A Cellular Mechanism for Nitric Oxide-mediated Cholinergic Control of Mammalian Heart Rate

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ABSTRACT The biochemical signaling pathways involved in nitric oxide (NO)-mediated cholinergic inhibition of L-type Ca^{2+} current ($I_{Ca[L]}$) were investigated in isolated primary pacemaker cells from the rabbit sinoatrial node (SAN) using the nystatin-perforated whole-cell voltage clamp technique. Carbamylcholine (CCh; 1 μM), a stable analogue of acetylcholine, significantly inhibited $I_{Ca(L)}$ after it had been augmented by isoproterenol (ISO; 1 μM). CCh also activated an outward K^+ current, $I_{K(ACh)}$. Both of these effects of CCh were blocked completely by atropine. Preincubation of the SAN cells with L-nitro-arginine methyl ester (L-NAME; 0.2–1 mM), which inhibits NO synthase (NOS), abolished the CCh-induced attenuation of $I_{Ca(L)}$ but had no effect on $I_{K(ACh)}$. Coincubation of cells with both L-NAME and the endogenous substrate of NOS, L-arginine (1 mM), restored the CCh-induced attenuation of $I_{Ca(L)}$, indicating that L-NAME did not directly interfere with the muscarinic action of CCh on $I_{Ca(L)}$. In the presence of ISO the CCh-induced inhibition of $I_{Ca(L)}$ could be mimicked by the NO donor 3-morpholino-sydnonimine (SIN-1; 0.1 mM). SIN-1 had no effect on its own or after a maximal effect of CCh had developed, indicating that it does not inhibit $I_{Ca(L)}$ directly. SIN-1 failed to activate $I_{K(ACh)}$, demonstrating that it did not activate muscarinic receptors. Both CCh and NO are known to activate guanylyl cyclase and elevate intracellular cGMP. External application of methylene blue (10 μM), which interferes with the ability of NO to activate guanylyl cyclase, blocked the CCh-induced attenuation of $I_{Ca(L)}$. However, it also blocked the activation of $I_{K(ACh)}$, suggesting an additional effect on muscarinic receptors or G proteins. To address this, a separate series of experiments was performed using conventional whole-cell recordings with methylene blue in the pipette. Under these conditions, the CCh-induced attenuation of $I_{Ca(L)}$ was blocked, but the activation of $I_{K(ACh)}$ was still observed. Methylene blue also blocked the SIN-1–induced decrease in $I_{Ca(L)}$. 6-anilino-5,8-quinolinedione (LY83583; 30 μM), an agent known to decrease both basal and CCh-stimulated cGMP levels, prevented the inhibitory effects of both CCh and SIN-1 on $I_{Ca(L)}$, but had no effect on the activation of $I_{K(ACh)}$ by CCh. In combination, these results show that CCh- and NO-induced inhibition of $I_{Ca(L)}$ is mediated by cGMP. CCh could not decrease $I_{Ca(L)}$ after it had been increased maximally by a membrane-permeable cAMP analogue, 8-(4-chlorophe-

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J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/95/07/45/21 $2.00
Volume 106 July 1995 45–65

nylthio)-adenosine-3′,5′-cyclic monophosphate (8-CPT-cAMP; 0.2 mM), which is resistant to the cAMP-specific phosphodiesterase (PDE). This finding suggests that neither the cAMP-dependent protein kinase (PKA) nor the biochemical events occurring subsequent to PKA activation are involved in the CCh- or NO-induced inhibition of $I_{Ca(L)}$. $I_{Ca(L)}$ was not changed significantly by the membrane-permeable cGMP analogues 8-bromoguanosine-3′,5′-cyclic monophosphate (8-Br-cGMP; 0.25–0.5 mM) and 8-(4-chlorophenylthio)-guanosine-3′,5′-cyclic monophosphate (8-CPT-cGMP; 0.25 mM). These compounds selectively activate cGMP-dependent protein kinase (PKG); their effects therefore rule out a role of PKG in this NO-mediated, CCh-induced inhibition of $I_{Ca(L)}$. A potent but nonspecific PDE inhibitor 3-isobutyl-1-methyl-xanthine (IBMX; 20 μM) completely prevented the CCh-induced attenuation of $I_{Ca(L)}$. Taken together, these findings suggest that in mammalian primary pacemaker SAN cells (in the presence of ISO), NO-mediated cholinergic inhibition of $I_{Ca(L)}$ is due to a cGMP-stimulated cAMP-specific PDE, which hydrolyzes cAMP and thus inhibits cAMP-dependent phosphorylation of $I_{Ca(L)}$ channels. Since $I_{Ca(L)}$ is essential for normal pacemaker activity, this biochemical pathway represents an important new mechanism for autonomic (cholinergic) control of mammalian heart rate.

INTRODUCTION

Rhythmic beating of the mammalian heart originates within primary pacemaker tissue in the sinoatrial node (SAN). Generation of each spontaneous pacemaker depolarization and action potential depends upon an interaction of several different ionic currents, and autonomic regulation of heart rate results from modulation of a number of these conductances by neurotransmitters (see Irisawa, Brown, and Giles, 1993). Activation of the sympathetic nervous system accelerates heart rate by releasing noradrenaline, which then binds to β-adrenergic receptors and increases both the L-type Ca^{2+} current, $I_{Ca(L)}$, and the hyperpolarization-activated inward current, I_f (see Giles and Shibata, 1981; Brown, 1982; Tsien et al., 1986; Hartzell, 1988; Irisawa et al., 1993; DiFrancesco, 1993). Conversely, stimulation of the parasympathetic (vagus) nerve releases acetylcholine (ACh), which decreases heart rate by (*a*) directly activating a K^+ current, $I_{K(ACh)}$, and inhibiting I_f and by (*b*) antagonizing the effects of noradrenaline by reducing $I_{Ca(L)}$, the so-called indirect effect (Hartzell, 1988: Irisawa et al., 1993; DiFrancesco, 1993). This negative chronotropic effect of ACh in mammalian heart is mediated by the M_2 subtype of muscarinic receptors (Hartzell, 1988).

A number of different membrane-bound, heterotrimeric GTP-binding proteins (G proteins) participate in the signal transduction cascade initiated by sympathetic and parasympathetic stimulation (Gilman, 1987; Brown, 1990; Eschenhagen, 1993). For example, the G protein G_s mediates β-adrenoceptor stimulation through two principal pathways. One of these is a membrane-delimited or direct pathway in which there are interactions between G_s and some of the ion-selective channels, e.g., I_f and $I_{Ca(L)}$ (Brown, 1990; but see Clapham, 1994). This direct pathway does not involve biochemical second messengers. In contrast, the indirect pathway mediated by G_s requires activation of adenylyl cyclase, which results in increased cAMP levels, stimulation of cAMP-dependent protein kinase (PKA), and an increase in the phosphorylation of a number of target proteins, including $I_{Ca(L)}$ channels

(Hartzell, 1988; McDonald, Pelzer, Trautwein, and Pelzer, 1994). Both the direct and the indirect stimulatory effects are decreased after activation of the inhibitory G protein (G_i), which couples M_2 receptors to at least three different membrane currents: $I_{K(ACh)}$ and I_f, which are inhibited directly, and $I_{Ca(L)}$, which is inhibited indirectly (Gilman, 1987; Hartzell, 1988; Brown, 1991; DiFrancesco, 1993; Irisawa et al., 1993; Eschenhagen, 1993; Clapham, 1994).

In the myocardium, the indirect action of ACh is thought to be mediated by a pertussis toxin-sensitive G protein, G_i, which is coupled to M_2 receptors (Hescheler, Kameyama, and Trautwein, 1986; Fischmeister and Shrier, 1989; Nakajima, Wu, Irisawa, and Giles, 1990). However, many details of the mechanism by which G_i inhibits $I_{Ca(L)}$ are still unresolved (Fleming, Wisler, and Watanabe, 1992; Eschenhagen, 1993). Although high concentrations of the α_i subunit of G_i directly inhibit adenylyl cyclase, this inhibitory effect is only partial and is dependent on the subtype of this enzyme (Taussig, Iniguez-Lluhi, and Gilman, 1993). The $\beta\gamma$ subunits from G_i can directly inhibit the type-I brain adenylyl cyclase, but this isoform has not been identified in heart (Tang and Gilman, 1991), suggesting that other mechanisms (in addition to adenylyl cyclase inhibition) must be involved in the ACh-induced inhibition of $I_{Ca(L)}$. A plausible alternative is that the ACh-induced attenuation of $I_{Ca(L)}$ is mediated by cGMP (Hartzell, 1988). cGMP interacts with at least three intracellular sites: cGMP-regulated ion channels, cGMP-dependent protein kinase (PKG), and cGMP-regulated cyclic nucleotide phosphodiesterases (PDEs) (Lincoln and Cornwell, 1993). A cGMP-stimulated PDE that hydrolyses both cAMP and cGMP is present in heart (Hartzell, 1988; Beavo and Reifsnyder, 1990). In amphibian cells, cGMP activates a cGMP-stimulated cAMP-specific PDE that hydrolyses cAMP and thereby decreases $I_{Ca(L)}$ after it has been augmented by cAMP-elevating agents, e.g., β-adrenergic agonists (see Hartzell, 1988). On the other hand, a cGMP-dependent protein kinase plays a major role in the cGMP-induced decrease in $I_{Ca(L)}$ in mammalian myocardium (Levi, Alloatti, and Fischmeister, 1989; Mery, Lohmann, Walter, and Fischmeister, 1991; Mery, Pavoine, Belhassen, Pecker, and Fischmeister, 1993).

