Mechanism of Acetylcholine-induced Inhibition of Ca Current in Bullfrog Atrial Myocytes

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ABSTRACT The mechanism of the anti-β-adrenergic action of acetylcholine (ACh) on Ca current, I_{Ca}, was examined using the tight-seal, whole-cell voltage clamp technique in single atrial myocytes from the bullfrog. Both isoproterenol (ISO) and forskolin increased I_{Ca} dose dependently. After I_{Ca} had been enhanced maximally by ISO (10^{-6} M), subsequent application of forskolin (50 μM) did not further increase I_{Ca}, suggesting that ISO and forskolin increase I_{Ca} via a common biochemical pathway, possibly by stimulation of adenylate cyclase. ACh (10^{-5} M) completely inhibited the effect of low doses of forskolin (2×10^{-6} M), as well as ISO, but it failed to block the effects of high doses of forskolin ($>5 \times 10^{-5}$ M). Intracellular application of cyclic AMP (cAMP) also increased I_{Ca}. ACh (10^{-5} M) failed to inhibit this cAMP effect, indicating that the inhibitory action of ACh occurs at a site proximal to the production of cAMP. ACh (10^{-5} M) also activated an inwardly rectifying K$^+$ current $I_{K(ACh)}$. Intracellular application of a nonhydrolyzable GTP analogue, GTPγS (5×10^{-4} M), activated $I_{K(ACh)}$ within several minutes; subsequent application of ACh (10^{-5} M) did not increase $I_{K(ACh)}$ further. These results demonstrate that a GTP-binding protein coupled to these K$^+$ channels can be activated maximally by GTPγS even in the absence of ACh. Intracellular application of GTPγS also strongly inhibited the effect of ISO on I_{Ca} in the absence of ACh. Pertussis toxin (IAP) completely prevented both the inhibitory effect of ACh on I_{Ca} and the ACh-induced activation of $I_{K(ACh)}$. GTPγS (50 μM–1 mM) alone did not increase I_{Ca} significantly; however, when ISO was applied first, GTPγS (5×10^{-4} M) gradually inhibited the ISO effect on I_{Ca}. These results indicate that ACh antagonizes the effect of ISO on I_{Ca} via a GTP-binding protein (G_i and/or G_o). This effect may be mediated through a direct inhibition by the α-subunit of G_i which is coupled to the adenylate cyclase.

INTRODUCTION

In the heart the Ca current (I_{Ca}), which plays an essential role in generating the action potential and controlling contractility, is regulated by autonomic transmitters

(Reuter, 1983; Tsien, 1983; Hartzell, 1988). β-Adrenergic agonists (e.g., ISO) increase I_{Ca} in both amphibian and mammalian myocardium. This effect is mediated by stimulation of adenylate cyclase (Fischmeister and Hartzell, 1986; Shibata et al., 1986). The resulting increase in intracellular cAMP activates cAMP-dependent protein kinase and increases I_{Ca} by phosphorylation of either the Ca channel itself or a protein near the Ca channel (Bean et al., 1984; Brum et al., 1984; Kameyama et al., 1985; Tsien et al., 1986).

Hormonal stimulation of adenylate cyclase requires molecular interactions between at least three separate entities: (a) β-adrenergic receptors, (b) a guanine nucleotide regulatory protein (G_s), and (c) the catalytic subunit of adenylate cyclase (Rodbell, 1980; Gilman, 1984). Activation of β-receptors by a β-agonist increases the rate of GDP release from, and GTP binding to G_s, which causes dissociation of G_s into α_s- and $\beta\gamma$-subunits. The α_s-subunit combined with GTP then activates the catalytic site of adenylate cyclase (Northup et al., 1983a). G_s can also be activated by nonhormonal substances such as cholera toxin and nonhydrolyzable GTP analogues (e.g., Gpp(NH)p, GTPγS). Like ISO, these substances can also increase I_{Ca} (Pappano et al., 1982; Hescheler et al., 1986).

In contrast to the apparently universal ability of β-adrenergic agonists to increase I_{Ca}, cholinergic agonists reduce I_{Ca} and consequently inhibit contractility of cardiac cells in different ways in amphibian and mammalian hearts. In mammalian ventricular cells ACh by itself lacks an inhibitory effect on either I_{Ca} or contractility; a significant inhibitory action of ACh occurs only in the presence of β-adrenergic stimulation (Biegon and Pappano, 1980; Hescheler et al., 1986). However, in chick ventricle (shortly after hatching) and frog myocardium ACh inhibits either I_{Ca} or contractility directly, i.e., even in the absence of β-adrenergic agonist (Antoni and Rotmann, 1968; Giles and Noble, 1976; Ten Eick et al., 1976; McAfee et al., 1978; Biegon and Pappano, 1980). Interestingly, in mammalian ventricle the inhibitory effect of ACh on I_{Ca} can be reversed by increasing concentrations of β-adrenergic agonist (Hescheler et al., 1986), while in frog cells this effect has not been observed (Fischmeister and Shrier, 1988).

A plausible mechanism for the muscarinic inhibition of I_{Ca} is that ACh can inhibit adenylate cyclase activity via activation of inhibitory GTP-binding proteins (G_i and/or G_o). In mammalian and chick cardiac cells there is evidence for this scheme (Hazeki and Ui, 1981; Endoh et al., 1985; Sorota et al., 1985; Hescheler et al., 1986). Two different possibilities have been suggested for the ACh-induced inhibition of adenylate cyclase mediated via G_i and/or G_o: (a) The $\beta\gamma$-subunits of G_i and/or G_o, which are released after stimulation of the muscarinic receptors coupled to them, may interact with free α_s-subunits from the stimulatory G_s protein to promote formation of an inactive G_s oligomer. This represents an indirect inhibition (Northup et al., 1983b; Katada et al., 1984a). (b) α_i but not α_o may compete with α_s for the same site on the adenylate cyclase (Katada et al., 1986). This effect has been referred to as direct inhibition. At present it is unclear which of these mechanisms may be involved in mediating the inhibitory effect of ACh on adenylate cyclase in vivo.

Other possible mechanisms for the muscarinic effects on I_{Ca} must also be considered. ACh may reduce cAMP levels by, for example, the activation of a cGMP-dependent phosphodiesterase (Fischmeister and Hartzell, 1987). Moreover, it

has been suggested that ACh may stimulate a protein phosphatase or a protein kinase, e.g., protein kinase C (Watanabe et al., 1984; Shibata et al., 1986; Lacerda et al., 1988).

We have attempted to clarify the mechanism(s) of ACh-induced inhibition of I_{Ca} by applying tight-seal voltage clamp techniques (Hamill et al., 1981; Hume and Giles, 1983) to single atrial myocytes from the bullfrog. Our results show that in frog atrial cells the activation of G_i and/or G_o by a muscarinic agonist (ACh) interferes with the signal transduction process from β-adrenergic receptor to Ca channel, and suggest that the inhibitory action of ACh on I_{Ca} is caused by the direct inhibitory effect on adenylate cyclase.

METHODS

Preparation

Single cells were isolated from bullfrog atrium using methods very similar to the ones described previously by Hume and Giles (1981) and Giles and Shibata (1985). Recent modifications will be described briefly. Adult bullfrogs, *Rana catesbeiana*, were killed and the atrium was removed and cut into 1-mm × 1-mm pieces in a Ca-free Ringer's solution (see below). These muscle segments were then treated with two enzyme-containing solutions: First, they were transferred into 5 ml of Ca^{2+}-free Ringer's solution containing 240 IU/ml of collagenase (Yakult Co. Ltd., Tokyo, Japan) together with 3,600 IU/ml of acetylated bovine pancreatic trypsin (type III; Sigma Chemical Co., St. Louis, MO) and incubated at room temperature (22–24°C) for 45 min. This enzyme-containing solution was then pipetted off and the atrial tissue segments were washed, first in Ca^{2+}-free Ringer's solution containing 0.1% bovine albumin (Sigma Chemical Co.) and then in Ca^{2+}-free Ringer's solution. Next, the pieces of atrium were reincubated in 5 ml of Ca^{2+}-free Ringer's solution containing 120 IU/ml of the Yakult collagenase for an additional 45–60 min. In the final step, the tissue segments were collected, resuspended in Ca^{2+}-free Ringer's solution, and then gently triturated (five times) using a large bore (2 mm in diameter) Pasteur pipette. This yielded an acceptable number of viable single cells. An aliquot of this cell suspension was transferred into the recording chamber, which contained HEPES-buffered Ringer's solution.

