Nitric Oxide Signaling Mediates Stimulation of L-Type Ca^{2+} Current Elicited by Withdrawal of Acetylcholine in Cat Atrial Myocytes

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ABSTRACT A perforated-patch whole-cell recording method was used to determine whether nitric oxide signaling participates in acetylcholine (ACh)-induced regulation of basal L-type Ca^{2+} current ($I_{Ca,L}$) in cat atrial myocytes. Exposure to 1 μM ACh for 2 min inhibited basal $I_{Ca,L}$ (−21 ± 3%), and withdrawal of ACh elicited rebound stimulation of $I_{Ca,L}$ above control (80 ± 13%) ($n = 23$). Stimulation of $I_{Ca,L}$ elicited by withdrawal of ACh (but not ACh-induced inhibition of $I_{Ca,L}$) was blocked by either 50 μM hemoglobin; 30 μM ODQ or 10 μM methylene blue, inhibitors of soluble guanylate cyclase; 10 μM W-7, a calmodulin inhibitor; or 10 μM L-NIO, an inhibitor of constitutive NO synthase (NOS). In cells incubated in 5 mM L-arginine, ACh-induced rebound stimulation of $I_{Ca,L}$ was enhanced compared with control responses. Histochemical assay (NADPH diaphorase) indicated that atrial myocytes express constitutive NOS. NO-donor, spermine/NO (SP/NO), >1 μM stimulated basal $I_{Ca,L}$. SP/NO-induced stimulation of $I_{Ca,L}$ was inhibited by 50 μM hemoglobin, 30 μM ODQ, or 5 μM H-89, an inhibitor of PKA, and was unchanged by 50 μM MnTBAP, a peroxynitrite scavenger. When $I_{Ca,L}$ was prestimulated by 10 μM milrinone, an inhibitor of cGMP-inhibited phosphodiesterase (type III) activity, SP/NO failed to further increase $I_{Ca,L}$. In cells incubated in pertussis toxin (3.4 μg/ml for 6 h; 36°C), ACh failed to affect $I_{Ca,L}$, but 100 μM SP/NO or 10 μM milrinone still increased basal $I_{Ca,L}$. These results indicate that in cat atrial myocytes NO signaling mediates stimulation of $I_{Ca,L}$ elicited by withdrawal of ACh but not ACh-induced inhibition of basal $I_{Ca,L}$. NO activates cGMP-induced inhibition of phosphodiesterase (type III) activity. Upon withdrawal of ACh, this mechanism allows cAMP to recover to levels above control, thereby stimulating $I_{Ca,L}$. Pertussis toxin–sensitive G-proteins couple M_2 muscarinic receptors to NO signaling. NO-mediated stimulation of $I_{Ca,L}$ elicited by withdrawal of ACh may be an important mechanism that rapidly restores cardiac pacemaker and contractile functions after cholinergic suppression of atrial activity.

KEY WORDS: electrophysiology • cyclic AMP • cyclic GMP • calmodulin • phosphodiesterase

INTRODUCTION

Nitric oxide is an important second messenger that mediates a variety of cardiovascular functions (Kelly et al., 1996), including cholinergic regulation of cardiac $I_{Ca,L}$ (Han et al., 1994, 1995, 1996; Balligand et al., 1995; Habuchi et al., 1996). For example, in the sinoatrial node (Han et al., 1995) or atrioventricular node (Han et al., 1996) cells, acetylcholine (ACh)[1] inhibits prestimulated $I_{Ca,L}$ via NO signaling mechanisms that activate cGMP-induced stimulation of phosphodiesterase (PDE) activity. This mechanism is thought to contribute to accentuated antagonism, where cholinergic inhibition of cardiac function is enhanced by earlier β-adrenergic stimulation.

Previous work has shown that in cat atrial myocytes exposure to ACh inhibits basal $I_{Ca,L}$ and withdrawal of ACh elicits a prominent rebound stimulation of $I_{Ca,L}$ above control (Wang and Lipsius, 1995). Rebound stimulation of $I_{Ca,L}$ elicited by ACh withdrawal is responsible for stimulation of atrial contractile (Wang and Lipsius, 1995) and pacemaker activities (Wang and Lipsius, 1996) above control, and can lead to the development of dysrhythmic atrial activity (Wang et al., 1997). A primary mechanism mediating the stimulatory effects of ACh withdrawal is a rebound increase in cAMP resulting from ACh-induced inhibition of PDE (type III) activity (Wang and Lipsius, 1995). Although NO-cGMP signaling is known to modulate PDE activity (Fischmeister and Mery, 1996), it is not known to what extent, if any, NO signaling contributes to ACh-induced regulation of basal $I_{Ca,L}$ in atrial myocytes. The purpose, therefore, of the present study was to determine whether NO signaling participates in ACh-induced regulation of $I_{Ca,L}$, especially in relation to stimulation of $I_{Ca,L}$ elicited by withdrawal of ACh.

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[1]*Abbreviations used in this paper:* Ach, acetylcholine; PDE, phosphodiesterase; PTX, pertussis toxin; SP/NO, spermine/NO.

MATERIALS AND METHODS

Cell Isolation

Details of the isolation and recording methods have been published previously (Wang and Lipsius, 1995). Atrial myocytes were isolated from cat heart. Adult cats of either sex were anesthetized with sodium pentobarbital (70 mg/kg i.p.). Hearts were perfused via a Langendorff apparatus with a bicarbonate-buffered Tyrode solution for ~5 min, followed by perfusion with a nominally Ca^{2+}-free Tyrode solution. After 5 min, the perfusion was switched to a low (36 μM) Ca^{2+} Tyrode solution containing 0.06% collagenase (type II; Worthington Biochemical Corp., Freehold, NJ) for 30–40 min. After collagenase perfusion, both atria were cut into small pieces and agitated in fresh collagenase and 0.01% protease. Experiments were performed on either right or left atrial cells, with no discernible differences. Cells studied were isolated on the morning of the experiment.

Electrophysiological Recording Methods

Cells used for study were transferred to a small tissue bath on the stage of an inverted microscope (Nikon Diaphot; Nikon Inc., Melville, NY) and superfused with a modified Tyrode solution containing (mM): 137 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 2.5 $CaCl_2$, 5 HEPES, and 11 glucose, titrated with NaOH to a pH of 7.4. Solution was perfused through a small (0.3 ml) chamber by gravity at ~5 ml/min. The system required ~20 s to completely exchange the bath contents. All experiments were performed at 35 ± 1°C. Cells selected for study were elongated and quiescent. Ionic currents were recorded using a nystatin-perforated patch (Horn and Marty, 1988) whole-cell recording method (Hamill et al., 1981). This method minimizes dialysis of intracellular contents with the internal pipette solution, thereby maintaining physiological buffering of intracellular Ca^{2+} and second messenger signaling pathways (Zhou and Lipsius, 1993). Nystatin was dissolved in DMSO at a concentration of 50 mg/ml, and then added to the internal pipette solution to yield a final nystatin concentration of 150 μg/ml. The pipette solution containing nystatin is strongly sonicated before use. The internal pipette solution contained (mM): 100 Cs-glutamate, 40 CsCl, 1.0 $MgCl_2$, 4 Na_2-ATP, 0.5 EGTA, 5 HEPES, titrated with CsOH to a pH of 7.2. To record $I_{Ca,L}$, ACh-activated K^+ currents were blocked by Cs^+ in the internal pipette solution and 20 mM CsCl in the external solution. Addition of CsCl to the external solution was not osmotically compensated. If ACh elicited changes in background K^+ conductance, the cell was discarded.

A single suction pipette was used to record ionic currents (discontinuous voltage clamp mode) using an Axoclamp 2A amplifier (Axon Instruments, Inc., Foster City, CA). Once a gigaseal was formed, the access resistance was monitored until it stabilized, which generally required ~5 min. In the voltage clamp mode, the amplifier sampling rate was ~10–12 kHz. A second scope was used to monitor the duty cycle to ensure complete settling of the voltage transient between samples. Computer software (pCLAMP; Axon Instruments, Inc.) was used to deliver voltage protocols and acquire and analyze data. In addition, all signals were digitally recorded on VCR tape.

In general, $I_{Ca,L}$ was activated by clamping cells from a holding potential of −40 mV, to inactivate fast Na^+ channels, to 0 mV for 200 ms every 5 s. Peak $I_{Ca,L}$ was measured with respect to steady state current and was not compensated for leak currents. Cells were exposed to ACh for 2 min to elicit maximum rebound stimulation of $I_{Ca,L}$. Consecutive exposures to ACh were performed at least 5 min apart to avoid any potential effects of desensitization. Preliminary experiments indicate that this time interval allowed reproducible effects of two consecutive ACh exposures on $I_{Ca,L}$. Raw data were analyzed for statistical significance using paired and unpaired Student's *t* tests and considered significant at $P < 0.05$. Data are expressed as mean ± SEM. In several experiments, the effects of ACh on $I_{Ca,L}$ were tested in the absence and then presence of a drug or compound that alters NO signaling. The percent change in $I_{Ca,L}$ induced by ACh in the presence of a drug or compound was determined in relation to the new baseline $I_{Ca,L}$ established by the drug or compound. The animal procedures used in this study were in accordance with the guidelines of the Animal Care and Use Committee of Loyola University Medical Center.