$I_{Ca(L)}$ is essential for normal pacemaker activity (Irisawa et al., 1993). Modulation of this current therefore represents an important mechanism for the autonomic control of heart rate. It is known that "physiological" vagal stimulation by a low dose of ACh significantly decreases the spontaneous firing rate without changing the maximum diastolic potential of the SAN, as expected for a selective inhibition of $I_{Ca(L)}$ (see Irisawa et al., 1993). However, the signal transduction pathway that leads to this ACh-induced attenuation of $I_{Ca(L)}$ has not been investigated in detail in the SAN of the mammalian heart (Petit-Jacques, Bois, Bescond, and Lenfant, 1993, 1994).

Muscarinic receptor activation by ACh is known to release nitric oxide (NO), and recent evidence has demonstrated that NO is an important second messenger involved in mediating the actions of neurotransmitters in the cardiovascular and central nervous systems (Bredt and Snyder, 1990; Bredt, Hwang, and Snyder, 1990; Moncada, Palmer, and Higgs, 1991; Finkel, Oddis, Jacobs, Watkins, Hattler, and Simmons, 1992; Calver, Collier, and Vallance, 1993; Dinerman, Lowenstein, and Snyder, 1993). The class of enzymes that form NO from arginine, NO synthases

(NOSs), have been identified in myocardial tissues (Klimaschewski, Kummer, Mayer, Couraud, Preissler, Philippin, and Heym, 1992) and cardiac myocytes (Schulz, Smith, Lewis, and Moncada, 1991; Schulz, Nava, and Moncada, 1992), but the physiological significance of NO synthesis remains unclear. In many different preparations, NO activates guanylyl cyclase and generates cGMP. Indeed, in cultured neonatal rat ventricular myocytes, agents known to inhibit NO synthase prevent the negative inotropic effect of ACh (Balligand, Kelly, Marsden, Smith, and Michel, 1993).

Recently, we (Han et al., 1994) have demonstrated that the cholinergic inhibition of $I_{Ca(L)}$ in isolated single SAN cells can be mimicked by an NO donor and that ACh cannot reduce $I_{Ca(L)}$ if NOS is inhibited. NO is therefore essential for the physiological control of heart rate occurring in the presence of adrenergic tone. However, the biochemical signaling pathways subsequent to NO generation remain undefined. In the current study, the following questions were addressed: (*a*) can NO that is released by cholinergic stimulation activate guanylyl cyclase? (*b*) Is cGMP involved in the carbamylcholine–NO inhibition of $I_{Ca(L)}$? (*c*) Can PKG or the cGMP-stimulated cAMP-specific PDE mediate carbamylcholine–NO attenuation of isoproterenol-stimulated $I_{Ca(L)}$?

METHODS

Cell Isolation

The technique used for isolation of single cells was the same as that described previously (Han, Habuchi, and Giles, 1994*a*; Han, Shimoni, and Giles, 1994*b*).

Solutions

The bicarbonate-buffered Tyrode's solution used for Langendorff perfusion of the whole heart contained (in millimolar): 121 NaCl, 5.0 KCl, 2.8 sodium acetate, 1.0 $MgCl_2$, 1.0 Na_2HPO_4, 24 $NaHCO_3$, 5.5 glucose, and 1.0 $CaCl_2$. This solution was equilibrated with 95% O_2 and 5% CO_2 (pH 7.4). The KB solution (Isenberg and Klockner, 1982) used for storing the cells contained (in millimolar): 90 potassium glutamate, 10 potassium oxalate, 25 KCl, 10 KH_2PO_4, 0.5 EGTA, 1.0 $MgSO_4$, 20 taurine, 5 HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), and 10 glucose (pH adjusted to 7.2 with KOH). The HEPES-buffered Tyrode's solution in which all experiments were performed contained (in millimolar): 145 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 1.0 Na_2HPO_4, 5.0 HEPES, 1.8 $CaCl_2$, and 10 glucose (pH adjusted to 7.4 with NaOH).

Recording Methods and Data Acquisition

In most experiments, the nystatin (0.4 mg/ml) perforated patch technique (Horn and Marty, 1988) in the whole-cell configuration (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) was used to current and voltage clamp these cells. The pipette solution contained (in millimolar): 140 KCl, 6 NaCl, 1 $MgCl_2$, and 5 HEPES (pH was adjusted to 7.2 with KOH). DC resistance was 1–3 MΩ. Nystatin was first dissolved in dimethylsulfoxide and then added to the pipette solution. In a few experiments, the conventional suction microelectrode ruptured patch whole-cell method was used. In these experiments, the pipette solution included (in millimolar): 95 potassium aspartate, 30 KCl, 5 HEPES, 3 Na_2phosphocreatine, 0.1 GTP (Na), 3 K_2ATP, 10 EGTA (ethyleneglycol-bis-(β-aminoethylether)-*N,N,N'N'*-tetraacetic acid), 1 $CaCl_2$, and 1 $MgCl_2$ (pH adjusted to 7.2 with KOH). The electrode resistance was between 2 and 4 MΩ. All recordings were made at 32–33°C.

Membrane currents were digitized at 2.5 kHz. The volume of the recording chamber was 0.5 ml, and a constant flow rate of 1.5 ml/min was used to superfuse the cells.

Drugs and Chemicals

Isoproterenol (ISO), carbamylcholine (CCh), L-arginine, nystatin, dimethylsulfoxide, methylene blue, 3-isobutyl-1-methyl-xanthine (IBMX), and 8-bromoguanosine-3′,5′-cyclic monophosphate (8-Br-cGMP) were purchased from Sigma Chemical Co. (St. Louis, MO). 3-Morpholino-sydnonimine (SIN-1) was obtained from Molecular Probes, Inc. (Eugene, OR). 8-(4-Chlorophenylthio)-guanosine-3′,5′-cyclic monophosphate (8-CPT-cGMP) and 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate (CPT-cAMP) were from BIOLOG (La Jolla, CA.). L-Nitro-arginine methyl ester (L-NAME) was purchased from BACHEM, Inc. (Torrance, CA). NG-monomethyl-L-arginine, monoacetate (L-NMMA) was obtained from Calbiochem Corp. (San Diego, CA). 6-Anilino-5,8-quinolinedione (LY83583) was a gift from Eli Lilly and Co. (Indianapolis, IN).

Statistics

Statistical significance was evaluated using an unpaired *t* test, where appropriate. Means with values of $P < 0.05$ were considered to be significantly different.

RESULTS

NO Involvement in Muscarinic Cholinergic Inhibition of $I_{Ca(L)}$

In a previous short communication (Han et al., 1994), we demonstrated that in rabbit SAN cells, inhibition of NOS by incubation in L-NMMA–containing Tyrode's solution for 10–20 min resulted in complete loss of the ability of CCh to attenuate $I_{Ca(L)}$. In the current study, we first confirmed this finding using a different NOS inhibitor, L-NAME (see Moncada, 1992). Fig. 1 shows a typical set of results. To obtain a consistent inhibitory effect, the SAN cells had to be incubated with relatively high concentrations of L-NAME (0.2–1 mM) for a long period (at least 3 h). This may be because L-NAME is an esterified analogue of arginine and must be deesterified before it is able to act on NOS (see Moncada, 1992). The rate of action of the appropriate intracellular esterase(s) may account for the necessity of high concentrations of L-NAME and lengthy exposure times, which have both been described previously (Balligand et al., 1993).

