

On the Mechanism of Basal and Agonist-induced Activation of the G Protein-gated Muscarinic K⁺ Channel in Atrial Myocytes of Guinea Pig Heart

HIROYUKI ITO, TSUNEAKI SUGIMOTO, ICHIRO KOBAYASHI, KATSUNOBU TAKAHASHI, TOSHIKI KATADA, MICHIO UI, and YOSHIHISA KURACHI

From the Second Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan; Department of Life Science, Faculty of Science, Tokyo Institute of Technology, Nagatsuda, Midori-ku, Yokohama 227, Kanagawa, Japan; Department of Physical Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan; and Division of Cardiovascular Diseases, Department of Internal Medicine, Mayo Clinic, Mayo Foundation, Rochester, Minnesota 55905

ABSTRACT Using the patch clamp technique, we examined the agonist-free, basal interaction between the muscarinic acetylcholine (m-ACh) receptor and the G protein (G_K)-gated muscarinic K⁺ channel ($I_{K,ACh}$), and the modification of this interaction by ACh binding to the receptor in single atrial myocytes of guinea pig heart. In the whole cell clamp mode, guanosine-5'-O-(3-thiotriphosphate) (GTP- γ S) gradually increased the $I_{K,ACh}$ current in the absence of agonists (e.g., acetylcholine). This increase was inhibited in cells that were pretreated with islet-activating protein (IAP, pertussis toxin) or *N*-ethylmaleimide (NEM). In inside-out patches, even in the absence of agonists, intracellular GTP caused openings of $I_{K,ACh}$ in a concentration-dependent manner in ~80% of the patches. Channel activation by GTP in the absence of agonist was much less than that caused by GTP- γ S. The agonist-independent, GTP-induced activation of $I_{K,ACh}$ was inhibited by the A promoter of IAP (with nicotinamide adenine dinucleotide) or NEM. As the ACh concentration was increased, the GTP-induced maximal open probability of $I_{K,ACh}$ was increased and the GTP concentration for the half-maximal activation of $I_{K,ACh}$ was decreased. Intracellular GDP inhibited the GTP-induced openings of $I_{K,ACh}$ in a concentration-dependent fashion. The half-inhibition of $I_{K,ACh}$ openings occurred at a much lower concentration of GDP in the absence of agonists than in the presence of ACh. From these results, we concluded (a) that the interaction between the m-ACh receptor and G_K is essential for basal stimulation of $I_{K,ACh}$, and (b) that ACh binding to the receptor accelerates the turnover of G_K and increases G_K's affinity to GTP analogues over GDP.

Address reprint requests to Dr. Y. Kurachi, Division of Cardiovascular Diseases, Department of Internal Medicine, Mayo Clinic, Mayo Foundation, Rochester, MN 55905.

INTRODUCTION

Agonist-binding to membrane receptors is essential for activation of G proteins to promote many cell-signaling systems, such as adenylate cyclase regulation and membrane phospholipid mobilization systems (Gilman, 1987; Neer and Clapham, 1988). The functional roles of agonist-bound receptors on G protein activation have been extensively studied in the adenylate cyclase regulation system: (a) Agonist-bound receptors stimulate GDP release and subsequent GTP binding on G protein (turn-on reaction), resulting in the functional dissociation of the G protein into its subunits (α -GTP and $\beta\gamma$), which in turn activate or inhibit the effectors. (b) Agonist-bound receptors also stimulate the GTPase activity of G protein α subunits (turn-off reaction). (c) Receptor-G protein interactions are specifically modified by bacterial toxins (e.g., cholera toxin and pertussis toxin).

In addition to enzymes like adenylate cyclase, G proteins also regulate various membrane ionic channels: the muscarinic K^+ channel (Breitwieser and Szabo, 1985; Pfaffinger, Martin, Hunter, Nathanson, and Hille, 1985; Kurachi, Nakajima, and Sugimoto, 1986*a, b*), the Na^+ channel (Schubert, VanDongen, Kirsch, and Brown, 1989), and the L-type Ca^{2+} channel (Yatani, Codina, Imoto, Reeves, Birnbaumer, and Brown, 1987) in cardiac myocytes, the N-type Ca^{2+} channel in neuronal cells (Hescheler, Rosenthal, Trautwein, and Schultz, 1987; Kasai and Aosaki, 1989), and the ATP-sensitive K^+ channel in pancreatic β cells, skeletal muscle, and cardiac muscle cells (Dunne, Bullett, Guodong, Wollheim, and Petersen, 1989; Parent and Coronado, 1989; Kirsch, Codina, Birnbaumer, and Brown, 1990). The molecular mechanisms underlying G protein regulation of ionic channels have been most extensively studied in the cardiac muscarinic K^+ channel system (Kurachi, Nakajima, and Sugimoto, 1986*c*; Logothetis, Kurachi, Galper, Neer, and Clapham, 1987; Yatani, Codina, Brown, and Birnbaumer, 1987; Logothetis, Kim, Northup, Neer, and Clapham, 1988; Yatani, Mattera, Codina, Graf, Okabe, Padrell, Iyenger, Brown, and Birnbaumer, 1988; Kurachi, Ito, Sugimoto, Katada, and Ui, 1989*a*; Kobayashi, Shibasaki, Takahashi, Tohyama, Kurachi, Ito, Ui, and Katada, 1990; for review see Nanavati, Clapham, Ito, and Kurachi, 1990). These reports focused on the roles played by G protein subunits in channel activation. However, the functional role of the muscarinic acetylcholine (m-ACh) receptor on the G protein-muscarinic K^+ channel system has not yet been fully studied. In this study, we examined the basal interaction between the m-ACh receptor and the muscarinic K^+ channel and the modification of this interaction by ACh binding to the receptor in cardiac atrial myocytes.

MATERIALS AND METHODS

Preparations

Single atrial cells of the guinea pig heart were obtained by an enzymatic dissociation method as described previously (Isenberg and Klöckner, 1982; Kurachi et al., 1986*b*). Briefly, collagenase (0.04% wt/vol, Sigma type 1; Sigma Chemical Co. St. Louis, MO) in nominally Ca^{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^+ /low- Cl^- solution (for composition, see below) at 4°C for later experiments. A small piece of the atrial

tissue was dissected and gently agitated in the recording chamber, which was filled with the control bathing solution. Spindle-like relaxed atrial cells showing clear striations were used for the experiments. All experiments were performed at 33–35°C.

Current Measurements

The GΩ seal-patch clamp technique was used in the whole cell, cell-attached patch, and inside-out patch configurations (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981). In the whole cell clamp conditions, the electrode resistance (2–4 MΩ) in series with the cell membrane was compensated.