Histochemical Methods

A histochemical assay (NADPH-diaphorase), as described previously (Prabhakar et al., 1993), was used to determine whether atrial myocytes express NOS activity. After cells were isolated, they were plated on microscope slides treated with laminin (Sigma Chemical Co., St. Louis, MO). Then cells were fixed by 10% formalin and washed in PBS at pH 7.4. Fixed cells from the same hearts were incubated for 1 h in PBS containing 0.3% Triton X-100, and 0.2 mM nitro blue tetrazolium, either in the absence (control) or presence of β-NADPH. In the presence of β-NADPH, NOS reduces tetrazolium to formazan, which appears as a dark blue stain.

Drugs and Chemicals

Drugs and chemicals included acetylcholine chloride (Sigma Chemical Co.), H-89 (*N*-[2-*p*-bromocinnamylamino]-5-isoquinolinesulfonamide) (Seikagaku America, Inc., Rockville, MD), MnTBAP (Mn tetrakis [4-benzoic acid] porphyrin chloride) (Calbiochem Corp., La Jolla, CA), hemoglobin (Sigma Chemical Co.), globin (Sigma Chemical Co.), W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride) (Sigma Chemical Co.), ODQ (1*H*-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one) (Sigma Chemical Co.), L-arginine and D-arginine (Sigma Chemical Co.); L-NIO (L-*N*5-(1-iminoethyl)-ornithine (Alexis Corp., San Diego, CA) and spermine/NO (Research Biochemicals, Inc., Natick, MA). Stock solutions of H-89 and ODQ were prepared in DMSO. Final DMSO concentrations were ≤0.05% and had no effect on basal $I_{Ca,L}$. Spermine/NO solutions were prepared fresh for each experiment and were not used for more than ~1 h.

RESULTS

ACh-induced Inhibition and Rebound Stimulation of $I_{Ca,L}$

Fig. 1, *A* and *B* illustrates the effects of ACh exposure and withdrawal on selected recordings of $I_{Ca,L}$ (*A*) and consecutive measurements of peak $I_{Ca,L}$ (*B*) obtained from a single atrial myocyte. Under control conditions, exposure to 1 μM ACh for 2 min inhibited $I_{Ca,L}$ by 36%. Within 30 s of withdrawing ACh, $I_{Ca,L}$ amplitude exhibited a prominent rebound stimulation of 141% above control. $I_{Ca,L}$ required ~4–5 min to return to a stable baseline. Similar ACh-induced changes in $I_{Ca,L}$ have been reported previously (Wang and Lipsius, 1995). As shown in Fig. 1 *B*, after the initial ACh exposure peak, $I_{Ca,L}$ generally stabilized at a level higher than the control baseline level. In other words, peak $I_{Ca,L}$ did not always fully recover from the stimulatory effects of ACh withdrawal even though the current reached a stable level. This was more evident when the rebound stimula-

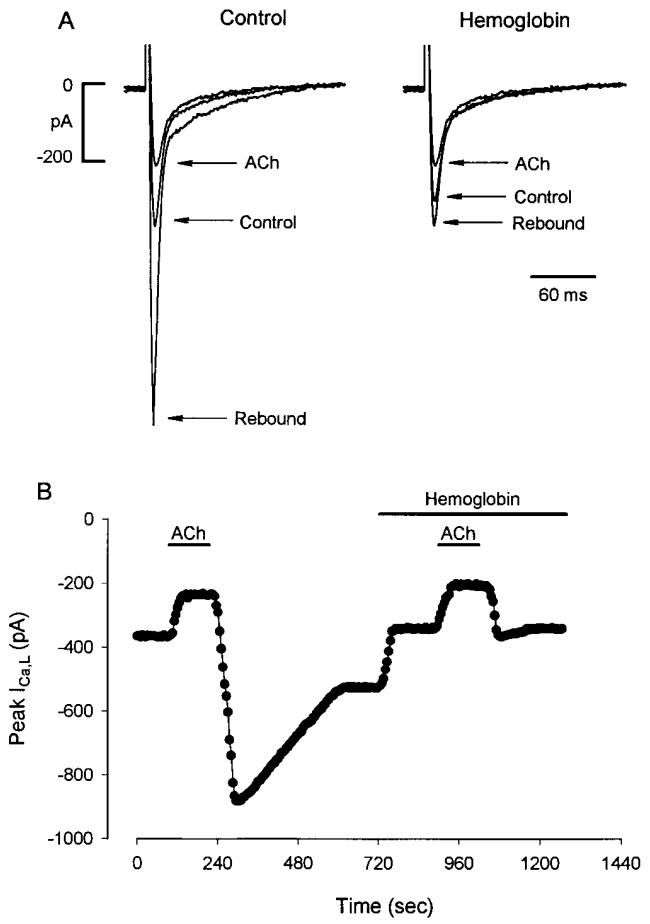

FIGURE 1. Effect of hemoglobin on ACh-induced regulation of $I_{Ca,L}$ (*A*) and consecutive measurements of peak $I_{Ca,L}$ amplitude throughout the same experiment (*B*). Under control conditions, exposure to 1 μM ACh inhibited $I_{Ca,L}$ and withdrawal of ACh elicited a prominent rebound stimulation of $I_{Ca,L}$. Hemoglobin (50 μM) in the external solution decreased $I_{Ca,L}$ to control levels. In the presence of hemoglobin, ACh inhibited $I_{Ca,L}$, but rebound stimulation of $I_{Ca,L}$ was abolished. All recordings were obtained from the same cell.

tory response was relatively large. This will be discussed in more detail below. The main focus of this study was to determine whether the ACh-induced changes in basal $I_{Ca,L}$ are mediated via NO signaling mechanisms.

Effects of Hemoglobin on ACh-induced Regulation of $I_{Ca,L}$

If ACh regulates $I_{Ca,L}$ via production of NO then hemoglobin, a well known scavenger of NO, should inhibit ACh-induced effects that are mediated by NO. Even though hemoglobin is restricted to the external space, because of its very high affinity for NO (Gibson and Roughton, 1957), hemoglobin can inhibit intracellular NO signaling mechanisms by acting as a "sink" for intracellular NO (Beasley et al., 1991; Balligand et al., 1993, 1995). We therefore tested ACh in the presence of hemoglobin. As shown in Fig. 1, *A* and *B*, once $I_{Ca,L}$ stabilized after the initial ACh exposure, exposure to 50 μM hemoglobin decreased $I_{Ca,L}$ (−35%) to control baseline levels. In the presence of hemoglobin, ACh decreased $I_{Ca,L}$ by 40% and rebound stimulation of $I_{Ca,L}$ elicited by withdrawal of ACh was essentially abolished (8%). In a total of five cells tested, in the absence and presence of 50 μM hemoglobin, ACh decreased $I_{Ca,L}$ by 21 ± 5% and 20 ± 6%, respectively, and ACh withdrawal stimulated $I_{Ca,L}$ by 106 ± 21% and 13 ± 6%, respectively, a reduction of 88% ($P < 0.02$). Hemoglobin alone decreased $I_{Ca,L}$ amplitude (−22 ± 5%) to a value that was not different from baseline control levels. In four additional cells, lowering the hemoglobin concentration to 10 μM blocked stimulation of $I_{Ca,L}$ elicited by ACh withdrawal by 50%, without affecting ACh-induced inhibition of $I_{Ca,L}$. Moreover, in three additional cells we found that 10 μM globin had no effect on basal $I_{Ca,L}$ or ACh-induced rebound stimulation of $I_{Ca,L}$, suggesting that the effects of hemoglobin were due to the binding of NO. These findings indicate that NO signaling is essential for rebound stimulation of $I_{Ca,L}$ elicited by ACh withdrawal but does not contribute to ACh-induced inhibition of basal $I_{Ca,L}$.

