Acetylcholine Reversal of Isoproterenol-Stimulated Sodium Currents in Rabbit Ventricular Myocytes

James J. Matsuda, Hon-Chi Lee, and Erwin F. Shibata

We have recently shown that β-adrenergic agonists enhance the cardiac sodium current (I_{Na}) in rabbits through dual G-protein regulatory pathways. To determine if muscarinic cholinergic receptor stimulation can also modulate I_{Na}, we studied the effects of acetylcholine (ACh) and carbachol on I_{Na} in enzymatically dispersed rabbit ventricular myocytes. Whole-cell patch-clamp experiments done at room temperature using 20 mM $[Na^+]_o$ showed that 100 nM isoproterenol increased I_{Na} and accelerated current decay as previously described. ACh (1 μM) or carbachol (1 μM) significantly reversed the stimulatory isoproterenol effects at test potentials throughout the I_{Na} activation range and at holding potentials negative to −80 mV. This effect was completely inhibited by atropine (1 μM) and was confirmed by studying single-channel I_{Na} from cell-attached patches. When I_{Na} was stimulated by forskolin (1 μM), carbachol (1 μM) significantly reversed the effect. The muscarinic-mediated inhibition of I_{Na} was inhibited by pertussis toxin (0.1 or 1.0 μg/ml) incubation (12–15 hours), suggesting that the effect was inhibitory G-protein dependent. Further investigation of the ACh inhibitory mechanism revealed that ACh alone had no effect on I_{Na} and that when cells were dialyzed with cAMP (5 μM), ACh failed to inhibit I_{Na}. Furthermore, cGMP failed to inhibit the effect of isoproterenol on I_{Na}. These data suggest that ACh acts at or proximal to adenylate cyclase stimulation. Thus, rabbit cardiac Na^+ channels are regulated by muscarinic agonists in a fashion similar to cardiac Ca^{2+} channels. (*Circulation Research* 1993;72:517–525)

KEY WORDS • sodium channels • cardiac myocytes • muscarinic receptors • β-adrenergic receptors

Isoproterenol (ISO), a β-adrenergic agonist, modulates several voltage-dependent ionic currents in the heart, including the calcium current (I_{Ca}), the delayed rectifier potassium current (I_K), the transient outward potassium current (I_{to}), and the pacemaker current (I_f) (see Reference 1 for review). The ISO-induced modulation of these currents contributes to increases in cardiac contractile strength (positive inotropic effect) and in the rate of the heart beat (positive chronotropic effect) (see Reference 1). For example, the ISO-induced stimulation of I_{Ca} leads to elevated cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) levels, causing an increase in cardiac contractility. We recently discovered that ISO also increases the voltage-dependent sodium current (I_{Na}) in the rabbit heart through dual G-protein regulatory pathways.[2] The physiological significance of the ISO-induced increase in I_{Na} has not been determined. However, continuous cable theory predicts that enhancements of I_{Na} should increase cardiac impulse conduction velocity (positive dromotropic effect).[3] It has also been suggested that the ISO-induced enhancement of I_{Na} might contribute to the positive inotropic effect of β-adrenergic agonists in the heart.[2] Muscarinic agonists have been shown to oppose the positive inotropic and chronotropic effects of β-adrenergic agonists in the heart. These effects are largely mediated by the reversal of β-adrenergic receptor stimulation of I_{Ca} and I_f by acetylcholine (ACh).[1,4] If cardiac I_{Na} regulation by neurotransmitters were a physiologically important process, parasympathetic agonists should also oppose the effects of ISO on I_{Na}. Direct evidence for the regulation of I_{Na} by muscarinic receptor activation, however, has not been shown. The purpose of this study was to determine if muscarinic agonists can modulate I_{Na} in isolated rabbit ventricular myocytes.

Since we previously demonstrated that ISO regulates I_{Na} and I_{Ca} by similar mechanisms,[2] our initial hypothesis was that ACh would also regulate these currents by similar signal transduction pathways in the rabbit heart. The muscarinic receptor–mediated inhibition of ISO-stimulated I_{Ca} in the heart has been clearly demonstrated.[1,4] The exact mechanism of this effect remains to be elucidated. Previous studies showed that ACh inhibits ISO-stimulated I_{Ca} in guinea pig[5] and frog[6,7] cardiac myocytes, whereas ACh alone had little or no effect on I_{Ca}. Other studies showed that ACh also inhibits forskolin-stimulated I_{Ca}.[5,7–10] However, when cells were dialyzed with cAMP, ACh did not affect I_{Ca}.[5–8] These results suggest that muscarinic inhibition of ISO-stimu-

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lated I_{Ca} takes place at or proximal to adenylate cyclase stimulation in the signal transduction pathway. Using nonhydrolyzable GTP analogues, Parsons et al[10] showed that the predominant site of ACh action in inhibiting ISO-stimulated I_{Ca} is adenylate cyclase. These findings agreed with biochemical studies that showed that ACh activation of cardiac muscarinic receptors antagonizes ISO-stimulated adenylate cyclase activity through the pertussis toxin–sensitive inhibitory G protein, G_i (see Reference 11 for review). Several models have been suggested for this effect including 1) muscarinic receptor–stimulated release of $\beta\gamma$ G-protein subunits from $G_{i\alpha}$, which inactivate $G_{s\alpha}$ (mass action hypothesis),[12] 2) direct inhibition of adenylate cyclase by $\beta\gamma$ subunits,[13] 3) direct inhibition of adenylate cyclase by $G_{i\alpha}$,[12] or 4) direct interaction of G_i with the β-adrenergic receptor, thus inhibiting its interaction with the stimulatory G protein, G_s.[14]

Other studies suggest that muscarinic inhibition of I_{Ca} may take place distal to adenylate cyclase activation. For instance, cGMP inhibited ISO- and cAMP-stimulated I_{Ca} in both the frog[15] and guinea pig[16] but did not affect basal I_{Ca} amplitude. It was suggested that muscarinic receptor stimulation increases intracellular cGMP levels and that this increase subsequently activates a cyclic nucleotide phosphodiesterase.[17] Recently, however, contrasting evidence by Ono and Trautwein[18] showed that cGMP potentiates the effect of ISO on I_{Ca} in guinea pig ventricular myocytes. Muscarinic receptor stimulation also increases inositol-1,4,5-trisphosphate formation and protein kinase C activation in heart via a phospholipase C–coupled G protein that is pertussis toxin insensitive.[11,19] The effect of this signaling pathway on Ca^{2+} channel function is not yet clear.