In experiments involving pretreatment with pertussis toxin (IAP) the isolated atrial cells were divided into two groups and incubated for 6 h at 30°C in normal Ringer's solution either with or without IAP (500 ng/ml) by placing culture dishes of these cell suspensions in an incubator under a humidified atmosphere of 95% air to 5% CO_2.

Solutions and Drugs

The composition of the superfusing solutions (in mM) was as follows. Normal Ringer's: NaCl 90, $NaHCO_3$ 20, KCl 2.5, $MgCl_2$ 5.0, $CaCl_2$ 2.5, glucose 8, saturated with 95% O_2 and 5% CO_2 (pH 7.4). HEPES Ringer's: NaCl 110, KCl 2.5, $MgCl_2$ 5.0, $CaCl_2$ 2.5, glucose 5, HEPES 5, buffered with NaOH to pH 7.4. Ca-free Ringer's: the same as the HEPES Ringer's solution except that $CaCl_2$ was added to a final concentration of 8×10^{-6} M. Recording microelectrodes were filled with a solution of the following composition (in mM): K-aspartate 90, KCl 20, EGTA-KOH 0.77, Na_2-ATP 3, $MgCl_2$ 1, HEPES 5, buffered to pH 7.3 with KOH.

The drugs used in these exeriments were ISO, ACh, forskolin, atropine, adenosine-3':5'-cyclic monophosphate (cAMP), propranolol, and IAP (Sigma Chemical Co.), and guanosine-5'-O-3-thiotriphosphate (GTPγS; Boehringer Mannheim, Biochemicals, Indianapolis, IN). Forskolin was dissolved in DMSO to make 10 mM stock solution, which was stored at $-10°C$.

In each experiment, just before use, the stock solution of the drug was diluted in HEPES Ringer's to the desired concentration. All experiments were performed at room temperatures (22–24°C).

Recording Technique

The gigaseal patch clamp technique was used in the whole-cell configuration (Hamill et al., 1981; Hume and Giles, 1983). The details of electronic circuits used for suction microelectrode voltage clamp have been described previously (Giles and Shibata, 1985; Clark and Giles, 1987). To prepare the suction microelectrodes, square bore borosilicate tubing (1 mm in diameter; Glass Co. of America, Bargaintown, NJ) was heated and pulled by gravity using a vertical micropipette puller (700D; David Kopf Instruments, New Haven, CT). The tips of the resulting micropipettes were smooth enough to make a gigaohm seal without being fire-polished. Their DC resistances varied between 3 and 6 MΩ when filled with the pipette solution. Under the whole-cell clamp conditions series resistances varied between 3 and 8 MΩ. This was electronically compensated to 1–6 MΩ. A hydraulic micromanipulator (MO-102; Narishige Scientific Instruments, Tokyo, Japan) which was mounted on the fixed stage of an inverted microscope (Nikon Diavert) was used to position the microelectrode near the cell. The seal resistance formed between electrode tip and cell membrane was between 5 and 30 GΩ. Membrane potential (V_m) was measured with a high input impedance unity gain amplifier (model 1600; A-M Systems Inc., Everett, WA). To measure current in the voltage clamp mode, a custom-designed circuit (Clark and Giles, 1987) was used in a current-to-voltage converter configuration. Transmembrane currents were measured as the voltage drop across a 100-MΩ feedback resistor.

Data Storage and Analysis

Membrane potential and current signals were monitored on a storage oscilloscope (model 5516ST, Kikusui, Tokyo, Japan) and stored in a microcomputer (IBM-AT) using an analogue-to-digital conversion board (Data Translation DT 2801-A) controlled by our acquisition and display software (Robinson and Giles, 1986). These records were also stored on video tape using an adaptor circuit for a digital VCR instrumentation recorder (model PCM-1; Medical System Corp., Great Neck, NY). The frequency-response of this device was DC-20 kHz. After the experiment, the stored data were sent to a DEC VAX 11-750 computer for analysis, graphing, and archiving.

RESULTS

ACh-induced Inhibition of Isoproterenol Effects on I_{Ca}

Fig. 1 shows the effect of a β-adrenergic agonist, ISO on I_{Ca}. The cell was held at −40 mV to inactivate the Na current, and voltage clamp pulses were applied at 0.2 Hz. As shown previously (Giles et al., 1989), in control conditions positive to approximately −30 mV, a TTX-resistant transient inward current, I_{Ca}, can be elicited (Fig. 1 A). This is followed by slower activation of an outward current, I_K. Upon repolarization to the holding potential, tail currents due to I_K deactivation are recorded. An inwardly rectifying background current, I_{K_1}, was elicited by hyperpolarizing pulses.

ISO (10^{-6} M) increased both I_{Ca} and I_K. Both of these effects of ISO were blocked by the β-antagonist propranolol (3×10^{-7} M; not shown), indicating that β-adrenergic receptors mediate these current changes (Giles et al., 1989). In the presence of

10^{-6} M ISO, the peak inward I_{Ca} (measured from the zero current level) increased from -110 ± 42.0 to $-1,034 \pm 380$ pA ($n = 10$) at 0 mV (Fig. 1 B). The effect of ISO on I_{Ca} was maximal at concentrations greater than 10^{-6} M.

The data in Fig. 2 show (a) the time-course of the ISO-induced stimulation of I_{Ca} and (b) the inhibitory action of ACh (10^{-5} M) on I_{Ca}. This cell was held at a membrane potential of -40 mV and depolarizing pulses to 0 mV were applied at 0.2 Hz. Superimposed I_{Ca} records are shown in Fig. 2 A. Fig. 2 B illustrates the time course of the changes in I_{Ca} and the holding current, measured from the zero current level (+ and □, respectively). ISO (10^{-6} M) increased I_{Ca} from -80 to -850
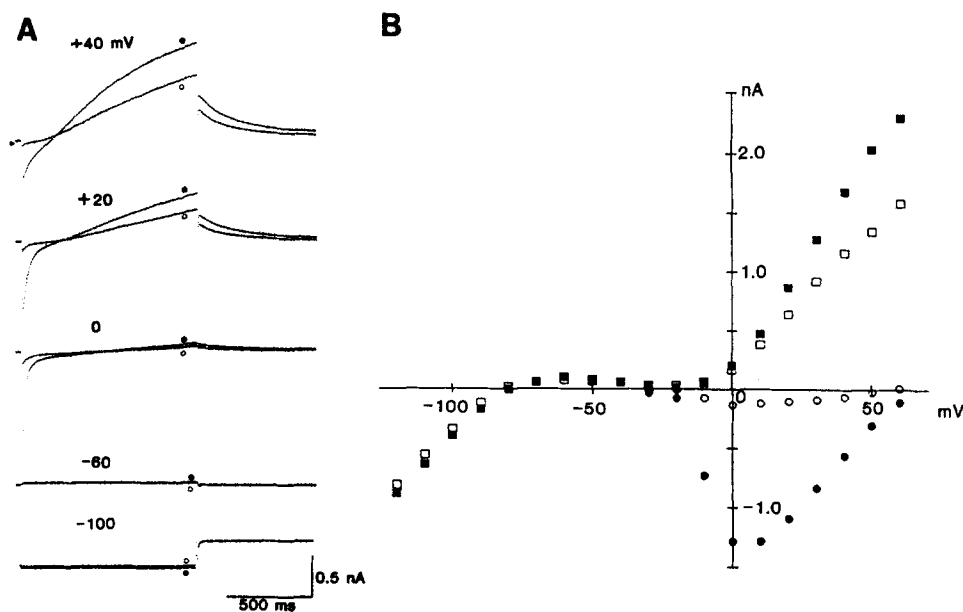