ACh-induced NO Acts via cGMP Signaling

A common pathway for NO signaling is through activation of soluble guanylate cyclase and the production of cGMP (Fischmeister and Mery, 1996). We therefore tested the effects of ACh in the absence and presence of ODQ, a potent and selective inhibitor of soluble guanylate cyclase activity (Brunner et al., 1996; Garthwaite et al., 1995). As shown in Fig. 2 *A*, under control conditions, 1 μM ACh elicited a typical inhibition of $I_{Ca,L}$ (−16%) and was followed, upon withdrawal of ACh, by stimulation of $I_{Ca,L}$ (60%) above control. Exposure to 30 μM ODQ alone slightly increased $I_{Ca,L}$ (3%). In the presence of ODQ, ACh inhibited $I_{Ca,L}$ by 22% and rebound stimulation of $I_{Ca,L}$ was abolished. In a total of four cells tested, in the absence and presence of ODQ, ACh-induced inhibition of $I_{Ca,L}$ was 16 ± 8% and 28 ± 8%, respectively, and stimulation of $I_{Ca,L}$ elicited by withdrawal of ACh was 95 ± 33% and 5 ± 6%, respectively ($P < 0.02$). ODQ alone increased $I_{Ca,L}$ by 9 ± 4%. Fig. 2 *B* shows the effects of 10 μM methylene blue, a relatively nonselective and weak inhibitor of soluble guanylate cyclase (Mayer et al., 1993), on the responses to ACh. Under control conditions, exposure to 1 μM ACh inhibited $I_{Ca,L}$ (−20%) and withdrawal of ACh stimulated $I_{Ca,L}$ (116%). Exposure to methylene blue alone slightly increased $I_{Ca,L}$. In the presence of methylene blue, ACh induced inhibition of $I_{Ca,L}$ (−34%) and withdrawal of ACh failed to stimulate $I_{Ca,L}$. In the four cells tested, in the absence and presence of methylene blue, ACh-induced inhibition of $I_{Ca,L}$ was 11 ± 3% and

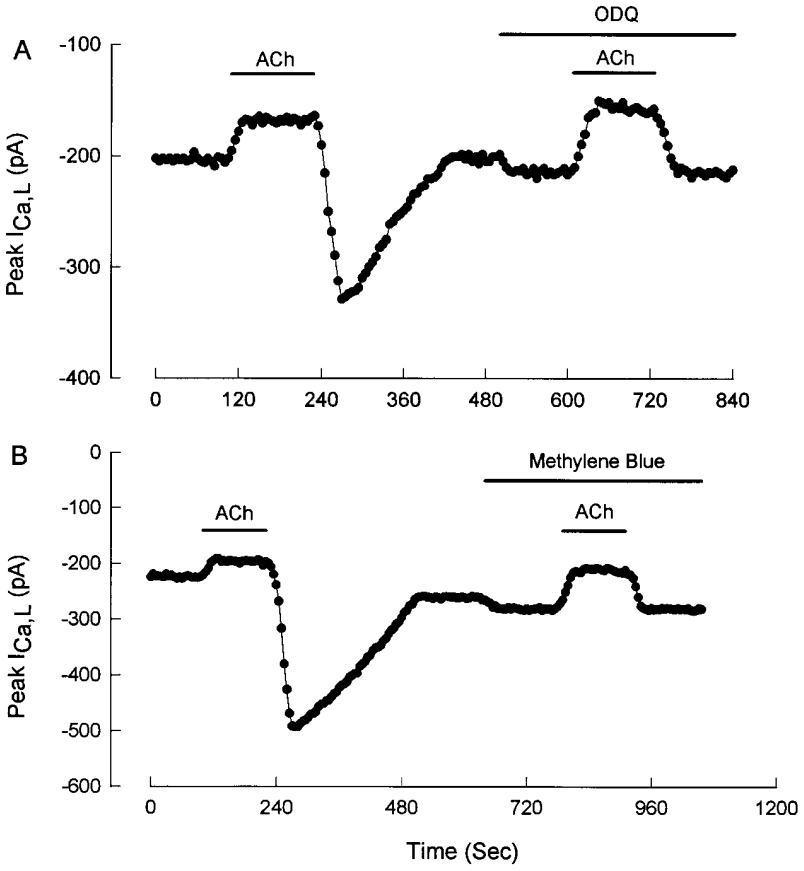

FIGURE 2. Effects of ODQ (*A*) and methylene blue (*B*), inhibitors of soluble guanylate cyclase, on ACh-induced regulation of $I_{Ca,L}$. Each graph shows consecutive measurements of peak $I_{Ca,L}$ amplitude. Effects of ODQ (*A*). Under control conditions, ACh (1 μM) exposure and withdrawal elicited inhibition and rebound stimulation of $I_{Ca,L}$, respectively. Exposure to 30 μM ODQ elicited a small increase in $I_{Ca,L}$. In the presence of ODQ, ACh-induced inhibition of $I_{Ca,L}$ was enhanced and rebound stimulation of $I_{Ca,L}$ was abolished. Effects of methylene blue (*B*). Under control conditions, ACh (1 μM) exposure and withdrawal elicited inhibition and rebound stimulation, respectively, of $I_{Ca,L}$. Methylene blue (10 μM) elicited a small increase in $I_{Ca,L}$. In the presence of methylene blue, ACh-induced inhibition of $I_{Ca,L}$ was enhanced and rebound stimulation of $I_{Ca,L}$ was abolished. Measurements of $I_{Ca,L}$ were obtained from two different cells.

30 ± 3%, respectively ($P < 0.005$), and withdrawal of ACh changed $I_{Ca,L}$ by 143 ± 37% and −7 ± 0.3%, respectively ($P < 0.05$). These findings provide further support for the idea that ACh-induced NO acts via soluble guanylate cyclase and presumably cGMP signaling to mediate rebound stimulation of $I_{Ca,L}$ elicited by withdrawal of ACh. Moreover, they indicate that NO-cGMP signaling does not mediate ACh-induced inhibition of basal $I_{Ca,L}$.

NOS Mediates ACh-induced Rebound Stimulation of $I_{Ca,L}$

If ACh acts via NO signaling then inhibition of constitutive NOS should block ACh-induced changes in $I_{Ca,L}$ mediated by NO. In Fig. 3, *A* and *B,* we tested the effect of L-NIO, an inhibitor of constitutive NOS (Rees et al., 1990) on ACh-induced regulation of $I_{Ca,L}$. The left portion of Fig. 3 *A* shows control responses to ACh exposure and withdrawal; inhibition (−18%) followed by prominent rebound stimulation of $I_{Ca,L}$ (74%). After recovery from ACh, exposure to 10 μM L-NIO alone decreased $I_{Ca,L}$ by 20%. In the presence of L-NIO, reexposure to ACh still inhibited $I_{Ca,L}$ (−16%) but rebound stimulation of $I_{Ca,L}$ was abolished. In the five cells tested, in the absence and presence of L-NIO (Fig. 3 *B*), ACh inhibited $I_{Ca,L}$ by 19 ± 5% and 23 ± 8%, respectively, and withdrawal of ACh stimulated $I_{Ca,L}$ by 111 ± 38% and 1 ± 7%, respectively ($P < 0.02$). L-NIO alone decreased $I_{Ca,L}$ amplitude by 28 ± 5%. As noted earlier, after withdrawal of the initial exposure to ACh, $I_{Ca,L}$ did not fully recover to its original control level. Therefore, the effect of L-NIO alone simply decreased $I_{Ca,L}$ back to control baseline levels. These results suggest that ACh-induced activation of constitutive NOS mediates rebound stimulation of $I_{Ca,L}$ but not inhibition of $I_{Ca,L}$. If L-NIO blocked ACh-induced rebound stimulation of $I_{Ca,L}$ by specifically inhibiting NOS activity, then elevated levels of L-arginine, the substrate used by NOS to synthesize NO (Mayer, 1995), should restore the effect of ACh. Therefore, in four additional cells we tested ACh in the presence of 10 μM L-NIO in cells incubated (3 h) in 5 mM L-arginine. Under these conditions, ACh elicited a typical rebound stimulation of $I_{Ca,L}$ (65 ± 4%).

Calmodulin-dependent NOS Mediates ACh-induced NO Signaling

Activation of constitutive NOS activity is Ca-calmodulin dependent (Bredt and Snyder, 1990). If ACh-induced activation of NO signaling is mediated via constitutive NOS, then rebound stimulation of $I_{Ca,L}$ elicited by ACh

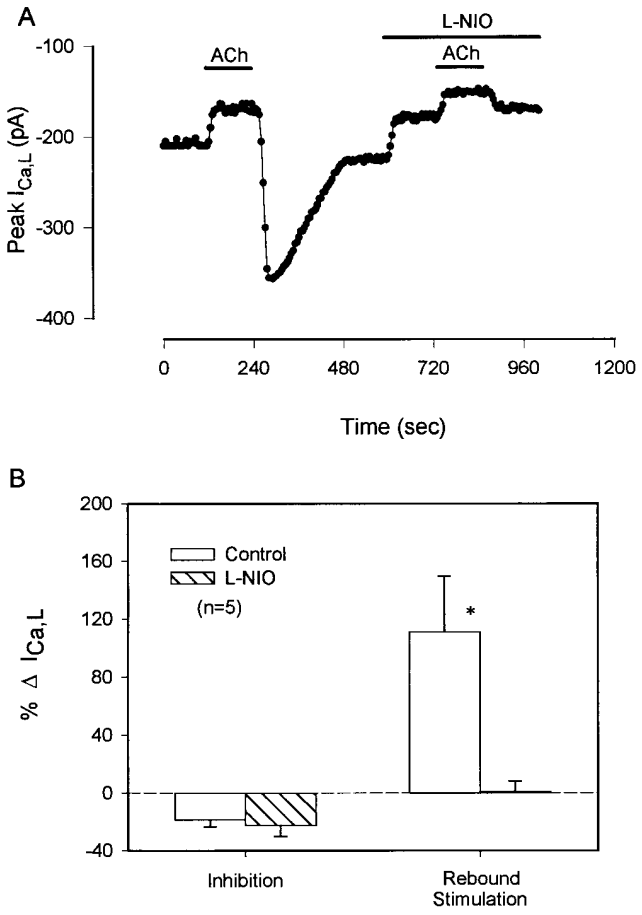

FIGURE 3. Effect of L-NIO, an inhibitor of constitutive NOS, on ACh-induced regulation of $I_{Ca,L}$. *A* shows consecutive measurements of peak $I_{Ca,L}$ amplitude, and *B* shows percent changes of ACh-induced inhibition and rebound stimulation of $I_{Ca,L}$ in the absence (*open bar*) and presence (*striped bar*) of 10 μM L-NIO (n = 5). Under control conditions, ACh exposure induced inhibition and ACh withdrawal induced rebound stimulation of $I_{Ca,L}$. L-NIO alone decreased $I_{Ca,L}$. In the presence of L-NIO, ACh inhibited $I_{Ca,L}$ and rebound stimulation of $I_{Ca,L}$ was abolished. *$P < 0.02$.