Solutions and Drugs

The control bathing solution contained (in mM): 136.5 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.53 MgCl₂, 0.33 NaH₂PO₄, 5.5 glucose, and 5.5 HEPES-NaOH buffer (pH 7.4). The composition of the high-K⁺/low-Cl⁻ solution was (in mM): 10 taurine, 10 oxalic acid, 70 glutamic acid, 25 KCl, 10 KH₂PO₄, 11 glucose, 0.5 EGTA, and 10 HEPES-KOH buffer (pH 7.3–7.4). In the whole cell clamp experiments, the pipettes were filled with the following solution (in mM): 140 KCl, 1 MgCl₂, 3 Na₂ATP, 5 EGTA-KOH, and 5 HEPES-KOH buffer (pH 7.2). GTP (Na salt; Sigma Chemical Co.) or guanosine-5'-O-(3-thiotriphosphate) (GTP-γS; Boehringer Mannheim GmbH, Mannheim, Germany) (100 μM) was also added to the solution.

In the patch clamp experiments, the composition of the pipette solution was (in mM): 145 KCl, 1 CaCl₂, 1 MgCl₂, and 5 HEPES-KOH (pH 7.4). Atropine (10 μM; Sigma Chemical Co.), theophylline (100 μM; Katayama Chemical, Osaka, Japan), or various concentrations of ACh (Sigma Chemical Co.) were added to the solution. In the inside-out patch clamp experiments, the bath was perfused with the following "internal" solution (in mM): 140 KCl, 1 MgCl₂, 5 EGTA-KOH, 5 HEPES-KOH buffer (pH 7.2), and various concentrations of GTP, GTP-γS, or GDP (Sigma Chemical Co.).

Islet-activating protein (IAP), pertussis toxin, was purchased from Funakoshi Chemical Ltd. (Tokyo, Japan). For pretreatment of the guinea pigs, 20 μg IAP was dissolved in 1 ml saline and given to the animals by intracardiac injection under ether anesthesia 2–3 d before the experiments. For treatment of inside-out patches, the A protomer of IAP was obtained by *in vitro* activation of IAP and applied to the patches with nicotinamide adenine dinucleotide (NAD; Sigma Chemical Co.) (Kurachi et al., 1986a, b). 50 μg IAP with 5 mM dithiothreitol was incubated in 5 ml of the internal solution with 3 mM ATP at 37°C for 15–20 min. The solution was diluted to 50 ml with the internal solution. Therefore, the final concentration of preactivated IAP was 1 μg/ml. *N*-Ethylmaleimide (NEM) was from Sigma Chemical Co. The isolated atrial myocytes were incubated in 10 μM NEM for 10–15 min.

βy subunits of IAP substrate G proteins were purified from bovine brain as described previously (Katada, Oinuma, and Ui, 1986b; Kobayashi et al., 1990).

Data Analysis

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). The data were reproduced and low-pass filtered using a FV-625A filter (NF Electronic Circuit Design) with Bessel characteristics (48 dB/octave slope attenuation) and analyzed on a computer (PC-9800VM2; NEC, Tokyo, Japan). For single channel analysis, the threshold to judge the open state was set at half of the unit amplitude of the single channel current (Colquhoun and Sigworth, 1983). The current density of GTP-γS-induced current in the whole cell experiments was calculated assuming that the specific cell membrane capacitance is 1 μF/cm². Statistical data were expressed as mean ± SD.

RESULTS

Induction of Muscarinic K⁺ Channel Current by GTP- γ S in the Whole Cell Atrial Myocyte and Its Inhibition by Islet-activating Protein and NEM

ACh and adenosine, when applied to guinea pig atrial cells, activate a specific K⁺ channel current ($I_{K_{ACh}}$) (Kurachi et al., 1986*a,b*). Fig. 1 *A* demonstrates the current of an isolated atrial myocyte upon exposure to ACh. The cell was whole cell voltage clamped at -40 mV. The patch pipette solution contained $100 \mu\text{M}$ GTP and 3 mM ATP. The patch membrane in the pipette was ruptured at the beginning of the current trace. In the absence of agonist, the holding current stayed at the initial steady level for >10 min, and no significant current flowing through $I_{K_{ACh}}$ was induced. Upon application of $11 \mu\text{M}$ ACh, an outward K⁺ current increased rapidly to a peak and then desensitized to a steady level (Carmeliet and Mubagwa, 1986; Kurachi, Nakajima, and Sugimoto, 1987). Upon washing out ACh, the current returned to the control level.

Fig. 1 *B* shows the effects of intracellular application of $100 \mu\text{M}$ GTP- γ S into atrial myocytes with or without pretreatment of IAP or NEM. In contrast to GTP, intracellular GTP- γ S caused a gradual increase of the outward K⁺ current in the absence of agonist in control untreated atrial myocytes (Fig. 1 *B, a*; see also Kurachi et al., 1987; Breitwieser and Szabo, 1988). The current reached the peak within 2–4 min after rupture of the patch membrane. This increase of the current probably represents the agonist-independent, basal turn-on reaction of the G protein which is coupled to $I_{K_{ACh}}$ (G_K). In the steady state, ACh did not further increase the current (see also Fig. 6 of Kurachi et al., 1987).

In Fig. 1 *B, b* and *c*, the effect of GTP- γ S on the K⁺ current was examined in atrial myocytes isolated from IAP-treated animals. In cells isolated from the animals 36 h after IAP administration, the K⁺ current induced by intracellular GTP- γ S was much smaller in magnitude ($\sim 40\%$ of the control) and slower in time course than that in untreated cells: the density and the half-time to reach the peak of the GTP- γ S-induced outward K⁺ current at -40 mV were $8.1 \pm 1.2 \mu\text{A}/\text{cm}^2$ and $57 \pm 10 \text{ s}$ ($n = 4$) in the untreated cells and $3.2 \pm 0.7 \mu\text{A}/\text{cm}^2$ and $257 \pm 194 \text{ s}$ ($n = 4$) in the IAP-treated cells. In cells isolated 72 h after IAP administration, GTP- γ S failed to evoke a significant outward current ($n = 8$ from three different animals). Addition of $11 \mu\text{M}$ ACh to the bathing solution failed to induce the K⁺ channel current in both *b* and *c*. In these cells, however, the full-sized L-type Ca²⁺ channel current was evoked by depolarizing voltage steps from -40 to 0 mV (not shown) and was augmented by isoprenaline (0.2 – $1.0 \mu\text{M}$), suggesting that G_s and the L-type Ca²⁺ channels were functioning. In addition, intracellular GTP- γ S failed to induce the K⁺ channel current in NEM-treated atrial cells (Fig. 1 *B, d*). IAP specifically ADP-ribosylates a certain population of G proteins, uncoupling them from their associated membrane receptors and preventing the transduction of signals from the receptors (Katada and Ui, 1982; Ui, 1984). At low concentrations, NEM, a sulfhydryl-alkylating agent, also mimics IAP in blocking the IAP-sensitive G proteins (Katada, Kurose, Oinuma, Hoshino, Shinoda, Asanuma, and Ui, 1986*a*; Kitamura and Nomura, 1987; Ueda, Misawa, Katada, Ui, Takagi, and Satoh, 1990) and inhibits ACh activation of $I_{K_{ACh}}$ (Nakajima, Irisawa, and Giles, 1990). Therefore, the inhibitory effects of IAP and NEM on the GTP- γ S-induced activation of $I_{K_{ACh}}$ current suggest that the interaction