FIGURE 1. The effect of ISO on calcium currents in single atrial cells. The membrane potential was held at -40 mV and 1.5-s pulses were applied to various membrane potentials at 0.2 Hz in 10-mV increments. A shows the original current traces before (O) and after (●) the application of ISO (10^{-6} M). The zero current level is shown by the triangle. These effects on the peak (O, ●) and the isochronal I-V relationships (at the end of the 1,500-ms pulse (□, ■) are illustrated in B. The I-V relationship after ISO is shown by solid symbols.

pA without a significant change in the holding current. Subsequent application of ACh (10^{-5} M) shifted the holding current in the outward direction by ~100 pA. This current, $I_{K(ACh)}$, has been described previously in atrial myocytes (Momose et al., 1984; Soejima and Noma, 1984; Kurachi et al., 1986b; and Simmons and Hartzell, 1987; Clark et al., 1990). When ACh was applied, I_{Ca} decreased rapidly at first and then more slowly (Fig. 2 B). After several minutes I_{Ca} reached a steady-state level well below the control. Subsequent application of a muscarinic antagonist (atropine, 10^{-6} M) quickly blocked both $I_{K(ACh)}$ and the inhibitory effect of ACh on I_{Ca}. However, the inhibitory effect of ACh on I_{Ca} could not be completely reversed by atropine. This
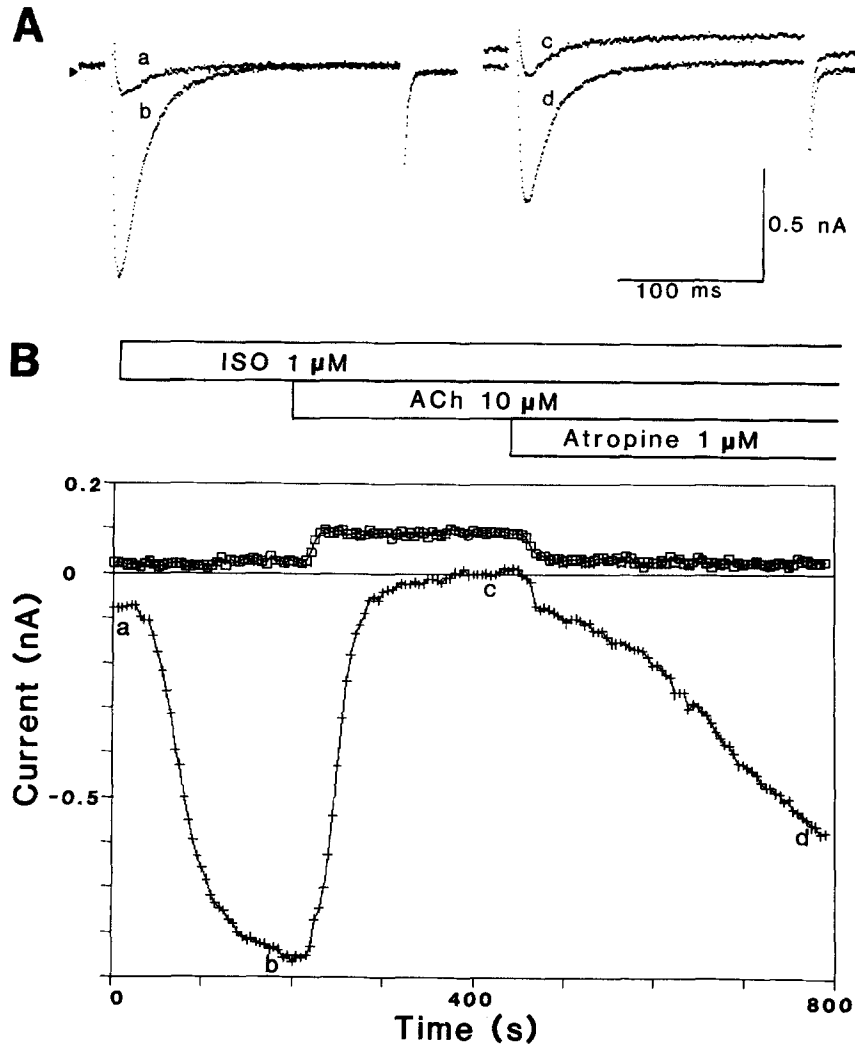
FIGURE 2.   Inhibitory effect of ACh on ISO-induced increase in I_{Ca}. In this and following figures, the membrane potential was held at -40 mV and a 200-ms depolarization pulse to 0 mV was applied at 0.2 Hz. The current traces illustrated in A were recorded in control (a), 10^{-6} M ISO (b), 10^{-6} M ISO plus 10^{-5} M ACh (c), and 10^{-6} M ISO plus 10^{-5} M ACh plus 10^{-6} atropine (d). The current traces $(a-d)$ were recorded at the times indicated by these letters in B. In this and subsequent figures the protocol (drug sequence) is shown in the upper part of B, and the lower part of B shows the time course of changes in the peak I_{Ca} $(+)$ and the holding current ($\square$) measured from the zero current level. Note that ACh $(10^{-5}$ M) shifted the holding current (and hence the peak of I_{Ca}) in the outward direction by ~100 pA, indicating that $I_{K(ACh)}$ was activated.

may be due to run-down of I_{Ca} or desensitization of isoproterenol response. When both ACh (10^{-5} M) and atropine (10^{-6} M) were applied simultaneously, the effect of ACh on I_{Ca} was completely abolished. These results indicate that the inhibitory effects of ACh on $I_{K(ACh)}$ and I_{Ca} are mediated by muscarinic receptors. Additional experiments demonstrated that 10^{-5} M ACh completely inhibited the effects of ISO, even when 10^{-5} M ISO was applied ($n = 3$). In contrast to this finding, Hescheler et al. (1986) have reported previously that the inhibitory effect of ACh on I_{Ca} in guinea pig ventricle can be overcome by increasing the concentration of ISO (see Discussion).

Effect of ACh on I_{Ca} in the Presence of cAMP

To determine whether the intracellular biochemical reactions corresponding to this ACh effect occur proximal or distal to the catalytic actions of adenylate cyclase, intracellular cAMP was increased by adding cAMP (10^{-4} M) to the recording pipette (Fig. 3). I_{Ca} increased progressively as cytosolic cAMP was increased via dialysis. Subsequent application of ACh (10^{-5} M) failed to significantly inhibit this increase in I_{Ca}. The activation of $I_{K(ACh)}$ caused an apparent outward shift in the peak of I_{Ca} of ~80 pA. Atropine (10^{-6} M) completely inhibited the effect of ACh on $I_{K(ACh)}$, and the baseline of I_{Ca} also shifted back to the inward direction. CdCl$_2$ (5×10^{-4} M), a Ca channel blocker, completely blocked cAMP-enhanced I_{Ca}. Similar results were observed in experiments using 10-fold lower (10^{-5} M) cAMP concentrations in the pipette ($n = 2$). These results provide strong evidence that the effect of ACh on I_{Ca} is not mediated by the mechanism distal to the catalytic actions of adenylate cyclase, in contrast to a preliminary report by Shibata et al. (1986).

Effect of Forskolin on I_{Ca}

Forskolin is known to stimulate adenylate cyclase (Seamon and Daly, 1986) and to increase I_{Ca} (Hescheler et al., 1986; Hartzell and Fischmeister, 1987). To test whether the ISO and forskolin effects on I_{Ca} are additive, maximal doses of ISO (2×10^{-6} M) and forskolin (5×10^{-5} M) were applied (Fig. 4). ISO dramatically increased I_{Ca}, but the subsequent application of forskolin failed to increase I_{Ca} any further. Thus, the effects of these two agents on I_{Ca} are not additive. The observed saturation of the ISO and forskolin actions suggests that the forskolin effect on I_{Ca} in frog heart is mediated by the adenylate cyclase cascade. This probably occurs through a direct activation of the catalytic subunit of adenylate cyclase, as is the case in other cell types (Hescheler et al., 1986; Hartzell and Fischmeister, 1987).