withdrawal should be blocked by inhibition of calmodulin. This idea was examined by testing the effects of ACh in the absence and then presence of W-7, a potent inhibitor of calmodulin (Hidaka et al., 1981; Asano, 1989). As shown in Fig. 4, *A* and *B*, under control conditions (*A*), 1 μM ACh induced a typical inhibition (−20%), and was followed, upon withdrawal of ACh, by a prominent stimulation of $I_{Ca,L}$ (89%). After recovery from ACh, the cell was exposed to 10 μM W-7 (Fig. 4 *B*), which decreased $I_{Ca,L}$ (−8%) to the control level. In the presence of W-7, ACh inhibited $I_{Ca,L}$ (−17%) and stimulation of $I_{Ca,L}$ typically elicited by withdrawal of ACh was essentially abolished. In the four cells tested, in the absence and presence of 10 μM W-7, ACh inhibited $I_{Ca,L}$ by 16 ± 3% and 16 ± 4%, respectively, and withdrawal of ACh stimulated $I_{Ca,L}$ by 60 ± 18% and 6 ± 8%, respectively ($P < 0.02$). W-7 alone decreased $I_{Ca,L}$ by 10 ± 2%, bringing $I_{Ca,L}$ amplitude to the control baseline level. These findings further support the conclusion that a constitutive Ca-calmodulin-dependent NOS mediates ACh-induced rebound stimulation of $I_{Ca,L}$ and does not mediate ACh-induced inhibition of $I_{Ca,L}$.

Effect of L-arginine on ACh-induced NO Signaling

As noted earlier, L-arginine is the substrate used by NOS to synthesize NO (Mayer, 1995). If NO signaling participates in ACh-induced regulation of $I_{Ca,L}$, then exposure to a relatively high concentration of L-arginine should accentuate the stimulatory response elicited by ACh withdrawal. In one approach, we tested 1 μM ACh in the absence and presence of 5 mM L-arginine in the same cell. The cell was acutely exposed to L-arginine for 8 min between the first and second exposures to ACh. In the absence of L-arginine, ACh decreased $I_{Ca,L}$ by 21 ± 5% and withdrawal of ACh stimulated $I_{Ca,L}$ by 49 ± 6% (not shown). Exposure to L-arginine elicited a small decrease in $I_{Ca,L}$ (−13 ± 6%). After 8 min in L-arginine, a second exposure to ACh inhibited $I_{Ca,L}$ by 23 ± 4% and withdrawal of ACh stimulated $I_{Ca,L}$ by 73 ± 13% (n = 8). Although the stimulation of $I_{Ca,L}$ elicited by ACh withdrawal was enhanced, it did not reach statistical significance ($P = 0.09$). It seemed possible that an 8-min exposure to L-arginine was not long enough to significantly raise intracellular L-arginine concentrations. Therefore, in a second approach, we incubated atrial myocytes in 5 mM L-arginine for at least 3 h before testing their response to ACh. Cells incubated in L-arginine for 3 h and those cells exposed to L-arginine for 8 min were obtained from the same two hearts. Cells incubated in L-arginine (3 h) exhibited basal $I_{Ca,L}$ amplitudes not significantly different from control cells. Fig. 5, *A* and *B* shows original $I_{Ca,L}$ traces from a typical experiment. In a cell not incubated in L-arginine (Fig. 5 *A*), exposure to ACh induced inhibition and withdrawal of ACh induced stimulation of $I_{Ca,L}$ (49%). In another cell incubated in L-arginine (Fig. 5 *B*), ACh-induced inhibition of $I_{Ca,L}$ was similar to control, but stimulation of $I_{Ca,L}$ elicited by withdrawal of ACh was enhanced (98%). In a total of six cells incubated in L-arginine, exposure to ACh decreased $I_{Ca,L}$ by 11 ± 5% and withdrawal of ACh stimulated $I_{Ca,L}$ by 109 ± 18%. Although ACh-induced inhibition of $I_{Ca,L}$ was smaller in cells incubated in L-arginine (−11 ± 5%) compared with control cells (−21 ± 5%), the differences in these unpaired data did not reach statistical significance. Stimulation of $I_{Ca,L}$ elicited by withdrawal of ACh, however, was significantly larger in L-arginine-treated cells (109 ± 18%) compared with control (49 ± 6%) ($P < 0.05$). To examine the stereo specificity of arginine, an additional experiment was performed where cells were incubated in 5 mM D-arginine. Cells

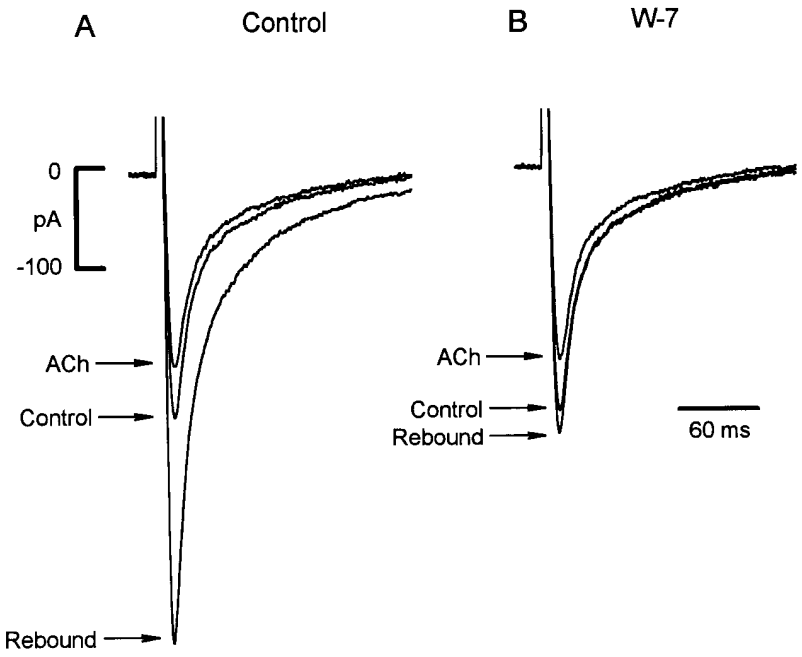

FIGURE 4. Effect of W-7, a calmodulin inhibitor, on ACh-induced regulation of basal $I_{Ca,L}$. Under control conditions (*A*), exposure to and withdrawal of 1 μM ACh elicited inhibition and rebound stimulation of $I_{Ca,L}$, respectively. After 4–5 min, $I_{Ca,L}$ recovered and stabilized (not shown). Exposure to 10 μM W-7 elicited a small decrease in $I_{Ca,L}$ to the control level (*B*). In the presence of W-7, ACh inhibited $I_{Ca,L}$ and rebound stimulation of $I_{Ca,L}$ elicited by withdrawal of ACh was essentially abolished. All recordings were obtained from the same cell.

incubated in D-arginine showed a 25% smaller rebound stimulation of $I_{Ca,L}$ induced by ACh withdrawal than control cells from the same heart ($n = 2$). The results are consistent with the view that additional substrate (L-arginine) for NO signaling augmented the stimulation of $I_{Ca,L}$ elicited by withdrawal of ACh. In our previous study, we noted that the increase in $I_{Ca,L}$ amplitude elicited by ACh withdrawal ranged between 5 and 232% (SD ± 49%; $n = 53$) in any given cell (Wang and Lipsius, 1995). Based on the present results, this variability in response could be due to the dependence of the rebound response on L-arginine concentration, which may vary in individual myocytes after isolation.