Fig. 1 compares results obtained under control conditions. (Fig. 1 *A*) and after incubation in L-NAME (Fig. 1 *B*). The three superimposed current traces in Fig. 1 *A* show that ISO (1 μM) enhanced $I_{Ca(L)}$ (*a* and *b*) and that CCh (1 μM) markedly attenuated this effect, as well as producing an outward shift in the holding current (*c*) that is due to activation of $I_{K(ACh)}$. Fig. 1 *B* shows corresponding current traces from a different cell that had been incubated in 1 mM L-NAME for 4 h. Under these conditions, ISO enhanced $I_{Ca(L)}$ (*a* and *b*), but CCh no longer attenuated it. Note, however, that the outward current, $I_{K(ACh)}$, still developed after CCh treatment (*c*). When the NOS inhibitor L-NMMA was used in this protocol, the pattern of results shown in Fig. 1, *A* and *B*, could be obtained in the same cell (Han et al., 1994). This could not be done with L-NAME, because the inhibitory action of this compound required a very long preincubation (before recording), and its effects were very slow to wash off. Fig. 1 *C* provides a summary of these effects, expressed as the per-

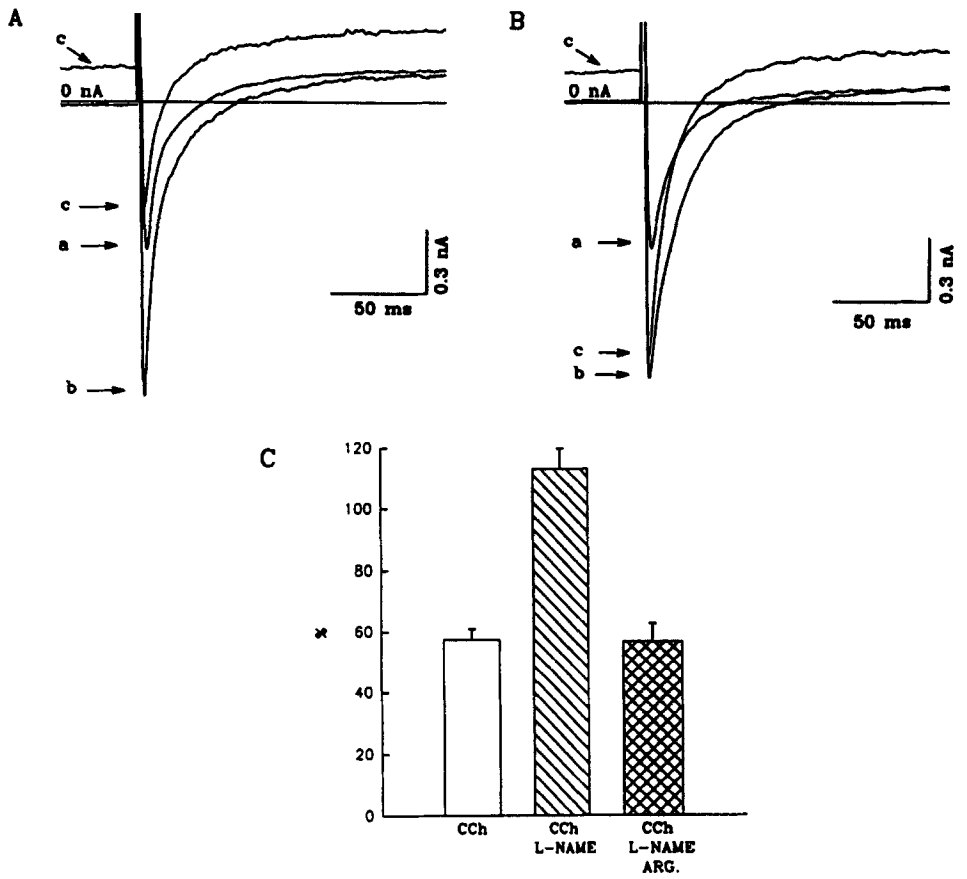

FIGURE 1. Effects of CCh on the ISO-stimulated $I_{Ca(L)}$. In this and subsequent experiments (Figs. 2–9), $I_{Ca(L)}$ was augmented by application of the β-adrenergic agonist ISO and was activated from a holding potential of −40 mV by applying 200-ms depolarizing steps to 0 mV at 0.067 Hz. (*A*) Superimposed current traces obtained in control (*a*), ISO (1 μM) (*b*), and ISO + CCh (1 μM) (*c*). The outward shift in the holding current reflects the activation of $I_{K(ACh)}$. (*B*) The same procedure was repeated in a different cell, which had been preincubated with 1 mM L-NAME for 4 h. In this case, ISO still enhanced $I_{Ca(L)}$ (*b*), and CCh shifted the holding current ($I_{K(ACh)}$), but did not attenuate $I_{Ca(L)}$ (*c*). (*C*) Summary of the mean (±SEM) changes in $I_{Ca(L)}$ (as a percentage of the current in ISO alone), produced by CCh alone, by CCh after incubation in L-NAME, and by CCh after coincubation in L-NAME and 1 mM L-arginine. Note that L-NAME incubation prevents the attenuation of $I_{Ca(L)}$ by CCh, and the addition of L-arginine to L-NAME restores the attenuation by CCh.

cent reduction (mean ± SEM) of ISO-stimulated $I_{Ca(L)}$. CCh alone reduced peak amplitude to 57 ± 4% of its magnitude in ISO (n = 24). After L-NAME incubation, peak amplitude of I_{Ca} was 113 ± 7% (n = 11), owing to the inhibitory effect of L-NAME on NOS and the resulting decreased NO production. Finally, note that the coincubation of cells in L-NAME with 1 mM L-arginine (the endogenous substrate of NOS) restored the ability of CCh to attenuate $I_{Ca(L)}$, i.e., $I_{Ca(L)}$ was reduced to

57 ± 6% (n = 4). The ability of CCh to attenuate $I_{Ca(L)}$ and of atropine to reverse this attenuation (data not shown), after up to 6 h of incubation in L-NAME, indicate that this NOS inhibitor does not directly interfere with other actions of the muscarinic agonist (e.g., its binding to receptors). Thus, although it has been suggested that L-NAME may act as a muscarinic antagonist (Buxton, Cheek, Eckman, Westfall, Sanders, and Keef, 1993), we find no evidence for this in SAN cells.

Our earlier work demonstrated that the NO donor SIN-1 had no effect in the absence of ISO, but that it attenuated ISO-stimulated $I_{Ca(L)}$ and did not further reduce $I_{Ca(L)}$ after maximal inhibition by CCh (Han et al., 1994). A prediction from these earlier results was that SIN-1, by directly supplying NO (bypassing NOS), should

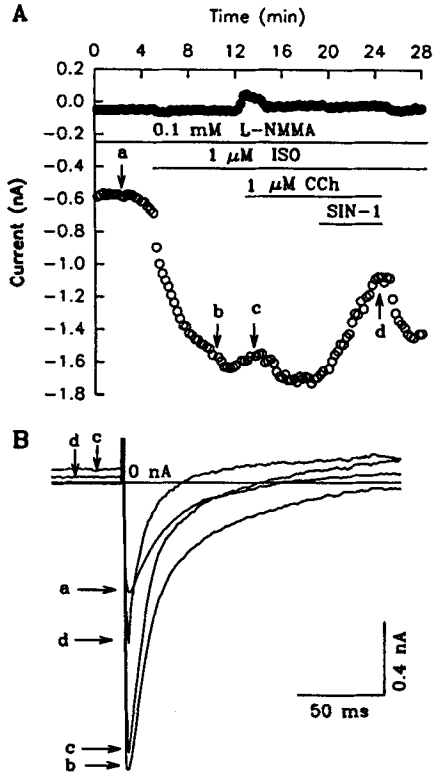

FIGURE 2. Effects of CCh (1 μM) and the NO donor SIN-1 (0.1 mM) on ISO-stimulated $I_{Ca(L)}$ in a SAN cell pretreated with L-NMMA. (*A*) Time course of changes in $I_{Ca(L)}$ and holding current in response to test agents. The horizontal bars denote the time during which each drug was applied. In this and subsequent figures, open circles (○) denote $I_{Ca(L)}$ and closed circles (●) denote the holding current level at −40 mV. The cell had been incubated with L-NMMA (0.1 mM) for 20 min before recording was initiated. The current records at the time points marked *a*, *b*, *c*, and *d* correspond to the superimposed traces in *B*. (*B*) Superimposed current traces obtained in control (*a*), ISO (*b*), ISO + CCh (*c*), and ISO + CCh + SIN-1 (*d*) solutions. Note that whereas L-NMMA treatment prevents the CCh attenuation of $I_{Ca(L)}$, it does not prevent the SIN-1-induced decrease in $I_{Ca(L)}$.

still attenuate $I_{Ca(L)}$ in the presence of the NOS inhibitors. In the current experiments (Fig. 2), we found when CCh-induced attenuation of $I_{Ca(L)}$ was inhibited by L-NMMA, addition of SIN-1 to the same cell indeed could still reduce $I_{Ca(L)}$.