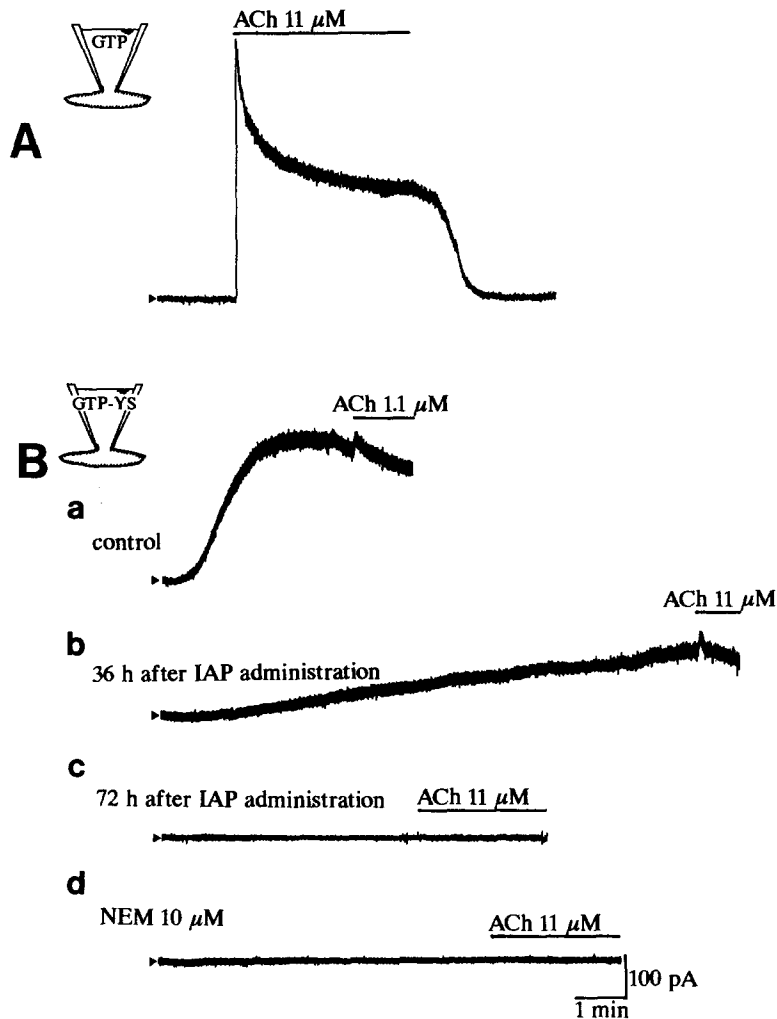


FIGURE 1. Effects of IAP and NEM on induction of the muscarinic K⁺ channel current by GTP-γS in the whole cell of atrial myocytes. (A) A typical example of the ACh-induced outward K⁺ current in atrial myocytes. 11 μM ACh was applied to the bathing solution as indicated by a bar above the current trace. The pipette solution contained 100 μM GTP and 3 mM ATP. (B) GTP-γS-induced muscarinic K⁺ channel current in the control and IAP- or NEM-treated atrial myocytes. The pipette solution contained 100 μM GTP-γS instead of GTP and 3 mM ATP. *a*, A control atrial myocyte; *b*, an atrial myocyte isolated from the animal of 36 h IAP treatment; *c*, an atrial myocyte isolated from the animal after 72 h of IAP treatment; *d*, an atrial myocyte pretreated with 10 μM NEM for 10–15 min. The holding potential was -40 mV throughout the experiments.

between the agonist-free membrane receptor and G_K is essential for the basal turn-on reaction of G_K. However, since bacterial toxin-mediated ADP ribosylation alters the amount of various G proteins in the cell membrane (Chang and Bourne, 1989; Watkins, Northup, and Malbon, 1989), loss of IAP substrate from the cardiac cell

membrane during the pretreatment of animals with IAP could also cause the decrease in the GTP- γ S-induced K^+ current in IAP-treated atrial myocytes.

Agonist-independent Basal Activation of the Muscarinic K^+ Channel in Cell-attached and Inside-out Patches

Fig. 2*A* demonstrates the ACh-induced activation of $I_{K_{ACh}}$ in cell-attached and inside-out patches. 0.1 μ M ACh was added to the pipette solution. Upon formation of the cell-attached patch, multi-levels of $I_{K_{ACh}}$ were observed. Once the membrane patch was excised from the cell into the GTP-free internal solution (inside-out patch), the channel activity disappeared completely. $I_{K_{ACh}}$ was promptly reactivated when GTP or GTP- γ S was applied to the intracellular side of the patch membrane. In the presence of ACh, the open probability of $I_{K_{ACh}}$ evoked by GTP was almost equal to that induced by GTP- γ S (see also Fig. 4).

In Fig. 2*B*, the pipette solution did not contain ACh but atropine (10 μ M) and theophylline (100 μ M) to block muscarinic and P_1 -purinergic receptors. Some channel openings were observed in the cell-attached patches, even though the antagonists instead of the agonists were present (basal activity; Sakmann, Noma, and Trautwein, 1983). These channel openings also disappeared upon formation of an inside-out patch. Upon application of 10 μ M GTP to the intracellular side of the cell membrane in these patches, openings of $I_{K_{ACh}}$ were induced in 77 of 98 patches (upper current trace in Fig. 2*B*) and were not induced at all in the remaining 21 of 98 patches (lower trace in Fig. 2*B*). In both cases, 10 μ M GTP- γ S reactivated the channel fully. However, even in patches where openings of $I_{K_{ACh}}$ were induced by GTP in the absence of agonist, the steady-state channel activity, $N \cdot P_o$ (where N is the number of channels in the patch and P_o is the probability that a channel is open), was much smaller than that seen in patches exposed to either GTP in the presence of ACh or GTP- γ S in the absence of agonist. In the upper current trace of Fig. 2*B*, the $N \cdot P_o$ of GTP (10 μ M)-induced channel openings relative to that induced by 10 μ M GTP- γ S was 0.18 (see also Fig. 4).

In Fig. 2*C*, the frequency histogram of the relative $N \cdot P_o$ of the channel openings induced by 10 μ M GTP in the absence of agonist was plotted in consecutive experiments of 19 patches from 7 different animals. GTP induced no significant openings of $I_{K_{ACh}}$ in 5 of 19 patches. The relative $N \cdot P_o$ of the remaining 14 patches ranged from 0.07 to 0.47 (0.22 ± 0.12 , $n = 14$). These agonist-independent, GTP-induced channel openings were inhibited by GDP and GDP- β S (see Fig. 5), suggesting the involvement of G_K .