ACh-induced Inhibition of Forskolin Effects on I_{Ca}

Low doses of forskolin (2×10^{-6} M) gradually increased I_{Ca} (Fig. 5). Addition of ACh (10^{-5} M) after forskolin treatment completely blocked the forskolin effect. Atropine (1 μM) reversed this effect of ACh on I_{Ca}. However, when higher doses of forskolin were applied, the inhibitory action of ACh was overcome. In the experiment shown in Fig. 6, ACh (10^{-5} M) completely inhibited the effect of ISO (10^{-6} M) on I_{Ca}, but subsequent application of forskolin (5.0×10^{-5} M) significantly increased I_{Ca}. Atropine (1 μM) completely blocked the effect of ACh on $I_{K(ACh)}$, which caused an apparent shift in the peak of I_{Ca} in the inward direction (by ~100 pA). However, I_{Ca}
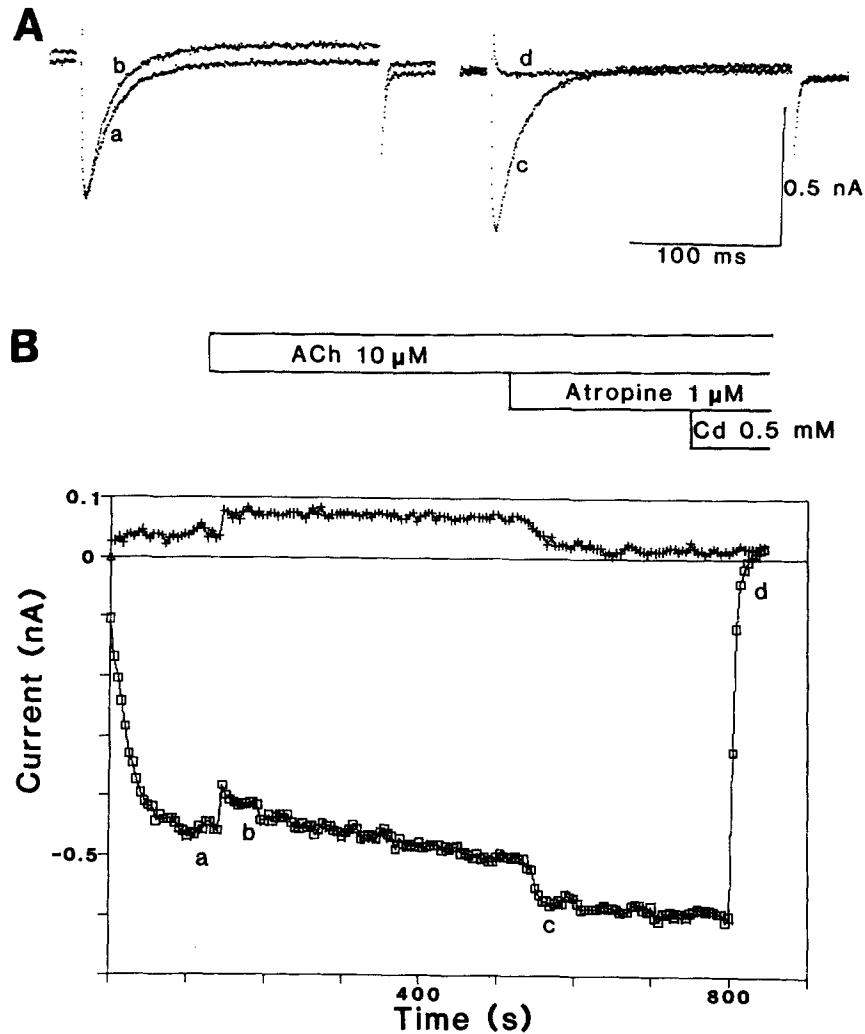
FIGURE 3.   Effect of ACh on I_{Ca} after intracellular application of cAMP. cAMP (10^{-4} M) was applied by dialysis through the recording micropipette. The current traces in A labeled a–d were recorded at the times indicated by these letters in B. Control (a) and after the application of (10^{-5} M) ACh (b), 10^{-5} M ACh plus 10^{-6} M atropine (c), and these drugs plus Cd^{2+} (0.5 mM) (d) are shown. Note that ACh did not have an inhibitory effect on I_{Ca} under these conditions.

itself was not increased by atropine. These results indicate that I_{Ca} can be activated maximally by forskolin (5 × 10^{-5} M) even after the application of ACh. Similar findings were obtained when ACh (10^{-5} M) was applied in the absence of ISO; i.e., ACh (10^{-5} M) had no significant effect on I_{Ca} in the presence of high doses of forskolin (>5 × 10^{-5} M), showing that maximal (saturating) concentrations of forskolin can overcome the effect of ACh on I_{Ca}. $CdCl_2$ (5 × 10^{-4} M) completely blocked the forskolin-induced enhancement of I_{Ca}. These results indicate that in frog
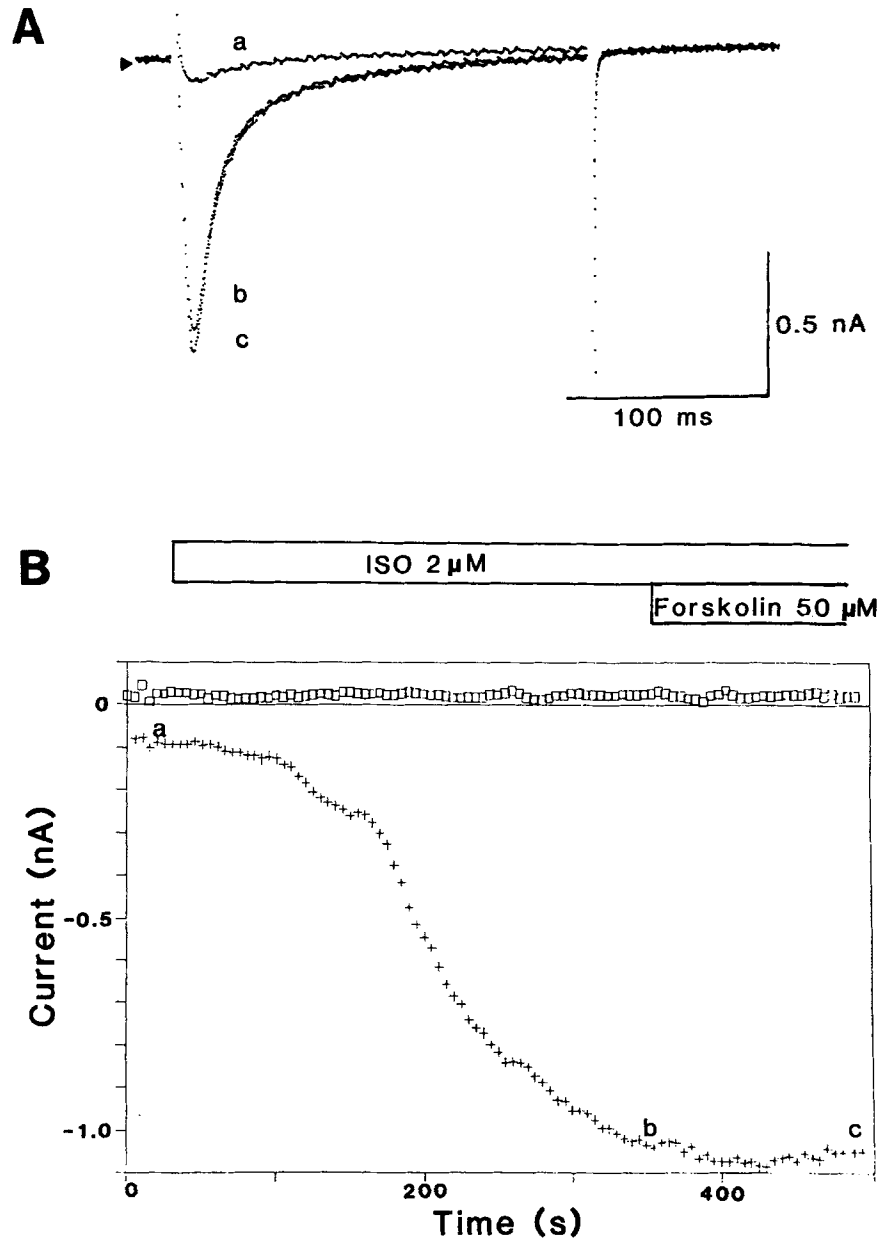
FIGURE 4. Saturation of the stimulatory effects of ISO and forskolin on I_{Ca}. The current traces shown in A were recorded in control (a), after 2×10^{-6} M ISO (b), and after 2×10^{-6} M ISO plus 5×10^{-5} M forskolin (c). The current traces in A (a–c) were recorded at the times indicated by these letters in B.
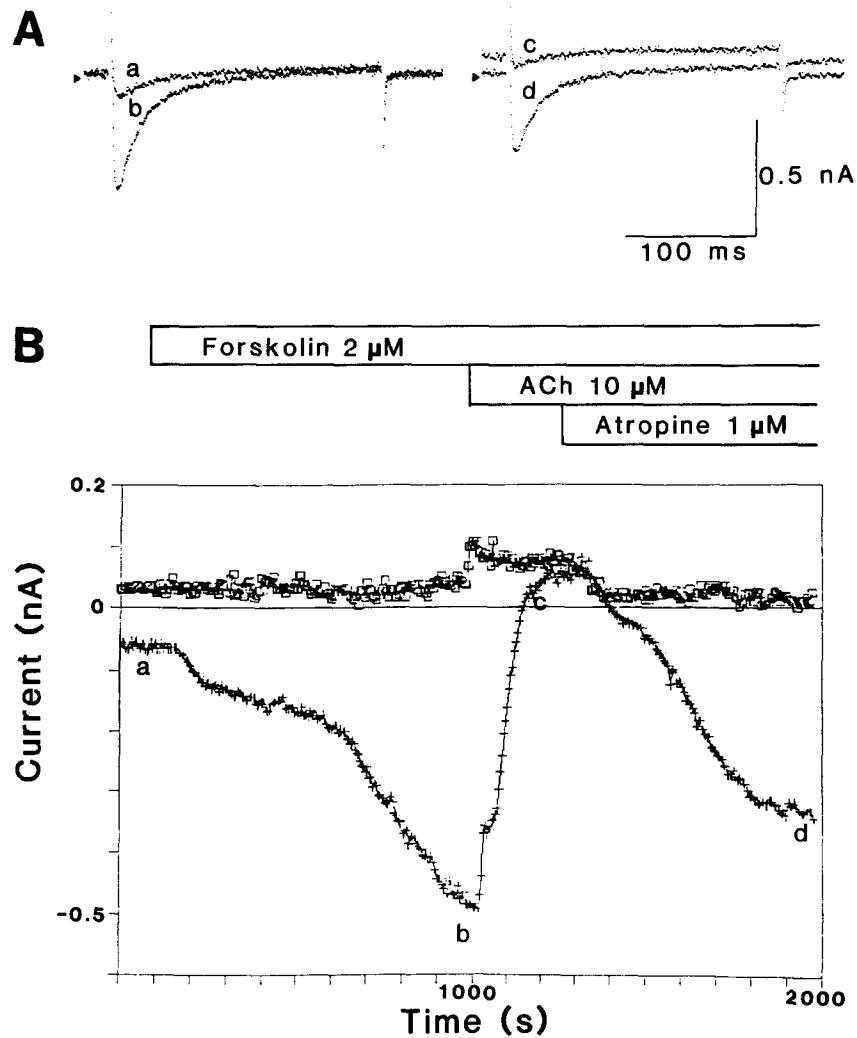
FIGURE 5. The effect of ACh on forskolin-enhanced I_{Ca}. The current traces shown in A were recorded in control (a), in 2×10^{-6} M forskolin (b), in forskolin (2×10^{-6} M) plus ACh (10^{-5} M) (c), and in forskolin (2×10^{-6} M) plus ACh (10^{-5} M) plus atropine (10^{-6} M) (d) at the times indicated by the corresponding letters in the graph in B.

