On the Mechanism of G Protein βγ Subunit Activation of the Muscarinic K+ Channel in Guinea Pig Atrial Cell Membrane

Comparison with the ATP-sensitive K+ Channel

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ABSTRACT The mechanism of G protein βγ subunit (G_{βγ})-induced activation of the muscarinic K+ channel (K_{ACb}) in the guinea pig atrial cell membrane was examined using the inside-out patch clamp technique. G_{βγ} and GTP-γS-bound α subunits (G_{αγ}) of pertussis toxin (PT)-sensitive G proteins were purified from bovine brain. Either in the presence or absence of Mg^{2+}, G_{βγ} activated the K_{ACb} channel in a concentration-dependent fashion. 10 nM G_{βγ} almost fully activated the channel in 132 of 134 patches (98.5%). The G_{βγ}-induced maximal channel activity was equivalent to or sometimes larger than the GTP-γS-induced one. Half-maximal activation occurred at ~6 nM G_{βγ}. Detergent (CHAPS) and boiled G_{βγ} preparation could not activate the K_{ACb} channel. G_{βγ} suspended by Lubrol PX instead of CHAPS also activated the channel. Even when G_{βγ} was pretreated in Mg^{2+}-free EDTA internal solution containing GDP analogues (24–48 h) to inactivate possibly contaminating G_{αγ}s, the G_{βγ} activated the channel. Furthermore, G_{βγ} preincubated with excessive GDP-bound G_{αγ} did not activate the channel. These results indicate that G_{βγ} itself, but neither the detergent CHAPS nor contaminating G_{αγ}, activates the K_{ACb} channel. Three different kinds of G_{αγ} at 10 pM–10 nM could weakly activate the K_{ACb} channel. However, they were effective only in 40 of 124 patches (32.2%) and their maximal channel activation was ~20% of that induced by GTP-γS or G_{βγ}.

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Thus, $G_{\alpha}$ activation of the $K_{ACb}$ channel may not be significant. On the other hand, $G_{\alpha}$'s effectively activated the ATP-sensitive $K^+$ channel ($K_{ATP}$) in the ventricular cell membrane when the $K_{ATP}$ channel was maintained phosphorylated by the internal solution containing 100 $\mu$M Mg$\cdot$ATP. $G_{\alpha}$ inhibited adenosine or mACh receptor-mediated, intracellular GTP-induced activation of the $K_{ATP}$ channel. $G_{\alpha}$'s also activated the phosphorylated $K_{ATP}$ channel in the atrial cell membrane, but did not affect the background $K_{ACb}$ channel. $G_{\beta}$ subsequently applied to the same patch caused prominent $K_{ACb}$ channel activation. The above results may indicate two distinct regulatory systems of cardiac $K^+$ channels by PT-sensitive $G$ proteins: $G_{\alpha}$ activation of the $K_{ATP}$ channel and $G_{\beta}$ activation of the $K_{ACb}$ channel.

**INTRODUCTION**

GTP-binding proteins ($G$) play a central role in the receptor-mediated regulation of cellular functions (Gilman, 1987; Neer and Clapham, 1988). They activate or inhibit a variety of effectors, such as adenyl cyclase and phospholipase C. Recently it was shown that plasma membrane ion channels can also be directly regulated by $G$ proteins. It was first reported that pertussis toxin (PT)-sensitive $G$ proteins are involved in the muscarinic acetylcholine (mACh) receptor-dependent activation of a specific inward-rectifying $K^+$ channel ($K_{ACb}$) current in cardiac atrial whole cells (Breitwieser and Szabo, 1985; Pfaffinger, Martin, Hunter, Nathanson, and Hille, 1985) and in the isolated atrial cell patch membrane (Kurachi, Nakajima, and Sugimoto, 1986a, b). Since then, it has been reported that the L-type $Ca^{2+}$ channel (Yatani and Brown, 1989), $Na^+$ channel (Schubert, VanDongen, Kirsch, and Brown, 1989), ATP-sensitive $K^+$ channel ($K_{ATP}$) (Kirsch, Codina, Birnbaumer, and Brown, 1990; Tung and Kurachi, 1990), and I$f$ channel (Yatani, Okabe, Codina, Birnbaumer, and Brown, 1990b) are directly regulated by $G$ proteins in cardiac myocytes.

$G$ proteins are heterotrimeric consisting of three subunits, $G_{\alpha}$, $G_{\beta}$, and $G_{\gamma}$. Agonist binding to the receptors stimulates GDP release and subsequent GTP binding on a $G$ protein, which results in the functional dissociation of the $G$ protein into its subunits, i.e., GTP-bound $G_{\alpha}(G_{\alpha}$GTP) and $G_{\beta}$. $G_{\gamma}$'s clearly differ among the members of $G$ proteins. Common $G_{\beta}$ may be shared among at least some $G_{\gamma}$'s to form the specific trimers. Therefore, it has been assumed that $G_{\alpha}$GTP mediates specific signals to effectors in various signal transduction systems, such as $G_{\alpha}$ activation of adenyl cyclase or transducin activation of cGMP phosphodiesterase (Gilman, 1987). However, both of the subunits can be the regulatory arm of the $G$ proteins to their effectors (Neer and Clapham, 1988).

Since the report of Logothetis, Kurachi, Galper, Neer, and Clapham (1987a) that $G_{\beta}$ purified from bovine brain activated the $K_{ACb}$ channel in the atrial cell patch membrane, specific roles of $G_{\beta}$ in cell signaling other than binding to $G_{\alpha}$GDP have been demonstrated: (1) Jelsema and Axelrod (1987) and Jelsema, Burch, Jaken, Ma, and Axelrod (1989) showed that transducin $G_{\beta}(G_{\gamma})$ activated phospholipase A$_2$ (PLA$_2$) in rod outer segments. Since the $G_{\beta}(G_{\gamma})$ activation of PLA$_2$ occurred even in the presence of GTP-$\gamma$S, which prevents reassociation of $G$ protein subunits, they concluded that the $G_{\beta}(G_{\gamma})$ activation was a direct effect, and was not caused by the binding of the $G_{\beta}(G_{\gamma})$ with an inhibitory $G_{i}$ thereby causing a removal of inhibition. (2) Katada, Kusakabe, Oinuma, and Ui (1987) showed that the $Ca^{2+}$-calmodulin
activation of rat brain adenylyl cyclase could be inhibited by 2–10 nM porcine brain G\textsubscript{i} or G\textsubscript{q}. They postulated that this inhibition was due to the formation of a calmodulin-G\textsubscript{i} or G\textsubscript{q} complex that was incapable of activating cyclase. (3) Whiteway, Hougan, Dignard, Thomas, Bell, Saari, Grant, O’Hara, and MacKay (1989) showed that in yeast, genetic mutants lacking the STE4 or STE18 genes (which encode for putative yeast G\textsubscript{i} and G\textsubscript{q}, respectively), were unable to respond to pheromone. On the basis of these experiments, they proposed that the putative yeast G\textsubscript{i} was directly involved in initiating the pheromone response. Also, in mutants in which the SCG1 gene (putative yeast G\textsubscript{q}) had been modified or was lacking, the pheromone pathway was constitutively activated, suggesting that the excess free G\textsubscript{i} stimulated the pathway.

Although the G\textsubscript{i} activation of the KA\textsubscript{Ch} channel in the cardiac atrial cell membrane was the first evidence for G\textsubscript{i} roles in various signal transduction pathways, exact roles of G\textsubscript{i} in channel regulation are still controversial (Codina, Yatani, Grenet, Brown, and Birnbaumer, 1987; Kirsch, Yatani, Codina, Birnbaumer, and Brown, 1988; Brown and Birnbaumer, 1990; Yatani, Okabe, Birnbaumer, and Brown, 1990a), despite several reports of further examination of the results (Logothetis, Kim, Northup, Neer, and Clapham, 1988; Kurachi, Ito, Sugimoto, Katada, and Ui, 1989a; Kobayashi, Shibasaki, Takahashi, Tohyama, Kurachi, Ito, Ui, and Katada, 1990; Nanavati, Clapham, Ito, and Kurachi, 1990). In this study we reexamined and contrasted the properties and mechanisms underlying activation of two cardiac K\textsuperscript{+} channels, K\textsubscript{ACa} and K\textsubscript{ATP}, by G protein subunits.

**MATERIALS AND METHODS**

**Preparations**

Single atrial and ventricular cells of the guinea pig heart were obtained by an enzymatic dissociation method as described previously (Isenberg and Klockner, 1982; Kurachi et al., 1986). Briefly, collagenase (0.04% wt/vol, Sigma type 1; Sigma Chemical Co., St. Louis, MO) in nominally Ca\textsuperscript{2+}-free bathing solution (for composition, see below) was perfused through the coronary arteries with a Langendorff apparatus for 20 min (37°C). The heart was then stored in the high-K\textsuperscript{+}, low-Cl\textsuperscript{−} solution (for composition, see below) at 4°C for later experiments. A small piece of cardiac tissue was dissected and gently agitated in the recording chamber filled with the control bathing solution. Quiescent relaxed atrial or ventricular cells showing clear striations were used for the experiments. All experiments were performed at 33–35°C.

