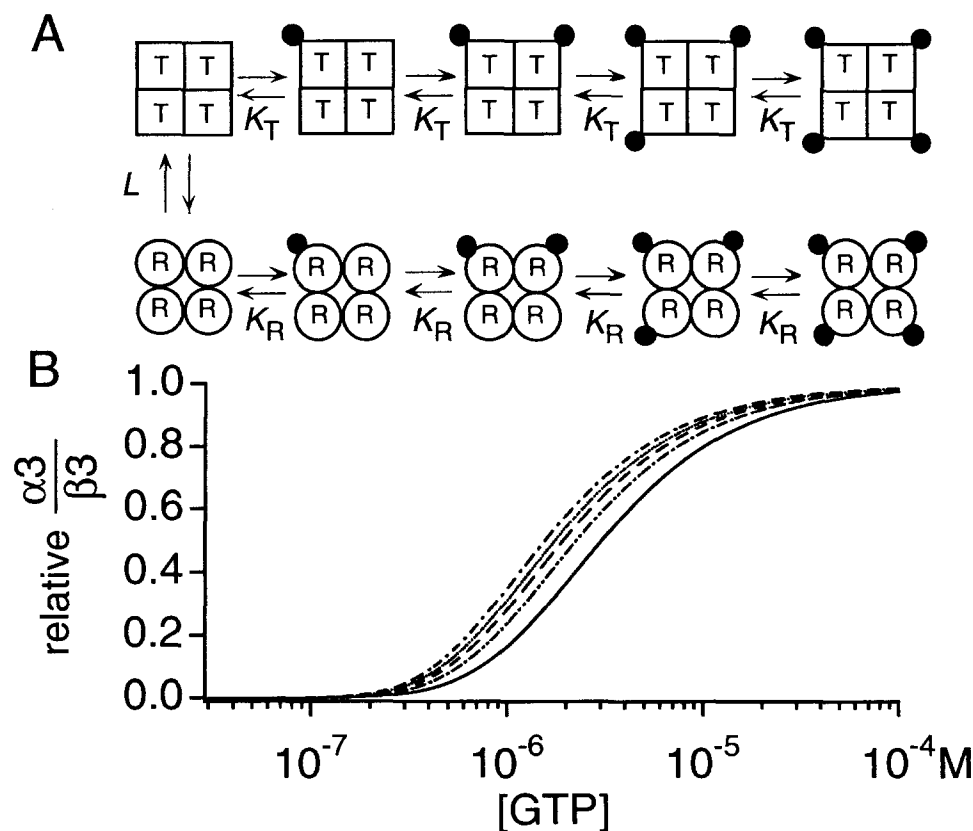


FIGURE 4. Relationship between $[GTP]$ and the K_{ACh} channel availability. (A) Concerted allosteric model of Monod, Wyman, and Changeux. Two different states of the protomers, tense (T) and relaxed (R) states, are represented by squares and circles, respectively. The latter has a higher affinity with the activated G protein subunit (G_K^*), which is represented by a small solid circle. In this illustration the K_{ACh} channel is supposed to be an oligomeric protein composed of four functionally identical protomers. (B) Relative α_3/β_3 ratio was plotted against $[GTP]$ with different presumed number of subunits ($m = 4, 5, 6, 7, 10$) of a K_{ACh} channel. ($m = 4$ —, 5 — — —, 6 — · — ·, 7 · · · · ·, and 10 — — — —).

ability of the functionally active K_{ACh} channel, was fitted by the Eq. 2 based on the MWC allosteric model (See Methods) with different presumed number of subunits ($m = 1-10$) of a K_{ACh} channel (Fig. 2 E). The [GTP]-availability relationship was well fitted by the MWC allosteric model when several subunits are assumed to compose the channel. In Table I, the parameters (L , K_T , K_R) and the chi-square value of each fitting were shown for each presumed number of subunits (m). The Chi square value was relatively large when m is one or two, but it became almost constant when m is four or more.

The relative α_3/β_3 ratio was calculated from the following equation:

$$relative \frac{\alpha_3}{\beta_3} = \frac{C^m(1+\alpha)^m}{(1+C\alpha)^m},$$

where $\alpha = [GTP]/K_R$.

In Fig. 4 B, relative α_3/β_3 , i.e., the ratio of the steady state probability of the A state to that of the U state, was calculated and plotted against [GTP] with different presumed number of subunits ($m = 4, 5, 6, 7, 10$) of a K_{ACh} channel. The relative α_3/β_3 ratio increased as [GTP] was raised. These data show that G_K^* enhances the channel activity through an increase in the number of functionally active channels by accelerating the rate of transition from state U to A (α_3) and/or decelerating the rate of transition from state A to U (β_3).

