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Regulation of Calcium Current by Low-K_m Cyclic AMP Phosphodiesterases in Cardiac Cells

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SUMMARY

The voltage-gated Ca^{2+} current (I_{Ca}) in cardiac myocytes is regulated by cAMP-dependent phosphorylation. Although the regulation of I_{Ca} via mechanisms involving modulation of cAMP synthesis is well understood, the regulation of cAMP degradation has been less thoroughly investigated. The goal of the present study was to investigate the participation of different subclasses of cAMP phosphodiesterase (PDE) in regulating cAMP-dependent phosphorylation of Ca^{2+} channels in frog ventricular myocytes. Cardiomyocytes were isolated enzymatically and mechanically and were patch-clamped using the whole-cell configuration of the patch-clamp technique. The effects of various low-K_m cAMP PDE inhibitors on I_{Ca} were examined. None of the inhibitors tested [milrinone, indolidan, 1-methyl 3-isobutyl xanthine (MIX), rolipram, or Ro 20-1724] were able to elevate I_{Ca} in the absence of elevated cAMP, although they all increased I_{Ca} in the presence of submaximal levels of cAMP. This result suggests that these compounds do not act directly on Ca^{2+} channels but rather modulate cAMP degradation. Half-maximal effects were observed with 1.4 μM milrinone and 3.4 μM MIX. Milrinone was effective when applied from either the extracellular or intracellular surface, whereas MIX was effective only when applied from the extracellular solution. In the presence of internal cGMP, which stimulates the cGMP-stimulated PDE, the low-K_m cAMP PDE inhibitors had no effect on I_{Ca}, whereas high concentrations of MIX, which inhibit the cGMP-stimulated PDE, increased I_{Ca}. This would support the hypothesis that cGMP-stimulated PDE either has a much stronger capacity to hydrolyze cAMP or is more efficiently coupled to Ca^{2+} channels than the low-K_m cAMP PDEs.

Phosphorylation of cardiac Ca^{2+} channels by cAMP-dependent protein kinase (1, 2) is the final step in a cascade of events that may be regulated at the locus of either cAMP production (i.e., adenylyl cyclase) or cAMP degradation (i.e., PDEs). Indeed, stimulation of adenylyl cyclase by β-adrenergic agonists or forskolin (3–5), external application of cAMP analogues or PDE inhibitors (6, 7), or intracellular application of cAMP or 8-bromo-cAMP (8–11) enhances I_{Ca} in cardiac cells of various species. Similarly, inhibition of adenylyl cyclase by acetylcholine (3, 10) or stimulation of a cAMP PDE by cyclic GMP (11, 12) reduces I_{Ca}. This evidence strongly suggests that I_{Ca} can be used as a good sensor of cAMP levels in cardiac cells. This is supported by the finding that the relationship between internally perfused cAMP concentration and I_{Ca} is well described by the mass action equation (12).

Although the regulation of I_{Ca} through mechanisms that modulate cAMP synthesis is relatively well documented, the regulation of cAMP hydrolysis is less well understood. According to current guidelines for PDE nomenclature (13–15), cAMP degradation is catalyzed by at least four classes of PDEs. 1) Ca-calmodulin-regulated PDEs hydrolyze both cAMP and cGMP. 2) cGMP-stimulated PDE hydrolyzes cAMP in a relatively high range of concentrations (10–100 μM). 3) cGMP-specific PDEs that have a ~50-fold lower K_m for cGMP than for cAMP have been described in photoreceptor, lung, and platelet but not in heart. 4) Low-K_m cAMP PDEs, all of which have a high selectivity for cAMP as substrate ($K_m < 1$ μM), exist as several different subclasses. One subclass is inhibited by low concentrations of cGMP and bipyridine PDE inhibitors such as milrinone and is termed cGMP-inhibited PDE. A second subclass is not regulated by cGMP and is inhibited by Ro 20-1724 and rolipram. It is interesting that cGMP stimulates one form of PDE and inhibits another form of PDE in the same cell (15–18), but how these different types of PDEs contribute to the regulation of cAMP levels and I_{Ca} remains unclear.