**Solutions and Chemicals**

The control bathing solution contained (mM): 136.5 NaCl, 5.4 KCl, 1.8 CaCl\textsubscript{2}, 0.53 MgCl\textsubscript{2}, 0.33 NaH\textsubscript{2}PO\textsubscript{4}, 5.5 glucose, and 5.5 HEPES-NaOH buffer (pH 7.4). The composition of the high-K\textsuperscript{+}, low-Cl\textsuperscript{−} solution was (mM): 10 taurine, 10 oxalic acid, 70 glutamic acid, 25 KCl, 10 KH\textsubscript{2}PO\textsubscript{4}, 11 glucose, 0.5 EGTA, and 10 HEPES-KOH buffer (pH 7.3–7.4). The composition of the pipette solution was (mM): 140 KCl, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, and 5 HEPES-KOH (pH 7.4). In the inside-out patch clamp experiments the bath was perfused with “internal” solution containing (mM): 140 KCl, 0.5–2 MgCl\textsubscript{2}, 5 EGTA-KOH, 5 HEPES-KOH buffer (pH 7.3). In Mg\textsuperscript{2+}-free internal solution, MgCl\textsubscript{2} was omitted and EGTA was replaced with equimolar EDTA.

GTP (Na salt), 5'-guanylylimidodiphosphate (GppNHp), ATP (Na or K salt), acetylcholine (ACh), atropine, nordihydroguaiaretic acid (NDGA), and trypsin (type I or II) were purchased
from Sigma Chemical Co. (St. Louis, MO). GDP, guanosine-5'-O-(2-thiodiphosphate) (GDP-
βS), and guanosine-5'-O-(3-thiotriphosphate) (GTP-γS) were from Boehringer Mannheim. AA-861 (2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone) was a gift from Takeda Pharmaceutical Co. (Osaka, Japan). Lipocortin I is a gift from Dr. Keizo Inoue (Faculty of Pharmaceutical Sciences, University of Tokyo).

Gα and Gβγ of PT-substrate G proteins were purified from bovine brain as described previ
ously (Katada, Oinuma, and Ui, 1986; Kobayashi et al., 1990). The purified Gβγ (6.5, 14.9,
or 20 μM), GαS (3.7 or 6.5 μM), GαQ (0.3 or 6.2 μM), Gαα (12.8 μM), and GαQ-GDP (1 μM) in 50 mM Na-HEPES (pH 7.4) solution containing 0.1 mM Na-EGTA and 0.7% (wt/vol) CHAPS (Dotite, Kumamoto, Japan) were stored at -80°C. The GαQ (20 μM) in the same buffer containing 0.5% (wt/vol) Lubrol PX instead of CHAPS was also used in several experiments. These subunits were dissolved in the internal solution at a concentration of 100 nM and stored at 4°C (stock preparations). The stock preparations were diluted by the internal solution to the desired concentrations just before use. The stock preparations were used within 5 d.

Current Measurements and Data Analysis

The GΩ seal patch clamp technique was used in the inside-out patch configuration (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981). Currents were measured by a patch clamp amplifier (EPC-7; List, Darmstadt, Germany) and monitored with a high-gain digital storage oscilloscope (VC-6025; Hitachi, Tokyo, Japan). The resistance of the patch electrodes ranged from 5 to 7 MΩ, and the tip of the electrode was coated with Sylgard and fire-polished. The data were stored in a video cassette recorder (BR6400; Victor, Tokyo, Japan) using a PCM converter system (RP-880; NF Electronic Circuit Design, Tokyo, Japan, or Instrutech, Elmont, NY, with a 10-kHz bandwidth), reproduced and low-pass filtered at 1.5–2 kHz (−3 dB) by a Bessel filter (FV-625A; NF Electronic Circuit Design; 48 dB/octave slope attenuation), sampled at 5 kHz, and analyzed off-line on a computer (PC-9800VM2; NEC, Tokyo, Japan, or MEGA-ST; Atari, Sunnyvale, CA). For single channel analysis, the threshold for judging the open state was set at half of the single channel amplitude (Colquhoun and Sigworth, 1983). Statistical data were expressed as mean ± SD.

RESULTS

Effects of Gβγ on the KACH Channel

Fig. 1 shows the effects of Gβγ on the KACH channel in an inside-out patch of guinea pig atrial cell membrane. With 0.3 μM ACh in the pipette solution, 100 μM GTP was perfused to the intracellular side of the inside-out patch, causing vigorous activity of the KACH channel (Kurachi et al., 1986a, b) (Fig. 1A), which disappeared after washing out GTP. Application of 10 nM Gβγ to the patch induced persistent openings of a K⁺ channel identical to the KACH channel. At a concentration of 10 nM, Gβγ almost fully and consistently activated the KACH channel within a significant lag time (<5 s) in 132 of 134 patches (98.5%). Activation of the KACH channel by GTP with agonists required intracellular Mg²⁺ (Kurachi, Nakajima, and Sugimoto, 1986c). In contrast, Gβγ could activate the KACH channel even in the 0 Mg²⁺-EDTA internal solution (14 of 14 patches) (see also Kurachi et al., 1989a). The KACH channel openings induced by GTP (100 μM) in the presence of 0.3 μM ACh and 2 mM Mg²⁺, Gβγ (10 nM) with 2 mM Mg²⁺, and Gβγ, in the absence of Mg²⁺ (EDTA solution) at various membrane potentials are shown in Fig. 1B. Similar
pulse-like channel openings were elicited by GTP and \( G_{\beta\gamma} \). With hyperpolarization, the unit amplitude of the channel increased. With depolarization, the amplitude decreased and became zero at around the K\(^+\) equilibrium potential \( (E_K \approx 0 \text{ mV in the present experimental condition}) \). At more positive potentials, small outward currents were observed in the internal solution containing Mg\(^{2+}\). In Mg\(^{2+}\)-free solution, outward currents activated by \( G_{\beta\gamma} \) were observed (Logothetis et al., 1987a). The unit conductance of GTP- and \( G_{\beta\gamma} \)-activated channels was \( \sim 40-45 \text{ pS} \) in symmetrical 150 mM K\(^+\) and showed a strong inward rectification with 2 mM Mg\(^{2+}\) in the internal solution. The current–voltage relationships were superimposable. The \( G_{\beta\gamma} \)-activated channel became linear in the absence of Mg\(^{2+}\), similar to that reported

**FIGURE 1.** Activation of \( K_{ACh} \) channel by GTP and \( G_{\beta\gamma} \). (A) After forming an inside-out patch in internal solution containing 2 mM MgCl\(_2\), 100 \( \mu \text{M} \) GTP activated the \( K_{ACh} \) channel with 0.3 \( \mu \text{M} \) ACh in the pipette solution. After washing out GTP, channel activity disappeared. Subsequent application of \( G_{\beta\gamma} \) activated the \( K_{ACh} \) channel irreversibly. Bars above the tracing indicate perfusing protocol. The patch was held at \(-80 \text{ mV}\). (B) Expanded recordings of the \( K_{ACh} \) channel at various holding potentials induced by GTP or \( G_{\beta\gamma} \) with or without 2 mM Mg\(^{2+}\) (indicated above each column). (C) Current–voltage \((I-V)\) relationship of the \( K_{ACh} \) channel. Filled triangles, open squares, and filled squares represent the \( I-V \) relationship induced by GTP with Mg\(^{2+}\) (2 mM), \( G_{\beta\gamma} \) with Mg\(^{2+}\) (2 mM), and \( G_{\beta\gamma} \) without Mg\(^{2+}\), respectively. Strong inward rectification was noted using GTP with Mg\(^{2+}\) and \( G_{\beta\gamma} \) with Mg\(^{2+}\). (D) Open-time histograms of the \( K_{ACh} \) channel currents induced by GTP and \( G_{\beta\gamma} \) at \(-80 \text{ mV}\). (E) Voltage-dependent channel activity in the absence of Mg\(^{2+}\) induced by \( G_{\beta\gamma} \) (filled circles) and GppNHp (open circles). The relative \( N'P_o \) was obtained in reference to the \( N'P_o \) induced by 10 nM \( G_{\beta\gamma} \) or GppNHp (10 \( \mu \text{M} \)) at \(-80 \text{ mV}\). The results were expressed as mean \( \pm \text{SD} \) (\( n = 3 \) each).
previously in the GppNHp-induced $K_{\text{ACH}}$ channel openings (Fig. 1, B and C; Horie and Irisawa, 1987; see also Logothetis et al., 1987a). The open-time histogram of the GTP- and $G_{\beta\gamma}$-activated channels at $-80$ mV could be fit by a single exponential curve with a time constant of $\sim 1$ ms in both cases (Fig. 1 D).