Islet-activating Protein and NEM Inhibit the Agonist-independent GTP-induced Activation of $I_{K_{ACh}}$ in the Inside-out Patches

To elucidate the molecular mechanisms underlying the GTP-induced openings of $I_{K_{ACh}}$ in the absence of agonist, we examined the effects of IAP and NEM on the GTP-induced activation of $I_{K_{ACh}}$ in inside-out patches. The pipette solution contained the antagonists, atropine and theophylline (Fig. 3). After activating $I_{K_{ACh}}$ with GTP, either the A protomer of IAP (with NAD) or NEM was added to the internal solution. Channel activity gradually disappeared within 1–2 min and failed to reappear after the removal of IAP or NEM, despite the continuous presence of GTP. GTP- γ S, on the other hand, could reactivate the channel (Fig. 3, *a* and *b*). However, when the patches

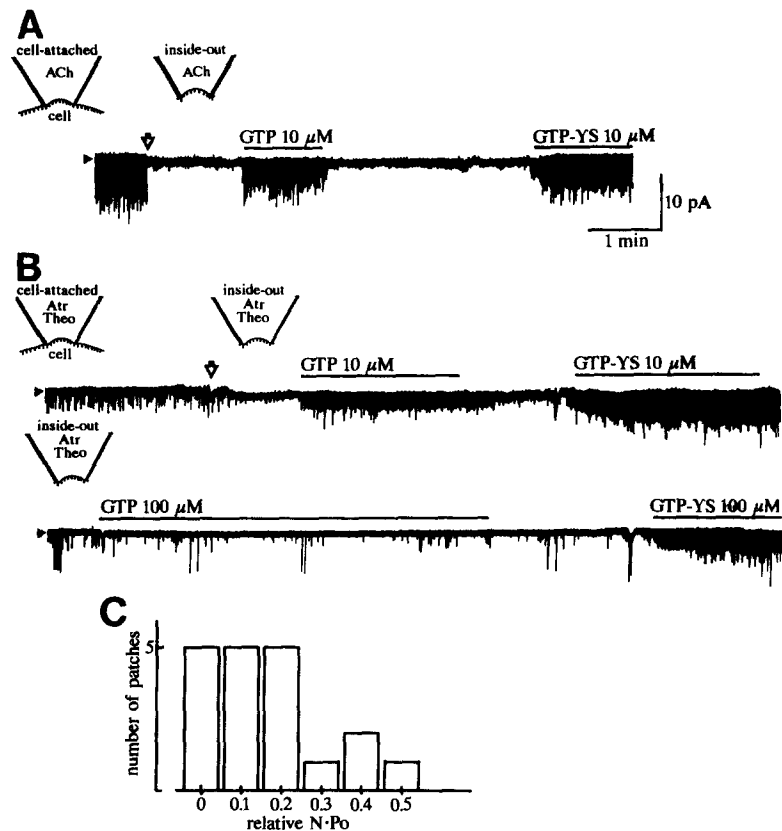


FIGURE 2. Agonist-independent basal activity of the muscarinic K⁺ channel in the cell-attached and inside-out patches. (A) An example of ACh-induced K⁺ channel current in the cell-attached and inside-out patches. The pipette solution contained 0.1 μ M ACh. In the inside-out patch, GTP and GTP- γ S induced the muscarinic K⁺ channel openings at around the same magnitude. (B) The pipette solution contained 10 μ M atropine and 100 μ M theophylline. In the cell-attached form, low-frequency channel openings were observed. In the inside-out patches, GTP applied to the inside of the patch membrane caused some openings of the channel in \sim 80% of the patches (*upper current trace*). In the remaining 20%, no openings were induced by GTP in the absence of agonists (*lower current trace*). The holding potential was -80 mV in the cell-attached patches (under perfusion of the internal solution) and in the inside-out patches. (C) Frequency histogram of the relative $N \cdot P_o$ of agonist-independent, GTP-induced openings of $I_{K,ACh}$ in inside-out patches. 10 μ M GTP and 10 or 100 μ M GTP- γ S were sequentially applied to each patch. The relative $N \cdot P_o$ of $I_{K,ACh}$ induced by GTP in each patch was calculated with reference to the GTP- γ S-induced activity.

were incubated with IAP or NEM for 7–10 min, GTP- γ S failed to reactivate the channel. Even in these patches, G protein $\beta\gamma$ subunits (10 nM, purified from bovine brain) could fully reactivate the channel (Kurachi et al., 1989a), indicating that neither the channel itself nor the interaction between the exogenously added G protein subunit and the channel was affected by these substances even after long incubation of the inside-out patches. We also observed that NAD (1–3 mM) did not

affect the GTP- or GTP- γ S-induced openings of $I_{K_{ACh}}$ ($n = 5$, not shown). NEM (30–50 μ M), applied after GTP- γ S activation of the channel, failed to inhibit the GTP- γ S-induced channel openings for > 10 min ($n = 5$, not shown). Therefore, these inhibitory effects of IAP and NEM on the agonist-independent openings of $I_{K_{ACh}}$ induced by GTP may not be due to the nonspecific blocking effects of these agents on

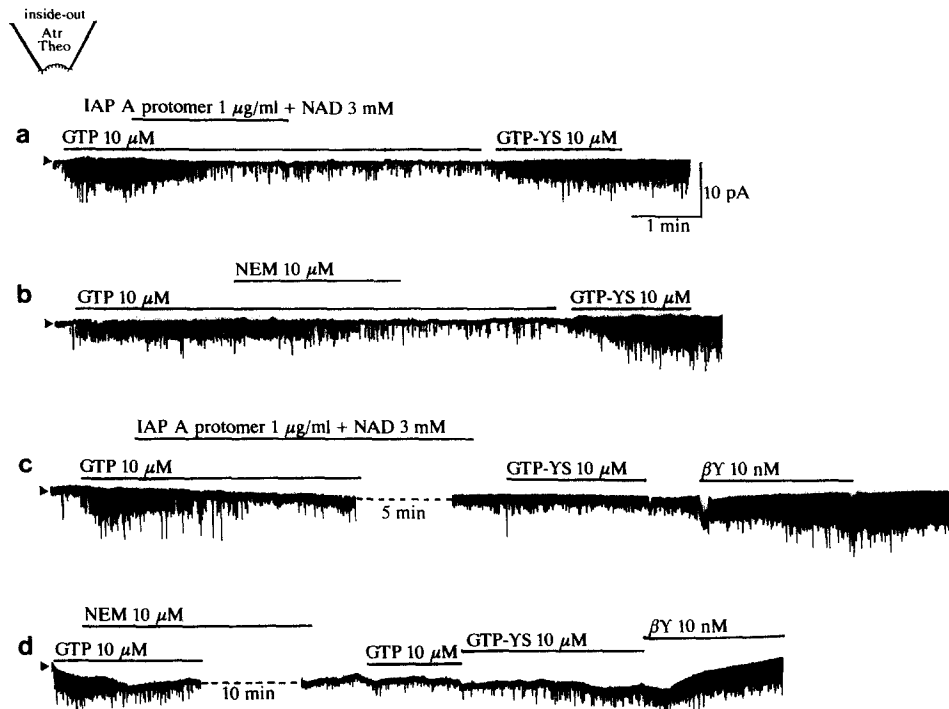


FIGURE 3. Effects of IAP and NEM on the GTP-induced K^+ channel activation in the absence of agonists. The pipette solution contained 10 μ M atropine and 100 μ M theophylline. In the inside-out patch condition, 10 μ M GTP added to the inside-side of the patch membrane caused activation of $I_{K_{ACh}}$. The A protomer of IAP with NAD (*a, c*) or NEM (*b, d*) was added to the GTP-containing solution, which inhibited the GTP-induced channel openings within 1–3 min. When GTP- γ S was added within several minutes after the inhibition of the channel by IAP or NEM, the channel openings reappeared (*a, b*). On the other hand, when IAP or NEM was perfused to the inside-out patch for 7–10 min, GTP- γ S failed to activate the channel. Even in this condition, G protein $\beta\gamma$ subunits activated the channel (*c, d*). The protocol for perfusing various substances in the inside of the patch membrane was indicated by bars above each current trace. The holding potential of patches was -80 mV.