DISCUSSION

The present study electrophysiologically examined the interaction between the active G protein subunit (G_K^*) and the cardiac K_{ACh} channel. The results indicate the following: (a) The channel function could be described with two independent mechanisms: a channel gate ($C_2 \leftrightarrow C_1 \leftrightarrow O$) and an availability of the functionally active channels ($U \leftrightarrow A$). G protein activation

shifted mainly the latter equilibrium in the rightward direction through a positive cooperative process. (b) Simulation with the MWC concerted allosteric model, with an assumption that $[G_K^*]$ in the membrane linearly increases as [GTP] is raised in the presence of a saturating concentration (0.5 μ M) of ACh, well explained the cooperativity and indicated that the K_{ACh} channel could be described as a multimer composed of four or more functionally identical subunits.

Kinetic Model

There has been no detailed kinetic analysis of the cardiac K_{ACh} channel, because a patch with only one channel could hardly be obtained in nodal or atrial myocytes. Earlier studies confirmed one open state with the time constant of ~ 1 ms (Sakmann et al., 1983; Kurachi et al., 1986b). Most reported two closed states, whose time constants clustered around values of 0.5–1.4, ~ 5 or 18–80 ms (Sakmann et al., 1983; Heidbüchel et al., 1990; Kim, 1991). It is usually not easy to reliably count the number of K_{ACh} channels in a patch, because of the relatively low P_o of this channel. Therefore, the assessment of the open and closed time constants of the K_{ACh} channel from single-channel recordings leaves some ambiguity. To overcome this problem, the spectral analysis was conducted in this study.

Based on the spectral analysis of current fluctuations at different [GTP], we formulated a kinetic model in which the K_{ACh} channel is controlled by two independent mechanisms: a GTP-independent fast gating ($C_2 \leftrightarrow C_1 \leftrightarrow O$) and a GTP-dependent slow transition of channel states ($U \leftrightarrow A$). Only in the A state, the channel can open when the gate reaches O. A similar model has been proposed for cardiac L-type Ca^{2+} channel to explain its modulation by β -adrenergic agents or dihydropyridine calcium channel blockers (Tsien et al., 1986; Kawashima and Ochi, 1988; Ochi and Kawashima, 1990). It was reported that β -adrenergic stimulation mainly modulates the slow gating process of L-type Ca^{2+} channel and increases the availability of the channel with small increase in open probability due to alterations of the fast kinetics (Tsien et al., 1986; Ochi and Kawashima, 1990). The mechanism of the slow gating has been related to phosphorylation of the Ca^{2+} channel by protein kinase A activated by the G protein-adenylyl cyclase cascade. Similarly, this study shows that G proteins increase the number of functionally active K_{ACh} channels by shifting the channel state equilibrium from unavailable to available. Different from the L-type Ca^{2+} channel, however, the state of the K_{ACh} channel is not regulated by channel phosphorylation but probably by direct action of G_K^* (Ito et al., 1992; Kurachi, 1994; Inanobe et al., 1995b; Kurachi, 1995).

Detailed analyses of membrane-delimited regulation of ion channels by G proteins similar to the K_{ACh} chan-

TABLE I
The Parameters and Chi Square Value of Each Fitting

No. of subunits	L	K_T	K_R	Chi square
1	9.251×10^4	7.203×10^{-7} M	5.753×10^{-12} M	1.2061
2	1.788×10^6	1.407×10^{-5} M	2.240×10^{-10} M	0.2602
3	3.985×10^7	1.999×10^{-6} M	7.130×10^{-10} M	0.2080
4	4.290×10^7	5.780×10^{-7} M	2.406×10^{-9} M	0.1874
5	3.286×10^8	3.393×10^{-7} M	3.128×10^{-9} M	0.1862
6	1.028×10^9	2.414×10^{-7} M	4.210×10^{-9} M	0.1862
7	2.761×10^9	1.884×10^{-7} M	5.214×10^{-9} M	0.1865
8	1.470×10^9	1.589×10^{-7} M	7.534×10^{-9} M	0.1868
9	8.435×10^8	1.393×10^{-7} M	9.932×10^{-9} M	0.1870
10	8.686×10^8	1.245×10^{-7} M	1.158×10^{-8} M	0.1872

The parameters (L , K_T , K_R) and the Chi-square value of each fitting were shown against the presumed number of subunits ($m = 1-10$).

nel have been performed for inhibition of neuronal N-type Ca^{2+} channel by various neurotransmitters (Hille, 1994). Activated G protein depresses the Ca^{2+} channel current and slows its activation. Kasai (1992) proposed that G protein induces an additional voltage-dependent gating whose transition is much slower than that of intrinsic activation gate. The fast gate is supposed to be G protein-independent. Boland and Bean (1993) presented another view that G protein shifts the activation curve of the fast gate to more depolarized potentials and makes the channel "reluctant" to open. They also suggested that activation of the G protein-bound channel would destabilize the interaction and accelerate unbinding of G protein. Our model is similar to Kasai's in that G protein modifies only the slow process which is independent of fast opening transitions, although the intrinsic fast gating of the K_{ACh} channel is voltage independent (Yamada and Kurachi, 1995).