Participation of cGMP-stimulated PDE in the regulation of I_{Ca} by cGMP has been recently demonstrated in frog ventricular cells, where intracellular perfusion with cGMP produces a decrease in I_{Ca} enhanced by a variety of treatments that elevate internal cAMP (11, 12). The hypothesis that I_{Ca} is regulated by cGMP-stimulated PDE is supported by considerable biochem-

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ABBREVIATIONS: PDE, phosphodiesterase; I_{Ca}, Ca^{2+} current; MIX, 1-methyl-3-isobutylxanthine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PIPES, piperazine-*N,N′*-bis(2-ethanesulfonic acid).

ical and electrophysiological evidence (2). This evidence includes 1) the characterization in frog ventricular cells of a cGMP-stimulated PDE activity with the same sensitivity to cGMP as I_{Ca} (19) and 2) a similar inhibition of the effects of cGMP on I_{Ca} and cGMP-stimulated PDE by high concentrations of MIX (12, 19). The fact that, in our experiments, cGMP only inhibits and does not stimulate I_{Ca} indicates either that cGMP-inhibited PDE does not participate in the regulation of I_{Ca} or that cAMP degradation under these conditions is dominated by cGMP-stimulated PDE. However, it seems likely that cGMP-inhibited PDE does participate in I_{Ca} regulation, because specific inhibitors of cGMP-inhibited PDE, which are among the most potent cardiotonic agents now available, produce an increase in Ca transients (20) and exert their positive inotropic effects partly through a stimulation of Ca influx (21–23).

In the present study, we attempted to clarify the participation of various subclasses of low-K_m cAMP PDEs in the regulation of I_{Ca} in cardiac cells. To do this, we investigated the effects of various low-K_m cAMP PDE inhibitors. The effects of milrinone (WIN 47203), indolidan (LY 195115), MIX, rolipram, and Ro 20-1724 were studied on whole-cell I_{Ca} recorded from frog ventricular cells. All these compounds stimulated I_{Ca} in a manner consistent with their inhibitory effects on PDEs. However, none of these compounds was able to elevate I_{Ca} when cGMP-stimulated PDE had been maximally stimulated by cGMP. This would support the assumption that cGMP-stimulated PDE either has a much stronger capacity to hydrolyze cAMP or is more efficiently coupled to Ca channels than the low-K_m cAMP PDEs. A preliminary report of some of these results has appeared elsewhere (24).

Materials and Methods

Electrophysiology. Experiments were performed both in Atlanta and in Orsay, with no significant differences in the results. In both places, ventricular cells were enzymatically dissociated from frog [*Rana catesbiana* (in Atlanta) or *Rana esculenta* (in Orsay)], according to methods published in detail elsewhere (5, 25). The cells were patch-clamped, superfused, and internally perfused as described in detail (3, 11, 12). Frog ventricular cells were depolarized every 8 sec from −80 mV holding potential to 0 mV for 200 or 400 msec. I_{Ca} was routinely measured on-line as the difference between peak inward current and the current at the end of the pulse (3). The experiments were performed at room temperature (19–24°).

Solutions. In all experiments, control external solution contained (in mM): 115 NaCl, 20 CsCl, 1.8 $MgCl_2$, 1.8 $CaCl_2$, 5 Na pyruvate, 5 glucose, 3×10^{-4} tetrodotoxin, and 10 HEPES, pH 7.4. Patch electrodes (0.5–2 MΩ) were filled with internal solution C.3M (Atlanta experiments) or MIG142 (Orsay experiments), with no significant differences in the results. The internal solutions contained (in mM): 1) C.3M: 116.8 CsCl, 3.04 $MgCl_2$, 3.26 Na_2ATP, 5 K_2EGTA, 5 Na_2CP, 0.00097 $CaCl_2$ (pCa, 10.42), and 10 K·PIPES, pH 7.15, adjusted with CsOH; or 2) MIG142: 119.8 CsCl, 4 $MgCl_2$, 3.1 Na_2ATP, 5 K_2EGTA, 5 Na_2CP, 0.42 Na_2GTP, 0.062 $CaCl_2$ (pCa 8.5), and 10 HEPES, pH 7.1, adjusted with KOH.