$I_{K_{ACh}}$. Since IAP specifically ADP-ribosylates a certain population of G proteins, and NEM alkylates G_i proteins to uncouple them from membrane receptors (Katada and Ui, 1982; Ui, 1984; Katada et al., 1986a; Kitamura and Nomura, 1987; Ueda et al., 1990), the failure of channel activation by GTP and GTP- γ S may be due to the functional uncoupling of G_K and the membrane receptor caused by IAP and NEM.

Concentration-dependent Activation of the Muscarinic K⁺ Channel by GTP and Inhibition by GDP in the Absence and Presence of ACh

To clarify the functional role of agonist binding in activation of the G_K-muscarinic K⁺ channel system, we examined the concentration-dependent effects of intracellular

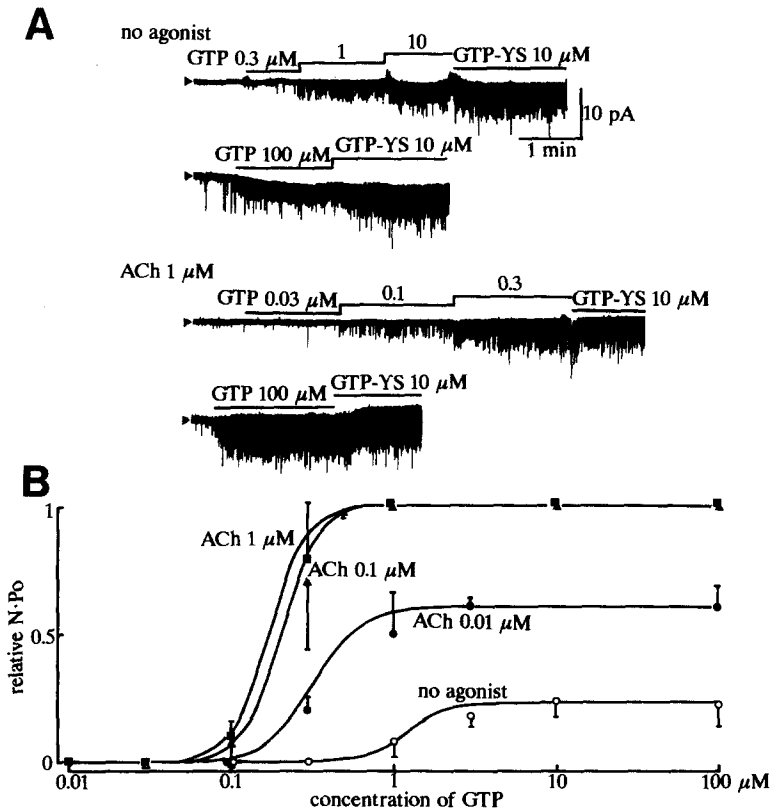


FIGURE 4. Concentration-dependent effect of GTP on the muscarinic K⁺ channel in the absence and presence of ACh. (A) Examples of inside-out patch experiments. The concentration of ACh in the pipette is 0 or 1 μM as indicated. The bar above each trace indicates the protocol for perfusing various concentrations of GTP and 10 μM GTP-γS. The membrane potential was -80 mV. Note that a 3–10-fold increase in GTP concentration resulted in a dramatic increase of *N·P_o* of *I_{K,ACh}*, indicating the existence of a highly cooperative process. (B) The relation between the concentration of GTP and the relative *N·P_o* of *I_{K,ACh}* with reference to the *N·P_o* induced by GTP-γS in each patch. Symbols and bars are mean ± SD. The continuous curves are fitted by the Hill equation with the least-squares method. (open circles) 0 μM ACh (*n* = 7); (filled circles) 0.01 μM ACh (*n* = 6); (filled triangles) 0.1 μM ACh (*n* = 6); (filled squares) 1 μM ACh (*n* = 6).

GTP and its inhibition by GDP at various concentrations of ACh in the pipette (Figs. 4 and 5).

Various concentrations of GTP from 0.01 to 100 μM were applied to the intracellular side of the patches. To avoid the effects of “run-down” in the measure-

ment of the GTP-dependent activation of $I_{K,ACh}$ (Kurachi et al., 1987; Kurachi, Ito, and Sugimoto, 1990), we applied a maximum of three concentrations of GTP to each patch. Especially high doses of GTP (10 and 100 μ M) were applied to separate patches in most experiments. 10 μ M GTP- γ S was applied at the end of each experiment to obtain the maximal $N \cdot P_o$ of $I_{K,ACh}$ in each patch. The pipette solution contained 0, 0.01, 0.1, and 1 μ M ACh. Fig. 4A demonstrates the $I_{K,ACh}$ channel currents measured with 0 and 1 μ M ACh in the pipette. Channel openings were evoked with 1 μ M GTP in the absence of agonist and 0.1 μ M GTP with 1 μ M ACh. Channel openings increased dramatically with increasing concentrations of GTP. They reached a maximal value at \sim 3–10 μ M GTP with 0 μ M ACh in the pipette, and 0.3–1 μ M GTP with 1 μ M ACh. The maximal channel activity induced by GTP in the absence of agonist was much less than, and that in the presence of 1 μ M ACh was equivalent to, the GTP- γ S (10 μ M)-induced channel activity.

Fig. 4B shows the GTP concentration dependence of the open probability of $I_{K,ACh}$. The data for each symbol were obtained from six or seven patches at various concentrations of ACh in the pipette. Relative $N \cdot P_o$ of $I_{K,ACh}$ was calculated with

TABLE I
Parameters of Hill Equation Fitted for the Relation between the GTP Concentration and the Relative $N \cdot P_o$ of $I_{K,ACh}$

ACh	K_d	V_{max}	H
μ M			
0	1.22	0.23	3.04
0.01	0.32	0.60	2.99
0.1	0.21	1.00	3.05
1	0.18	1.00	3.05

K_d , GTP concentration at the half-maximal activation of $I_{K,ACh}$; V_{max} , maximal relative $N \cdot P_o$; H , Hill coefficient.

reference to the $N \cdot P_o$ of 10 μ M GTP- γ S-induced channel openings in each patch. Each concentration–response relation was fitted by the Hill equation using the least-squares method (Yamaoka, Tanigawara, Nakagawa, and Uno, 1981):

$$y = V_{max} / \{1 + (K_d/[GTP])^H\}$$

where y is the relative $N \cdot P_o$, V_{max} is the maximal relative $N \cdot P_o$, K_d is the GTP concentration at the half-maximal activation of the channel, and H is the Hill coefficient.