The effects of ACh on the fast inward cardiac I_{Na} have not been directly studied. Cheng et al[20] showed that ISO reduced the fast component of the action potential maximal upstroke velocity in depolarized guinea pig cardiac tissue and that ACh reversed this effect. In this study, we examine directly the effects of muscarinic agonists on cardiac I_{Na} and explore the mechanisms responsible for these effects. We show that the effects of ACh on I_{Na} are similar to those reported for I_{Ca}.

Materials and Methods

Cell Isolation

Rabbit ventricular myocytes were enzymatically dissociated as previously described.[2] Briefly, rabbit hearts were retrogradely perfused on a modified Langendorff apparatus with 0.017 mg/ml protease (type XXIV, Sigma Chemical Co., St. Louis, Mo.) for 10 minutes at 37°C. Small pieces of ventricular tissue (2 mm×2 mm) were then removed from the right ventricle and put in vials containing 0.6 mg/ml collagenase (Sigma type I) in a nominally zero $CaCl_2$ solution containing (mM) NaCl 140, $MgCl_2$ 1.0, KCl 4.5, HEPES 10, and glucose 5.55 (pH 7.35). This solution was also used as the cell storage solution. The pieces of ventricular tissue were shaken for 5 minutes at 35°C and then rinsed and stored at room temperature in the zero Ca^{2+} storage solution without enzyme. Single cells were then dissociated by mild mechanical trituration. The cells were Ca^{2+} tolerant, had resting potentials between −89 and −75 mV at room temperature, and remained viable up to 18 hours after cell isolation.

Experimental Procedures

The patch-clamp apparatus and techniques[21] were similar to those previously described.[2] Whole-cell currents were filtered at 2 kHz and sampled at 25 kHz; single-channel currents were filtered at 2 kHz and sampled at 10 kHz. Uncompensated pipette resistances were 0.5–1.0 MΩ for whole-cell recordings and 3–5 MΩ for single-channel recordings; 85%–95% of the whole-cell series resistances were compensated for by use of the Axopatch 200 (Axon Instruments, Foster City, Calif.) patch-clamp amplifier. All experiments were performed at room temperature (23°–25°C).

Ensemble averaging of single-channel currents was accomplished using pCLAMP software (Axon Instruments, version 5.5). Leak subtraction of single-channel currents was done as previously described,[2] and all test pulses for single-channel experiments were preceded by 500-msec hyperpolarizing prepulses to −140 mV. The time course of current decay was determined using a FORTRAN IV version of the DISCRETE program developed by Provencher[22] as previously described.[2] We have previously determined that the I_{Na} current decay at test potentials of −30 mV and below are best fit by one exponential and that two time constants were determined to be a better fit at more depolarized potentials.[2] In the present study, we determined the time constant of I_{Na} current decay only at test potentials negative to −30 mV. Therefore, only one time constant is reported.

External Na^+ concentration was lowered to 20 mM, and test pulses for most experiments were restricted to potentials where I_{Na} was not fully activated. Even under these conditions I_{Na} was frequently too large to adequately voltage clamp. Therefore, we only used cells in which the I_{Na} amplitude was less than 1.5 nA. The membrane voltage error was thus <2 mV, and the data were therefore not corrected. These cells had an average cell capacitance of 74.7±3.4 pF (mean±SEM, n=14). The time constant of capacitative current decay after series resistance compensation was 89±0.01 μsec (mean±SEM, n=14). Time-dependent changes of I_{Na} (see Reference 23 for review) were frequently observed in both whole-cell and single-channel experiments. In our experiments, these shifts typically occurred during the first 20 minutes of recording. We waited at least 20 minutes for I_{Na} to reach steady state (no visible change in I_{Na} amplitude at a given test potential over a 5-minute period) before data were taken. Internal perfusion of the patch pipette was done according to the method of Neher and Eckert.[24] All data are expressed as mean±SEM, and significance was determined by a paired t test at $p<0.05$.

Solutions and Drugs

The whole-cell recording pipette solution contained (mM): cesium aspartate 130, HEPES 10, Na_2-ATP 5.0, GTP 0.5, EGTA 5.0, $CaCl_2$ 0.5, and $MgCl_2$ 2.0 (pH 7.25 with CsOH; pCa ≈8). Whole-cell bath solutions contained (mM) tetramethylammonium chloride (TMA) 100, tetraethylammonium chloride (TEA) 20, KCl 4.5, $MgCl_2$ 1.0, $CaCl_2$ 1.0, $BaCl_2$ 0.5, $CdCl_2$ 0.5, HEPES 10, and glucose 5.55 (pH 7.35). In some experiments 120 mM Tris-hydrochloride was used as a Na^+ replacement

in the bath solution instead of TEA and TMA. The pipette solution for all single-channel recordings contained (mM): NaCl 140, KCl 4.5, $CaCl_2$ 1.0, $MgCl_2$ 1.0, and HEPES 10 (pH 7.35). Bath solutions for the single-channel cell-attached experiments contained (mM) K^+-aspartate 140, EGTA 5, $MgCl_2$ 2.0, glucose 5.55, and HEPES 10 (pH 7.35) to depolarize the cells to 0 mV.