Histochemical Assay for NOS Activity

So far, the results indicate that NO signaling underlies the stimulation of $I_{Ca,L}$ elicited by withdrawal of ACh. In the next several experiments, we sought to determine whether atrial myocytes exhibit constitutive NOS activity, and whether exogenous NO stimulates $I_{Ca,L}$ in a way similar to that elicited by withdrawal of ACh. In Fig. 6, *A* and *B*, we used a histochemical technique (NADPH-diaphorase assay; Prabhakar et al., 1993) to demonstrate the presence of NO synthase activity. Isolated atrial cells were fixed, permeabilized, and then incubated in nitro blue tetrazolium in the absence or pres-

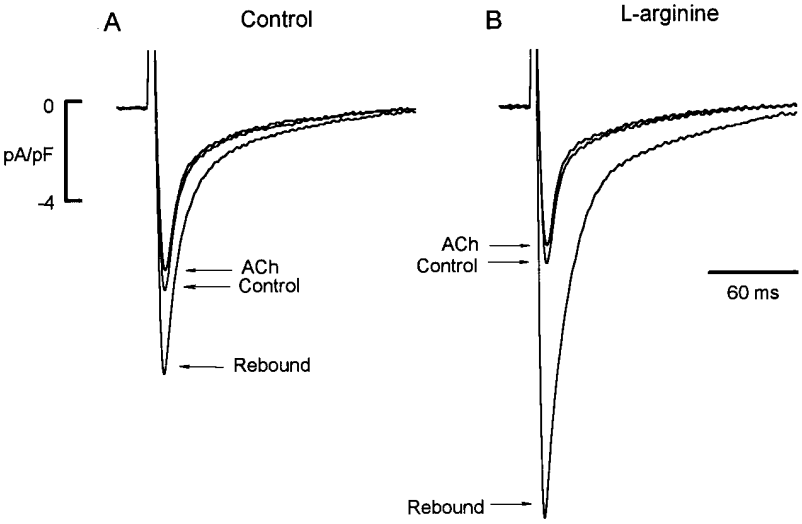

FIGURE 5. Effect of L-arginine on ACh-induced regulation of $I_{Ca,L}$. In control cells (not incubated in L-arginine), ACh induced a typical inhibition followed by rebound stimulation of $I_{Ca,L}$ elicited by ACh withdrawal (*A*). In another cell incubated in 5 mM L-arginine for 3 h, withdrawal of ACh elicited a significantly larger rebound stimulation of $I_{Ca,L}$ (*B*).

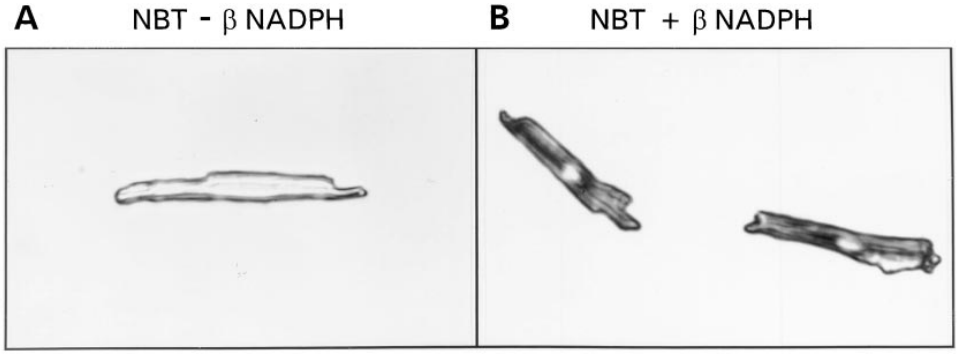

FIGURE 6. Histochemical assay for NOS activity. Atrial myocytes were exposed to nitro blue tetrazolium (*NBT*) in either the absence (*A*) or presence (*B*) of β-NADPH. In the presence of β-NADPH, NOS reduces tetrazolium to formazan, a compound that appears as a dark stain. In the absence of β-NADPH (*A*), atrial cells appear clear (control) and in the presence of β-NADPH cells appear darkly stained, indicating the presence of a constitutive form of NOS activity.

ence of β-NADPH. Cells prepared in the absence of β-NADPH (Fig. 6 *A*) failed to exhibit a positive staining reaction and those treated in the presence of β-NADPH stained positively (Fig. 6 *B*). Similar results were obtained in cells isolated from three hearts.

Effect of NO Donors on $I_{Ca,L}$

Spermine NO (SP/NO) spontaneously releases NO without the production of other biologically active by-products or intermediates (Maragos et al., 1991) and therefore was used in the present study as our typical NO donor. Fig. 7, *A* and *B* shows the effect of 100 μM SP/NO on original $I_{Ca,L}$ recordings (*A*) and the current–voltage relationship of $I_{Ca,L}$ (*B*). As shown in Fig. 7 *A*, SP/NO elicited a reversible increase in basal $I_{Ca,L}$ amplitude (127%). SP/NO increased $I_{Ca,L}$ throughout the voltage range and shifted peak $I_{Ca,L}$ ~5 mV more negative without affecting the reversal potential. Because SP/NO spontaneously releases NO and therefore decomposes to some extent over time (Maragos et al., 1991), we thought the construction of a dose–response relationship would be quantitatively equivocal. However, to obtain a qualitative understanding of the effects of SP/NO, we tested different concentrations of SP/NO (0.3, 1, 30, 100, and 300 μM) on basal $I_{Ca,L}$ amplitude. These experiments indicated that concentrations of SP/NO $\leq$ 1 μM have no significant effect on $I_{Ca,L}$ and concentrations of SP/NO $>$ 1 μM each elicited consistent dose-dependent increases in $I_{Ca,L}$. Concentrations of SP/NO $\geq$ 100 μM were essentially maximal. To ensure that the carrier compound, spermine, was not responsible for the observed effects of SP/NO, we tested 300 μM spermine alone and found no effect on $I_{Ca,L}$ ($n = 3$) (not shown). These results indicate that NO stimulates basal $I_{Ca,L}$. Moreover, they support the results presented above that NO signaling mediates stimulation of $I_{Ca,L}$ elicited by withdrawal of ACh.

Effects of Hemoglobin and MnTBAP

The experiments shown in Fig. 8, *A* and *B* were performed to establish that the stimulatory effect of SP/

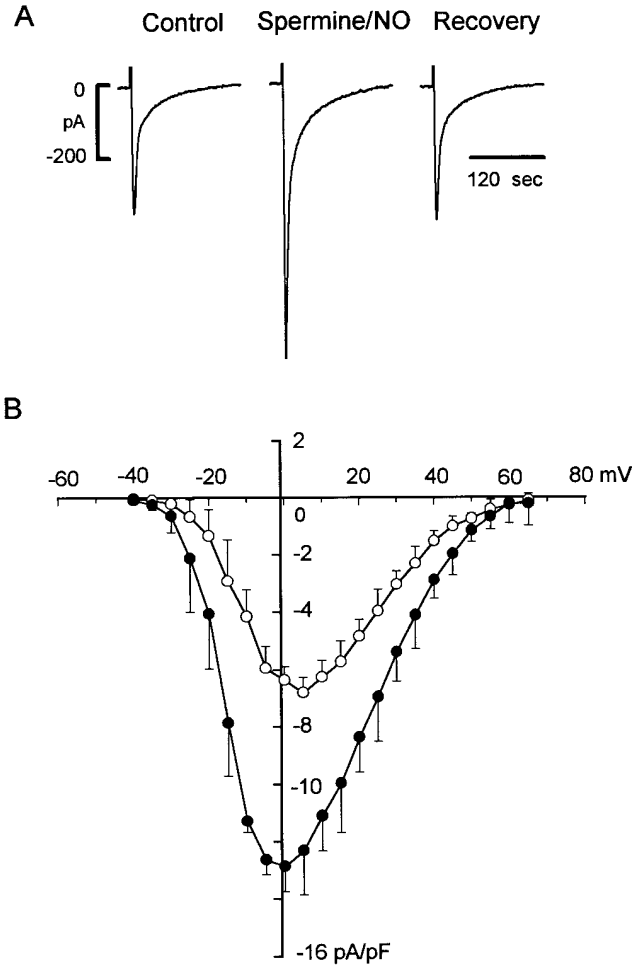

FIGURE 7. Effect of spermine/NO on basal $I_{Ca,L}$ (*A*) and the current–voltage relationship of $I_{Ca,L}$ (*B*). Spermine/NO (100 μM) elicited a reversible increase in $I_{Ca,L}$ amplitude throughout the voltage range, and shifted peak $I_{Ca,L}$ to more negative voltages without affecting the reversal potential. The recovery of $I_{Ca,L}$ after washout of spermine/NO is not shown in the current–voltage relationship for clarity.