Is cGMP Involved in These Actions of NO?

In many tissues, NO can activate guanylyl cyclase(s) (GC), which then generates cGMP. We therefore attempted to inhibit the action of NO on $I_{Ca(L)}$ in myocytes from rabbit SAN by applying methylene blue, a compound that can block NO-induced activation of GC (Michel and Smith, 1993; Brady, Warren, Poole-Wilson, Williams, and

Harding, 1993; Mery et al., 1993). In six cells, 5–10 μM methylene blue completely blocked the action of CCh on $I_{Ca(L)}$ in SAN cells. The peak amplitude of $I_{Ca(L)}$ was 97 ± 3% (n = 6) of the value in ISO plus methylene blue. However, in addition to blocking the inhibitory effect of CCh on $I_{Ca(L)}$, the activation of $I_{K(ACh)}$ was also inhibited completely. This pattern of results is illustrated in Fig. 3, *A* and *B*. When CCh was

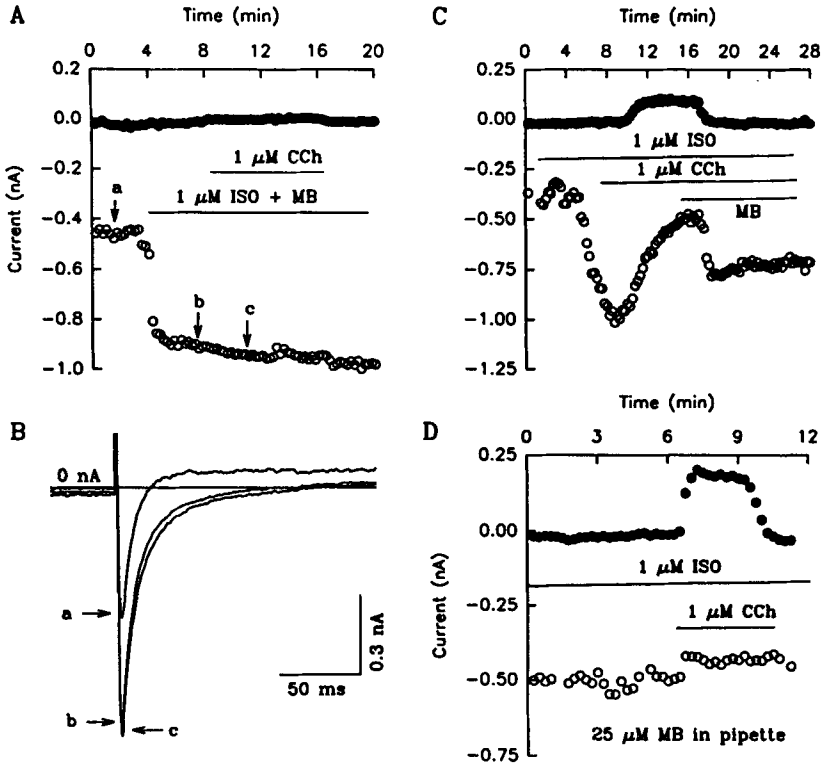

FIGURE 3. Effects of methylene blue (MB; 10 μM) on CCh (1 μM)–induced inhibition of $I_{Ca(L)}$. (*A*) Methylene blue blocks the CCh-induced inhibition of ISO-stimulated $I_{(Ca(L)}$. Note that there is also no activation of $I_{K(ACh)}$, indicated by the lack of shift in the holding current. The horizontal bars denote the time during which each drug was applied. (*B*) Superimposed current records at the time points marked *a*, *b*, and *c* in *A*. Traces are from control (*a*), ISO + MB (*b*), and ISO + MB + CCh (*c*) solutions. (*C*) Addition of methylene blue after CCh increases $I_{Ca(L)}$ and reduces $I_{K(ACh)}$, thus, reversing both effects of CCh (different cell). (*D*) The effects of CCh in a cell in which methylene blue (25 μM) was included in the pipette solution (whole-cell recording without nystatin). The outward shift in holding current was present, yet the attenuation of $I_{Ca(L)}$ was still prevented by methylene blue. (See text).

applied first, the subsequent addition of methylene blue reversed both CCh effects; i.e., it increased $I_{Ca(L)}$, and shifted the holding current inward, to its pre-CCh level. This result, seen in three cells, is shown in Fig. 3 *C*. A plausible explanation for these findings is that methylene blue can act as an antagonist at the muscarinic receptor. If this is the case, methylene blue-induced blockade of CCh effects cannot be taken as

convincing evidence for an NO–induced activation of guanylyl cyclase in the muscarinic action of CCh. To address this question, two series of experiments were performed. In the first, the suction electrode whole-cell method of recording was used, to enable intracellular dialysis with methylene blue. Although the rundown of $I_{Ca(L)}$ tends to be faster when using this method, the pipette solution used (see Methods) allowed sufficiently stable recordings over the required time period (15–25 min).

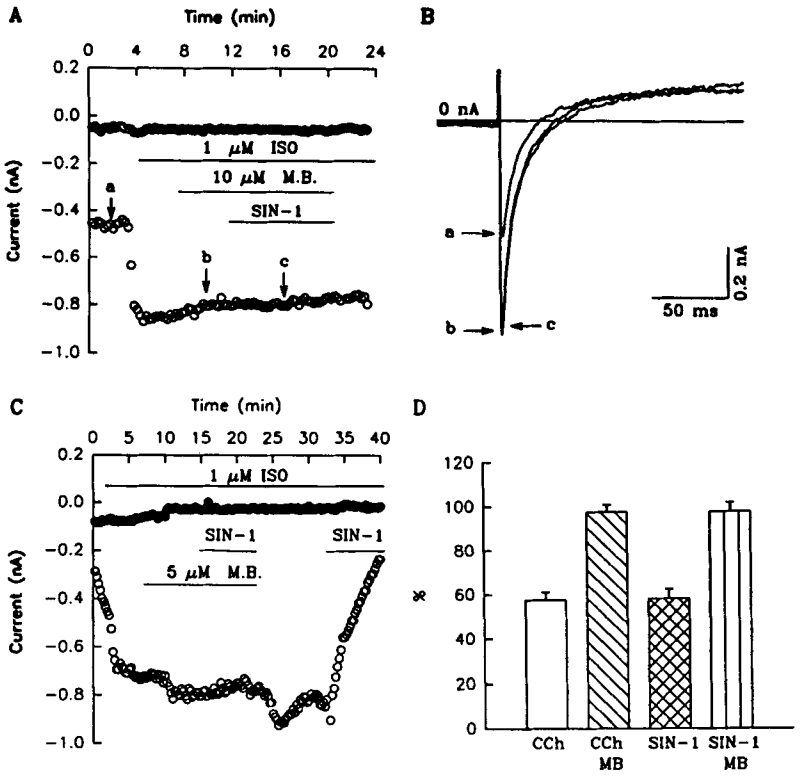

FIGURE 4. Effects of methylene blue (MB) on the action of the NO donor SIN-1. (*A*) Time course of $I_{Ca(L)}$ (○) and holding current (●) before (*a*) and after addition of ISO (*b*). The subsequent addition of 10 μM methylene blue had no direct effect, but prevented the attenuation of $I_{Ca(L)}$ by 0.1 mM SIN-1 (*c*). (*B*) Superimposed current traces from corresponding times *a*, *b*, and *c* in *A*. (*C*) In a different cell, 5 μM methylene blue prevented the attenuation of ISO-stimulated $I_{Ca(L)}$ by 0.1 mM SIN-1. After methylene blue washoff, 0.1 mM SIN-1 in the same cell greatly attenuated $I_{Ca(L)}$. (*D*) Summary of the effects of methylene blue on the attenuation of $I_{Ca(L)}$ by CCh and SIN-1. The bars represent the amplitude of $I_{Ca(L)}$ as a percentage, relative to that in ISO alone.