The $K_{\text{ACH}}$ channel activity induced by a nonhydrolyzable GTP analogue, GppNHp, and $G_{\beta\gamma}$ were measured at various potentials. To measure the channel activity at potentials positive to 0 mV, the Mg$^{2+}$-free/EDTA internal solution was perfused after activation of the channel by GppNHp or $G_{\beta\gamma}$. The relative $N$-$P_o$ of the channel (where $N$ is the number of the channels in the patch, and $P_o$ is the open probability of each channel) at each membrane potential was obtained with reference to the $N$-$P_o$ value at $-80$ mV in each patch. The relative $N$-$P_o$ of the GppNHp- and $G_{\beta\gamma}$-activated $K_{\text{ACH}}$ channels decreased as the membrane potential was depolarized to more positive than $E_K$ ($\sim 0$ mV) (Fig. 1 E).

Fig. 2 shows the steady-state relationship between the concentration of $G_{\beta\gamma}$ and the $K_{\text{ACH}}$ channel activity. Each symbol represents a different patch. Various concentrations (0.1-100 nM) of $G_{\beta\gamma}$ were sequentially applied to each patch. Steady-state activity of the channels was determined by perfusing each concentration of the subunit for $\sim 10$ min. The $K_{\text{ACH}}$ channel activity induced by $G_{\beta\gamma} > 10$ nM reached a steady level within 0.5 min. At 0.3–3 nM $G_{\beta\gamma}$, the channel activity gradually progressed and reached an apparently steady level within 5–10 min. Further perfusion of the subunit did not induce significant increase of the channel activity. It was also confirmed in five other patches that 0.1 nM $G_{\beta\gamma}$ did not cause any channel openings until $\sim 30$ min of perfusion. The channel activity at various concentrations of $G_{\beta\gamma}$ was normalized to the $N$-$P_o$ of the channel activity induced by 100 $\mu$M GTP (in the presence of ACh in the pipette) or 10–100 $\mu$M GTP-$\gamma$S (in the presence or absence of ACh in the pipette) in each patch. The minimum concentration of bovine brain $G_{\beta\gamma}$ required to activate the channel was $\sim 300$ pM, which is 10 times larger than that previously reported for rat brain $G_{\beta\gamma}$ (Kurachi et al., 1989a). The half-maximal activation of the channel occurred at $\sim 6$ nM $G_{\beta\gamma}$, the maximal channel activity induced by $G_{\beta\gamma}$ was equivalent to or sometimes greater than that by 10–100 $\mu$M GTP-$\gamma$S.

**Specificity of the $G_{\beta\gamma}$ Activation of the $K_{\text{ACH}}$ Channel**

The detergent CHAPS was used to suspend the hydrophobic $G_{\beta\gamma}$. The concentration of CHAPS used to suspend 10 nM $G_{\beta\gamma}$ was either 5.7, 7.6, or 17.5 $\mu$M. Fig. 3 A shows that CHAPS (10–200 $\mu$M), purchased either from Dotite or from Sigma Chemical Co., alone did not activate the $K_{\text{ACH}}$ channel, but subsequent application of GTP or $G_{\beta\gamma}$ suspended in CHAPS activated the $K_{\text{ACH}}$ channel in the same patch ($n = 10$ each; Logothetis et al., 1988; Kurachi et al., 1989a; Nanavati et al., 1990), suggesting that (1) CHAPS itself did not activate the $K_{\text{ACH}}$ channel and (2) the negative effect of CHAPS was not due to vesicle formation of the patch (Kirsch et al., 1988). The buffer solution for $G_{\beta\gamma}$ also did not activate the $K_{\text{ACH}}$ channel ($n = 4$, not shown). When the $G_{\beta\gamma}$ preparation was boiled at 100°C for 5 min, the preparation did not activate the $K_{\text{ACH}}$ channel ($n = 4$), while nonboiled $G_{\beta\gamma}$ activated the channel in the same patch (Fig. 3 B). It was reported that Lubrol PX did not activate the $K_{\text{ACH}}$ channel (Kirsch et al., 1988) but blocked the channel activity at higher concentrations (Logothetis et al., 1988).
Therefore, we also tested the effects of $G_{\beta\gamma}$ suspended in another detergent, Lubrol PX, on the $K_{ACb}$ channel in Fig. 3 C. The $K_{ACb}$ channel was activated in a concentration-dependent manner by $G_{\beta\gamma}$ suspended in Lubrol PX ($n = 5$). The concentration of Lubrol PX in 10 nM $G_{\beta\gamma}$ solution was $2.5 \times 10^{-4}\%$. Lubrol PX at this concentration did not affect the receptor-mediated, GTP-induced activation of

![Graph A](image)

**Figure 2.** Concentration-dependent activation of the $K_{ACb}$ channel by $G_{\beta\gamma}$. After forming an inside-out patch, $G_{\beta\gamma}$ induced the $K_{ACb}$ channel activity in a concentration-dependent fashion. Either 100 nM GTP (in the presence of 1 nM ACh) in the beginning of the experiment or 10–100 nM GTP-\(\gamma\)S at the end of experiment was added to the internal side of the membrane to obtain maximal activation of the $K_{ACb}$ channel of each patch. The patch was held at -80 mV. The bottom graph shows the relationship between the relative $N'P_o$ of the $K_{ACb}$ channel and the concentration of $G_{\beta\gamma}$ obtained from four patches (represented by different symbols). The continuous line is a curve fit to the Hill equation using the nonlinear, least-squares regression method, MULTI (Yamaoka, Tanigawara, Nakagawa, and Uno, 1981): \[
y = \frac{V_{MAX}}{1 + \left(\frac{K_d}{[G_{\beta\gamma}]}\right)^H}\]

where $y$ is the relative $N'P_o$, $V_{MAX}$ is the maximal relative $N'P_o$, $K_d$ is the $G_{\beta\gamma}$ concentration at which half-maximal channel activation occurred, and $H$ is the Hill coefficient. In the graph, the Hill coefficient was 3.12 and the half-maximal channel activation occurred at 6 nM $G_{\beta\gamma}$, $\gamma = 0.95$. The $N'P_o$ obtained from each concentration of $G_{\beta\gamma}$ was normalized with reference to the maximum $N'P_o$ induced by 100 nM GTP (with 1 nM ACh) or GTP-\(\gamma\)S in each patch.

the $K_{ACb}$ channel ($n = 5$), but blocked it at a high concentration ($10^{-3}\%$) ($n = 8$). These results indicate that activation of the $K_{ACb}$ channel induced by $G_{\beta\gamma}$ preparation is attributable only to the effects of heat-sensitive materials, including $G_{\beta\gamma}$ itself, but not to the detergent (CHAPS) or unknown heat-resistant substances contaminating the preparation.
It was also argued that the effects of the G_{\beta y} preparation on the K_{ACH} channel may be due to the contaminating preactivated (or GTP-\gamma S-bound) G_{\alpha y} (Birnbaumer and Brown, 1987; Codina et al., 1987). To eliminate the possibility of contamination of G_{\alpha y} in the G_{\beta y} preparation, G_{\beta y} was preincubated in the Mg^{2+}-free EDTA solution containing 2–10 \mu M GDP or GDP-\beta S for 24–48 h at 4°C, as it is known that G_{\alpha} is unstable in the Mg^{2+}-free solution (Codina, Hildebrandt, and Birnbaumer, 1984). Fig. 4A shows that the G_{\beta y}, pretreated with Mg^{2+}-free EDTA solution containing 2 \mu M GDP for 24 h, activated the K_{ACH} channel in the Mg^{2+}-free internal solution. Under the same conditions, neither G_{\alpha y}10, G_{\alpha y}20, nor G_{\alpha y}x (up to 10 nM) activated the K_{ACH} channel (n = 6 or 7 for each G_{\alpha y}; see also Fig. 2 of Kurachi et al., 1989a). These results suggest that the channel activation by the exogenous G_{\beta y} was not due to contaminating G_{\alpha y}. We also examined the effects of GDP-\beta S on activation of the channel by G_{\beta y} (Fig. 4B). GDP-\beta S (100 \mu M) inhibited the GTP-induced activation of the K_{ACH} channel (1.1 \mu M ACh in the pipette). G_{\beta y}, preincubated in Mg^{2+}-free EDTA solution containing 10 \mu M GDP-\beta S, activated the K_{ACH} channel in the continuous presence of 100 \mu M GDP-\beta S in the internal solution. Since GDP-\beta S is expected to block activation of the native G proteins by binding to G_{\alpha} (Gilman, 1987; Neer and Clapham, 1988), the involvement of native G proteins in the exogenous G_{\beta y}-induced activation of the K_{ACH} channel is also unlikely.