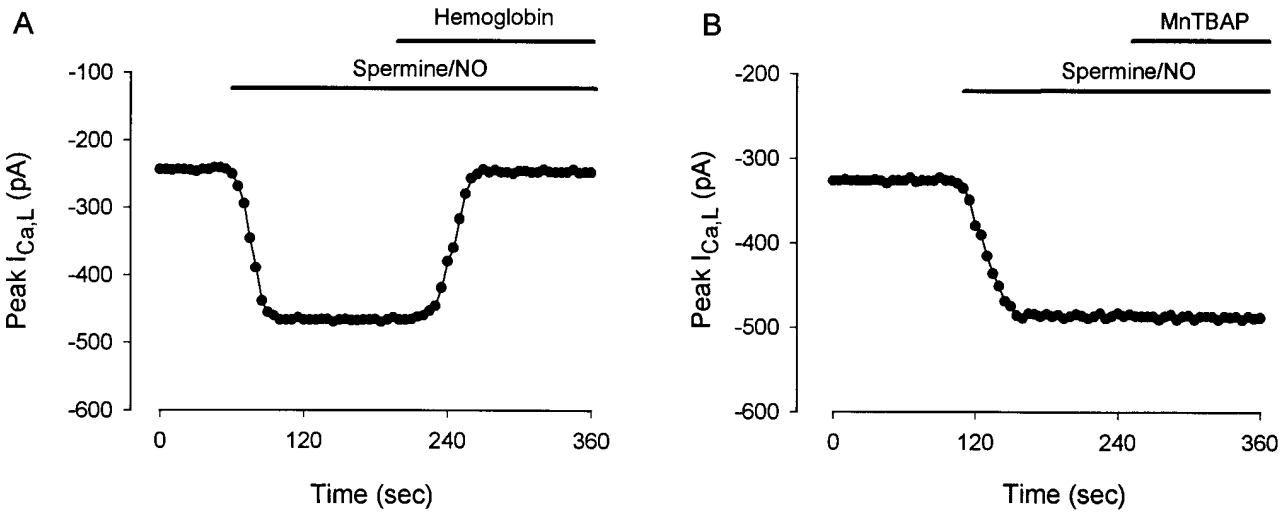

FIGURE 8. Effects of hemoglobin (*A*) and MnTBAP (*B*) on spermine/NO-induced stimulation of basal $I_{Ca,L}$. Each graph shows consecutive measurements of peak $I_{Ca,L}$ amplitude recorded throughout an experiment. Spermine/NO (50 μM) elicited a sustained increase in $I_{Ca,L}$ that was blocked by addition of 50 μM hemoglobin to the external spermine/NO-containing solution (*A*). Spermine/NO (50 μM) elicited a sustained increase in $I_{Ca,L}$ (*B*). Addition of 50 μM MnTBAP failed to affect spermine/NO-induced stimulation of $I_{Ca,L}$. MnTBAP (50 μM) alone had no significant effect on basal $I_{Ca,L}$ amplitude.

NO on basal $I_{Ca,L}$ is due to NO. As shown in Fig. 8 *A*, 50 μM SP/NO elicited an increase in $I_{Ca,L}$ that was maintained during exposure to SP/NO. Addition of 50 μM hemoglobin to the external solution inhibited the effects of SP/NO and returned $I_{Ca,L}$ to control levels. When hemoglobin was removed in the continued presence of SP/NO, $I_{Ca,L}$ increased once again (not shown), indicating that the effects of hemoglobin were reversible. In a total of four cells tested, SP/NO alone increased $I_{Ca,L}$ by 61 ± 11% and hemoglobin returned $I_{Ca,L}$ to levels not different from control ($P < 0.001$). The fact that hemoglobin blocked the stimulation of $I_{Ca,L}$ elicited by both exogenous NO and ACh withdrawal supports the idea that both effects are mediated by a common mechanism; i.e., NO signaling.

NO can react with oxygen free radicals to produce peroxynitrite, a biological active compound (Stamler et al., 1992*b*). Although SP/NO does not generate oxygen free radicals (Maragos et al., 1991), it is possible that oxygen free radicals produced endogenously may react with NO released by SP/NO to affect $I_{Ca,L}$. To examine this point, we tested SP/NO in the absence and presence of MnTBAP, a superoxide dismutase mimetic and peroxynitrite scavenger (Day et al., 1995; Szabo et al., 1996). As shown in Fig. 8 *B*, exposure to 50 μM SP/NO elicited a sustained increase in $I_{Ca,L}$. Addition of 50 μM MnTBAP to the external solution failed to prevent the effects of SP/NO. In the three cells tested, SP/NO alone increased $I_{Ca,L}$ by 54 ± 17% and the addition of MnTBAP had no significant effect on $I_{Ca,L}$ amplitude (56 ± 17%). In four additional cells, MnTBAP alone exerted no significant effect on basal $I_{Ca,L}$ amplitude. These findings, in conjunction with those presented in Fig. 8 *A*, suggest that SP/NO is acting via NO, and not peroxynitrite, to stimulate basal $I_{Ca,L}$. These results, however, do rule out the possibility that NO may form biologically active intermediates, such as S-nitrosothiols (Ignarro et al., 1981; Stamler et al., 1992*a*; Arnelle and Stamler, 1995), that may stimulate $I_{Ca,L}$, as reported in ferret ventricular myocytes (Campbell et al., 1996). In ferret myocytes, however, the stimulatory effects of S-nitrosothiols were mediated by direct S-nitrosylation and/or disulfide reactions and not via second messenger signaling mechanisms. The experiments presented below will establish that in cat atrial myocytes NO is acting via second messenger (cGMP) signaling.

NO Stimulates $I_{Ca,L}$ via Soluble Guanylate Cyclase

The next question we addressed was whether exogenous NO acts via soluble guanylate cyclase and the production of cGMP. We therefore tested the effect of ODQ on SP/NO-induced stimulation of $I_{Ca,L}$. The original records in Fig. 9 show the effects of 300 μM SP/NO on $I_{Ca,L}$ in the absence (control) and presence of 30 μM ODQ. Under control conditions, SP/NO elicited a marked increase in $I_{Ca,L}$ (129%) that reversed on washout. In the presence of ODQ, the stimulatory effects of SP/NO on $I_{Ca,L}$ were abolished. In a total of three cells tested, SP/NO increased $I_{Ca,L}$ by 130 ± 33% in the absence and by 15 ± 6% in the presence of ODQ ($P < 0.05$). ODQ alone had little effect on $I_{Ca,L}$ amplitude. These experiments indicate that NO is acting via soluble guanylate cyclase and presumably cGMP to

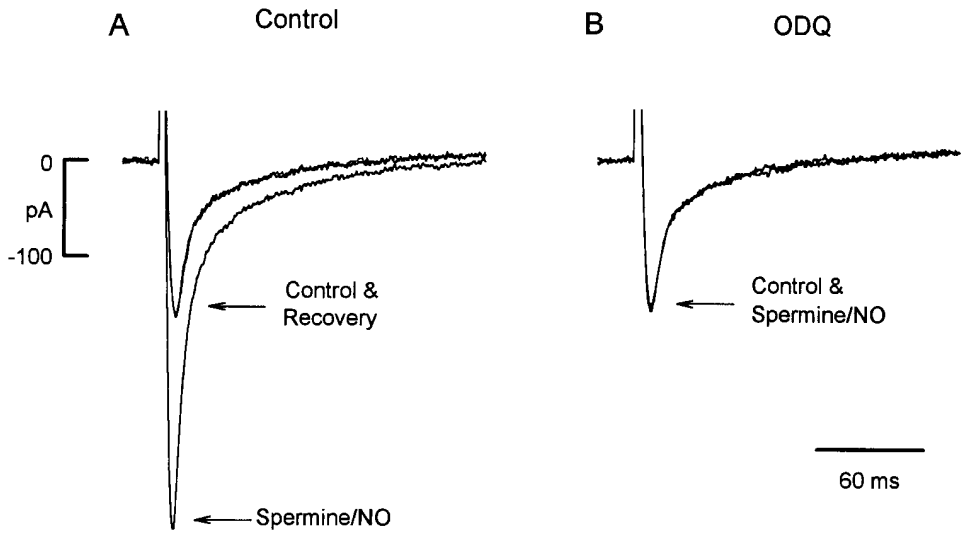

FIGURE 9. Effect of ODQ on spermine/NO-induced stimulation of basal $I_{Ca,L}$. Under control conditions (*A*), 300 μM spermine/NO elicited a reversible increase in $I_{Ca,L}$ amplitude. In the presence of 30 μM ODQ (*B*), the effects of spermine/NO on $I_{Ca,L}$ were abolished. All currents were recorded from the same cell.

stimulate basal $I_{Ca,L}$. Moreover, they are consistent with the results presented above that NO acts via second messenger (cGMP) signaling to mediate stimulation of $I_{Ca,L}$ elicited by withdrawal of ACh.

NO Acts via Inhibition of PDE and Stimulation of cAMP/PKA

Our previous study showed that milrinone, an inhibitor of PDE (type III) activity (Harrison et al., 1986) attenuates rebound stimulation of $I_{Ca,L}$ elicited by ACh withdrawal (Wang and Lipsius, 1995). If SP/NO also is acting to stimulate $I_{Ca,L}$ via inhibition of PDE (type III), then earlier stimulation of $I_{Ca,L}$ by milrinone should prevent SP/NO from exerting any additional stimulation of $I_{Ca,L}$. As shown in Fig. 10, 100 μM SP/NO stimulated $I_{Ca,L}$ (118%). Washout of SP/NO returned $I_{Ca,L}$ toward control. Exposure to 10 μM milrinone stimulated $I_{Ca,L}$ to about the same extent as SP/NO (122%). This finding indicates that cat atrial myocytes express PDE (type III) activity, as reported previously (Wang and Lipsius, 1995). In the presence of milrinone, reexposure to SP/NO failed to further increase $I_{Ca,L}$, and, in fact, elicited a small inhibition of $I_{Ca,L}$ (−17%). In all