All pharmacological agents were obtained from Sigma. Stock solutions of ISO, ACh, and carbamylcholine (carbachol [CCh]) were dissolved in water and made on a daily basis. Forskolin was initially dissolved in ethanol at a concentration of 1 mM and then diluted 1:1,000 into the bath solution, giving a 1 μM forskolin solution with a solvent concentration of 0.1%. This concentration of ethanol alone in control experiments did not affect I_{Na}.[2] cAMP and cGMP were directly dissolved in the pipette solution at the indicated concentrations. Dibutyryl cGMP and dibutyryl cAMP were directly dissolved in the bath solution. Pertussis toxin was dissolved directly into the cell storage solution (0.1 or 1.0 μg/ml). Treated cells were incubated in pertussis toxin at room temperature for 10–14 hours, and control cells were incubated in storage solution without pertussis toxin for equal time periods.

Results

Muscarinic agonists affect several ionic currents in cardiac myocytes (see Reference 1), which could potentially complicate our analysis of I_{Na}. Therefore, we tested the effect of ACh on the net ionic currents throughout a wide range of potentials (from −120 to +40 mV). Using our whole-cell bath and pipette solutions, ACh (1–10 μM) did not activate a current characteristic of $I_{K,ACh}$, the ACh-activated potassium current (n=4, data not shown). A muscarinic receptor–activated time-independent Na^+ inward current that is sensitive to high agonist concentrations (K_d of 12 μM for CCh) has been described in mammalian cardiac myocytes.[25] Under our experimental conditions, 300 μM CCh did not activate this current at any potential (n=4, data not shown), suggesting that our ionic conditions (i.e., low $[Na^+]_o$) sufficiently reduced this current, making it undetectable. Therefore, we believe that the effects of ACh and CCh described below are due to direct effects on the fast inward I_{Na} and not due to activation of any background current.

Whole-cell experiments done from a holding potential of −100 mV and a test potential of −30 mV revealed that ISO (100 nM) increased I_{Na} and that the addition of ACh (1 μM) to the ISO-containing solution partially reversed this effect (see Figure 1). The ACh response was blocked by the muscarinic receptor antagonist atropine (1 μM). Initial experiments were done in 20 mM $[Na^+]_o$ using 20 mM TEA and 100 mM TMA as a Na^+ replacement. Under these conditions, 100 nM ISO significantly increased I_{Na} by 36.9±5.4% (mean±SEM, p<0.001, n=7), and 1 μM ACh significantly reversed the ISO stimulation by 54.0±13.7% (p<0.001; 100% reversal represents complete reversal of ISO effect). A recent study by Caulfield[26] showed that TEA inhibits the effects of muscarinic agonists and antagonists on I_{Ca} in a glioma cell line. Therefore, we tested this finding in cardiac cells with solutions void of TEA using 120 mM Tris-hydrochloride as a Na^+ replacement. Under these conditions, 100 nM

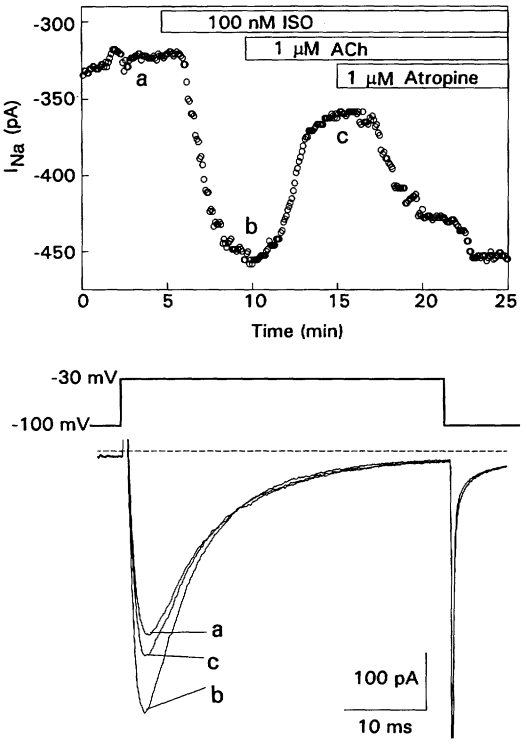

FIGURE 1. *The effect of acetylcholine (ACh) on isoproterenol (ISO)–stimulated sodium current (I_{Na}) in a rabbit ventricular myocyte. Top panel: Peak whole-cell I_{Na} amplitude is plotted against time during bath perfusion of ISO (100 nM), ACh (1 μM), and atropine (1 μM) at the times indicated by the horizontal bars. Stimulation rate was 0.2 Hz. Bottom panel: Raw current tracings from the same experiment are shown at times a–c. Three consecutive tracings were averaged at each time point. The dashed line represents zero current level.*

ISO increased I_{Na} by 33.9±4.9% (p<0.001, n=7), and 1 μM ACh reversed the ISO stimulation by 79.0±3.6% (p<0.001; holding potential, −100 mV; test potential, −30 mV). The augmented ACh effect in Tris-containing solutions supports the observation that TEA inhibits muscarinic agonists. In our previous study,[2] ISO not only enhanced I_{Na} amplitude but it also accelerated the rate of I_{Na} decay. In the present study, ISO also decreased the time constant of I_{Na} decay by 20.9±3.3% (p<0.05), and ACh reversed this effect by 75.1±6.7% (p<0.05, n=7). In seven experiments, ACh alone (1 μM) had no significant effect on I_{Na} (see Figure 2), and CCh at concentrations up to 300 μM also had no significant effect on I_{Na} (n=3).