Fig. 5 shows the effects of G_{\alpha y}-GDP on G_{\beta y} activation of the K_{ACH} channel. In Fig. 5A, G_{\beta y} was preincubated with an excessive amount of G_{\alpha y}-GDP for 5 min at 35°C. G_{\beta y} preincubated with G_{\alpha y}-GDP did not activate the K_{ACH} channel (n = 5), while boiled
GTP, 3 nM preincubated with GDP 2 μM/Mg2+0 mM

GTP-γ-S 10 μM

1 min

10 pA

βγ 10 nM preincubated with GDP-γ-S/Mg2+0 mM

GTP-γ-S 10 μM

2 min

1 min

10 pA

FIGURE 4. Effects of pretreatment of Gαγ by Mg2+-free EDTA solution with GDP and GDP-γ-S. (A) After washing out GTP, 3 nM Gαγ preincubated with 0 mM Mg2+-EDTA/GDP (2 μM) was dissolved in Mg2+-free EDTA internal solution and superfused to the bath. The Gαγ activated the KACh channel. Subsequent application of 10 μM GTP-γ-S in the Mg2+-containing internal solution further increased the channel activity. (B) When 10 μM GTP was applied to the inside-out patch, it induced significant activation of the KACh channel, which was totally suppressed by 100 μM GDP-γ-S. In the presence of 100 μM GDP-γ-S, Gαγ preincubated with 0 mM Mg2+-EDTA and 10 μM GDP-γ-S caused maximal activation of the KACh channel. The patch solution contained 1.1 μM ACh and the patch was held at -80 mV.

Gαγ-GDP did not prevent Gαγ activation (n = 5; not shown). However, when Gαγ-GDP was applied after activation of the KACh channel by Gβγ, the channel activation was not affected by Gαγ-GDP (n = 4; Fig. 5 B). This indicates that the inhibitory effects of Gαγ-GDP did not reside in its direct inhibition of the channel, but rather Gαγ-GDP might associate with Gβγ to form inactive trimeric G proteins during preincubation. This observation strongly suggests that Gβγ alone activated the KACh channel.

FIGURE 5. Effects of Gαγ-GDP on Gβγ activation of KACh channel. (A) After washing out GTP, 5 nM Gαγ preincubated with 10 nM Gαγ-GDP was perfused to the internal side of the membrane. The Gαγ preparation did not induce the KACh channel activity. However, further application of Gβγ alone activated the KACh channel. (B) After the channel was activated by 5 nM Gβγ, application of Gαγ-GDP (10 nM) did not suppress the channel activity. The patch solution contained 1.1 μM ACh and the patch was held at -80 mV.
**G protein Activation of the **$K_{ACb}$ **Channel Is Not Mediated by Phospholipase A2**

Kim, Lewis, Graziadei, Neer, Bar-Sagi, and Clapham (1989), inspired by findings that arachidonic acid activated the $K_{ACb}$ channel (Kurachi, Ito, Sugimoto, Shimizu, Miki, and Ui, 1989b) and $G_{Pro}$ stimulated PLA$_2$ (Jelsema and Axelrod, 1987; Jelsema et al., 1989), proposed that $G_{Pro}$ activated the $K_{ACb}$ channel via stimulation of PLA$_2$. Their evidence was based on the findings that (1) arachidonic acid and lipoxygenase metabolites activated the channel in cell-attached patches, (2) an antibody to PLA$_2$ known to inhibit PLA$_2$ activity blocked $G_{Pro}$ activation, and (3) an inhibitor of arachidonic acid metabolism (NDGA; 1 $\mu$M) blocked $G_{Pro}$-induced channel activity. Although we have not examined effects of the antibody to PLA$_2$, we have tested this hypothesis by examining the effects of various arachidonic acid metabolism inhibitors, lipocortin I (a PLA$_2$ inhibitor), NDGA (a lipoxygenase inhibitor), and AA-861 (a specific 5-lipoxygenase inhibitor) on the $G_{Pro}$ activation of the $K_{ACb}$ channel. Fig. 6 shows that none of these agents prevented activation of the $K_{ACb}$ channel by $G_{Pro}$ ($n = 5–7$ for each inhibitor). In guinea pig atrial myocytes, 1–5 $\mu$M NDGA and 3 $\mu$M

![Figure 6](image)
AA-861 prevented arachidonic acid metabolite-mediated activation of the K_ACh channel by arachidonic acid, \( \alpha_1 \)-adrenergic agonists, and platelet-activating factor (PAF) in cell-attached patches (Kurachi et al., 1989b; Kurachi, Ito, Sugimoto, Shimizu, Miki, and Ui, 1989c; Nakajima, Sugimoto, and Kurachi, 1991). Therefore, these results suggest that PLA2-eicosanoid cascade is not involved in \( G_{B,\gamma} \) activation of the K_ACh channel, although other unknown intermediate steps still cannot be ruled out.

The Trypsin-sensitive Activation Site of the K_ACh Channel Is Not Involved in the \( G_{B,\gamma} \) Activation of the K_ACh Channel

It was recently reported that intracellular application of trypsin irreversibly activated the K_ACh channel in the absence of agonists and GTP analogues (Kirsch and Brown, 1989). It was postulated that trypsin activated the K_ACh channel by removing an inhibitory particle or mechanism from the channel. The relationship between the trypsin-sensitive activation site and \( G_{B,\gamma} \) activation of the K_ACh channel was examined.

In Fig. 7, when perfused to the intracellular side of the inside-out patch, trypsin (type I or II, 0.25–1.0 mg/ml) gradually activated the K_ACh channel within 0.5–1 min in 26 of 28 patches. ACh (0.3 \( \mu \)M) was present in the pipette solution. It was noted that trypsin induced persistent channel activation if the enzyme was washed out within 5 min. This channel activation was not suppressed by GDP-\( \beta \)S (\( n = 3 \), not shown), and did not require GTP analogues in the internal side of the patch (Kirsch and Brown, 1989). Prolonged application of trypsin, however, gradually reduced the channel activity within 10–20 min (Fig. 7A; Kirsch and Brown, 1989). After channel activity disappeared, GTP or GTP-\( \gamma \)S (10–100 \( \mu \)M) could not induce channel activation.
probably due to inactivation or denaturation of native G proteins in the membrane. In these patches, however, G_{\beta\gamma} could still reactivate the K_{ACH} channel (n = 7). When G_{\beta\gamma} was applied to the patch at the steady activation of the channel induced by 1–2 min perfusion of trypsin, the K_{ACH} channel was further activated to the full activation level induced by 100 \mu M GTP (n = 5; Fig. 7 B). These results suggest that G_{\beta\gamma} can activate the K_{ACH} channel at a site independent of the trypsin-sensitive activation site. G_{\beta\gamma}’s, on the other hand, could not reactivate the channel in five trypsin-treated patches. However, since the success rate of G_{\beta\gamma} activation of the K_{ACH} channel was low (~30% as stated below), we could not conclude whether G_{\beta\gamma} could activate the channel in the patches after trypsin treatment.

**Roles of G_{\beta\gamma} in the K_{ACH} Channel Activation**

Fig. 8, A–C, shows the effects of three different G_{\beta\gamma}’s (10–100 pM) on the K_{ACH} channel. Three heterogenous G_{\beta\gamma}’s, which were purified from bovine brain and preactivated with GTP-\gamma S, weakly activated the K_{ACH} channel with a lag time of several minutes when applied to the internal side of the patch membrane (Kobayashi...
et al., 1990). The $G_{i\alpha}$ activation of the $K_{ACh}$ channel started with a lag time of 3–5 min and reached a quasi-steady state at ~10 min. The channel activation occurred at 1–3 pM $G_{i\alpha}$'s and saturated at ~30–100 pM $G_{i\alpha}$'s. Application of 1–10 nM $G_{i\alpha}$'s did not induce further increase of the channel activity (not shown; see also Kobayashi et al., 1990 and Nanavati et al., 1990). The saturated level of activation of the $K_{ACh}$ channel achieved by $G_{i\alpha}$'s was 18.5 ± 9.2% ($n = 14$) of the 10 μM GTP-γS-induced channel activity. Further application of GTP-γS (10 μM) or $G_{py}$ (10 nM) maximally activated the channel in the same patches. This weak channel activation by $G_{i\alpha}$'s (100 pM–10 nM) was observed in 40 of 124 patches (32%). The success rate of $G_{i\alpha}$'s for activation of the $K_{ACh}$ channel did not differ significantly between the concentrations of 100 pM and 10 nM. Thus, $G_{i\alpha}$ activation of the $K_{ACh}$ channel was inconsistent and much weaker than $G_{py}$ activation (Nanavati et al., 1990).