Drugs. The drugs used in the experiments were (−)-isoprenaline (Sigma Chemical Co., St. Louis, MO), MIX (Sigma), cyclic AMP (Sigma), and forskolin (Calbiochem, San Diego, CA). The PDE inhibitors were generously supplied as follows: milrinone (WIN 47203) [1,6-dihydro-2-methyl-6-oxo(3,4′-bipyridine)-5-carbonitrile], Dr. A. E. Soria, Sterling-Winthrop; indolidan (LY 195115) [1,3-dihydro-3,3-dimethyl-5-(1,4,5,6-tetrahydro-6-oxo-3-pyridazinyl)-2*H*-indol-2-one], Dr. M. L. Johnson, Lilly Research Laboratories; Ro 20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidione], Dr. P. F. Sorter, F. Hoffman LaRoche; and rolipram [4-[3-(cyclo-pentoxy)-4-methoxyphenyl]-2-pyrrolidinone], Berlex Laboratories. All PDE inhibitors were prepared as stock solutions of 10 mM in anhydrous dimethyl sulfoxide. Forskolin was prepared as a stock solution of 10 mM in anhydrous ethanol. Control solutions contained the same amount of dimethyl sulfoxide or ethanol as the solutions containing PDE inhibitors or forskolin.

Statistical analysis. Data are expressed as mean ± standard error of the number of points indicated.

Results

Effects of milrinone and MIX on I_{Ca}. Fig. 1 shows the effects of external application of 1 and 10 μM milrinone, an inhibitor of cGMP-inhibited PDE, on I_{Ca} recorded from a frog ventricular cell. When the cell was exposed to milrinone under basal conditions, I_{Ca} was not significantly modified. In six cells exposed to 10 μM milrinone under basal conditions, I_{Ca} was 141.6 ± 17.4 pA before and 151.0 ± 21.1 pA after an average 2.8 ± 0.5-min exposure to milrinone. MIX (1–30 μM), which is a nonselective PDE inhibitor at high micromolar concentrations but inhibits the Ca-calmodulin-stimulated PDE, the rolipram-sensitive PDE, and the cGMP-inhibited PDE with K_i values of 2.5, 15, and 2 μM, respectively (15, 18), did not modify basal I_{Ca} either (Fig. 2; second exposure to MIX at 25 min). The absence of effects of milrinone and MIX on basal I_{Ca} suggests that these compounds do not directly activate Ca channels. If milrinone and MIX act by inhibiting PDE activity, one would expect them to increase I_{Ca} only when cAMP levels are elevated.

We, therefore, examined the effect of milrinone and MIX on I_{Ca}, which had been increased by various manipulations to elevate intracellular cAMP. Milrinone and MIX significantly enhanced forskolin-, isoprenaline-, or cAMP-elevated I_{Ca} (Figs. 1 and 2), in contrast to the absence of effect on basal I_{Ca}. As shown in Fig. 2, the relative stimulatory effects of these PDE inhibitors could possibly vary, depending on the stimulatory pathway used to enhance I_{Ca}. Therefore, we chose the most direct pathway to stimulate I_{Ca}, i.e., intracellular perfusion with

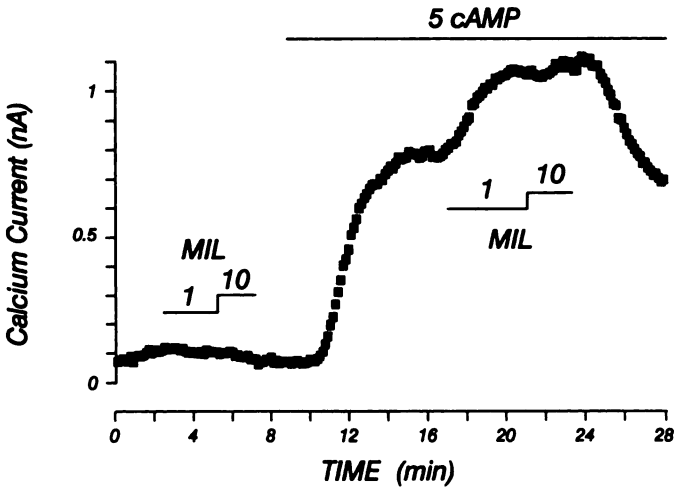

Fig. 1. Effect of milrinone (*MIL*) on I_{Ca}. I_{Ca} was elicited by 400-msec pulses from −80 mV to 0 mV once every 8 sec. Each *square* represents the net I_{Ca}, measured as the difference between the peak inward current and the current at the end of the pulse. K currents were blocked with intracellular and extracellular Cs and the fast Na current was blocked with tetrodotoxin, as described (3).