The effect of ISO on I_{Na} was mimicked by agents that increase intracellular cAMP levels, such as forskolin.[2] Figure 3 shows that forskolin (1 μM) increased I_{Na} and that 1 μM CCh partially reversed the effect. Using TEA and TMA as Na^+ replacements, forskolin (1 μM) increased I_{Na} by 47.1±11.4% (p<0.01), and CCh (1 μM) significantly reversed this effect by 28.4±3.3% (p<0.05; holding potential, −100 mV; test potential, −30 mV; n=4). When Na^+ was replaced by Tris, forskolin increased I_{Na} by 52.2±9.8% (p<0.01), and CCh reversed the forskolin effect by 49.6±5.0% (p<0.005, n=5). The time course of current decay in these experiments was

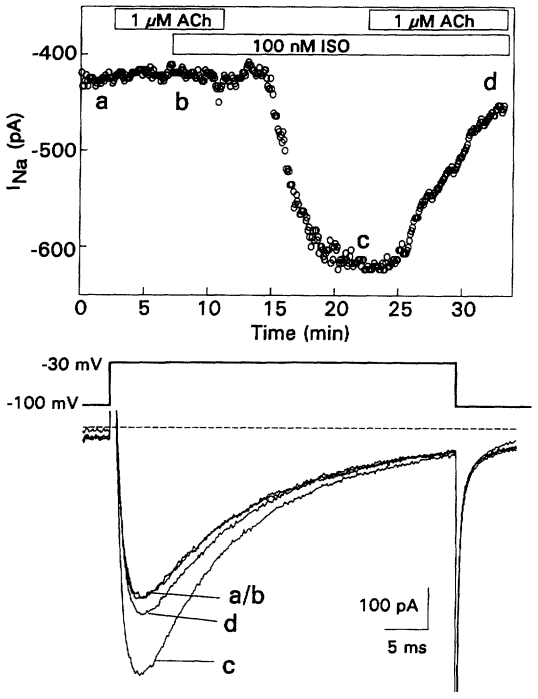

FIGURE 2. *The effect of acetylcholine (ACh) alone on isoproterenol (ISO)-stimulated sodium current (I_{Na}) in a rabbit ventricular myocyte. Top panel: Peak whole-cell I_{Na} amplitude is plotted vs. time during bath perfusion of ACh (1 μM) and ISO (100 nM) at the times indicated by the horizontal bar. Stimulation rate was 0.2 Hz. Bottom panel: Raw current tracings from the same experiment are shown at times a–d. Three consecutive tracings were averaged at each time point. The dashed line represents zero current level.*

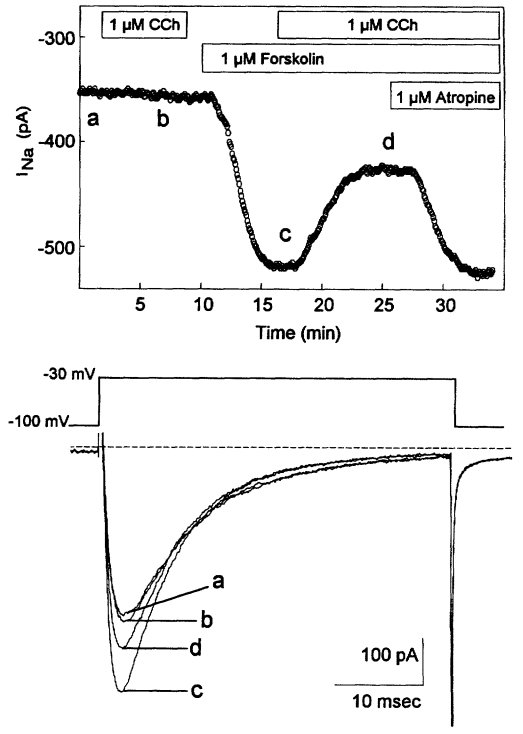

FIGURE 3. *The effect of carbachol (CCh) on control and on forskolin-stimulated sodium current (I_{Na}) in a rabbit ventricular myocyte. Top panel: Peak whole-cell I_{Na} amplitude is plotted vs. time during bath perfusion of CCh (1 μM), forskolin (1 μM), and atropine (1 μM). Stimulation rate was 0.2 Hz. Bottom panel: Raw current tracings from the same experiment are shown at times a–d. Three consecutive tracings were averaged at each time point. The dashed line represents zero current level.*

decreased by 26.2±2.5% ($p<0.05$) with forskolin, and CCh reversed this forskolin effect by 40.3±11.8% ($p<0.05$).

To determine if the effect of ACh was G-protein (G_i or G_o) dependent, cells were incubated in pertussis toxin (1.0 μg/ml) for at least 12 hours. In the pertussis toxin–treated cells, ISO increased I_{Na} by 35.4±6.3%, but ACh or CCh in concentrations up to 100 μM did not affect the ISO-stimulated I_{Na} ($n=6$, see Figure 4). Similar results were found when cells were incubated in lower concentrations of pertussis toxin (0.1 μg/ml). Control cells that were not incubated in pertussis toxin but were kept in storage solution for at least 12 hours showed normal responses to both ISO and ACh ($n=5$), similar to the acutely dissociated cells.

We have previously shown that ISO increased I_{Na} throughout the activation range.[2] Figure 5 shows the averaged peak current–voltage relation ($n=4$) for whole-cell I_{Na} before and after ISO and ISO+CCh application. All currents were normalized to the largest current for each cell (i.e., the current recorded at −15 mV after ISO application). The effects of ISO and ISO+CCh were significant at test potentials from −30 to 0 mV ($p<0.05$). These results confirm our previous findings and show that CCh (1 μM) reverses the effect of ISO at test potentials throughout the activation range.

Our previous study also showed that the effect of ISO on I_{Na} is holding potential dependent, such that the largest ISO responses are seen at hyperpolarized holding potentials.[2] The data in Figure 6 confirm this result and show that CCh significantly reverses the ISO effect at holding potentials negative to −80 mV ($n=4$, $p<0.05$ for −85 mV, $p<0.01$ for prepulse potentials of −90 to −120 mV). These data were normalized to construct the steady-state inactivation curve shown in Figure 7. The steady-state inactivation curve was obtained by normalizing currents to the maximal I_{Na} obtained at a prepulse of −120 mV. The curve was then fit using a conventional Boltzmann distribution equation of the following form:

$$h_\infty = \{1+\exp[(V_m - V_{1/2})/k]\}^{-1}$$

where h_∞ is the steady-state activation of I_{Na}, V_m is membrane voltage, $V_{1/2}$ is the half-inactivation potential, and k is the slope factor. Neither $V_{1/2}$ nor k was significantly altered by ISO (100 nM) or ISO+CCh (1 μM) ($n=4$). These experiments were repeated using a test potential on the positive slope portion of the current–voltage relation, and similar results were obtained. At a test potential of 0 mV, $V_{1/2}$ values were −74.5 mV for the control condition, −73.4 mV for ISO, and −76.0 for ISO+CCh ($n=2$, data not shown). The respective slope factors were 5.0, 4.8, and 5.1 mV.