In Fig. 8 D, we examined the effects of low concentrations (30–200 pM) of $G_{py}$ on the GTP-induced $K_{ACh}$ channel activation. If native $G_{in\text{-GTP}}$ mediates the signal from the muscarinic receptor to the $K_{ACh}$ channel, it would be expected that exogenous $G_{py}$ inhibits the GTP activation of the channel by accelerating formation of inactive trimeric G proteins. Although Okabe, Yatani, Evans, Ho, Codina, Birnbaumer, and Brown (1990) reported that 100 pM–1 nM $G_{py}$ completely suppressed the agonist-mediated, GTP-induced activation of the $K_{ACh}$ channel, in our experiments 10–200 pM exogenous bovine brain $G_{py}$ did not affect the GTP-induced channel activation in the time range of 4–8 min ($G_{py}$ 10–50 pM, $n = 5$; 100 pM, $n = 7$; 200 pM, $n = 5$). After washing out GTP from the bath, the channel activity disappeared. Subsequent application of 3 nM $G_{py}$ to the same inside-out patches caused prominent activation of the $K_{ACh}$ channel.

From the above results, we conclude that $G_{in\text{-GTP}}$ cannot fully account for the receptor-mediated, GTP-induced activation of the $K_{ACh}$ channel.

**Effects of $G_{i\alpha}$ on the Phosphorylated ATP-sensitive K⁺ Channel ($K_{ATP}$) in Ventricular and Atrial Myocytes**

Since it was reported that the $K_{ATP}$ channel could be activated by PT-sensitive $G_{i\alpha}$'s in rat ventricular cell membrane (Kirsch et al., 1990; see also Tung and Kurachi, 1990), we examined effects of $G_{i\alpha}$'s purified from bovine brain on the channel in the guinea pig ventricular cell membrane. In the guinea pig ventricular cell membrane, the $K_{ACh}$ channel cannot be activated by either GTP-γS or G protein subunits, probably because the $K_{ACh}$ channel is not expressed. In the ATP-free internal solution, the $K_{ATP}$ channel disappeared spontaneously, which was referred as "run down." The run down or an inoperative state of the $K_{ATP}$ channel is assumed to be caused by dephosphorylation of the channel (Ohno-Shosaku, Zunkler, and Trube, 1987; Tung and Kurachi, 1991). Therefore, inside-out patches of ventricular cell membrane were formed in the internal solution containing 100 μM ATP and 0.5 mM MgCl₂ to maintain the phosphorylated state of the $K_{ATP}$ channel in this series of experiments. Channel phosphorylation could be confirmed by washing out ATP from the perfusate at the end of each experiment, which would result in openings of the $K_{ATP}$ channel if the $K_{ATP}$ channels were phosphorylated during the experiment. If no channel openings were observed by perfusing ATP-free internal solution, it was assumed that
dephosphorylation rendering the channel inactive had occurred; these experiments were discarded from the analysis.

When \( G_{i1} \) or \( G_{i2a} \) (100 pM) was perfused to the internal surface of the patch membrane, bursting openings of a \( K^+ \) channel were induced (Fig. 9, A and B). This channel showed a unitary conductance of \( \sim 90 \) pS and a mean open time of \( \sim 1.5 \) ms (Fig. 10, B and C). Channel activity could be suppressed by 2 mM ATP (Fig. 9 B) or 1 \( \mu \)M glibenclamide (a specific \( K_{ATP} \) channel blocker; not shown), confirming that the channel was the \( K_{ATP} \) channel (Ashcroft, 1988). With reference to the background openings \( (N'P_o) \) of the \( K_{ATP} \) channel in the presence of ATP (100 \( \mu \)M), \( G_{i1a} \) and \( G_{i2a} \) increased the \( N'P_o \) of the channel by a factor of 12.2 \( \pm \) 10.4 \( (n = 4) \) of \( N'P_o \) and 15.8 \( \pm \) 12.0 \( (n = 5) \), respectively. \( G_{i8} \) activated the channel in two of five patches. In Fig. 9 C, when the pipette solution contained adenosine (10 \( \mu \)M), the \( N'P_o \) of the \( K_{ATP} \) channel was increased from 1.47 to 3.26 after application of GTP (100 \( \mu \)M), probably via activation of native \( G \) proteins in the patch membrane. In contrast to the \( K_{ACH} \) channel in the atrial cell membrane, further application of 10 nM \( G_{Py} \) inhibited the GTP-induced increase of the \( K_{ATP} \) channel activity and reduced the \( N'P_o \) value to 1.26, which was comparable to the baseline activity of the \( K_{ATP} \) channel in this patch. Similar observations were obtained in five other patches in the presence of either adenosine (10 \( \mu \)M, \( n = 3 \)) or ACh (1 \( \mu \)M, \( n = 2 \)) in the pipette. \( G_{Py} \) itself did not affect the background \( K_{ATP} \) channel activity in the presence of 100 \( \mu \)M Mg-ATP (not shown; \( n = 7 \)). These results indicate that \( \alpha \) subunits of the PT-sensitive \( G \) proteins activate the \( K_{ATP} \) channel in ventricular cell membrane.
Figure 10. (A) Effects of Gsia and Goß on the KATP and KAcH channels in the atrial cell membrane. The pipette solution contained 1 μM ACh. The inside-out patch was formed at the arrow above the current trace in the internal solution containing 100 μM ATP and 0.5 mM MgCl2. Gsia (300 pM) was first applied to the internal side of the patch, which clearly induced openings of the KATP channel (~90 pS) (4, 2) without affecting background activity of the KAcH channel. Subsequently, Goß (10 nM) was applied to the patch, which caused a dramatic increase of 45 pS KAcH channel openings in the same patch (4, 3, 4). Numbers above the current trace indicate the location of each expanded current trace below. In the expanded current trace, the dotted line is the first level of the KAcH channel and the continuous line is that of the KATP channel. The arrowhead at each trace is the zero current level. (B) The current-voltage relation of Gsia-induced KATP channel in ventricular (open squares) and atrial (open circles) cell membrane, and Goß-induced KAcH channel in the atrial cell membrane (closed circles). (C) The open-time histograms of Gsia-induced KATP channel (in ventricle) and Goß-induced KAcH channel (in atrium) at −80 mV.
In Fig. 10A, we compared the effects of $G_{ia}$ and $G_{ib}$ on the $K_{ATP}$ and $K_{AC}$ channels in the atrial cell membrane where both channels are expressed. In the cell-attached form, the $K_{AC}$ channel was activated vigorously by ACh (1 μM) in the pipette. In the inside-out patch condition, the openings of the $K_{AC}$ channel decreased to a minimal background level. It was noted that the background channel activity in 100 μM Mg-ATP solution was higher than that in the internal solution without Mg-ATP. When $G_{ia}$ (300 pM) was applied to the internal side of the membrane, bursting $K^+$ channel openings with a conductance of ~90 pS were clearly induced. On the other hand, the background openings of the $K_{AC}$ channel were not affected significantly (Fig. 10A, 2). Openings of the 90-pS $K^+$ channel were blocked by 1 μM glibenclamide (not shown), indicating that this was the $K_{ATP}$ channel.

Subsequent application of $G_{ib}$ (10 nM) to the patch dramatically increased openings of the 40–45-pS $K_{AC}$ channel in the same patch membrane (Fig. 10A, 3 and 4). The $G_{ib}$-induced openings of the $K_{AC}$ channel were not affected by glibenclamide (not shown). The same results were obtained in two other patches.

The $K_{ATP}$ channel activated by $G_{ia}$ and $G_{ib}$ in both ventricular and atrial cell membranes had a conductance of ~90 pS in the inward direction, while the $K_{AC}$ channel activated by $G_{ib}$ in the atrial cell membrane had a conductance of ~40–45 pS (Fig. 10B). The $K_{AC}$ channel showed a prominent inward-rectifying property, although the $K_{ATP}$ channel did not rectify significantly in the presence of 500 μM MgCl$_2$ (Horie, Irisawa, and Noma, 1987). The open-time histogram of the $K_{ATP}$ and $K_{AC}$ channels at ~80 mV could be fit by a single exponential curve with a time constant of 1.4 ms for the $K_{ATP}$ channel and 1.1 ms for the $K_{AC}$ channel, respectively (Fig. 10C). These conductance and kinetic properties of the $K_{ATP}$ and $K_{AC}$ channels are consistent with those values previously reported (Noma, 1983; Kurachi et al., 1986a, b; Tung and Kurachi, 1991).

**DISCUSSION**

The major findings of the present study are: (1) $G_{ib}$ of PT-sensitive G proteins purified from bovine brain activated the $K_{AC}$ channel in a concentration-dependent fashion in guinea pig atrial myocytes. $G_{ib}$-activated $K_{AC}$ channel activity appeared to be voltage dependent in a way similar to the GppNHp-activated channel activity. (2) $G_{ib}$ activation of the $K_{AC}$ channel was independent of the effects of detergent, was not mediated by activation of the PLA$_2$-eicosanoid pathway, and was independent of the trypsin-sensitive activation site on the channel. (3) Three kinds of $G_{ib}$ purified from bovine brain weakly activated the $K_{AC}$ channel but to a much lower level and much less consistently than $G_{ib}$. (4) In contrast, $G_{ib}$'s could activate the phosphorylated $K_{ATP}$ channel, and $G_{ib}$ suppressed receptor-mediated, GTP-induced activation of the $K_{ATP}$ channel in the ventricular cell membrane.