As the concentration of ACh in the pipette was raised from 0 to 0.01, 0.1, or 1 μ M, the following points were observed (Table I): (a) the threshold concentration of GTP necessary to induce openings of the channel decreased; (b) the GTP concentration for half-maximal activation of the channel (K_d) decreased; and (c) the maximal relative $N \cdot P_o$ (V_{max}) increased; however, (d) the Hill coefficient of the fitted curve was constant around 3 and was independent of the ACh concentration. These results indicate that ACh binding increased both the maximal response and the apparent affinity of $I_{K,ACh}$ for GTP.

Fig. 5 shows the effects of GDP on the GTP (10 μ M)-induced openings of $I_{K,ACh}$. The pipette solution contained 0 or 0.3 μ M ACh. In inside-out patches, 10 μ M GTP induced the openings of $I_{K,ACh}$. The GTP-induced channel openings in the presence of 0.3 μ M ACh in the pipette were nearly the same as the GTP- γ S-induced openings, while openings in the absence of agonist were much less frequent (see also Fig. 4). In both cases, GDP, when added to the internal solution, inhibited channel openings in

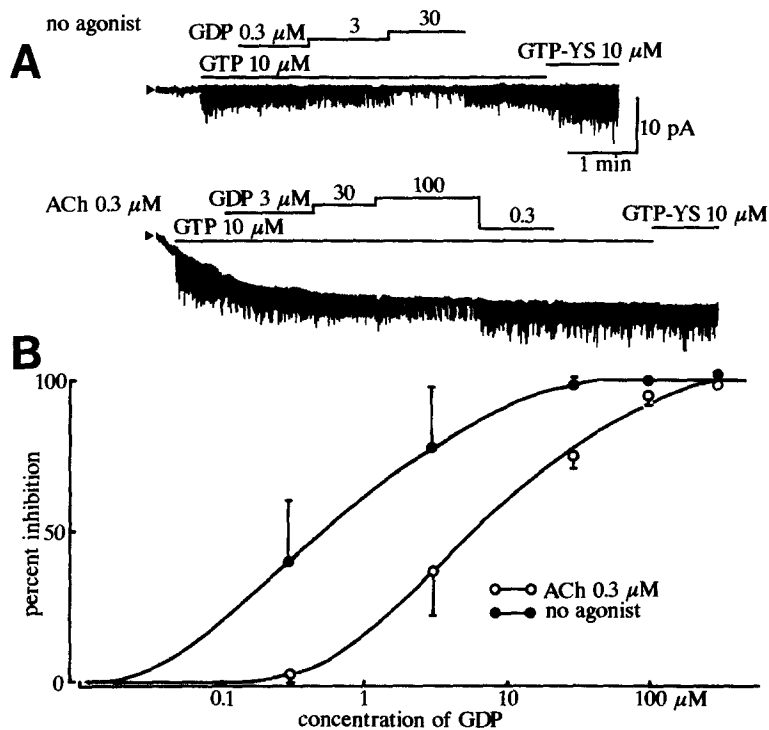


FIGURE 5. GDP inhibition of the GTP-induced activation of the muscarinic K⁺ channel in the absence and presence of ACh. (A) Examples of inside-out patch experiments. The concentration of ACh in the pipette is indicated at each current trace. Protocol for perfusing 10 μ M GTP, various concentrations of GDP, and 10 μ M GTP- γ S is indicated above each trace. (B) The relations between the concentration of GDP and percent inhibition of GTP (10 μ M)-induced openings of $I_{K,ACh}$ by GDP in the absence and presence of ACh are shown. The symbols and bars are means \pm SD ($n = 5$ for no agonist, $n = 6$ for ACh 0.3 μ M). The relations were fitted by the Hill equation with the least-squares method. See text for details.

a concentration-dependent manner. In the examples shown in Fig. 5A, in the absence of agonist, 0.3, 3, and 30 μ M GDP decreased channel activity by 51.8, 86.6, and 99.2%, respectively. With 0.3 μ M ACh in the pipette, 30 and 100 μ M GDP decreased the GTP-induced channel openings by 60.7 and 92.0%, respectively. Upon washout of GDP, the GTP-induced channel activity returned to the initial level.

The relationship between the concentration of GDP and the inhibition of GTP-induced channel openings at 0 and 0.3 μM ACh is presented in Fig. 5 B. The percent inhibition of $N \cdot P_o$ of the channel by various concentrations of GDP was expressed with reference to $N \cdot P_o$ in the absence of GDP in each patch. The concentration–response relation was fitted by the Hill equation using the least-squares method. The IC_{50} was 0.51 μM GDP in the absence of agonist and 5.6 μM GDP in the presence of 0.3 μM ACh. The Hill coefficient was ~ 1 in both cases. These results indicate that ACh binding increases the affinity of G_k for GTP over GDP.

DISCUSSION

The major findings of this study are that (a) in the absence of agonists, a basal stimulation of the muscarinic K^+ channel ($I_{k,ACh}$) occurs through G_k ; (b) IAP and NEM inhibit the basal stimulation of $I_{k,ACh}$ induced by GTP and GTP- γS both in the whole cell and in the inside-out patch configurations; (c) agonist binding to the receptor facilitates the activity of $I_{k,ACh}$ by increasing both the maximal response and the apparent affinity of the channel for GTP over GDP. This facilitation of channel opening is probably due to activation of G_k by agonist binding to the receptor; (d) the Hill coefficient for the relationship between the GTP concentration and $N \cdot P_o$ of $I_{k,ACh}$ was ~ 3 , independent of the concentration of ACh.

Activation of G_k occurs at a basal level even in the absence of agonist. This was confirmed by the gradual activation of $I_{k,ACh}$ in whole atrial cells exposed to intracellular GTP- γS (Fig. 1) and by the low frequency of openings induced by intracellular GTP in inside-out patches (Fig. 2). This basal stimulation of $I_{k,ACh}$ via G_k was inhibited by IAP or NEM both in whole cells and in inside-out patches (Figs. 1 and 3). NEM (30–50 μM) did not affect the GTP- γS -induced channel openings for > 10 min ($n = 5$, not shown), when applied to the patch after channel activation by GTP- γS . NAD (1–3 mM), which was applied to the inside-out patch without the preactivated IAP, did not affect the GTP- or GTP- γS -induced openings of $I_{k,ACh}$ ($n = 5$, not shown). Therefore, these inhibitory effects of IAP and NEM on the agonist-independent, GTP-induced openings of $I_{k,ACh}$ are probably not a result of the nonspecific blocking effects of these agents on $I_{k,ACh}$. Furthermore, purified G protein $\beta\gamma$ subunits could reactivate $I_{k,ACh}$ even after the treatment of inside-out patches by IAP or NEM for 7–10 min. This observation indicates that IAP and NEM affect neither the channel itself nor the interaction between the exogenously applied G protein subunit and the channel (see also Nakajima et al., 1990). Therefore, the functional uncoupling of G_k and the membrane receptor may have progressed time-dependently during incubation of the inside-out patches by IAP and NEM (Katada and Ui, 1982; Ui, 1984; Katada et al., 1986a; Kitamura and Nomura, 1987; Ueda et al., 1990), resulting in loss of activation of $I_{k,ACh}$ by GTP and then finally by GTP- γS (Fig. 3). We interpret these results as indicating that complete uncoupling of the receptor and G_k induced by a long incubation with IAP and NEM may have eliminated the basal level of turnover of G_k . Thus, the functional coupling of the receptor and G_k may be essential for the agonist-independent activation of $I_{k,ACh}$ induced by intracellular GTP.