Studies on the L-type Ca^{2+} current in the heart have shown that ACh does not affect I_{Ca} when the cells are

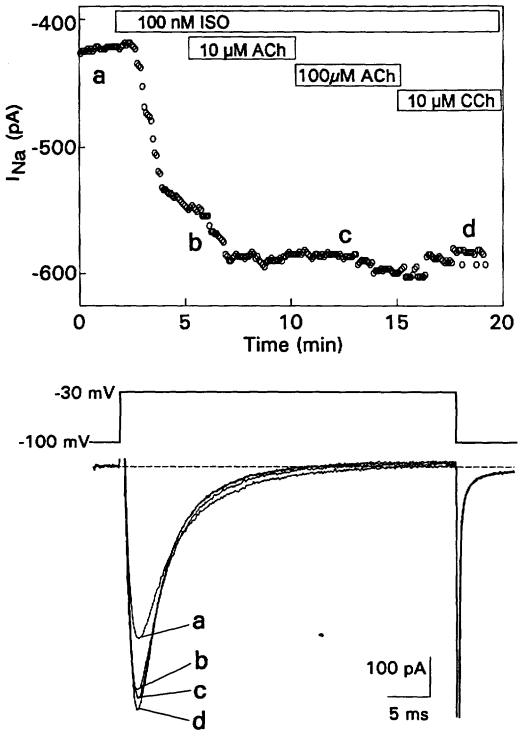

FIGURE 4. *The effect of pertussis toxin incubation on acetylcholine (ACh) regulation of sodium current (I_{Na}) in a rabbit ventricular myocyte. Top panel: Whole-cell I_{Na} amplitude is plotted vs. time during bath perfusion of isoproterenol (ISO, 100 nM), ACh (10 or 100 μM), and carbachol (CCh, 10 μM). The cell had been incubated in pertussis toxin (1 μg/ml) for 13 hours. Stimulation rate was 0.2 Hz. Bottom panel: Raw current tracings from the same experiment are shown at times a–d. Three consecutive tracings were averaged for each time point. The dashed line represents zero current level.*

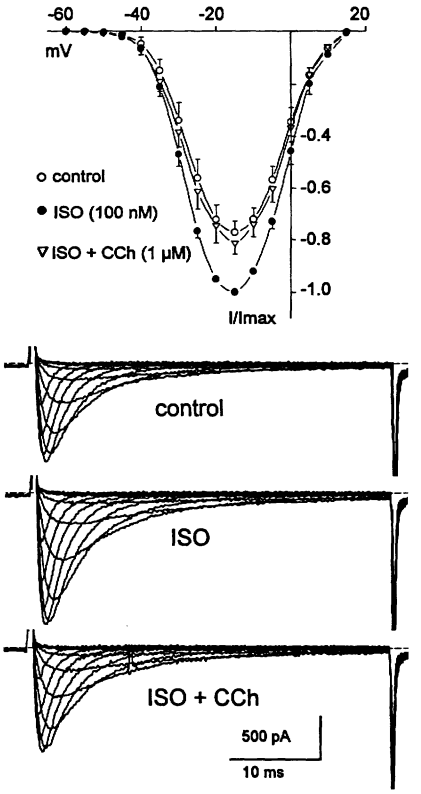

FIGURE 5. *The effect of isoproterenol (ISO) and carbachol (CCh) on the current–voltage relation. Top panel: Normalized peak whole-cell sodium current (I_{Na}) amplitude (I/I_{max}) is plotted vs. test potential for the control condition (open circles), 100 nM ISO (closed circles), and ISO+1 μM CCh (open triangles). Experiments were performed from a holding potential of −100 mV in 20 mM $[Na^+]_o$. Values are mean±SEM* (n=4). *Data points without error bars indicate that the SEM was smaller than the symbol size. The effects of ISO and ISO+CCh were significant at test potentials from −30 to 0 mV* ($p<0.05$). *Bottom panel: Raw current tracings are shown from a representative cell under the indicated conditions. Test potentials ranged from −60 to 10 mV.*

dialyzed with cAMP, suggesting that ACh affects cAMP production and not cAMP degradation.[5–7] To determine if ACh affects cAMP degradation in rabbit ventricular myocytes, we studied the effects of ACh on I_{Na} in cAMP-dialyzed cells. Figure 8 shows that dialysis of cAMP (5 μM) into the pipette increased I_{Na}, whereas subsequent application of ACh (1 μM) did not alter the cAMP-enhanced I_{Na}. The effect of cAMP reached a steady state at approximately 5 minutes after intracellular perfusion was initiated and remained constant up to 15 minutes after the perfusion was stopped. In three cells, 5 μM cAMP increased I_{Na} by 45.4±9.6% ($p<0.05$), but 1 μM ACh did not affect the cAMP-enhanced currents. ACh also had no effect on I_{Na} in cells in which I_{Na} was elevated by bath application of 2 mM dibutyryl cAMP ($n=3$, data not shown).

It has been suggested that the effects of ACh are mediated by cGMP in heart (see Reference 1). In our experiments, bath application of a membrane-permeable analogue of cGMP, dibutyryl cGMP (5 mM), had no effect on ISO-stimulated I_{Na} ($n=3$, data not shown). Furthermore, intracellular perfusion of cGMP (10 or 100 μM) into the recording pipette also did not alter ISO-stimulated I_{Na} and did not inhibit the effect of ACh on I_{Na} ($n=5$). In separate experiments, intracellular perfusion of cGMP (10 or 100 μM) had no effect on ISO-stimulated I_{Na} ($n=5$). Figure 9 shows the effect of cGMP and ACh on ISO-stimulated I_{Na} in a representative cell.