With this protocol, in eight cells, CCh activated $I_{K(ACh)}$ (because no methylene blue was present in the perfusate), yet it failed to attenuate $I_{Ca(L)}$ (Fig. 3 *D*). The mean value of peak $I_{Ca(L)}$ after CCh addition was 95 ± 3% of the value in ISO alone, which strongly supports NO involvement in CCh action. In the second series of experiments, SIN-1, an NO donor, was used in an independent test of this interpretation of the effects of methylene blue. SIN-1 directly releases NO, thus bypassing muscarinic

receptor and G protein activation. In the presence of ISO, SIN-1 reduced peak $I_{Ca(L)}$ to 58 ± 4% of the amplitude in ISO alone (n = 19). This effect of SIN-1 was blocked by methylene blue (n = 5; Fig. 4, *A* and *B*), as shown previously by Mery et al. (1993). The peak $I_{Ca(L)}$ in SIN-1 in the presence of ISO and methylene blue, was 98 ± 4% of its magnitude in ISO and methylene blue. Fig. 4 *C* shows an example in which SIN-1 failed to affect $I_{Ca(L)}$ in the presence of methylene blue. Thereafter, methylene blue

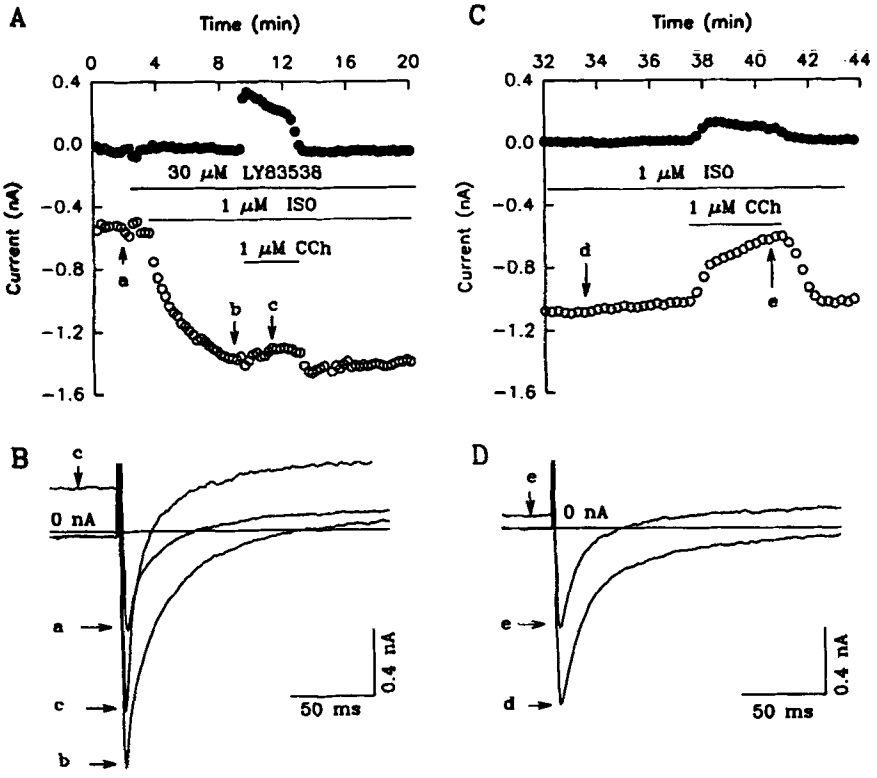

FIGURE 5. Effects of LY83583 (30 μM) on CCh (1 μM)–induced inhibition of $I_{Ca(L)}$. (*A*) LY83583 did not affect the increase in current by ISO (*b*), but blocked the CCh-induced inhibition of $I_{(Ca(L)}$ (*c*). The horizontal bars denote the time during which each drug was applied. (*B*) Superimposed current records at the time points marked *a* (*control*), *b* (ISO + LY83538), and *c* (ISO + LY83538 + CCh) in *A*. Note that LY83583 did not prevent the CCh-induced activation of $I_{K(ACh)}$. (*C*) In the same cell, LY83538 was washed off and CCh was reapplied in the presence of ISO alone. In the absence of LY83538, CCh reduced $I_{Ca(L)}$. (*D*) Superimposed current traces corresponding to *C*, from points *d* (ISO) and *e* (ISO + CCh), showing the reduction in $I_{Ca(L)}$.

was washed out, and a second exposure to SIN-1 then strongly attenuated the current. Fig. 4 *D* summarizes data describing the reduction in $I_{Ca(L)}$ by CCh and SIN-1 (as a percentage relative to the amplitude of $I_{Ca(L)}$ in ISO), in the presence and absence of methylene blue.

Guanylyl cyclase activation will elevate intracellular cGMP, which is one of the mediators of the muscarinic cholinergic effects on $I_{Ca(L)}$ in working myocardium

(Watanabe and Besch, 1975; Hartzell, 1988; Levi et al., 1989). To determine if this biochemical cascade is involved in the SAN, cells were exposed to ISO and CCh in the presence of LY83538, a compound that strongly inhibits both basal and CCh-stimulated cGMP production (Schmidt, Sawyer, Truex, Marshall, and Fleisch, 1984; Diamond and Chu, 1985; Mulsch, Luckhoff, Pohl, Busse, and Bassenge, 1989). In 11 cells, LY83538 (30 μM) did not significantly change the enhancement of $I_{Ca(L)}$ by

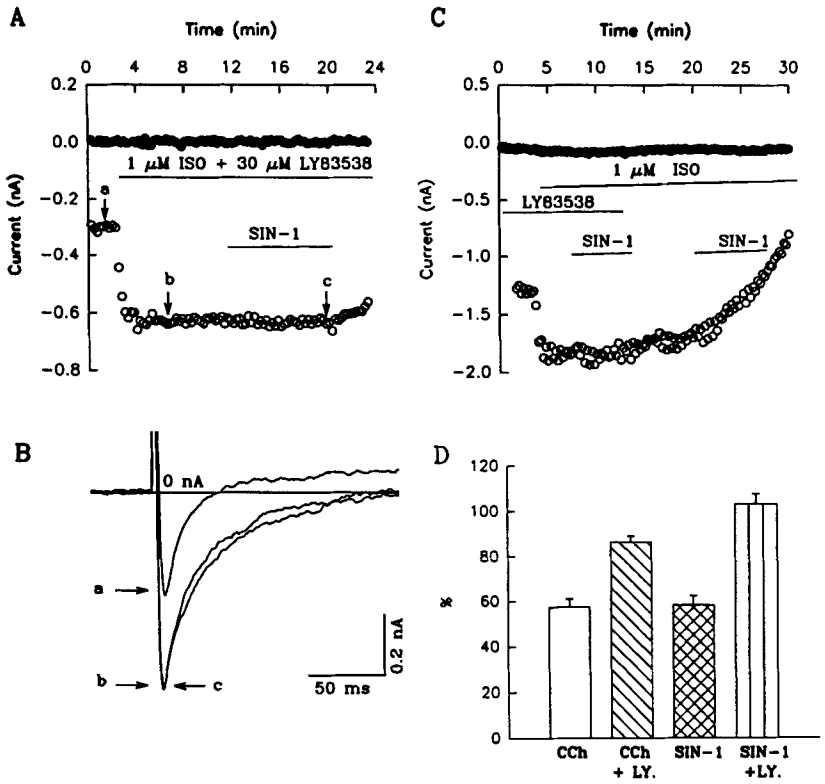

FIGURE 6. Effects of LY83538 on SIN-1 attenuation of $I_{Ca(L)}$. (*A*) Time course of $I_{Ca(L)}$ (○) and holding current (●) in control (*a*), ISO + LY83538 (*b*), and after exposure to 0.1 mM SIN-1 (*c*). (*B*) Superimposed current traces corresponding to points *a*, *b*, and *c* in *A*. (*c*), In a different cell, 0.25 mM SIN-1 was without effect on $I_{Ca(L)}$ in the presence of ISO + LY83538; however, SIN-1 attenuated $I_{CA(L)}$ after washout of LY83538. (*D*) Summary of the effects of LY83538 on the attenuation of $I_{Ca(L)}$ by CCh and by SIN-1. Note that LY83538 prevents attenuation of $I_{Ca(L)}$ by CCh and SIN-1.

ISO, but it blocked the inhibitory action of CCh on this current, without altering the activation in $I_{K(ACh)}$. The mean value of ISO-stimulated $I_{Ca(L)}$ after exposure to CCh, in the presence of LY83538, was 92 ± 5% of the value in ISO. An example of this pattern of results is shown in Fig. 5. Fig. 5 *A* shows the time course of changes in the holding current and in $I_{Ca(L)}$ after exposure to CCh. Fig. 5 *B* shows current traces recorded at the times indicated. In this and four other cells, LY83538 was

washed off and the cell was reexposed to CCh. This restored the attenuating effect of CCh on $I_{Ca(L)}$, as shown in Fig. 5, *C* and *D*. These findings strongly suggest that in rabbit SAN, CCh triggers the production of cGMP and that this elevation in cGMP can inhibit $I_{Ca(L)}$.