$G_{ib}$ Activates the $K_{AC}$ Channel in Atrial Myocytes

Since the initial report on G protein regulation of the $K_{AC}$ channel function in the whole-cell condition (Breitwieser and Szabo, 1985; Pfaffinger et al., 1985), this concept was further strengthened by the evidence that the channel could be activated in inside-out patches of atrial cell membrane by intracellular GTP in the presence of
agonists (ACh or adenosine), by nonhydrolyzable GTP analogues (Kurachi et al., 1986a, b, c), and by purified G protein subunits in the absence of agonists (Codina et al., 1987; Logothetis et al., 1987a, 1988; Yatani, Codina, Brown, and Birnbaumer, 1987; Cerba, Klöckner, and Isenberg, 1988; Kirsch et al., 1988; Kurachi et al., 1989a). However, opinions differ as to which G protein subunit, G\(_i3\) or G\(_p\), plays a major role in the K\(_{AC}\) channel activation. Logothetis et al. (1987a) first reported that G\(_p\), purified from bovine brain activated the K\(_{AC}\) channel in chick embryonic atrial cells, while G\(_i3\)'s from human erythrocytes were found to activate the K\(_{AC}\) channel (Codina et al., 1987; Yatani et al., 1987). It was proposed that G\(_i3\) but not G\(_p\) activated the K\(_{AC}\) channel, and that the activating effects of G\(_i3\) on the K\(_{AC}\) channel shown by Logothetis et al. (1987a, 1988) and Kurachi et al. (1989a) were due to either (1) contamination of G\(_i3\) or (2) the detergent CHAPS, which was used to suspend G\(_i3\) (Kirsch et al., 1988; Brown and Birnbaumer, 1990; Yatani et al., 1990a).

In this study we reexamined the properties and possible mechanisms underlying G\(_i3\) activation of the K\(_{AC}\) channel.

G\(_p\) with and without Mg\(^{2+}\) activated a population of K\(^+\) channels with properties similar to the K\(_{AC}\) channel: a unitary channel conductance of ~40–45 pS at -80 mV and a mean channel open time of ~1 ms (Fig. 1, B-D). At membrane potentials positive to E\(_K\) (~0 mV), the channel currents induced by both GTP and G\(_p\), in the presence of 2 mM Mg\(^{2+}\) showed strong inward rectification. The inward rectification of the channel currents activated by G\(_p\), as well as by nonhydrolyzable GTP analogues such as GTP-\(\gamma\)S and GppNHp disappeared when the Mg\(^{2+}\)-free EDTA solution was perfused to the inside-out patches (Fig. 1, B and C; Horie and Irisawa, 1987; Logothetis et al., 1987a). It was also noted that G\(_p\)-induced channel activity (expressed as \(N'P_o\)) in the absence of Mg\(^{2+}\) decreased in a voltage-dependent manner when the holding potential was more positive than E\(_K\), similar to the GppNHp- or GTP-\(\gamma\)S-induced K\(_{AC}\) channel activity (Fig. 1 E). Therefore, the exogenous G\(_p\)-activated K\(_{AC}\) channel showed the same conductance and kinetic properties as those activated by GTP analogues.

It was reported that CHAPS was able to activate the K\(_{AC}\) channel in a Mg\(^{2+}\)-dependent manner (Kirsch et al., 1988; Yatani et al., 1990a). G\(_p\) could activate the K\(_{AC}\) channel in the Mg\(^{2+}\)-free EDTA solution as effectively as in the Mg\(^{2+}\)-containing solution (Fig. 4), which should be independent of the effects of CHAPS. Furthermore, we found that CHAPS (10–200 \(\mu\)M) with Mg\(_{Cl_2}\) (0.5–2 mM) and the buffer solution for G\(_p\) did not activate the channel as reported previously (Logothetis et al., 1987a, 1988; Logothetis, Kurachi, Galper, Neer, and Clapham, 1987b; Kurachi et al., 1989a). When boiled G\(_p\) (10 nM) was perfused to the patch, it did not activate the channel. However, subsequent application of G\(_p\) activated the channel, indicating that a heat-labile substance, such as G\(_p\), was responsible for the channel activation. Furthermore, 1–10 nM G\(_p\), suspended in 2.5 \times 10^{-5} or 2.5 \times 10^{-4}\% Lubrol PX solution, activated the channel. These results ruled out the possibility that CHAPS or buffer solution alone activated the K\(_{AC}\) channel.

If G\(_p\) activation of the channel was due to G\(_i3\) contamination, as previously suggested by Birnbaumer and Brown (1987), then preincubation of G\(_p\) in Mg\(^{2+}\)-free EDTA solution containing 2–10 \(\mu\)M GDP or GDP-\(\beta\)S for 24–48 h (at 4°C) should render G\(_p\) inactive, since G\(_i3\)'s are unstable in the Mg\(^{2+}\)-free condition (Codina et al.,
However, we found that Mg\textsuperscript{2+}-free GDP (or GDP-βS)-treated G\textsubscript{βγ} was as effective as nontreated G\textsubscript{βγ} in activating the K\textsubscript{ACH} channel (Fig. 4), suggesting that the effects of G\textsubscript{βγ} on the channel were not due to G\textsubscript{α} contamination.

The specific effect of G\textsubscript{βγ} on the K\textsubscript{ACH} channel was further confirmed by the observation that G\textsubscript{βγ}, when preincubated with excessive G\textsubscript{αGDP}, could not activate the channel, while G\textsubscript{βγ} alone subsequently activated the channel (see also Logothetis et al., 1988). During preincubation, G\textsubscript{βγ} may have bound to G\textsubscript{αGDP} to form an inactive heterotrimer (Gilman, 1987; Neer and Clapham, 1988). However, once the K\textsubscript{ACH} channel was activated by G\textsubscript{βγ}, the channel activity could not be suppressed or reversed by G\textsubscript{αGDP} (Fig. 5 B). This result clearly indicates that the suppressive effect of G\textsubscript{αGDP} on G\textsubscript{βγ} activation was not due to the direct inhibition of the K\textsubscript{ACH} channel. It also suggests that (1) the exogenously applied, more hydrophobic G\textsubscript{βγ} might bind to a site in the more hydrophobic center of lipid bilayer membrane, which G\textsubscript{αGDP} could not access, or (2) exogenous G\textsubscript{βγ} activated the channel through some unknown intermediate steps which caused irreversible channel activation. Logothetis et al. (1988) showed that G\textsubscript{αGDP} reversed the K\textsubscript{ACH} channel activation in a patch pretreated with G\textsubscript{βγ} or GTP-γS. This observation suggests that the functional activating arm of G\textsubscript{R} to the K\textsubscript{ACH} channel is G\textsubscript{βγ}.

### Roles of G\textsubscript{α} in the K\textsubscript{AC} and K\textsubscript{ATP} Channel Activation

We previously reported that three heterogenous G\textsubscript{δ1,2,3α}'s purified from bovine brain reassociated with G\textsubscript{βγ} in the GDP-bound form and served as substrates for PT-catalyzed ADP ribosylation, and that G\textsubscript{α}’s inhibited the G\textsubscript{α}’s activated adenylyl cyclase activity in S40 cell membrane, G\textsubscript{α} being the most effective inhibitor (Kobayashi et al., 1990). In this study we examined the effects of these G\textsubscript{α}’s on the phosphorylated K\textsubscript{ATP} channel (in the presence of 100 μM ATP and 0.5 mM MgCl\textsubscript{2}) and the K\textsubscript{ACH} channel. Since the K\textsubscript{ATP} channel in atrial myocytes is low in density and mostly dephosphorylated compared with ventricular myocytes (unpublished data), ventricular cells were mainly used to examine the effects of G\textsubscript{α} on the K\textsubscript{ATP} channel openings. In ventricular cells, G\textsubscript{α}’s effectively activated the K\textsubscript{ATP} channel, and G\textsubscript{βγ} inhibited the receptor-mediated, GTP-induced activation of the K\textsubscript{ATP} channel (Fig. 9). Therefore, G\textsubscript{in-GTP} may be the activating arm of the G proteins for the K\textsubscript{ATP} channel.