To confirm the whole-cell experiments, we used cell-attached patch single-channel recording techniques in which series resistance artifacts are reduced. Test pulses to −50 mV were preceded by hyperpolarizing prepulses to −140 mV for 500 msec to avoid problems associated with hyperpolarizing shifts in steady-state inactivation. Figure 10 shows five sequential tracings from a cell-attached patch during the control period, during bath application of ISO (100 nM), and during ISO+ACh administration (1 μM). The ensemble currents (150 tracings) from the same cell are shown in the lower panel of Figure 10. ISO increased the amplitude of the ensemble current by 66.5±31.7% ($p<0.01$, $n=3$) and decreased the time constant of current decay by 38.9±7.0% ($p<0.05$). ACh reversed the ISO effect on I_{Na} amplitude by 88.3±15.2% ($p<0.01$) and reversed the effect on current decay by 89.7±17.4% ($p<0.01$).

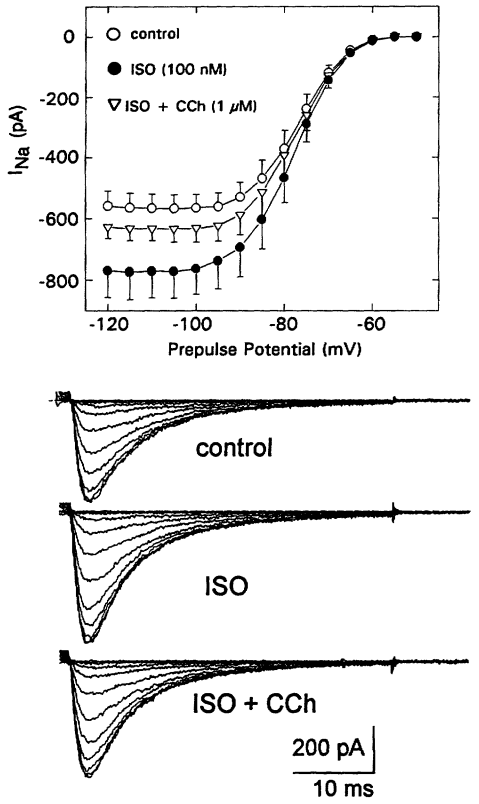

FIGURE 6. *The effect of holding potential on the isoproterenol (ISO) and carbachol (CCh) effect. Top panel: Whole-cell sodium current (I_{Na}) amplitude is plotted vs. prepulse potential for the control condition (open circles), 100 nM ISO (closed circles), and ISO+1 μM CCh (open triangles). Values are mean±SEM (n=4). Data points without error bars indicate that the SEM was smaller than the symbol size. See Figure 7 for pulse protocol. The effects of ISO and ISO+CCh were significant at all prepulse potentials negative to −80 mV ($p<0.05$ for prepulse potential of −85 mV, $p<0.01$ for prepulses from −95 to −120 mV). Bottom panel: Raw current tracings are shown from a representative cell under the indicated conditions.*

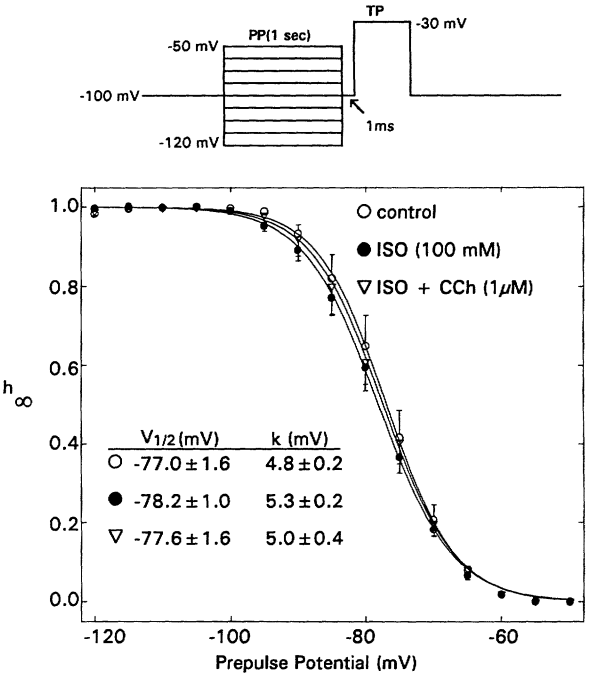

FIGURE 7. *The effect of isoproterenol (ISO) and carbachol (CCh) on sodium current (I_{Na}) steady-state inactivation h_∞. Top panel: Double-pulse protocol used for this and the previous figure. PP, prepulse potential; TP, test potential. Bottom panel: I_{Na} inactivation curves generated by normalization of the data in Figure 5 for control (open circles), ISO (closed circles), and ISO+CCh (open triangles). The data were fit to a Boltzmann equation with half-inactivation values ($V_{1/2}$) and slope factors (k) as indicated (mean±SEM, n=3).*

Discussion

The present study provides the first direct evidence that muscarinic agonists inhibit the cardiac fast inward I_{Na} only after being stimulated by ISO. This is similar to the muscarinic-mediated regulation of I_{Ca}[1] and I_f[27] in the heart. Findings that paralleled the regulation of cardiac I_{Ca} were as follows: 1) ACh alone did not affect I_{Na}. 2) The effect of ACh was blocked by pertussis toxin. 3) ACh also reversed the effect of forskolin on I_{Na}. 4) ACh did not affect I_{Na} when cells were dialyzed with cAMP. Therefore, we believe that ACh regulates cardiac I_{Ca} and I_{Na} through similar mechanisms.