atrial cells inhibitory muscarinic effects of ACh on I_{Ca} can override the stimulatory effects of ISO, and that the site of the action of ACh may be the same as the site for forskolin stimulation of I_{Ca}, i.e., the catalytic subunit of adenylate cyclase (see Discussion).

Involvement of GTP-binding Proteins in ACh Effects

To study whether the effect of ACh on I_{Ca} involves activation of the inhibitory GTP-binding proteins (G_i and/or G_o), atrial cells were pretreated with IAP, an
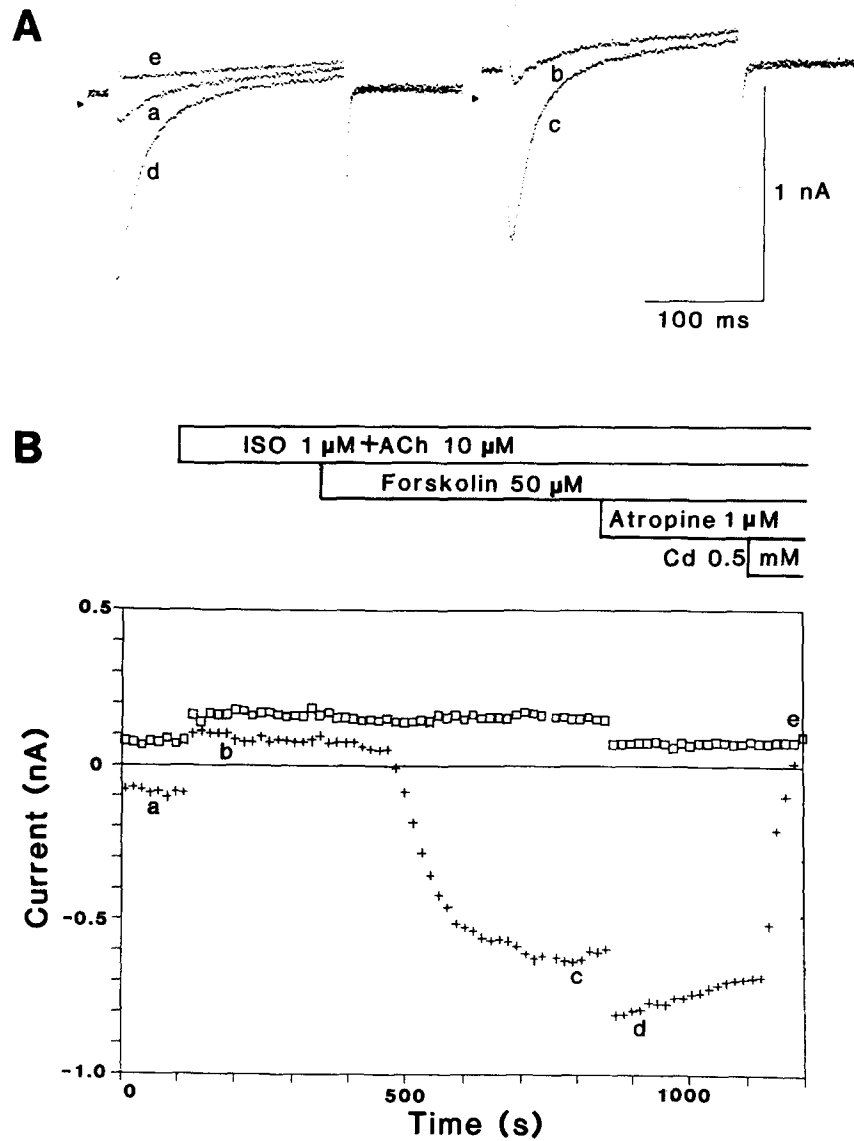
FIGURE 6.   Effects of ACh on ISO- and forskolin-induced increases in I_{Ca}. The original current traces shown in A were recorded in control (a), 10^{-6} M ISO plus 10^{-5} M ACh (b), 10^{-6} M ISO plus 10^{-5} M ACh plus 5.0×10^{-5} M forskolin (c), 10^{-6} M ISO plus 10^{-5} M ACh plus 5.0×10^{-5} M forskolin plus 10^{-6} M atropine (d), and after additional application of 0.5 mM Cd^{2+} (e). The current traces in A (a–e) were recorded at the times indicated by the corresponding letters in B.
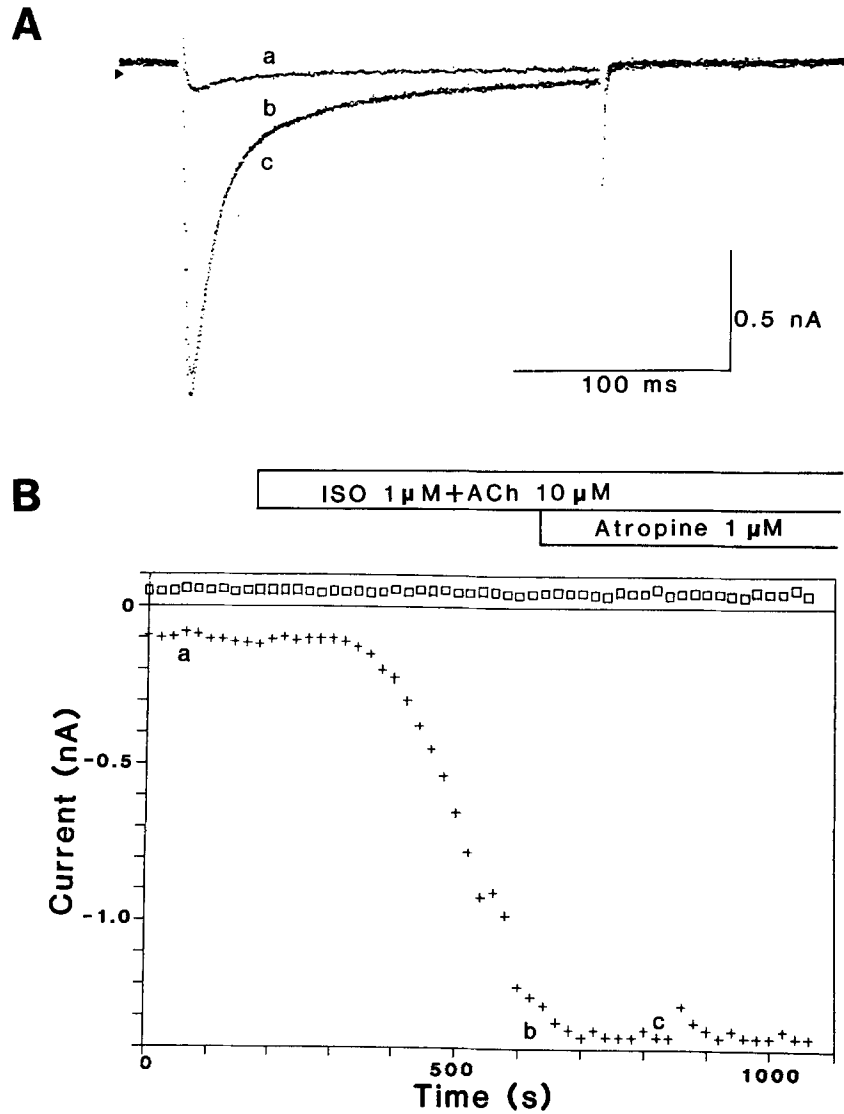
FIGURE 7.   Pretreatment with IAP blocks the effects of ACh on both $I_{K(ACh)}$ and I_{Ca} in bullfrog atrial cells. The current traces shown in A were recorded from IAP-treated cells in control (a), after 10^{-6} M ISO plus 10^{-5} M ACh (b), and after 10^{-6} M ISO plus 10^{-5} M ACh plus 10^{-6} M atropine (c). The current traces in A (a–c) were recorded by times indicated by the corresponding letters on the graph in B.

