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

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We have recently shown that β-adrenergic agonists enhance the cardiac sodium current (INa) in rabbits through dual G-protein regulatory pathways. To determine if muscarinic cholinergic receptor stimulation can also modulate INa, we studied the effects of acetylcholine (ACh) and carbachol on INa 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 INa 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 INa 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 INa from cell-attached patches. When INa was stimulated by forskolin (1 μM), carbachol (1 μM) significantly reversed the effect. The muscarinic-mediated inhibition of INa 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 INa and that when cells were dialyzed with cAMP (5 μM), ACh failed to inhibit INa. Furthermore, cGMP failed to inhibit the effect of isoproterenol on INa. These data suggest that ACh acts at or proximal to adenylyl cyclase stimulation. Thus, rabbit cardiac Na+ channels are regulated by muscarinic agonists in a fashion similar to cardiac Ca2+ 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 (ICa), the delayed rectifier potassium current (IK), the transient outward potassium current (Ito), and the pacemaker current (Ip) (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 ICa leads to elevated cytoplasmic Ca2+ ([Ca2+]i) levels, causing an increase in cardiac contractility. We recently discovered that ISO also increases the voltage-dependent sodium current (INa) in the rabbit heart through dual G-protein regulatory pathways. The physiological significance of the ISO-induced increase in INa has not been determined. However, continuous cable theory predicts that enhancements of INa should increase cardiac impulse conduction velocity (positive dromotropic effect). It has also been suggested that the ISO-induced enhancement of INa might contribute to the positive inotropic effect of β-adrenergic agonists in the heart. 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 ICa and Ip by acetylcholine (ACh). If cardiac INa regulation by neurotransmitters were a physiologically important process, parasympathetic agonists should also oppose the effects of ISO on INa. Direct evidence for the regulation of INa by muscarinic receptor activation, however, has not been shown. The purpose of this study was to determine if muscarinic agonists can modulate INa in isolated rabbit ventricular myocytes.

Since we previously demonstrated that ISO regulates INa and ICa by similar mechanisms, 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 ICa in the heart has been clearly demonstrated. The exact mechanism of this effect remains to be elucidated. Previous studies showed that ACh inhibits ISO-stimulated ICa in guinea pig and frog cardiac myocytes, whereas ACh alone had little or no effect on ICa. Other studies showed that ACh also inhibits forskolin-stimulated ICa. However, when cells were dialyzed with cAMP, ACh did not affect ICa. These results suggest that muscarinic inhibition of ISO-stimu-
lated I\textsubscript{ca} takes place at or proximal to adenylate cyclase stimulation in the signal transduction pathway. Using nonhydrolyzable GTP analogues, Parsons et al\textsuperscript{19} showed that the predominant site of ACh action in inhibiting ISO-stimulated I\textsubscript{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\textsubscript{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\textsubscript{i}a, which inactivate G\textsubscript{ia} (mass action hypothesis),\textsuperscript{12} 2) direct inhibition of adenylate cyclase by \( \beta \gamma \) subunits,\textsuperscript{13} 3) direct inhibition of adenylate cyclase by G\textsubscript{ia},\textsuperscript{12} or 4) direct interaction of G\textsubscript{i} with the \( \beta \)-adrenergic receptor, thus inhibiting its interaction with the stimulatory G protein, G\textsubscript{s}.\textsuperscript{14}

Other studies suggest that muscarinic inhibition of I\textsubscript{ca} may take place distal to adenylate cyclase activation. For instance, cGMP inhibited ISO- and cAMP-stimulated I\textsubscript{ca} in both the frog\textsuperscript{15} and guinea pig\textsuperscript{16} but did not affect basal I\textsubscript{ca} amplitude. It was suggested that muscarinic receptor stimulation increases intracellular cGMP levels and that this increase subsequently activates a cyclic nucleotide phosphodiesterase.\textsuperscript{17} Recently, however, contrasting evidence by Ono and Trautwein\textsuperscript{18} showed that cGMP potentiates the effect of ISO on I\textsubscript{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.\textsuperscript{11,19} The effect of this signaling pathway on Ca\textsuperscript{2+} channel function is not yet clear.

The effects of ACh on the fast inward cardiac I\textsubscript{Na} have not been directly studied. Cheng et al\textsuperscript{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\textsubscript{Na} and explore the mechanisms responsible for these effects. We show that the effects of ACh on I\textsubscript{Na} are similar to those reported for I\textsubscript{ca}.

### Materials and Methods

**Cell Isolation**

Rabbit ventricular myocytes were enzymatically dissociated as previously described.\textsuperscript{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 Ca\textsuperscript{2+} solution containing (mM) NaCl 140, MgCl\textsubscript{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\textsuperscript{2+} storage solution without enzyme. Single cells were then then dissociated by mild mechanical trituration. The cells were Ca\textsuperscript{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\textsuperscript{21} were similar to those previously described.\textsuperscript{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°C–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,\textsuperscript{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\textsuperscript{22} as previously described.\textsuperscript{2} We have previously determined that the I\textsubscript{Na} current decay at test potentials of −30 mV and below is best fit by one exponential and that two time constants were determined to be a better fit at more depolarized potentials.\textsuperscript{2} In the present study, we determined the time constant of I\textsubscript{Na} current decay only at test potentials negative to −30 mV. Therefore, only one time constant is reported.