Our finding that ACh does not modulate I_{Na} in cells dialyzed with cAMP suggests that ACh acts at or proximal to adenylate cyclase activation. One possibility is that the $\beta\gamma$ subunits released from $G_{i\alpha}$ subsequent to muscarinic receptor stimulation bind and inactivate $G_{s\alpha}$ released from β-adrenergic stimulation.[12] This hypothesis predicts that ACh will only affect I_{Na} if activated $G_{s\alpha}$ is present to stimulate I_{Na}. All of our findings are consistent with this hypothesis except the data showing that ACh reversal of forskolin-stimulated I_{Na}. Since forskolin directly activates adenylate cyclase, its effects do not require the presence of G proteins.[28] Recent studies, however, show that forskolin's potency and efficacy in activating adenylate cyclase can be increased by the participation of $G_{s\alpha}$.[29] The $\beta\gamma$ subunits released from $G_{i\alpha}$ by muscarinic stimulation might inactivate basal levels of $G_{s\alpha}$, thus rendering forskolin less effective in activating adenylate cyclase. Further experiments need to be done to test this hypothesis.

Another possibility is that the $\beta\gamma$ subunits released from $G_{i\alpha}$ directly inhibit adenylate cyclase. Recently, it has been shown that G-protein $\beta\gamma$ subunits directly inhibit calmodulin-activated (type I) adenylate cyclase only after stimulation by $G_{s\alpha}$.[13,30] Type I adenylate cyclase, however, is thought to be expressed exclusively in the central nervous system.[31] Type IV (calmodulin-insensitive) adenylate cyclase has been identified in heart tissue as well as in other tissues including brain.[32] This adenylate cyclase subtype is stimulated by $\beta\gamma$ G-protein subunits after $G_{s\alpha}$ activation.[13,31,33] It should be noted that the adenylate cyclase assays in these studies were performed in heterologous cell lines transfected with adenylate cyclase subtypes. Whether or not adenylate cyclases respond similarly to $\beta\gamma$ in vivo remains to be determined. It is also possible that there is an adenylate cyclase isozyme in heart that is inhibited by $\beta\gamma$ subunits after $G_{s\alpha}$ stimulation that has not been identified.

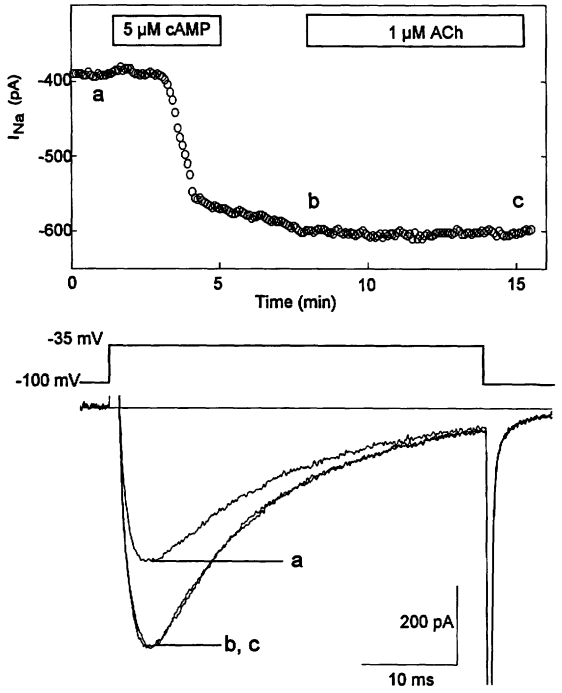

FIGURE 8. *The effect of acetylcholine (ACh) on cAMP-stimulated sodium current (I_{Na}). Top panel: I_{Na} peak amplitude is plotted vs. time during intracellular perfusion of 5 μM cAMP and bath application of 1 μM ACh at the times indicated by the horizontal bars. Stimulation rate was 0.2 Hz. Bottom panel: Raw current tracings from the same cell are plotted at times a–c. Three consecutive tracings are averaged for each data point. Horizontal line represents zero current level.*

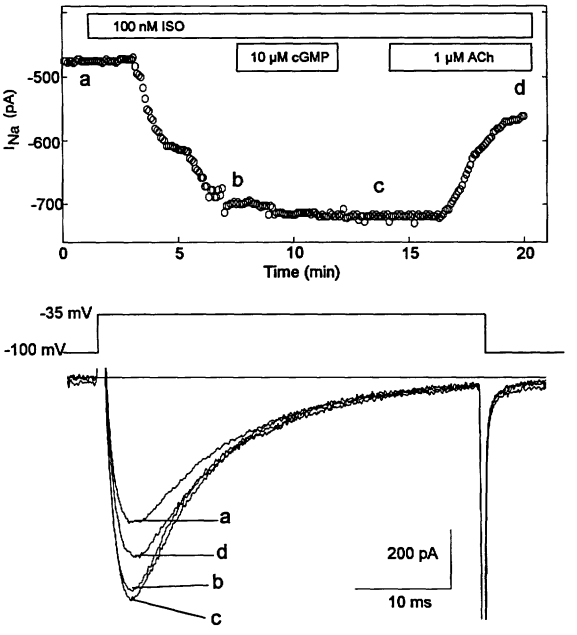

FIGURE 9. *The effect of cGMP and acetylcholine (ACh) on isoproterenol (ISO)–stimulated sodium current (I_{Na}). Top panel: I_{Na} peak amplitude is plotted vs. time during bath application of 100 nM ISO, during intracellular perfusion of 10 μM cGMP, and during bath application of 1 μM ACh at the times indicated by the horizontal bars. Stimulation rate was 0.2 Hz. Bottom panel: Raw current tracings from the same cell are plotted at times a–d. Three consecutive tracings were averaged for each time point. Horizontal line indicates zero current level.*