Functional vs. Molecular Biological Analysis

We assumed that with saturating concentrations of ACh, $[\text{G}_\text{K}^*]$ increases linearly as $[\text{GTP}]$ is raised in the range of concentrations used in this study. The results shown in Fig. 3 may indicate that in the presence of saturating concentrations of ACh, the response to GTP reaches the maximum plateau because channels are saturated with active G protein subunits (G_K^*), although it is difficult to unequivocally verify this assumption. It was shown that the exogenous $\text{G}_{\beta\gamma}$, either purified from brain membrane or recombinant, activate the K_{ACh} channel almost irreversibly, possibly because they are hydrophobic and may partition into and hardly escape from the membrane lipid bilayer (Logothetis et al., 1987; Ito et al., 1992; Wickman et al., 1994). They would accumulate in the membrane patch; hence, at least theoretically, exogenous $\text{G}_{\beta\gamma}$ would fully activate the channel at any concentrations higher than a certain threshold concentration in a time-dependent manner. It was, however, also shown that the K_{ACh} channel activity increased and reached the maximum plateau level in a dose-dependent fashion as the concentration of exogenous $\text{G}_{\beta\gamma}$ was raised (Ito et al., 1992; Wickman et al., 1994). At this maximum plateau level, channels were probably saturated with the exogenous $\text{G}_{\beta\gamma}$. Furthermore, we have found that $\text{G}_{\beta\gamma}$ purified from brain membranes apparently activated the K_{ACh} channel in a positive cooperative manner, when the activities were isochronally measured (Ito et al., 1992).

Hydrophilic transducin $\beta\gamma$ subunits, which reversibly opened the channel, also showed a positive cooperative activation of the channel (Yamada et al., 1994a). In both cases, the Hill coefficients were ~ 3 , which is close to the value obtained for the channel activation by GTP (Kurachi et al., 1990; Ito et al., 1991). It is also known that the stoichiometry of GTP-binding to a G protein is 1:1 and that G proteins are usually present in large excess of their effector (Gilman, 1987). Taken above results together, it would be reasonable to assume that $[\text{G}_\text{K}^*]$ in the patch membrane increased almost linearly as $[\text{GTP}]$ was raised in a range of 0.1–10 μM in the presence of a saturating concentration of ACh (0.5 μM).

With this assumption, the positive cooperative activation of the K_{ACh} channel by GTP could be well explained by the MWC concerted allosteric model. The simulation indicated that the channel could be well described as a multimer composed of four or more functionally identical subunits. This prediction is consistent with the current hypothesis that inwardly rectifying K^+ channels are tetrameric, after the analogy of voltage-dependent K^+ channels (Jan and Jan, 1994; Inanobe et al., 1995a). The result of this functional analysis, however, could not be simply translated into molecular terms. It was recently proposed that the cardiac K_{ACh} channel is composed of at least two different inwardly rectifying K^+ channel subunits, products of GIRK1 and CIR (GIRK4) (Krapivinsky et al., 1995). It was also suggested that the brain K_{ACh} channel would be formed by GIRK1 with other clones, such as GIRK2 or GIRK3 (Duprat et al., 1995; Kofuji et al., 1995). GIRK4 or GIRK2 alone produced a novel K^+ channel current activated by $\text{G}_{\beta\gamma}$ (Krapivinsky et al., 1995; Kofuji et al., 1995). Thus, it is possible that G_K^* also interacts with the cardiac K_{ACh} channel protein through subunits other than GIRK1. At present, the functional role for the GIRK4 or GIRK2 polypeptide in the interaction of the K_{ACh} channel with $\text{G}_{\beta\gamma}$ is not fully understood. This information should definitely be incorporated to improve the model. Despite these ambiguities, our functional model well described the positive cooperative activation of the K_{ACh} channel by G protein and predicted the multimeric structure of the channel. Therefore, the model may provide a novel functional basis to further clarify the molecular interaction between G protein and the K_{ACh} channel.

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