In other experiments (12 cells), LY83538 (30 μM) blocked the inhibitory action

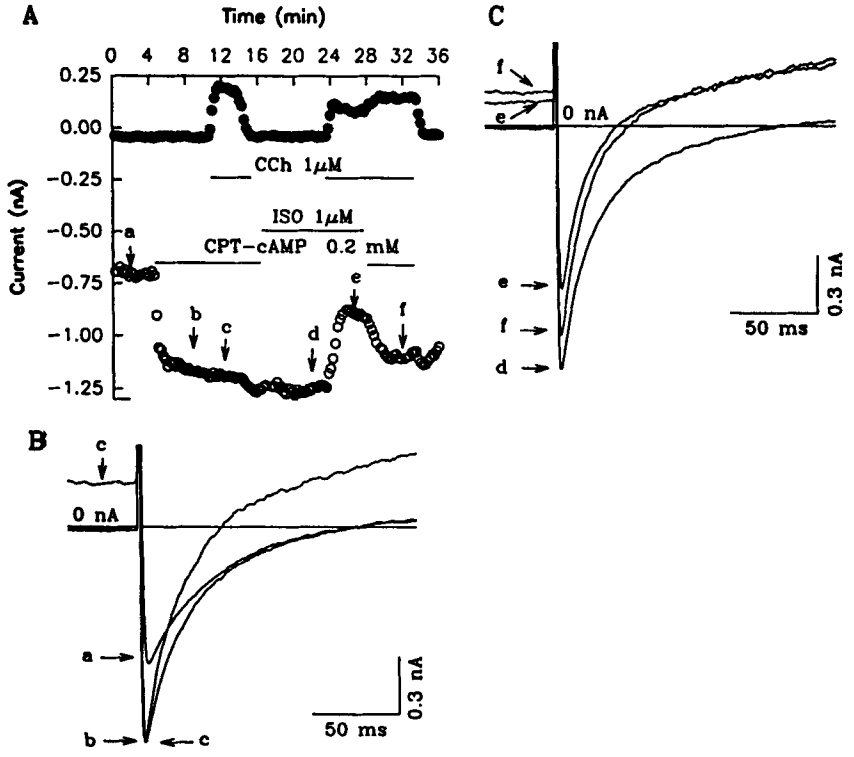

FIGURE 7. Effects of the membrane-permeable, nonhydrolyzable cAMP analogue CPT-cAMP on CCh action. (*A*) Time course of changes in $I_{Ca(L)}$ (○) and holding current (●) in response to each drug application. The horizontal bars indicate the time during which each drug was applied. The current records denoted by *a–f* correspond to the traces in *B* and *C*. Addition of 0.2 mM CPT-cAMP to the superfusate increased $I_{Ca(L)}$ (*b*), and the subsequent addition of CCh (1 μM) did not attenuate it (*c*), although CCh did activate $I_{K(ACh)}$ (outward shift in holding current). When CPT-cAMP was replaced by ISO (*d*), a second exposure to CCh produced the normal $I_{Ca(L)}$ attenuation (*e*). Switching back to CPT-cAMP (in the presence of CCh) reversed this attenuation (*f*), suggesting that $I_{Ca(L)}$ can be attenuated only when the degradable cAMP is present. (*B*) Superimposed current records corresponding to *a–c* in *A*. (*C*) Superimposed current traces corresponding to *d–f* in *A*.

of SIN-1 on ISO-stimulated $I_{Ca(L)}$. The mean magnitude of $I_{Ca(L)}$ in SIN-1 alone was 58 ± 4% of its magnitude in ISO; and after application of LY83538, it was 103 ± 4% (Fig. 6, *A* and *B*). In two cells, LY83538 was washed off, and a second exposure to SIN-1 also produced the expected attenuation in $I_{Ca(L)}$ (Fig. 6 *C*). A summary of the effects of LY83538 on the attenuation of $I_{Ca(L)}$ by both CCh and SIN-1 is shown in

Fig. 6 *D*. These findings further support our working hypothesis, in which NO production leads to an elevation of cGMP, which then results in the attenuation of $I_{Ca(L)}$ (see Discussion and Fig. 10).

To obtain more information about the biochemical mechanisms responsible for the observed muscarinic attenuation of $I_{Ca(L)}$, the biochemical events following elevation in cGMP were investigated. In frog myocardium, cGMP stimulates a PDE that augments cAMP breakdown (Hartzell, 1988; Simmons and Hartzell, 1988; Mery et al., 1993), thereby attenuating the ISO-stimulated $I_{Ca(L)}$. In mammalian ventricle, on the other hand, cGMP activates (a cGMP-dependent protein kinase) PKG, which leads to a reduction of $I_{Ca(L)}$ (Levi et al., 1989; Mery et al., 1991). We therefore studied whether either of these two mechanisms are operative in mammalian cardiac primary pacemaker cells.

Effects of Cyclic Nucleotide Analogues

Cyclic nucleotide analogues that are not hydrolyzed by intracellular PDE provide a useful experimental approach for evaluating the possible involvement of cyclic nucleotide–dependent PDE in the observed effects of NO. We exposed SAN cells ($n = 6$) to a membrane-permeable analogue of cAMP, CPT-cAMP, which directly activates PKA and is not degraded by PDE, thus resulting in a marked increase in $I_{Ca(L)}$. The effect of this compound on $I_{Ca(L)}$ is similar to that of β-adrenergic agonists. However, it bypasses the receptor-dependent, G_s-mediated activation of adenylyl cyclase, and its effects are maintained, as opposed to transient. In six cells, the mean augmentation of $I_{Ca(L)}$ was 183.5 ± 25.8% of the control magnitude. Moreover, after CPT-cAMP (0.2 mM), $I_{Ca(L)}$ was no longer reduced by CCh. The mean current amplitude after CCh treatment was 96.1 ± 4.0% of the prior value (Fig. 7). In two cells, CPT-cAMP was washed out and ISO was then introduced (point *d* in Fig. 7, *A* and *C*). Under these conditions (in the presence of normal β-adrenergic activation), CCh markedly reduced $I_{Ca(L)}$ (point e in Fig. 7, *A* and *C*). Subsequently, after ISO was removed and CPT-cAMP was added, the inhibitory action of CCh on $I_{Ca(L)}$ was partially reversed (point *f* in Fig. 7, *A* and *C*). This set of results implies that $I_{Ca(L)}$ can be attenuated by CCh only when cAMP can be degraded by a PDE. Note that the outward current, $I_{K(ACh)}$, activated by CCh is present under both conditions (CPT-cAMP and ISO), i.e., it is not dependent on these cyclic nucleotide levels. These results rule out any site of action that is at or subsequent to the activation of PKA in the CCh/NO-induced attenuation of $I_{Ca(L)}$. For example, the enhancement of phosphatase activity and the resulting dephosphorylation of the $I_{Ca(L)}$ channel would still occur in the presence of CPT-cAMP.

CCh-induced inhibition of $I_{Ca(L)}$ may result from an inhibition of adenylyl cyclase, as has been suggested previously in mammalian ventricle and atrium (Hescheler et al., 1986; Nakajima et al., 1990). This is thought to occur through muscarinic activation of G_i, which directly inhibits the G_s stimulation of adenylyl cyclase resulting from β-adrenergic receptor occupation. This possibility was evaluated ($n = 5$) in rabbit SAN cells, to which ISO was added with (or after exposure to) 10–20 μM IBMX, a nonselective inhibitor of a number of different PDEs. IBMX was applied with ISO to ensure that adenylyl cyclase was also activated. The combination re-

sulted in a mean increase in $I_{Ca(L)}$ to 162.5 ± 8.5% of its control value. Subsequent addition of CCh produced no inhibitory effect on $I_{Ca(L)}$ (the mean value after CCh was 118.8 ± 5.7% of the value before CCh); however, an outward shift in the holding current, signifying activation of $I_{K(ACh)}$, was present. IBMX was then washed out