There are several studies suggesting that the effects of ACh are mediated by cGMP (see Reference 1). In frog ventricular myocytes, intracellular application of cGMP antagonizes I_{Ca} stimulated by cAMP.[6,15] These findings suggest that cGMP activates a cyclic nucleotide phosphodiesterase that promotes cAMP hydrolysis. In mammalian cardiac preparations, cGMP has varied effects on I_{Ca}. Levi et al[16] showed that cGMP antagonizes cAMP-stimulated I_{Ca} in guinea pig ventricular myocytes and suggested that cGMP acts through a cGMP-dependent protein kinase. This hypothesis was supported by Mery et al,[34] who showed that intracellular application of cGMP-dependent protein kinase inhibited I_{Ca} in rat ventricular myocytes. Another recent study, however, showed that cGMP (1–10 μM) enhances I_{Ca} stimulated by ISO, forskolin, or cAMP but not I_{Ca} stimulated by hydrolysis-resistant cAMP analogues in guinea pig ventricular myocytes.[18] It was hypothesized that cGMP inhibits a phosphodiesterase, causing an elevation in intracellular cAMP. In this same study, higher concentrations of cGMP (100–1,000 μM), which would activate cGMP-dependent protein kinase, inhibited I_{Ca} in 25% of their cells. Intracellular application of cGMP-dependent protein kinase, however, failed to give a consistent result. Our experiments show that dibutyryl cGMP (5 mM) in the bath did not antagonize the effect of ISO on I_{Na}. Furthermore, intracellular application of cGMP (10 or 100 μM) did not affect basal I_{Na} or ISO-stimulated I_{Na} and did not prevent ACh from inhibiting ISO-stimulated I_{Na}. It is possible that 100 nM ISO saturates the cell with cAMP, preventing cGMP from having any noticeable effect. However, the observed increase in I_{Na} with 100 nM ISO (36.9±5.4%) is significantly lower than what we previously observed for 1 μM ISO (55.9±11.8%),[2] suggesting that 100 nM ISO does not saturate the cell with cAMP.

Another possible regulatory site distal to adenylate cyclase is at the level of a protein phosphatase. Ahmad et al[35] showed that muscarinic agonists can attenuate the effects of ISO and forskolin by enhancement of type 1 protein phosphatase activity in heart. Perhaps ACh stimulates a similar phosphatase that is responsible for Na^+ channel dephosphorylation in the rabbit heart. Our previous article showed that the phosphatase calcineurin decreased baseline Na^+ channel activity, suggesting that Na^+ channels are phosphorylated in the basal state.[2] If ACh indeed stimulates a phosphatase, we would expect to see not only an effect of ACh alone on I_{Na} but also an effect of ACh on cAMP-dialyzed myocytes. Neither of these effects was observed in the present study.

Protein kinase C activation has also been hypothesized to be responsible for ACh inhibition of I_{Ca} in the heart (see Reference 1). Muscarinic receptor activation of protein kinase C in the heart is thought to occur through a pertussis toxin–insensitive G protein coupled to phospholipase C.[11,19] The physiological role of this pathway is not clear, but its effects appear to show positive inotropy and require high agonist concentra-

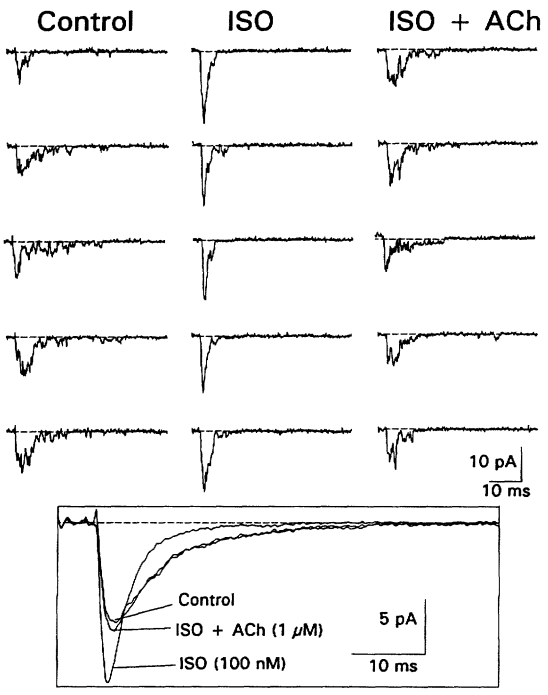

FIGURE 10. *The effects of isoproterenol (ISO) and acetylcholine (ACh) on single-channel sodium current (I_{Na}). Top panel: Five consecutive sweeps from a cell-attached patch are shown for the control condition and after bath application of 100 nM ISO and ISO+1 μM ACh. Stimulation rate was 1 Hz. Test pulses to −50 mV (40 msec) were preceded by hyperpolarizing prepulses to −140 mV (500 msec). Bottom panel: Ensemble-averaged current tracings (n=150) from the same experiment are shown under the indicated conditions.*

tions (>10 μM CCh).[1] An ionic current associated with this pathway has been described in several cardiac preparations. This muscarinic-stimulated pertussis toxin–insensitive current is a Na^+-dependent time-independent inward current with a K_d of 12 μM for CCh.[25] We did not observe any ACh-associated changes in holding current in our experiments using ACh (or CCh) concentrations as high as 300 μM. A recent study showed that pertussis toxin treatment can completely inhibit the effect of CCh on ISO-stimulated I_{Ca} in rabbit ventricular myocytes.[36] The effects of ACh and CCh on ISO-stimulated I_{Na} were similarly inhibited by pertussis toxin treatment in our experiments. Therefore, it is unlikely that the muscarinic effects on I_{Na} were mediated by protein kinase C.

In summary, we have shown that muscarinic agonists inhibit ISO-stimulated I_{Na}. This effect is mediated by a pertussis toxin–sensitive G protein and is independent of cGMP. Our data suggest that the ACh-mediated inhibition of I_{Na} does not involve activation of a phosphodiesterase or a phosphatase. Rather, we believe that ACh antagonizes ISO's stimulatory effect on adenylate cyclase. The exact mechanism of this effect needs to be further investigated. These results also confirm our previous findings[2] that ISO stimulates I_{Na} and further support the hypothesis that the regulation of I_{Na} by neurotransmitters is a physiologically important process.

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