islet-activating protein from the bacterium *Bordetella pertussis*. In IAP-treated cells ($n = 3$; Fig. 7), application of ISO (10^{-6} M) significantly increased I_{Ca} (150 ± 20 pA in control to 1,350 ± 200 pA, $n = 3$) even in the presence of ACh (10^{-5} M). The effect of ACh on $I_{K(ACh)}$ was also abolished after IAP treatment. Atropine (10^{-6} M) failed to further increase I_{Ca}, indicating that IAP completely removed the effect of ACh on I_{Ca}. These results indicate that the GTP-binding proteins that are ADP-ribosylated by IAP mediate the effects of ACh on I_{Ca} and $I_{K(ACh)}$. An earlier abstract from our laboratory (Shibata et al., 1986) reported that IAP treatment did not block the activation of $I_{K(ACh)}$. These incorrect findings appear to have resulted from the exposure time to IAP, which was too short at room temperature.

To obtain further insight into the molecular mechanisms of these ACh effects, a nonhydrolyzable GTP analogue, GTPγS, was dialyzed into the cell from the recording pipette (Fig. 8). GTPγS (5×10^{-4} M) activated $I_{K(ACh)}$ gradually, as indicated by the slow increase in the holding current in the outward direction. In addition, the application of GTPγS almost completely inhibited the effect of ISO (10^{-6} M) on I_{Ca} even in the absence of ACh. However, I_{Ca} could still be increased by subsequent application of forskolin (5×10^{-5} M). These results and the data in Fig. 6 show (*a*) that forskolin (50 µM) can overcome the inhibitory effects of either ACh or GTPγS on I_{Ca}, and (*b*) that GTPγS can activate the G_i and/or G_o protein directly, i.e., even without an agonist being present. After the effects of GTPγS were fully developed, subsequent application of ACh did not activate $I_{K(ACh)}$ any further, indicating that the G_i and/or G_o proteins were maximally activated by this GTPγS concentration (5×10^{-4} M). These results also suggest that in bullfrog atrial cells, maximum activation of G_i and/or G_o proteins can overcome the effects of activation of G_s.

GTPγS was also dialyzed into IAP-treated atrial cells. Application of GTPγS (500 µM) activated $I_{K(ACh)}$ and also inhibited the effect of ISO (10^{-6} M) on I_{Ca} (not shown, $n = 3$), which suggests that IAP blocks the coupling between the muscarinic receptors and GTP-binding proteins, but that it does not affect the coupling between G proteins and adenylate cyclase or $I_{K(ACh)}$. In summary, our results are consistent with a molecular pharmacological scheme in which ACh exerts its inhibition effect on adenylate cyclase via the activation of G_i and/or G_o proteins. The ACh-induced effect, mediated by G_i and/or G_o, can override that of the stimulatory G protein (G_s) effect. In contrast, activation of the catalytic subunit of adenylate cyclase by forskolin can overcome the inhibitory effects of even maximal doses of ACh.