When purified muscarinic receptors and G_i proteins were reconstituted in phospholipid vesicles, background turnover rates of IAP-treated and nontreated G_i

proteins were the same (Kurose, Katada, Haga, Haga, Ichiyama, and Ui, 1986). This indicated that the functional interaction between the agonist-unbound receptor and G_i had no effect on the function of G_i in this system. On the other hand, GTPase activity of G_s was stimulated in the absence of agonist only when β-adrenergic receptor was reconstituted with purified G_s-adenylate cyclase system in the phospholipid vesicle (Cerione, Codina, Benovic, Lefkowitz, Birnbaumer, and Caron, 1984). Thus, the basal stimulation of G_K induced by the functional interaction between the agonist-unbound receptor and G_K may represent one of the characteristic properties of the atrial G_K-muscarinic K⁺ channel system.

In this study, the GTP-induced activation of $I_{K_{ACh}}$ without agonist varies from patch to patch (Fig. 2). The 5-lipoxygenase metabolites of arachidonic acid activate the atrial G_K-muscarinic K⁺ channel system in the absence of agonists in a [GTP]_i-dependent manner (Kurachi, Ito, Sugimoto, Shimizu, Miki, and Ui, 1989b). Since it might be expected that phospholipase A₂, which mediates the release of arachidonic acid from cell membranes, is activated to various degrees in isolated atrial cells (Needleman, Turk, Jakschik, Morrison, and Lefkowitz, 1986), arachidonic acid metabolites may be responsible for the variation of the GTP-induced activation of $I_{K_{ACh}}$ in the absence of agonists.

Agonist binding to the receptor is the initial step in G protein-mediated signal transduction systems (Gilman, 1987; Neer and Clapham, 1988). It is followed by the release of GDP from G protein and binding of GTP, resulting in functional dissociation of the protein into its subunits (α-GTP and βγ). The functionally dissociated subunits activate or inhibit various effectors. Although a low level of basal turnover of G proteins has been reported in several transduction systems including the G_K-muscarinic K⁺ channel (Cerione et al., 1984; Kurachi et al., 1987; Breitwieser and Szabo, 1988), it is generally observed that agonist binding to membrane receptors markedly stimulates the process. In this study, the channel openings induced by intracellular GTP were enhanced by ACh in a concentration-dependent fashion (Fig. 4). The magnitude of the maximal response as well as the apparent affinity of $I_{K_{ACh}}$ openings for intracellular GTP increased with an increase in ACh concentration. The Hill coefficient for the relationship between the channel activity and the concentration of GTP remained constant at ~3 independent of the ACh concentration. These observations suggest that the ACh-induced alteration in the magnitude of channel activation and the apparent affinity for GTP may be due to the facilitation of functional dissociation of G_K induced by agonist binding to the muscarinic ACh receptors (Kurose et al., 1986), and that the positive cooperative effect of GTP on the channel open probability may be derived from the intrinsic properties of the functional steps between G protein subunits and the K⁺ channel (Kurachi et al., 1990).

Although GDP inhibited the GTP-induced openings of $I_{K_{ACh}}$ in both the presence and absence of ACh, the half-maximal inhibition occurred at a much higher concentration of GDP in the presence of ACh than in the absence of agonist (Fig. 5). Since the Hill coefficient for GDP inhibition of the GTP-induced channel activation was ~1 with 0 or 0.3 μM ACh, it can be assumed that GDP and GTP compete for the same site on G protein in a one-to-one reaction. These results are consistent with the

biochemical measurement of GDP inhibition of activated G proteins (Gilman, 1987; Neer and Clapham, 1988).

For the positive cooperative effect of intracellular GTP on the channel activation, two possibilities may exist (Kurachi et al., 1990): (a) a functional unit of the K^+ channels may have three binding sites for the G protein subunits; or (b) G protein subunits may stimulate an unknown amplification process that in turn activates $I_{K,ACH}$. Recently, Kirsch and Brown (1989) showed that intracellular application of trypsin causes opening of the K^+ channel in the absence of agonists and intracellular GTP. They proposed that trypsin-sensitive factors, located in the K^+ channel itself or in its vicinity, may tonically inhibit the K^+ channel. Removal of the inhibitory effects of the factors may be involved in the activation of $I_{K,ACH}$ by G proteins (see also Logothetis et al., 1987). In this case, the positively cooperative activation of the K^+ channel by intracellular GTP may possibly represent the properties of the inhibitory factors of the K^+ channel. However, the role of trypsin-sensitive activation of $I_{K,ACH}$ has not yet been elucidated.

Another possibility is that some unknown amplification process is involved in G protein subunit activation of $I_{K,ACH}$. It was shown that tetradecanoyl phorbol acetate did not cause activation of $I_{K,ACH}$, which excluded the possibility that diacylglycerol activation of protein kinase C is involved in the G protein activation of the channel (Logothetis et al., 1987; Yatani et al., 1987). Recently it was proposed that phospholipase A_2 and arachidonic acid metabolites are involved in the activation of $I_{K,ACH}$ by exogenously applied G protein $\beta\gamma$ subunits (Kim, Lewis, Graziadei, Neer, Bar-Sagi, and Clapham, 1989). However, the mechanisms of how arachidonic acid metabolites stimulate the G protein–muscarinic K^+ channel system have not yet been clarified (Kurachi et al., 1989b; Scherer and Breitwieser, 1990). Arachidonic acid metabolites themselves are probably not involved in the native G protein activation of $I_{K,ACH}$ (Kurachi et al., 1989b; Kim et al., 1989). Further studies are needed to elucidate the intermediate amplification steps in the physiological G protein subunit activation of the K^+ channel.

The authors thank Prof. Edward Carmeliet (University of Leuven, Leuven, Belgium), Dr. David Clapham (Mayo Foundation, Rochester, MN), and Chaya Nanavati (Mayo Foundation) for their kind comments on the manuscript, and Monica Zeien for secretarial assistance.

This work was supported by grants from the Ministry of Education, Culture and Science of Japan, the Research Program on Cell Calcium Signalling in the Cardiovascular System, and Eisai Pharmaceutical Co. (Isshin-Kai), by a grant for the cardiovascular diseases (1A-1) from the Ministry of Health and Welfare of Japan, and by Mayo funding.

Original version received 1 August 1990 and accepted version received 10 April 1991.

REFERENCES

- 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.
- Breitwieser, G. E., and G. Szabo. 1988. Mechanism of muscarinic receptor-induced K^+ channel activation as revealed by hydrolysis-resistant GTP analogues. *Journal of General Physiology*. 91:469–493.