In the atrial cell membrane in the absence of Mg-ATP, G\textsubscript{α}’s did not affect the dephosphorylated, inoperative K\textsubscript{ATP} channel. They activated the K\textsubscript{AC} channel in ~30% of the patches to ~20% of the GTP-γS-induced channel activity, which cannot totally account for the physiological G\textsubscript{R} activation of the channel (Fig. 8). In the presence of 100 μM ATP and 5 mM MgCl\textsubscript{2}, G\textsubscript{α1α} activated the phosphorylated K\textsubscript{ATP} channel without significant alteration of the background openings of the K\textsubscript{AC} channel in three patches. Subsequent application of G\textsubscript{βγ} to the same inside-out patches resulted in dramatic activation of the K\textsubscript{AC} channel (Fig. 10). Therefore, we conclude that the G protein subunit activates the K\textsubscript{ATP} channel and the K\textsubscript{AC} channel differentially in the cardiac cell membrane: G\textsubscript{in-GTP} activates the K\textsubscript{ATP} channel and G\textsubscript{βγ} activates the K\textsubscript{AC} channel (Fig. 11). The former mechanism exists in both ventricular and atrial cells, while the latter may exist in atrial but not in ventricular cells. Since cardiac myocytes contain millimolar concentrations of intracellular ATP, the G protein activation of the K\textsubscript{ATP} channel system may not be operative under physiolog-
ical conditions. However, the system might play a significant role in the ischemia-induced shortening of the cardiac action potential. Although we cannot completely exclude the possibility that the $G_{\text{mb}}$-GTP pathway may partly contribute to the $G_{\text{k}}$ activation of the $K_{\text{ACh}}$ channel, the pathway cannot be the major regulatory mechanism for the $K_{\text{ACh}}$ channel.

**Mechanism of $G_{\beta\gamma}$ Activation of the $K_{\text{ACh}}$ Channel**

It was recently reported that arachidonic acid and its 5-lipoxygenase metabolites stimulate the $K_{\text{ACh}}$ channel activity in the cell-attached patches of atrial cells (Kim et al., 1989; Kurachi et al., 1989b). Kim et al. (1989) showed that (1) inhibition of the eicosanoid cascade by a monoclonal antibody against PLA$_2$ and 1 $\mu$M NDGA blocked the $G_{\beta\gamma}$ activation but not the receptor-mediated, GTP-induced activation of the $K_{\text{ACh}}$ channel, and that (2) arachidonic acid metabolites directly activated the $K_{\text{ACh}}$ channel when applied to the internal side of the isolated patches. They suggested that the exogenous $G_{\beta\gamma}$ activation of the $K_{\text{ACh}}$ channel in inside-out patches was mediated by the arachidonic acid–eicosanoid pathway, which may not be involved in the physiological $G_{\text{k}}$ activation of the $K_{\text{ACh}}$ channel. However, our observations in the present as well as previous studies were inconsistent with those of Kim et al. (1989) in several major points: (1) We observed that $G_{\beta\gamma}$ activated the $K_{\text{ACh}}$ channel in inside-out patches in which the PLA$_2$–eicosanoid pathway was inhibited by lipocortin I (15 $\mu$g/ml, a PLA$_2$ inhibitor), NDGA (1–5 $\mu$M, a lipoxygenase inhibitor), and AA-861 (3 $\mu$M, a 5-lipoxygenase inhibitor). The concentrations of these inhibitors were sufficiently high to block arachidonic acid–, $\alpha$-adrenergic agonist–, and PAF-induced, arachidonic acid metabolite–mediated activation of the $K_{\text{ACh}}$ channel in the guinea
pig atria (Kurachi et al., 1989b, c; Nakajima et al., 1991). (2) Arachidonic acid and its metabolites are far less effective in activating the K_{ACCh} channel than G_{βγ}. We previously showed that arachidonic acid-α-adrenergic agonist-, or PAF-induced, arachidonic acid metabolite-mediated K_{ACCh} channel activity in the cell-attached patch was ~30–40% of the GTP-γS- or G_{βγ}-induced channel activity. Kim et al. (1989) also reported only an 11-fold increase of the K_{ACCh} channel activity over the basal level by 5-HPETE and a 20-fold increase by LTD_{4}, but a 180-fold increase by G_{βγ} and a 175-fold increase by GTP-γS in rat neonatal atrial cells. These two results indicate that arachidonic acid metabolite activation of the K_{ACCh} channel cannot quantitatively account for the G_{βγ}-induced activation of the channel. (3) Arachidonic acid or leukotriene C_{4} superfused to the internal side of the patch membrane did not activate the K_{ACCh} channel in the absence of GTP in our experiments (Kurachi et al., 1989b), which is different from the observation by Kim et al. (1989). (4) The stimulating effects of arachidonic acid metabolites on the K_{ACCh} channel clearly required intracellular GTP in our study (Kurachi et al., 1989b). Nakajima et al. (1991) suggested that arachidonic acid metabolites cause a persistent stimulation of G_{K}, but not the K_{ACCh} channel itself, resulting in receptor-independent activation of the K_{ACCh} channel by intracellular GTP. The GTP dependence of arachidonic acid and leukotriene C_{4} effects of the basal K_{ACCh} channel activity was also reported in frog atrial whole cells (Scherer and Breitwieser, 1990). Although these experiments may be complicated by the use of lipophilic compounds and rather nonspecific inhibitors, our data support a direct activation of the K_{ACCh} channel by G_{βγ}.

It was recently suggested that trypsin, when applied to the internal side of the membrane, could remove the inhibitory mechanism(s) on the K_{ACCh} channel, thereby resulting in channel activation (Kirsch and Brown, 1989), and the G protein subunit might target the trypsin-sensitive site to activate the channel. However, our present results (Fig. 7) suggest that G_{βγ} activates the K_{ACCh} channel either directly or at a site downstream from the trypsin activation site. Thus, the trypsin-sensitive site may not be directly involved in the G_{βγ} activation of the K_{ACCh} channel.

If the effect of exogenous G_{βγ} on the K_{ACCh} channel is irreversible and G_{βγ} activates the channel directly, it would be expected that any concentration of G_{βγ} should eventually cause the maximal activation of the channel. This, however, does not appear to be the case in the G_{βγ} activation of the K_{ACCh} channel in the present study (Fig. 2). Similarly, we recently reported that intracellular GTP-γS, which activates G_{K} irreversibly, increased the K_{ACCh} channel activity in a concentration-dependent fashion: the steady-state relationship between the concentration of intracellular GTP-γS and the channel activity could be fit by the Hill equation with a Hill coefficient of 3–4 and a K_{D} of 0.06–0.08 μM GTP-γS (Nakajima, Sugimoto, and Kurachi, 1992; see also Kurachi, Ito, and Sugimoto, 1990; Ito, Sugimoto, Kobayashi, Takahashi, Katada, Ui, and Kurachi, 1991). These results clearly imply that other factors, not yet understood, are affecting the steps between the G_{K} subunit and the K_{ACCh} channel.

In summary, G_{βγ} activates the K_{ACCh} channel effectively and consistently, and may play a major role in the muscarinic activation of the K_{ACCh} channel. However, further studies are necessary to elucidate the molecular mechanisms underlying interaction between G_{βγ} and the K_{ACCh} channel.
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REFERENCES

Ashcroft, F. M. 1988. Adenosine 5'-triphosphate-sensitive potassium channels. Annual Review of Neuroscience. 11:97–118.

Birnbaumer, L., and A. M. Brown. 1987. G protein opening of K+ channels. Nature. 327:21–22.

Breitwieser, G. E., and G. Szabo. 1985. Uncoupling of cardiac muscarinic and β-adrenergic receptors from ion channels by a guanine nucleotide analogue. Nature. 317:538–540.

Brown, A. M., and L. Birnbaumer. 1990. Ionic channels and their regulation by G protein subunits. Annual Review of Physiology. 52:197–213.

Cerbai, E., U. Klöckner, and G. Isenberg. 1988. The α-subunit of the GTP-binding protein activates muscarinic potassium channels of the atrium. Science. 240:1782–1783.

Codina, J., J. D. Hildebrandt, and L. Birnbaumer. 1984. Effects of guanine nucleotides and Mg on human erythrocytes N1 and N2, the regulatory components of adenylyl cyclase. Journal of Biological Chemistry. 259:11408–11418.

Codina, J., A. Yatani, D. Grenet, A. M. Brown, and L. Birnbaumer. 1987. The α subunit of the GTP binding protein Gα1 opens atrial potassium channels. Science. 236:442–445.

Colquhoun, D., and F. J. Sigworth. 1983. Fitting and statistical analysis of single-channel records. In Single-Channel Recording. B. Sakmann, E. Neher, editors. Plenum Publishing Corp., New York. 191–263.

Gilman, A. G. 1987. G proteins: transducers of receptor-generated signals. Annual Review of Biochemistry. 56:615–649.

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

Horie, M., and H. Irisawa. 1987. Rectification of muscarinic K+ current by magnesium ion in guinea pig atrial cells. American Journal of Physiology. 253:H210–H214.