DISCUSSION

Summary of Findings

These experiments have confirmed that in frog atrium ACh activates an inwardly rectifying K^+ current, $I_{K(ACh)}$, and that this effect is mediated by the inhibitory GTP-binding protein(s) (G_i and/or G_o), which can be ADP-ribosylated by IAP and thus uncoupled from the muscarinic receptor (cf. Clark et al., 1990). ACh can antagonize the stimulatory effects of β-adrenergic agonists on I_{Ca}. This effect is also mediated via G_i and/or G_o. The effects of G_i and/or G_o appear to be able to override those of the stimulatory G protein (G_s) on adenylate cyclase activity, since ACh can almost completely inhibit the effect of β-adrenergic agonists on I_{Ca}.
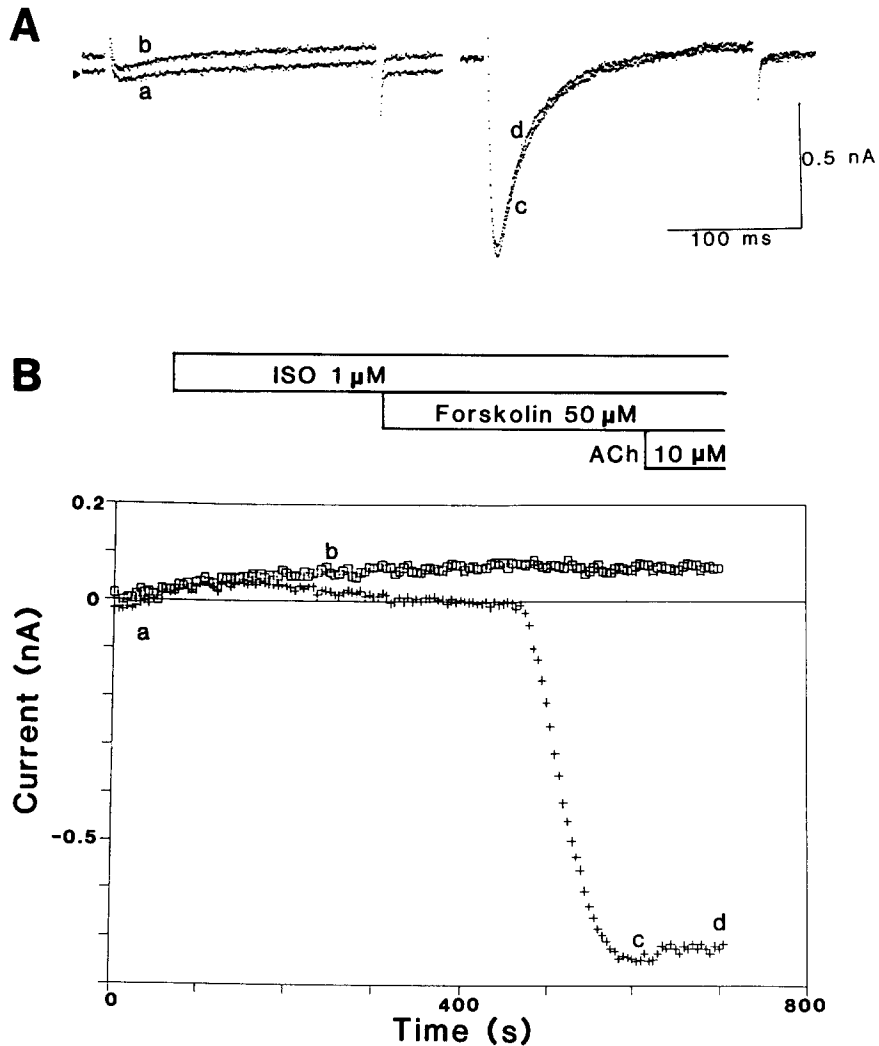
FIGURE 8.   Effect of a nonhydrolyzable GTP analogue (GTPγS) on the ISO- and forskolin-induced increase in I_{Ca}. GTPγS (500 μM) was applied by dialysis through the recording electrode. The current trace (a) was recorded immediately after impalement of the cell. After GTPγS had diffused into the cell $I_{K(ACh)}$ (□) gradually increased. Subsequent application of 10^{-6} M ISO (b) failed to increase I_{Ca} significantly, but 5×10^{-5} M forskolin still augmented I_{Ca} (c). However, the subsequent application of 10^{-5} M ACh (d) did not increase $I_{K(ACh)}$ any further and also failed to block the effect of forskolin on I_{Ca}. The current traces shown in A were recorded at the times indicated by the corresponding letters in B.

Direct Inhibition of Adenylate Cyclase by ACh

A direct inhibitory action of the dissociated subunit of a G protein (probably the α_i-subunit) on the catalytic activity of the adenylate cyclase, appears to be involved in the inhibitory effect of ACh on I_{Ca}. Several possible mechanisms for the G_i- or G_o-mediated inhibitory effects of ACh on adenylate cyclase have been proposed. One possibility is that the $\beta\gamma$-subunit that is released from G_i and/or G_o after stimulation of muscarinic receptors may interact indirectly with free α_s-subunit of G_s to promote formation of an inactive G_s oligomer. Alternatively, α_i may compete directly with G_s for the same locus on the catalytic site of adenylate cyclase (Katada et al., 1984, 1986). It is still not clear whether the effect of ACh on adenylate cyclase is mediated by the direct inhibition of its catalytic subunit (C), whether it is mediated indirectly via changes in the efficacy of G_s-C interaction, or whether a combination of both mechanisms is involved (Gilman, 1984; Katada et al., 1986; Birnbaumer, 1987). In frog atrial cells our results favor the direct mechanism. If the indirect action of $\beta\gamma$-subunit was predominant, the activation of G_s by GTPγS would be expected to irreversibly stimulate the adenylate cyclase and increase I_{Ca} even after activation of G_i and/or G_o by ACh or GTPγS, since the $\beta\gamma$-subunit could not associate with α_s, which would be bound to GTPγS (Fleming and Watabe, 1988). However, as shown in Fig. 8, in frog atrial cells GPTγS (50 μM–1 mM) by itself activated $I_{K(ACh)}$, but I_{Ca} was not increased significantly. Moreover, when GTPγS (500 μM) was applied after pretreatment with ISO (10^{-6} M) to activate G_s (Schramm and Rodbell, 1975) (Fig. 9), I_{Ca} was first increased (by ISO), but as cytosolic GTPγS levels increased I_{Ca} gradually decreased, and $I_{K(ACh)}$ was also activated. Thus, we conclude that in frog atrial cells ACh inhibits the effect of β-adrenergic agonists, mainly by a direct action of the α-subunit of G_i on adenylate cyclase. This direct action of ACh on adenylate cyclase can also inhibit the effect of forskolin.

Activation of $I_{K(ACh)}$ by the Nonhydrolyzable GTP Analogue, GTPγS

ACh activates a specific inwardly rectifying K^+ channel in frog atrial cells (Momose et al., 1984; Breitwieser and Szabo, 1985; Simmons and Hartzell, 1987; Clark et al., 1990) and in mammalian atrial cells (Soejima and Noma, 1984; Kurachi et al., 1986a, b). Recent studies on mammalian atrial cells have shown that the GTP-binding proteins are essential for the activation of these K^+ channels by muscarinic agonists. The following findings support this hypothesis. When nonhydrolyzable GTP analogues (e.g., GTPγS, Gpp(NH)p) are applied, a persistent activation of $I_{K(ACh)}$ is observed (Kurachi et al., 1986b; Breitwieser and Szabo, 1985). IAP, which ADP-ribosylates the G proteins (G_i and/or G_o) (Katada and Ui, 1982; Ui, 1984), eliminates this activation of the K^+ channel by ACh (Pfaffinger et al., 1985; Kurachi et al., 1986b). Recent demonstrations that the bath applications of the $\beta\gamma$-subunit of G_i and/or G_o can activate $I_{K(ACh)}$ (Logothetis et al., 1987; Yanati et al., 1987a) may provide further insight into the mechanism(s) of activation of this K^+ current by ACh.

Pfaffinger et al. (1985) and Breitwieser and Szabo (1985) have shown that G proteins are involved in the activation of $I_{K(ACh)}$. Our results confirm these findings. The application of the nonhydrolyzable GTP analogue (GTPγS) consistently activated $I_{K(ACh)}$, and IAP abolished the effect of ACh-induced K^+ current. This result is

somewhat different from those of Breitwieser and Szabo (1985) and Simmons and Hartzell (1987). In both of these studies a different nonhydrolyzable GTP analogue (Gpp(NH)p) failed to activate $I_{K(ACh)}$ in the absence of ACh. It is possible that this difference can be explained by the choice of GTP analogues and/or their concentrations. The failure of Gpp(NH)p to activate $I_{K(ACh)}$ may indicate that the ratio of Gpp(NH)p/GTP in their preparations was too low to allow significant agonist-independent activation, since they used low pipette concentrations of Gpp(NH)p,
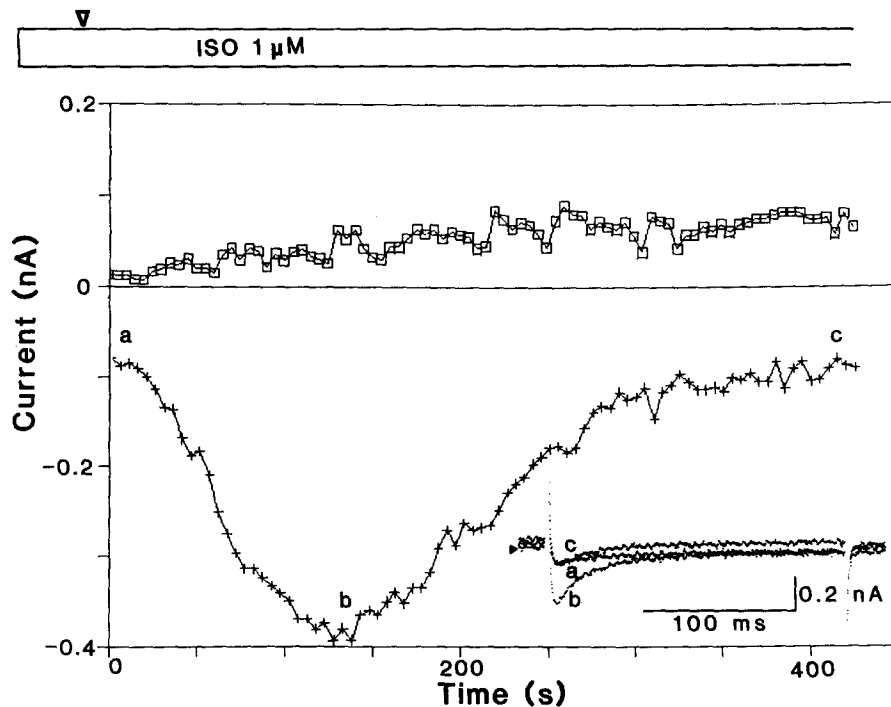