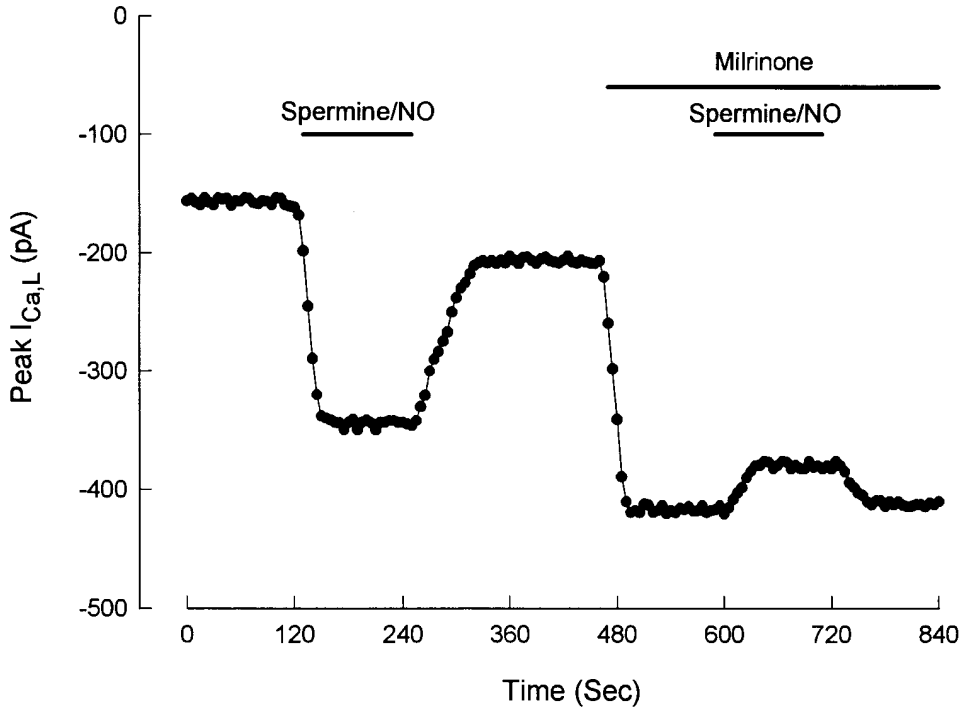

FIGURE 10. Effect of spermine/NO on $I_{Ca,L}$ prestimulated by milrinone, an inhibitor of PDE (type III) activity. Under control conditions, spermine/NO (100 μM) stimulated basal $I_{Ca,L}$. After washout of spermine/NO, exposure to 10 μM milrinone elicited a prominent increase in $I_{Ca,L}$. In the presence of milrinone, spermine/NO failed to elicit an additional stimulation of $I_{Ca,L}$ and, in fact, elicited a small inhibition of $I_{Ca,L}$.

cells tested, SP/NO alone increased $I_{Ca,L}$ by 117 ± 6% ($n = 3$), milrinone alone increased $I_{Ca,L}$ by 113 ± 33% ($n = 5$), and the addition of SP/NO in the presence of milrinone failed to further stimulate $I_{Ca,L}$ (−17 ± 2%) ($n = 5$). Interpretation of these experiments is based on the premise that milrinone does not maximally stimulate $I_{Ca,L}$. In this regard, we have previously reported in atrial myocytes that when $I_{Ca,L}$ is prestimulated by 10 μM milrinone, 1 μM isoproterenol elicits an additional increase in $I_{Ca,L}$ of 101 ± 26% ($n = 5$) (Wang and Lipsius, 1995), indicating that $I_{Ca,L}$ is not maximally stimulated by milrinone. These results, in conjunction with those described earlier, suggest that NO-cGMP signaling acts to inhibit PDE (type III) activity and thereby stimulate basal $I_{Ca,L}$ via elevation of cAMP.

We previously reported that inhibition of cAMP-dependent PKA activity abolishes rebound stimulation of $I_{Ca,L}$ elicited by ACh withdrawal (Wang and Lipsius, 1995). To determine whether exogenous NO stimulates $I_{Ca,L}$ via cAMP, SP/NO was tested in the absence and presence of H-89, an inhibitor of cAMP-dependent PKA activity (Chijiwa et al., 1990). As shown in the bar graph in Fig. 11, 100 μM SP/NO elicited a mean increase in $I_{Ca,L}$ of 108 ± 35%. In the presence of 5 μM H-89, the stimulatory effect of SP/NO on $I_{Ca,L}$ (36 ± 7%) was significantly reduced compared with control ($P < 0.05$; $n = 5$). In three additional cells, the concentration of SP/NO was lowered (30 μM) to a level that elicited an increase in $I_{Ca,L}$ comparable to that achieved by ACh withdrawal. In the absence and presence of H-89, 30 μM SP/NO elicited a mean increase in $I_{Ca,L}$ of 61 ± 5% and 14 ± 5%, respectively ($P < 0.001$). These findings indicate that NO stimulates $I_{Ca,L}$ primarily by increasing cAMP and are consistent with the role of NO signaling in stimulation of $I_{Ca,L}$ elicited by ACh withdrawal.

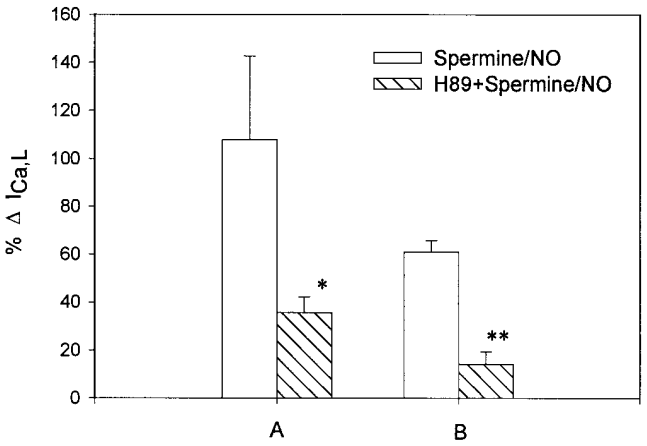

FIGURE 11. Effect of H-89, an inhibitor of PKA, on spermine/NO-induced stimulation of $I_{Ca,L}$. The graph shows the percent change in $I_{Ca,L}$ amplitude elicited by either 100 μM spermine/NO (*A*) or 30 μM spermine/NO (*B*) in the absence (*open bar*) and presence (*striped bar*) of 5 μM H-89. Spermine/NO-induced stimulation of $I_{Ca,L}$ was significantly blocked by inhibition of PKA activity. In addition, H-89 exerted a more effective block at lower spermine/NO concentrations. *$P < 0.05$; **$P < 0.001$.

ACh-induced NO Signaling Is Pertussis Toxin–sensitive

Previous findings indicate that pertussis toxin (PTX) blocks both ACh-induced inhibition and rebound stimulation of $I_{Ca,L}$ (Wang and Lipsius, 1995). Based on the present results, those findings indicate that PTX-sensitive G-proteins mediate ACh-induced NO signaling mechanisms. To ensure that PTX does not interfere with steps in the NO signaling pathway downstream from G-protein sites, we tested the effects of 1 μM ACh, 100 μM SP/NO, and 10 μM milrinone on atrial myocytes incubated in PTX (3.4 μg/ml for 6 h; 36°C). As expected, in cells not incubated in PTX, ACh exposure and withdrawal elicited inhibition (−16 ± 9%) and stimulation (124 ± 32%) of $I_{Ca,L}$, respectively ($n = 4$), and SP/NO (208%; $n = 2$) and milrinone (137%; $n = 2$) also stimulated $I_{Ca,L}$. In cells preincubated in PTX from the same hearts, the effects of ACh on $I_{Ca,L}$ were abolished ($n = 5$), whereas SP/NO and milrinone still increased $I_{Ca,L}$ by 117 ± 35% and 99 ± 41%, respectively ($n = 4$). These results indicate that PTX does not interfere with NO signaling sites downstream from NOS, and suggests that PTX-sensitive G-proteins couple M_2 muscarinic receptors to constitutive NOS.

DISCUSSION

In cat atrial myocytes, exposure to ACh inhibits basal $I_{Ca,L}$ and withdrawal of ACh elicits rebound stimulation of $I_{Ca,L}$ above control (Wang and Lipsius, 1995). Stimulation of $I_{Ca,L}$ elicited by ACh withdrawal was shown to involve ACh-induced inhibition of PDE (type III) activity. The present results extend our previous findings by demonstrating that ACh-induced modulation of PDE is mediated via NO-cGMP signaling and that this is the primary mechanism responsible for stimulation of $I_{Ca,L}$ elicited by ACh withdrawal. Thus, pharmacological interventions (hemoglobin, L-NIO, W-7, ODQ) that specifically block various steps in the NO-cGMP signaling pathway each blocked rebound stimulation of $I_{Ca,L}$, whereas interventions expected to enhance NO signaling (incubation in L-arginine) enhanced stimulation of $I_{Ca,L}$ elicited by ACh withdrawal. In addition, activation of NO signaling by exogenous NO (SP/NO) stimulated $I_{Ca,L}$, and its mechanism of action was essentially the same as that underlying the stimulation of $I_{Ca,L}$ elicited by ACh withdrawal. In other words, SP/NO stimulated $I_{Ca,L}$ via cGMP-induced inhibition of PDE (type III) activity and elevation of cAMP. A similar mechanism has been reported for NO-induced stimulation of basal $I_{Ca,L}$ in human atrial myocytes (Kirstein et al., 1995). The

present study also provides histochemical evidence that atrial myocytes express a constitutive form of NOS activity that can act as a substrate for the actions of ACh. The same histochemical staining method has been used to identify constitutive NOS in neuronal tissue (Hope et al., 1991) and cardiac ventricular myocytes (Zakharov et al., 1996). More specific methods of immunohistochemistry, Northern blot analysis, or in situ hybridization have revealed eNOS in human atrial tissue (Wei et al., 1996), rat atrial (Seki et al., 1996), and rabbit AV nodal cells (Han et al., 1996). It is interesting to note that in guinea pig ventricular myocytes, withdrawal of ACh also elicits rebound stimulation of cAMP-regulated chloride current (Zakharov and Harvey, 1997). However, even though these cells express constitutive NO synthase activity (Zakharov et al., 1996), ACh-induced rebound stimulation of chloride current does not involve NO-cGMP signaling mechanisms (Zakharov and Harvey, 1997).