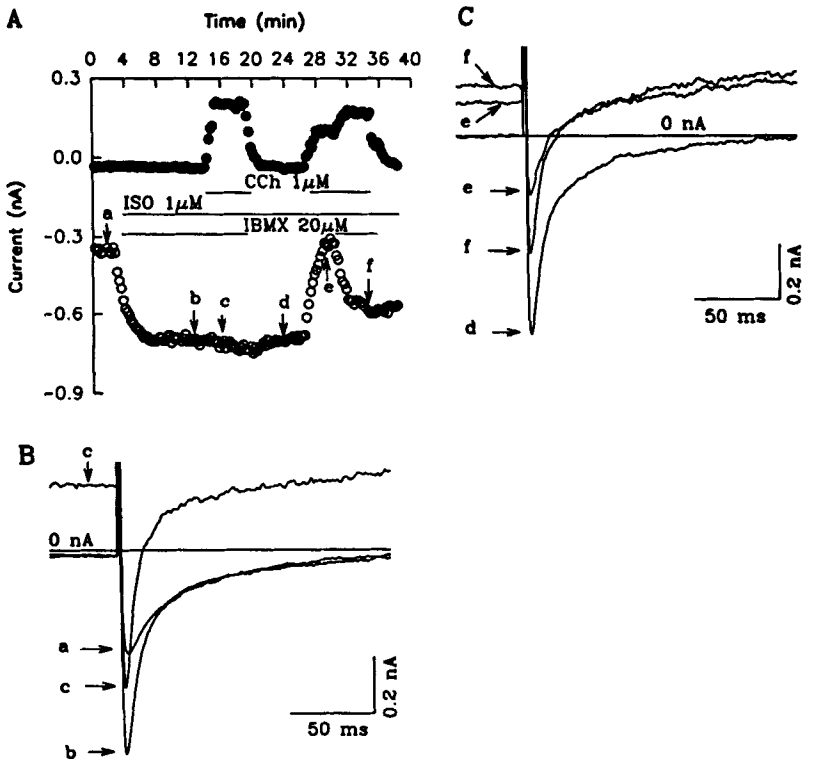

FIGURE 8. Effect of the nonspecific PDE inhibitor IBMX on CCh actions. (*A*) Time course of changes in $I_{Ca(L)}$ (○) and holding current (●) in response to each drug. The horizontal bars indicate the time during which each drug was applied. The current records denoted by the *a–j* correspond to the traces in *B* and *C*. In this cell, ISO was added with 20 μM IBMX, resulting in an increase in $I_{Ca(L)}$ (*b*). CCh (1 μM) did not change $I_{Ca(L)}$ under these conditions (*c*) but still activated $I_{K(ACh)}$. When IBMX was washed out (still in ISO; *d*), a second exposure to CCh strongly inhibited $I_{Ca(L)}$ (*e*). Reintroducing IBMX reversed this CCh-induced attenuation (*f*), presumably by preventing the stimulation of PDE by CCh (see text). Note that IBMX seems to potentiate the effects of CCh on $I_{K(ACh)}$ (the outward shift in *c* is unusually large, and at *f*, IBMX produces a secondary shift, with no change in CCh concentration). (*B*) Superimposed current records corresponding to *a–c*. (*C*) Superimposed current traces corresponding to points *d–f*.

(point *d* in Fig. 8, *A* and *C*), and when CCh was added for a second time, strong attenuation of $I_{Ca(L)}$ was observed (point *e* in Fig. 8, *A* and *C*) . Thereafter, reapplying IBMX reversed this effect of CCh (point *f* in Fig. 8, *A* and *C*). These results suggest that CCh does not reduce $I_{Ca(L)}$ by directly inhibiting adenylyl cyclase, because this

would have occurred even in the presence of IBMX. These findings also support the hypothesis that CCh inhibits $I_{Ca(L)}$ only when cAMP breakdown is enhanced, e.g., by activating the cGMP-stimulated PDE.

In our final series of experiments, two membrane-permeable analogues of cGMP,

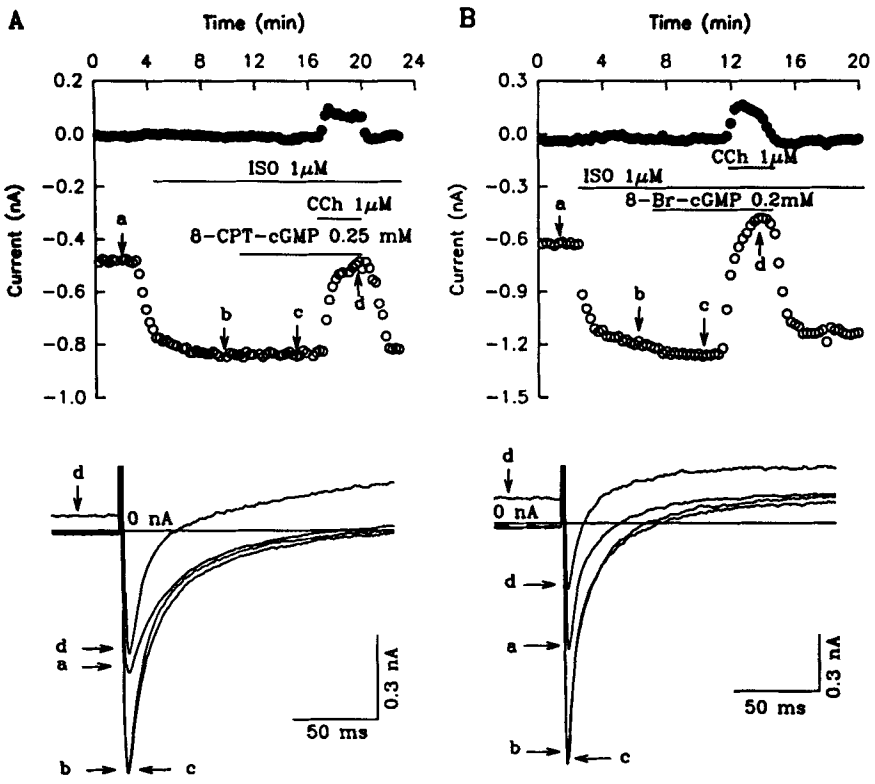

FIGURE 9. Effects of two membrane-permeable, nonhydrolyzable analogues of cGMP, 8-bromo-cGMP and 8-CPT-cGMP, on CCh actions. (*A*) 8-CPT-cGMP (0.25 mM) has no inhibitory effect on ISO-stimulated $I_{Ca(L)}$. (*B*) 8-Br-cGMP (0.2 mM) also does not inhibit the ISO-stimulated $I_{Ca(L)}$. In both *A* and *B*, the top row shows the time course of changes in $I_{Ca(L)}$ (○) and holding current (●) in response to each test agent and the bottom row shows the superimposed current traces obtained at the time points marked *a–d* above. The horizontal bars denote the time during which each agent was applied. Neither 8-CPT-cGMP (*c*) nor 8-Br-cGMP (*c*) inhibits ISO-stimulated $I_{Ca(L)}$ (*b*). Addition of CCh (1 μM) produced the expected attenuation of $I_{Ca(L)}$ along with the activation of $I_{K(ACh)}$ (*d*).

8-Br-cGMP and 8-CPT-cGMP, were used. Both of these analogues strongly and selectively activate PKG and are insensitive to breakdown by PDE (Lincoln and Cornwell, 1993; Geiger, Nolte, Butt, Sage, and Walter, 1992). Results from these experiments are shown in Fig. 9. Neither 8-Br-cGMP nor CPT-cGMP, when added after ISO, had an inhibitory effect on ISO-stimulated $I_{Ca(L)}$. The mean ($n = 5$) peak $I_{Ca(L)}$ amplitude in 8-Br-cGMP was 103.8 ± 2.2% (relative to ISO alone), and that in

8-CPT-cGMP was 93.9 ± 3.0% of the amplitude in ISO alone (n = 4), suggesting that PKG activation cannot attenuate $I_{Ca(L)}$. In the presence of 8-Br-cGMP to CCh reduced $I_{Ca(L)}$ to 56.2 ± 7.1% of the value in ISO; and in the presence of 8 CPT-cGMP $I_{Ca(L)}$ was reduced to 61.4 ± 9.6%. These findings suggest that cGMP resulting from CCh actions can reduce $I_{Ca(L)}$, presumably by activation of a PDE which enhances