Horie, M., H. Irisawa, and A. Noma. 1987. Voltage-dependent magnesium block of adenosine-triphosphate-sensitive potassium channel in guinea-pig ventricular cells. Journal of Physiology. 387:251–272.

Isenberg, G., and U. Klöckner. 1982. Calcium tolerant ventricular myocytes prepared by preincubation in a "KB medium." Pflügers Archiv. 395:6–18.

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. Journal of General Physiology. 98:517–533.
Jelsema, C. L., and J. Axelrod. 1987. Stimulation of phospholipase A2 activity in bovine rod outer segments by the βγ subunits of transducin and its inhibition by the α subunit. Proceedings of the National Academy of Sciences, USA. 84:3623–3627.

Jelsema, C. L., R. M. Burch, S. Jaken, A. D. Ma, and J. Axelrod. 1989. Modulation of phospholipase A2 activity in rod outer segments of bovine retina by G protein subunits, guanine nucleotides, protein kinases and calpactin. Neurology and Neurobiology. 49:25–41.

Katada, T., K. Kusakabe, M. Oinuma, and M. Ui. 1987. A novel mechanism for the inhibition of adenylate cyclase via inhibitory GTP-binding proteins. Calmodulin-dependent inhibition of the cyclase catalyst by the βγ-subunits of GTP-binding proteins. Journal of Biological Chemistry. 262:11897–11900.

Katada, T., M. Oinuma, and M. Ui. 1986. Two guanine nucleotide-binding proteins in rat brain serving as the specific substrate of islet-activating protein, pertussis toxin. Journal of Biological Chemistry. 261:8182–8191.

Kim, D., D. L. Lewis, L. Graziadei, E. J. Neer, D. Bar-Sagi, and D. E. Clapham. 1989. G protein βγ-subunits activate the cardiac muscarinic K+ channel via phospholipase A2. Nature. 337:557–560.

Kirsch, G. E., and A. M. Brown. 1989. Trypsin activation of atrial muscarinic K+ channels. American Journal of Physiology. 257:H354–H358.

Kirsch, G. E., J. Codina, L. Birnbaumer, and A. M. Brown. 1990. Coupling of ATP-sensitive K+ channels to A1 receptors by G proteins in rat ventricular myocytes. American Journal of Physiology. 259:H820–H826.

Kobayashi, I., S. Shibasaki, K. Takahashi, K. Tohyama, Y. Kurachi, H. Ito, M. Ui, and T. Katada. 1990. Purification and characterization of five different α-subunits of guanosine-nucleotide-binding proteins in bovine brain membranes: their physiological properties to the activities of adenylate cyclase and atrial muscarinic K+ channels. European Journal of Biochemistry. 191:499–506.

Kurachi, Y., H. Ito, and T. Sugimoto. 1990. Positive cooperativity in activation of the cardiac muscarinic K+ channel by intracellular GTP. Pflügers Archiv. 416:216–218.

Kurachi, Y., H. Ito, T. Sugimoto, T. Katada, and M. Ui. 1989a. Activation of atrial muscarinic K+ channels by low concentrations of βγ-subunits of rat brain G protein. Pflügers Archiv. 413:325–327.

Kurachi, Y., H. Ito, T. Sugimoto, T. Shimizu, I. Miki, and M. Ui. 1989b. Arachidonic acid metabolites as intracellular modulators of the G protein-gated cardiac K+ channel. Nature. 337:555–557.

Kurachi, Y., H. Ito, T. Sugimoto, T. Shimizu, I. Miki, and M. Ui. 1989c. α-Adrenergic activation of the muscarinic K+ channel is mediated by arachidonic acid metabolites. Pflügers Archiv. 414:102–104.

Kurachi, Y., T. Nakajima, and T. Sugimoto. 1986a. Acetylcholine activation of K+ channels in cell-free membrane of atrial cells. American Journal of Physiology. 251:H681–H684.

Kurachi, Y., T. Nakajima, and T. Sugimoto. 1986b. On the mechanism of activation of muscarinic K+ channels by adenosine in isolated atrial cells: involvement of GTP-binding proteins. Pflügers Archiv. 407:264–274.

Kurachi, Y., T. Nakajima, and T. Sugimoto. 1986c. Role of intracellular Mg2+ in the activation of muscarinic K+ channel in cardiac atrial cell membrane. Pflügers Archiv. 407:572–574.

Logothetis, D. E., D. Kim, J. K. Northup, E. J. Neer, and D. E. Clapham. 1988. Specificity of action of guanine nucleotide-binding regulatory protein subunits on the cardiac muscarinic K+ channel. Proceedings of the National Academy of Sciences, USA. 85:5814–5818.

Logothetis, D. E., Y. Kurachi, J. Galper, E. J. Neer, and D. E. Clapham. 1987a. The βγ subunits of GTP-binding proteins activate the muscarinic K+ channel in heart. Nature. 325:321–326.
Logothetis, D. E., Y. Kurachi, J. Galper, E. J. Neer, and D. E. Clapham. 1987b. G protein opening of K⁺ channels. *Nature.* 327:21–22.

Nakajima, T., T. Sugimoto, and Y. Kurachi. 1991. Platelet-activating factor activates cardiac Go via arachidonic acid metabolites. *FEBS Letters.* 289:239–243.

Nakajima, T., T. Sugimoto, and Y. Kurachi. 1992. Effects of anions on the G protein–mediated activation of the muscarinic K⁺ channel in the cardiac atrial cell membrane. Intracellular chloride-inhibition of the GTPase activity of Go. *Journal of General Physiology.* 99:665–682.

Nanavati, C., D. E. Clapham, H. Ito, and Y. Kurachi. 1990. A comparison of the roles of purified G protein subunits in the activation of the cardiac muscarinic K⁺ channel. In *G Proteins and Signal Transduction.* N. M. Nathanson and T. K. Harden, editors. The Rockefeller University Press, New York. 30–41.

Neer, E. J., and D. E. Clapham. 1988. Role of G protein subunits in transmembrane signalling. *Nature.* 333:129–134.

Noma, A. 1983. ATP-regulated K⁺ channels in cardiac muscle. *Nature.* 305:147–148.

Ohno-Shosaku, T., B. J. Zunkle, and G. Trube. 1987. Dual effects of ATP on K⁺ current of mouse pancreatic ß-cells. *Pflügers Archiv.* 408:133–138.

Okabe, K., A. Yatani, T. Evans, Y. Ho, J. Codina, L. Birnbaumer, and A. M. Brown. 1990. βγ dimers of G proteins inhibit atrial muscarinic K⁺ channels. *Journal of Biological Chemistry.* 265:12854–12858.

Pfaffinger, 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.

Scherer, R. W., and G. E. Breitwieser. 1990. Arachidonic acid metabolites alter G protein–mediated signal transduction in heart. Effects on muscarinic K⁺ channels. *Journal of General Physiology.* 96:735–755.

Schubert, B., A. VanDongen, G. E. Kirsch, and A. M. Brown. 1989. β-Adrenergic inhibition of cardiac sodium channels by dual G-protein pathways. *Science.* 245:516–519.

Tung, R. T., and Y. Kurachi. 1990. G protein activation of the cardiac ATP-sensitive K channel. *Circulation.* 82:III–462.

Tung, R. T., and Y. Kurachi. 1991. On the mechanism of nucleotide diphosphate activation of the ATP-sensitive K⁺ channel in ventricular cell of guinea-pig. *Journal of Physiology.* 437:239–256.

Whiteway, M., L. Hougan, D. Dignard, D. Y. Thomas, L. Bell, G. C. Saari, F. J. Grant, P. O’Hara, and V. L. Mackay. 1989. The STE4 and STE18 genes of yeast encode potential β and γ subunits of the mating factor receptor-coupled G protein. *Cell.* 56:467–477.

Yamaoka, K., Y. Tanigawara, T. Nakagawa, and T. Uno. 1981. A pharmacokinetic analysis program (MULTI) for microcomputer. *Journal of Pharmacobiodynamics.* 4:879–890.

Yatani, A., and A. M. Brown. 1989. Rapid β-adrenergic modulation of cardiac calcium channel currents by a fast G protein pathway. *Science.* 245:71–74.

Yatani, A., J. Codina, A. M. Brown, and L. Birnbaumer. 1987. Direct activation of mammalian atrial muscarinic potassium channels by GTP regulatory protein Go. *Science.* 235:207–211.

Yatani, A., K. Okabe, L. Birnbaumer, and A. M. Brown. 1990a. Detergents, dimeric Gβγ, and eicosanoid pathways to muscarinic atrial K⁺ channels. *American Journal of Physiology.* 258:H1507–H1514.

Yatani, A., K. Okabe, J. Codina, L. Birnbaumer, and A. M. Brown. 1990b. Heart rate regulation by G protein acting on the cardiac pacemaker channel. *Science.* 249:1163–1166.