FIGURE 9. The effects of GTPγS on ISO-enhanced I_{Ca}. 10^{-6} M ISO was bath applied before the disruption of the membrane as indicated by the open triangle at the top of the figure. GTPγS (500 μM) was applied by dialysis through the recording micropipette. Current traces taken at the times indicated by the letters (a–c) are superimposed in the inset of the lower panel. Note that after application of ISO (10^{-6} M) I_{Ca} first increased (b), but then gradually decreased (c) as GTPγS diffused into the cell. $I_{K(ACh)}$ also increased (b–c) as GTPγS diffused into the cell.

and did not "clamp" the GTP concentration (Breitwieser and Szabo, 1988). Our results show that GTPγS (500 μM) can consistently activate $I_{K(ACh)}$ even in the absence of ACh, and that this effect reaches a steady-state level within several minutes. Subsequent application of ACh did not further increase $I_{K(ACh)}$, indicating that the GTP-binding protein coupled to $I_{K(ACh)}$ was activated maximally by the (GTPγS). In addition, our results confirm that IAP selectively uncouples the G protein(s) from the muscarinic receptor (Cote et al., 1984; Logothetis et al., 1987),

because even in IAP-treated cells the application of GTPγS could activate $I_{K(ACh)}$ in the absence of ACh.

Muscarinic Inhibition of I_{Ca}

In frog atrial and ventricular cells in normal Ringer's only a single type of Ca current can be detected. It is sensitive to dihydropyridines and resembles L type Ca current in other preparations, including mammalian cardiac cells (Argibay et al., 1988; Campbell et al., 1988).

It is now well known that in frog and mammalian myocardial cells ACh can inhibit I_{Ca} in the presence of β-adrenergic agonists (Breitwieser and Szabo, 1985; Fischmeister and Hartzell, 1986; Shibata et al., 1986). In frog atrium ACh appears to inhibit the action of β-adrenergic agonists in a noncompetitive manner, since ACh (10^{-5} M) almost completely inhibited the ISO-induced increase in I_{Ca}, even in the presence of saturating concentrations of ISO ($\geq 10^{-6}$ M), consistent with the findings of Fischmeister and Shrier (1989) in frog ventricular cells. We attempted to determine whether the mechanism of this inhibition is different in frog cells than in mammalian myocytes. Biochemical studies have shown that in mammalian ventricular cells muscarinic agonists inhibit the effects of β-adrenergic agonists on the adenylate cyclase by an indirect action of the βγ-subunits released from G proteins (G_i and/or G_o) (Fleming and Watanabe, 1988). Consistent with this hypothesis, (*a*) basal adenylate cyclase activity and (*b*) activation of this enzyme by the nonhydrolyzable GTP analogues could not be inhibited by muscarinic agonists. Hescheler et al. (1986) have shown that ACh can antagonize the activation of adenylate cyclase by β-agonists via mechanisms involving GTP-binding proteins (probably G_i) and that it can inhibit Ca channel phosphorylation and thus decrease I_{Ca}. Complementary biochemical evidence for this hypothesis has also been obtained (Hazeki and Ui, 1981; Endoh et al., 1985; Sorota et al., 1985). In frog atrial cells, Breitwieser and Szabo (1985) found that the anti-β-adrenergic effect of ACh is mediated by a GTP-binding protein, and Shibata et al. (1986) have shown that GTPγS, can inhibit the stimulatory effect of ISO on I_{Ca}. Our results confirm that ACh inhibits β-adrenergic effects on I_{Ca}, and show that this inhibition is mediated by a GTP-binding protein, probably G_i and/or G_o, as it is in mammalian myocytes.

These findings differ from some of the results obtained using mammalian heart cells (Hescheler et al., 1986). In guinea pig ventricular cells the application of a nonhydrolyzable GTP analogue (Gpp(NH)p) increased I_{Ca}, an effect that is opposite to our finding. It is unlikely that it is due solely to the difference of nonhydrolyzable GTP analogues, since we have also observed that in rabbit ventricular cells GTPγS (100–300 μM) alone can increase I_{Ca} irreversibly (unpublished results). These different responses to nonhydrolyzable GTP analogues could be due to differences between frog atrial cells and mammalian ventricular myocytes in potency for stimulation and/or inhibition of the adenylate cyclase via the stimulatory G protein or the inhibitory G protein. It is possible that in frog atrial cells maximal activation of G_i by GTPγS can overcome the effects of GTPγS on G_s, while in mammalian ventricular cells this cannot happen. This hypothesis is also compatible with the observation that in mammalian myocytes the inhibitory effect of ACh could be overcome by large concentrations of ISO (Hescheler et al., 1986), while in frog atrial

and/or ventricular cells (Fischmeister and Shrier, 1989) this effect of ISO has not been observed.

The inhibitory effects of ACh on I_{Ca} have been hypothesized to be caused by several different mechanisms: (a) activation of a cyclic GMP-dependent phosphodiesterase (Fischmeister and Hartzell, 1987); (b) stimulation of a protein phosphatase (Watanabe et al., 1984); and/or (c) activation of protein kinase C (Shibata et al., 1986; Lacerda et al., 1988). All of these hypotheses require the inhibitory effects of ACh to be mediated by biochemical events distal to adenylate cyclase. Our results indicate that this is unlikely. Thus, the application of cAMP increased I_{Ca} in a dose-dependent manner; however, simultaneous application of ACh did not affect the time course of this cAMP-induced increase of I_{Ca} even when very low doses of cAMP (10^{-5} M) were used. This result is somewhat similar to the finding of Fischmeister and Hartzell (1986). Furthermore, ACh can block the effect of isoproterenol on I_{Ca} even in the presence of the phosphodiesterase inhibitor IBMX (5×10^{-4} M, results not shown). In combination, these results suggest that the site of ACh action is mainly proximal to cAMP production. The role of changes in cGMP in these muscarinic actions of ACh remains unclear (Keely and Lincoln, 1978; Shibata et al., 1986; Fischmeister and Hartzell, 1987).

Recently it has been reported that in mammalian ventricular cells G_s can directly activate Ca channels, independently of cAMP (Yatani et al., 1987b; Brown and Birnbaumer, 1988; Yatani and Brown, 1989; Pelzer et al., 1990). Our data suggest that this direct coupling may not be present in frog atrium. Thus, in our experiments the time course of the effect of isoproterenol on I_{Ca} is different from that of ACh on $I_{K(ACh)}$, which is the best-known example of a direct effect of a G protein. In addition, the application of GTPγS did not significantly increase I_{Ca}, even though $I_{K(ACh)}$ was activated by GTPγS. From these results it seems unlikely that Ca channels are directly modulated by activation of G_s, as is the case for the activation of G_i and/or G_o, which is involved in modulation of the ACh-dependent K^+ channel. However, a small direct effect of G_s and I_{Ca} cannot be ruled out by our whole-cell data.

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