External Na\textsuperscript{+} concentration was lowered to 20 mM, and test pulses for most experiments were restricted to potentials where I\textsubscript{Na} was not fully activated. Even under these conditions I\textsubscript{Na} was frequently too large to adequately voltage clamp. Therefore, we only used cells in which the I\textsubscript{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\textsubscript{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\textsubscript{Na} to reach steady state (no visible change in I\textsubscript{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.\textsuperscript{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\textsubscript{2}ATP 5.0, GTP 0.5, EGTA 5.0, CaCl\textsubscript{2} 0.5, and MgCl\textsubscript{2} 2.0 (pH 7.25 with CsOH; pCa =8). Whole-cell bath solutions contained (mM) tetrathylammonium chloride (TEA) 100, tetraethylammonium chloride (TEA) 20, KCl 4.5, MgCl\textsubscript{2} 1.0, CaCl\textsubscript{2} 1.0, BaCl\textsubscript{2} 0.5, CdCl\textsubscript{2} 0.5, HEPES 10, and glucose 5.55 (pH 7.35). In some experiments 120 mM Tris-hydrochloride was used as a Na\textsuperscript{+} 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 INa. CAMP and cGMP were directly dissolved in the pipette solution at the indicated concentrations. Dibutyryl cAMP 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 INa. 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 INa, 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$_0$ of 12 μM for CCh) has been described in mammalian cardiac myocytes.  

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$^+$]), 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 INa 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 INa, 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$^{+}$], using 20 mM TEA and 100 mM TMA as a Na$^+$ replacement. Under these conditions, 100 nM ISO significantly increased INa 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 showed that TEA inhibits the effects of muscarinic agonists and antagonists on INa in a glomus 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 ISO increased INa 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, ISO not only enhanced INa amplitude but it also accelerated the rate of INa decay. In the present study, ISO also decreased the time constant of INa decay by 29.9±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 INa (see Figure 2), and CCh at concentrations up to 300 μM also had no significant effect on INa (n=3).

The effect of ISO on INa was mimicked by agents that increase intracellular cAMP levels, such as forskolin. Figure 3 shows that forskolin (1 μM) increased INa, and that 1 μM CCh partially reversed the effect. Using TEA and TMA as Na$^+$ replacements, forskolin (1 μM) increased INa by 47.1±11.4% (p<0.01), and CCh (1 μM) significantly reversed this effect by 28.4±3.5% (p<0.05; holding potential, −100 mV; test potential, −30 mV; n=4). When Na$^+$ was replaced by Tris, forskolin increased INa 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

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**FIGURE 1.** The effect of acetylcholine (ACh) on isopropenol (ISO)–stimulated sodium current (INa) in a rabbit ventricular myocyte. Top panel: Peak whole-cell INa 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.
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, or Gs) dependent, cells were incubated in pertussis toxin (1.0 μg/ml) for at least 12 hours. In the pertussis toxin–treated cells, ISO increased INa by 35.4±6.3%, but ACh or CCh in concentrations up to 100 μM did not affect the ISO-stimulated INa (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 INa throughout the activation range. Figure 5 shows the averaged peak current–voltage relation (n=4) for whole-cell INa 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 INa is holding potential dependent, such that the largest ISO responses are seen at hyperpolarized holding potentials. 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 INa obtained at a prepulse of -120 mV. The curve was then fit using a conventional Boltzmann distribution equation of the following form:

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

where $h_s$ is the steady-state activation of INa, $V_m$ 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 mV for ISO+CCh (n=2, data not shown). The respective slope factors were 4.0, 4.8, and 5.1 mV.

Studies on the L-type Ca2+ current in the heart have shown that ACh does not affect ICa, when the cells are...
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 \( \mu M \)) into the pipette increased \( I_{Na} \), whereas subsequent application of ACh (1 \( \mu M \)) 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 \( \mu M \) cAMP increased \( I_{Na} \) by 45.4±9.6% (p<0.05), but 1 \( \mu M \) 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, dibutyl cGMP (5 mM), had no effect on ISO-stimulated \( I_{Na} \) (n=3, data not shown). Furthermore, intracellular perfusion of cGMP (10 or 100 \( \mu M \)) 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 \( \mu M \)) 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 predules 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 \( \mu M \)). 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).
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. It is similar to the muscarinic-mediated regulation of $I_{Na}$ and $I_{Na}$ in the heart. Findings that the regulation of cardiac $I_{Na}$ 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 a proximal to adenylate cyclase activation. One possibility is that the $\beta Y$ subunits released from $G_{sa}$ subsequent to muscarinic receptor stimulation bind and inactivate $G_{sa}$ released from $\beta$-adrenergic stimulation. This hypothesis predicts that ACh will only affect $I_{Na}$ if activated $G_{sa}$ 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.

Recent studies, however, show that forskolin's potency and efficacy in activating adenylate cyclase can be increased by the participation of $G_{sa}$. The $\beta Y$ subunits released from $G_{sa}$ by muscarinic stimulation might inactivate basal levels of $G_{sa}$, thus rendering forskolin less effective in activating adenylate cyclase. Further experiments need to be done to test this hypothesis.

Another possibility is that the $\beta Y$ subunits released from $G_{sa}$ directly inhibit adenylate cyclase. Recently, it has been shown that G-protein $\beta Y$ subunits directly inhibit calmodulin-activated (type I) adenylate cyclase only after stimulation by $G_{sa}$. Type I adenylate cyclase, however, is thought to be expressed exclusively in the central nervous system. Type IV (calmodulin-insensitive) adenylate cyclase has been identified in heart tissue as well as in other tissues including brain.

Adenylate cyclase subtype is stimulated by $\beta Y$ G-protein subunits after $G_{sa}$ activation. 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 $\beta Y$ in vivo remains to be determined. It is also possible that there is an adenylate cyclase isozyme in heart tissue that is inhibited by $\beta Y$ subunits after $G_{sa}$ stimulation that has not been identified.
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 ACh.\(^5\)\(^6\)\(^15\) 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.\(^24\) 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 hydrolys-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-
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