The present findings also indicate that NO-cGMP signaling does not contribute to ACh-induced inhibition of basal $I_{Ca,L}$. Thus, none of the drugs that blocked NO signaling and rebound stimulation of $I_{Ca,L}$ prevented ACh-induced inhibition of $I_{Ca,L}$. This finding provides further evidence that NO signaling plays a specific role in stimulation of basal $I_{Ca,L}$ elicited by ACh withdrawal. In our previous study, we reported preliminary experiments where L-NMMA, an inhibitor of NOS activity, blocked ACh-induced inhibition of $I_{Ca,L}$, suggesting that NO signaling may play a role in ACh-induced inhibition of basal $I_{Ca,L}$ (Wang and Lipsius, 1995). Clearly, that finding is not supported by the weight of evidence obtained in the present study. To reexamine the role of NOS in ACh-induced regulation of $I_{Ca,L}$, we found in the present study that the NOS blocker L-NIO did not prevent ACh-induced inhibition of $I_{Ca,L}$, although it abolished stimulation of $I_{Ca,L}$ elicited by ACh withdrawal. Moreover, the fact that elevated levels of L-arginine restored ACh-induced stimulation of $I_{Ca,L}$ indicates that L-NIO acted specifically to inhibit NOS. These results are consistent with all the other findings in this study, which indicate that NO signaling does not play a role in ACh-induced inhibition of basal $I_{Ca,L}$. Others have reported that NO signaling plays a critical role in the inhibitory effects of ACh on cardiac $I_{Ca,L}$ (Mery et al., 1991, 1993; Han et al., 1994, 1995, 1996; Levi et al., 1994; Wahler and Dollinger, 1995; Balligand et al., 1995). However, this inhibitory effect of NO signaling is obtained only when $I_{Ca,L}$ is prestimulated by elevated cAMP. Under these conditions, NO-cGMP signaling is thought to inhibit $I_{Ca,L}$ via stimulation of PDE (Han et al., 1995, 1996) or activation of PKG activity (Mery et al., 1991; Levi et al., 1994; Wahler and Dollinger, 1995). Neither of these NO signaling mechanisms appear to play a role in ACh-induced inhibition of basal $I_{Ca,L}$ in cat atrial myocytes. We have found, however, that when $I_{Ca,L}$ is prestimulated by isoproterenol, SP/NO inhibits rather than stimulates $I_{Ca,L}$ in cat atrial myocytes (our unpublished observations). This finding may explain the present observation that SP/NO inhibited $I_{Ca,L}$ in cells prestimulated by milrinone (Fig. 10), which acts by raising cAMP.

In the present experiments, ACh-induced inhibition of basal $I_{Ca,L}$ is evident (even enhanced) in the presence of methylene blue in the external solutions. In rabbit SA node pacemaker cells (Han et al., 1995), external application of methylene blue blocked ACh-induced inhibition of prestimulated $I_{Ca,L}$ as well as ACh-induced activation of K^+ current. However, when applied intracellularly, methylene blue only blocked ACh-induced inhibition of $I_{Ca,L}$. From these findings, it was concluded that methylene blue blocks muscarinic receptors. A similar finding has been reported in rat ventricular myocytes (Abi Gerges et al., 1997). The present results indicate that in cat atrial myocytes methylene blue does not block muscarinic receptors. It may be that methylene blue exerts nonspecific receptor blocking effects that are species dependent. Another interesting aspect of these experiments is that methylene blue very effectively blocked the stimulation of $I_{Ca,L}$ elicited by ACh withdrawal even though it is considered a weak inhibitor of soluble guanylate cyclase activity. This may be explained by the fact that methylene blue also is reported to inhibit NOS (Mayer et al., 1993), and therefore may act to block two critical sites in the NO signaling pathway activated by ACh.

Each of the pharmacological compounds used in the present study to manipulate different steps in the NO signaling pathway exerted some direct effects on $I_{Ca,L}$. These effects could be interpreted to indicate that these cells produce endogenous levels of NO that could potentially modulate basal $I_{Ca,L}$. However, even though $I_{Ca,L}$ stabilized after the initial ACh exposure peak, $I_{Ca,L}$ generally did not fully recover to control levels, indicating some residual stimulatory effects of ACh. Therefore, $I_{Ca,L}$ was not in a true basal state during the administration of each test compound. Those NO-blocking compounds that inhibited $I_{Ca,L}$ returned $I_{Ca,L}$ amplitude to control baseline values. Their actions, therefore, could be interpreted as blocking the residual NO-induced stimulatory effects exerted by the initial ACh exposure. However, other compounds that blocked NO signaling exerted small stimulatory effects on $I_{Ca,L}$. Their actions are less clear and may involve more complex effects on $I_{Ca,L}$. In any case, these experiments were not designed to examine the direct effects of these drugs on basal NO signaling mechanisms. Moreover, regardless of the direct drug effects, the results of these experiments are consistent with the conclusions that NO signaling mediates rebound stimulation of $I_{Ca,L}$ elicited by ACh withdrawal.

Together with our previous work (Wang and Lipsius, 1995), the present results suggest that exposure to ACh acts via M_2 muscarinic receptors coupled to PTX-sensitive G-proteins to inhibit basal $I_{Ca,L}$, primarily via inhibition of the adenylate cyclase/cAMP/PKA cascade. At the same time, ACh acts via PTX-sensitive G-proteins coupled to constitutive NOS to elicit NO-cGMP-induced inhibition of PDE (type III) activity. Upon withdrawal of ACh from the receptor, we speculate that adenylate cyclase/cAMP activity recovers more rapidly than PDE activity, resulting in an increase in cAMP above baseline and rebound stimulation of $I_{Ca,L}$. These findings are supported by direct measurements of cAMP in chick heart cells where withdrawal of cholinergic agonist acts via PTX-sensitive G-proteins to elicit rebound stimulation of adenylate cyclase activity and increases in cAMP concentrations above control levels (Linden, 1987).

Functionally, $I_{Ca,L}$ plays a critical role in the regulation of cardiac pacemaker and contractile activities. In fact, rebound stimulation of $I_{Ca,L}$ elicited by ACh withdrawal stimulates SA node pacemaker cell rate (Wang and Lipsius, 1996) and atrial cell contraction (Wang and Lipsius, 1995) above control levels. Rebound stimulation of $I_{Ca,L}$ elicited by ACh withdrawal also has been reported in catecholamine-treated Purkinje fibers (Ehara and Mitsuiye, 1984). This stimulatory effect of ACh on $I_{Ca,L}$ probably serves to rapidly restore cardiac pacemaker and contractile functions after periods of cholinergic suppression. Moreover, background β-adrenergic stimulation augments rebound stimulation of cAMP (Linden, 1987) and $I_{Ca,L}$ (Wang and Lipsius, 1995). This suggests that in the presence of β-adrenergic stimulation NO signaling may mediate both enhanced ACh-induced inhibition of $I_{Ca,L}$ (i.e., accentuated antagonism) and enhanced rebound stimulation of $I_{Ca,L}$. In addition to stimulating Ca^{2+} influx via $I_{Ca,L}$, ACh-induced stimulation of cAMP stimulates Ca^{2+} uptake into the sarcoplasmic reticulum (Wang et al., 1997). By loading intracellular Ca^{2+} stores, withdrawal of ACh can Ca^{2+} overload the sarcoplasmic reticulum, thereby eliciting Ca^{2+}-mediated delayed afterdepolarizations and spontaneous/triggered atrial activities (Wang et al., 1997). This arrhythmogenic aspect of ACh withdrawal may be enhanced by other preexisting conditions that raise intracellular Ca^{2+}. This suggests that the NO signaling mechanisms presented here may contribute to vagally induced Ca^{2+}-mediated atrial dysrhythmias. NO signaling, therefore, may be a potential target for antiarrhythmic drug therapies. The relevance of the present results to human cardiac function is emphasized by the fact that basal $I_{Ca,L}$ in cat atrial myocytes and human atrial myocytes (Kirstein et al., 1995) exhibit similar stimulatory responses to NO signaling. Whether human atrial myocytes exhibit similar responses to ACh remains to be determined.

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