- Carmeliet, E., and K. Mubagwa. 1986. Desensitization of the acetylcholine-induced increase of potassium conductance in rabbit Purkinje fibres. *Journal of Physiology*. 371:239–255.
- Cerione, R. A., J. Codina, J. L. Benovic, R. J. Lefkowitz, L. Birnbaumer, and M. G. Caron. 1984. The mammalian β_2 -adrenergic receptor: reconstitution of functional interactions between pure receptor and pure stimulatory nucleotide binding protein of adenylate cyclase system. *Biochemistry*. 23:4519–4525.
- Chang, F., and H. R. Bourne. 1989. Cholera toxin induces cAMP-independent degradation of G^{*}. *Journal of Biological Chemistry*. 264:5352–5357.
- Colquhoun, D., and F. J. Sigworth. 1983. Fitting and statistical analysis of single channel record. In *Single Channel Recording*. B. Sakmann and E. Neher, editors. Plenum Publishing Corp., New York. 191–263.
- Dunne, M. J., M. J. Bullett, L. Guodong, C. B. Wollheim, and O. H. Petersen. 1989. Galanin activates nucleotide-dependent K⁺ channels in insulin-secreting cells via a pertussis toxin-sensitive G protein. *EMBO Journal*. 8:413–420.
- 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 recording from cells and cell-free membrane patches. *Pflügers Archiv*. 391:85–100.
- Hescheler, J., W. Rosenthal, W. Trautwein, and G. Schultz. 1987. The GTP-binding protein, G_o, regulates neuronal calcium channels. *Nature*. 325:445–447.
- Isenberg, G., and U. Klöckner. 1982. Calcium tolerant ventricular myocytes prepared by preincubation in a "KB medium." *Pflügers Archiv*. 395:6–18.
- Kasai, H., and T. Aosaki. 1989. Modulation of Ca-channel current by an adenosine analogue mediated by a GTP-binding protein in chick sensory neurons. *Pflügers Archiv*. 414:145–149.
- Katada, T., H. Kurose, M. Oinuma, S. Hoshino, M. Shinoda, S. Asanuma, and M. Ui. 1986a. Role of GTP-binding proteins in coupling of receptors and adenylate cyclase. *Gunma Symposia on Endocrinology*. 23:45–67.
- Katada, T., M. Oinuma, and M. Ui. 1986b. Two guanine nucleotide-binding proteins in rat brain serving as a specific substrate of islet-activating protein, pertussis toxin. *Journal of Biological Chemistry*. 261:8182–8191.
- Katada, T., and M. Ui. 1982. Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. *Proceedings of the National Academy of Sciences, USA*. 79:3129–3133.
- Kim, D., D. L. Lewis, L. Graziadei, E. J. Neer, D. Bar-Sagi, and D. E. Clapham. 1989. G-protein $\beta\gamma$ -subunits activate the cardiac muscarinic K⁺-channel via phospholipase A₂. *Nature*. 337:557–560.
- Kirsh, G. E., and A. M. Brown. 1989. Trypsin activation of atrial muscarinic K⁺ channels. *American Journal of Physiology*. 257:H334–H338.
- Kirsh, G. E., J. Codina, L. Birnbaumer, and A. M. Brown. 1990. Direct G-protein effects on ATP-sensitive K⁺ channels in rat ventricular myocytes. *Biophysical Journal*. 57:290a. (Abstr.)
- Kitamura, Y., and Y. Nomura. 1987. Uncoupling of rat cerebral cortical α_2 -adrenoceptors from GTP-binding proteins by N-ethylmaleimide. *Journal of Neurochemistry*. 49:1894–1901.
- Kobayashi, I., H. Shibasaki, K. Takahashi, K. Tohyama, Y. Kurachi, H. Ito, M. Ui, and T. Katada. 1990. Purification and characterization of five different α -subunits of guanine-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 $\beta\gamma$ 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., 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.
- Kurachi, Y., T. Nakajima, and T. Sugimoto. 1987. Short-term desensitization of muscarinic K⁺ channel current in isolated atrial myocytes and possible role of GTP-binding proteins. *Pflügers Archiv.* 410:227–233.
- Kurose, H., T. Katada, T. Haga, K. Haga, A. Ichiyama, and M. Ui. 1986. Functional interaction of purified muscarinic receptors with purified inhibitory guanine nucleotide regulatory proteins reconstituted in phospholipid vesicles. *Journal of Biological Chemistry.* 261:6423–6428.
- 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. 1987. The $\beta\gamma$ subunits of GTP-binding proteins activate the muscarinic K⁺ channel in heart. *Nature.* 325:321–326.
- Nakajima, T., H. Irisawa, and W. Giles. 1990. N-Ethylmaleimide uncouples muscarinic receptors from acetylcholine-sensitive potassium channels in bullfrog atrium. *Journal of General Physiology.* 96:887–903.
- 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. 29–41.
- Needleman, P., J. Turk, B. A. Jakschik, A. R. Morrison, and J. B. Lefkowitz. 1986. Arachidonic acid metabolism. *Annual Review of Biochemistry.* 55:69–102.
- Neer, E., and D. E. Clapham. 1988. Role of G protein subunits in transmembrane signalling. *Nature.* 333:129–134.
- Parent, L., and R. Coronado. 1989. Reconstitution of the ATP-sensitive potassium channel of skeletal muscle. Activation by a G-protein dependent process. *Journal of General Physiology.* 94:445–463.
- 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.
- Sakmann, B., A. Noma, and W. Trautwein. 1983. Acetylcholine activation of single muscarinic K⁺ channels in isolated pacemaker cells of the mammalian heart. *Nature.* 303:250–253.
- 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. M. J. 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.
- Ueda, H., H. Misawa, T. Katada, M. Ui, H. Takagi, and M. Satoh. 1990. Functional reconstitution of purified G_i and G_o with μ -opioid receptors in guinea pig striatal membranes pretreated with micromolar concentrations of N-ethylmaleimide. *Journal of Neurochemistry.* 54:841–848.

- Ui, M. 1984. Islet-activating protein, pertussis toxin: a probe for functions of the inhibitory guanine nucleotide regulatory component of adenylate cyclase. *Trends in Pharmacological Sciences*. 5:277–279.
- Watkins, D. C., J. K. Northup, and C. C. Malbon. 1989. Pertussis toxin treatment *in vivo* is associated with a decline in G-protein β -subunits. *Journal of Biological Chemistry*. 264:4186–4194.
- 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., J. Codina, A. M. Brown, and L. Birnbaumer. 1987. Direct activation of mammalian atrial muscarinic potassium channels by GTP regulatory protein G_K . *Science*. 235:207–211.
- Yatani, A., J. Codina, Y. Imoto, J. Reeves, L. Birnbaumer, and A. Brown. 1987. A G protein directly regulates mammalian cardiac calcium channels. *Science*. 238:1288–1292.
- Yatani, A., R. Mattera, J. Codina, R. Graf, K. Okabe, E. Padrell, R. Iyenger, A. M. Brown, and L. Birnbaumer. 1988. The G protein-gated atrial K⁺ channel is stimulated by three distinct G α -subunits. *Nature*. 336:680–682.