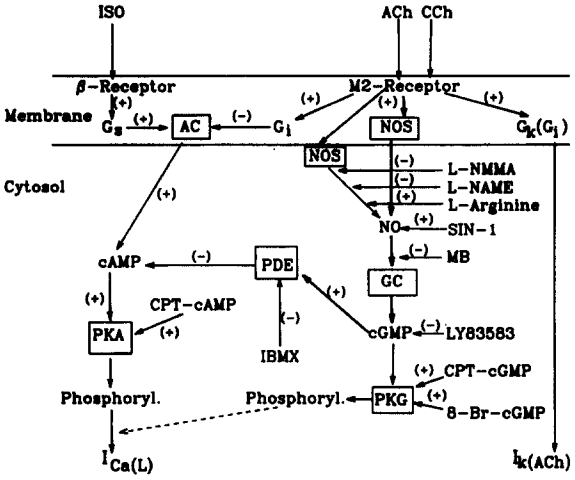

FIGURE 10. Diagram of our working hypothesis describing the cellular signaling pathways for NO-mediated cholinergic inhibition of $I_{Ca(L)}$ in the presence of adrenergic tone in rabbit SAN. Activation of NOS after the binding of CCh to the M_2 receptor results in NO production. This is blocked by L-NMMA or L-NAME, but this inhibition can be reversed by application of excess L-arginine. The NO donor SIN-1 directly provides NO, which stimulates guanylyl cyclase (*GC*) and elevates cGMP levels. Guanylyl cyclase activation is blocked by methylene blue (*MB*), and formation of cGMP is more selectively inhibited by LY83538. In this scheme, cGMP stimulates both the cGMP-dependent protein kinase (PKG) and the cGMP-stimulated cAMP-specific PDE. Blocking PDE by IBMX or the application of a nonhydrolyzable cAMP analogue, CPT-cAMP, prevents the CCh- or NO-induced reduction of $I_{Ca(L)}$. Two cGMP analogues that activate PKG without affecting PDE activity do not prevent the reduction of $I_{Ca(L)}$ by CCh. In this scheme, it is the cGMP-activated cAMP-specific PDE that hydolyzes ISO-elevated cAMP and decreases the $I_{Ca(L)}$.

cAMP breakdown. In contrast, the nonhydrolyzable analogues of cGMP that do not stimulate this class of PDE are without effect on $I_{Ca(L)}$.

DISCUSSION

Summary of Findings

Our results from primary pacemaker (SAN) cells of rabbit heart can be summarized using a pharmacological/biochemical working hypothesis that describes the cascade of events involved in cholinergic inhibition of L-type Ca^{2+} current, $I_{Ca(L)}$. This scheme and the interventions that we have used to test its validity are shown in Fig. 10. Under physiological conditions, the attenuation of $I_{Ca(L)}$, which is essential for the slowing of heart rate (Irisawa et al., 1994), takes place in the presence of a steady or background adrenergic tone. Under these conditions, modulation of $I_{Ca(L)}$ can occur at very low concentrations of ACh, which activate little if any outward K^+ current, $I_{K(ACh)}$ (see Irisawa et al., 1994). In all of our protocols, adrenergic tone was mimicked by application of ISO; and a relatively high concentration of

the muscarinic agonist CCh (1 μM) was used to ensure that $I_{K(ACh)}$ was also activated. $I_{K(ACh)}$, which was measured as an outward shift in the holding current (at −40 mV), provided an internal control so that a functional muscarinic response could be verified in all recordings.

Three important issues concerning the physiological modulation of $I_{Ca(L)}$ have been clarified by our results. First, the involvement of NO as an obligatory mediator of muscarinic cholinergic responses in the SAN in the presence of ISO was confirmed (see Han et al., 1994). This was done using two different inhibitors of NOS, either L-NAME or L-NMMA. Preincubation of cells with either compound consistently prevented the inhibitory action of CCh on $I_{Ca(L)}$ and this effect could be overcome by coincubation with an excess of L-arginine, the endogenous substrate of NOS (Figs. 1 and 2). In addition, the NO donor SIN-1, which can mimic the inhibitory effect of CCh (Han et al., 1994), was shown to retain its effect in the presence of L-NAME or L-NMMA (Fig. 2). Our results also demonstrate that the effects of CCh and of SIN-1 can be blocked by methylene blue, which is known to interfere with NO-induced activation of guanylyl cyclase and to decrease cGMP levels (Figs. 3 and 4). However, methylene blue blocked the effects of CCh on both $I_{Ca(L)}$ and $I_{K(ACh)}$, indicating that this compound has nonspecific effects (possibly muscarinic receptor blockade) in addition to inhibition of guanylyl cyclase.

The second important outcome from our work is the demonstration that in mammalian SAN cells the effects of CCh and of SIN-1 on $I_{Ca(L)}$ can be inhibited by the compound LY83538. Because LY83538 significantly reduces intracellular cGMP levels, this result strongly suggests that the NO-mediated inhibition of $I_{Ca(L)}$ involves the generation of cGMP in rabbit SAN. Furthermore, LY83583 had no effect on activation of $I_{K(ACh)}$, a muscarinic response that is not dependent on cGMP levels.

Third, our findings also suggest that cGMP-dependent activation of a cAMP-specific PDE is an important step in the indirect muscarinic modulation of $I_{Ca(L)}$. Increased activity of this isoform of PDE would be expected to decrease cAMP levels and reduce the phosphorylation of the L-type Ca^{2+} channels (Simmons and Hartzell, 1988). Accordingly, in the presence of nonhydrolyzable analogues of cAMP or the nonselective PDE inhibitor IBMX, CCh was without effect (Figs. 6 and 7; see also Wang and Lipsius, 1994). This finding rules out any direct inhibition of a cAMP-dependent protein kinase (PKA) by CCh, or a modulation of biochemical events subsequent to activation of PKA, e.g., enhanced dephosphorylation of the L-type Ca^{2+} channel by phosphatases. The lack of an inhibitory effect of CCh on $I_{Ca(L)}$ in the presence of ISO and IBMX or the nonhydrolyzable analogue of cAMP or CPT-cAMP strongly suggests that the inhibition of adenylyl cyclase by CCh (or elevated cGMP) does not play a significant role in the attenuation of $I_{Ca(L)}$ in rabbit pacemaker cells. However, it is conceivable that intracellular cAMP reaches saturating levels after exposure to IBMX and ISO, leading to maximal augmentation of $I_{Ca(L)}$. Under these conditions, CCh-induced decreases in the rate of cAMP formation might go undetected, because a sufficiently high concentration of cAMP may still be present to give maximal $I_{Ca(L)}$. To test this possibility, we applied IBMX after CCh (Fig. 8, *e* and *f*) . Under these conditions, if the major effect of CCh was to inhibit ISO-stimulated adenylyl cyclase, cAMP levels would be very low, IBMX-induced inhibition of PDE would have very small effects, and the resulting increase

in cAMP and $I_{Ca(L)}$ would be expected to be small. In fact, the effects of IBMX on $I_{Ca(L)}$ were very similar whether it was applied before or after CCh (see Fig. 8), implying that CCh does not act mainly on the adenylyl cyclase in rabbit SAN. Although elevated cGMP levels in response to CCh application and/or NO production can stimulate PKG, this enzyme does not appear to play a significant role in the cholinergic attenuation of $I_{Ca(L)}$ in rabbit SAN (Fig. 9), because membrane-permeable analogues of cGMP failed to change $I_{Ca(L)}$ significantly. This is in contrast to the effects of CCh, which markedly reduced $I_{Ca(L)}$.

Relation to Previous Work

The muscarinic modulation of $I_{Ca(L)}$ is known to involve two different biochemical pathways depending upon the species or cardiac tissue being studied. In mammalian ventricular cells (Levi et al., 1989; Mery et al., 1991) cGMP-dependent activation of a protein kinase (PKG) is necessary for the reduction in $I_{Ca(L)}$. On the other hand, in frog myocardium (Hartzell, 1988; Mery et al., 1993), the most important biochemical reaction appears to be cGMP-induced stimulation of a PDE, which then selectively breaks down cAMP. The resulting reduction in cAMP levels attenuates $I_{Ca(L)}$. Our data show that in mammalian pacemaker cells the biochemical scheme that has been identified in frog ventricle is operative.

Limitations of Our Approach

Our data are consistent with, but do not unequivocally prove, the working hypothesis outlined in Fig. 10. For example, if IBMX or 8-CPT-cAMP can inhibit adenylyl cyclase indirectly, perhaps through a negative feedback control mechanism activated by very high levels of cAMP (see Iyengar, 1993), no further inhibition of adenylyl cyclase by CCh would be observed. In addition, a cGMP-induced inhibition of PKA or a cGMP-dependent activation of a phosphatase that acts directly on $I_{Ca(L)}$ channels might be effective in the presence of cAMP, but conceivably may not occur in the presence of the cAMP analogue CPT-cAMP. The isoform of the PDE activated by cGMP needs to be identified in SAN cells. Further work is also required to elucidate how NOS is activated after application of muscarinic agonists.

We thank Dr. D. Severson of the Departments of Pharmacology and Therapeutics for useful discussions and constructive comments. We are grateful to K. Burrell and C. Collins for skilled secretarial assistance.

This work was supported by the Medical Research Council of Canada (MRC), the Heart and Stroke Foundation of Alberta, and the Alberta Heritage Foundation for Medical Research (AHFMR), and the Canadian Diabetes Association. X. Han holds a postdoctoral fellowship from the MRC, and W. R. Giles is an AHFMR Medical Scientist.

Original version received 27 May 1994 and accepted version received 1 February 1995.

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