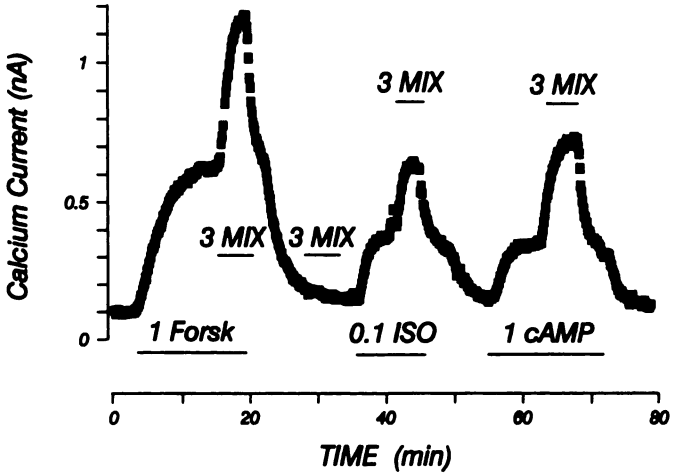

Fig. 2. Effect of MIX on I_{Ca} elevated by intracellular forskolin (*Forsk*), isoprenaline (*ISO*), or intracellular cAMP. The cell was first exposed extracellularly to combinations of 1 μM forskolin, 0.1 μM isoproterenol, and 3 μM MIX, as shown. The cell was then perfused internally with 1 μM cAMP for the period shown.

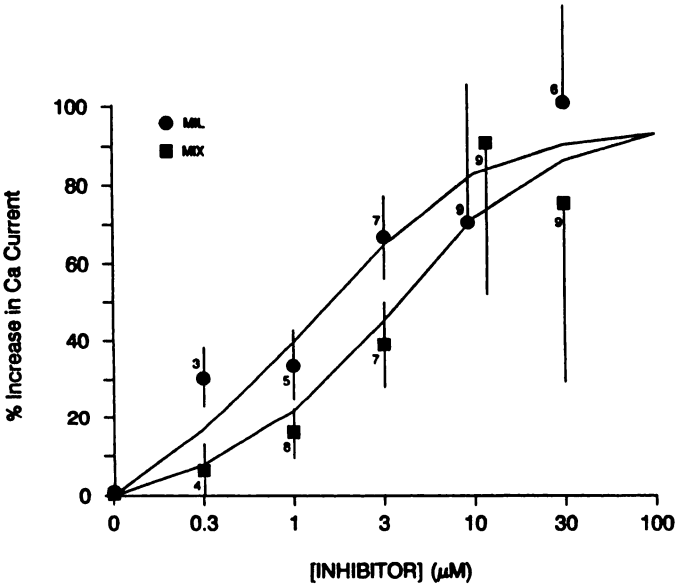

Fig. 3. Concentration-response curves for the effect of milrinone and MIX on I_{Ca} elevated by intracellular perfusion with cAMP. Cells were perfused internally with 1 μM cAMP and then exposed to various concentrations of either milrinone (MIL) (●) or MIX (■), as shown on the *x*-axis. The effect of the drugs is expressed as the percentage increase relative to the current in the presence of cAMP alone. *Error bars*, standard error; *numbers adjacent to the symbols*, number of cells. *Lines*, nonlinear least-mean-squares fits to the Michaelis equation. The best-fit parameters are given in the text.

cAMP, for performing a quantitative analysis of the effects of milrinone and MIX on I_{Ca}.

Fig. 3 shows the concentration-response curves for the stimulatory effects of milrinone and MIX on I_{Ca}. The data represent the percentage of stimulation of I_{Ca} above the current amplitude elevated by 1 μM cAMP. This concentration of cAMP by itself elevated I_{Ca} by 342.1 ± 38.5% (n = 30) over basal. Significant effects were observed with 0.3 μM milrinone and 1 μM MIX. MIX, at 1 μM, should not significantly affect cGMP-stimulated PDE, which is inhibited by MIX with a K_i of >30 μM (15, 18, 19). The data of Fig. 3 were fitted using a nonlinear least-mean-squares regression of the means to the Michael equation. Maximal stimulation of I_{Ca} (E_{max}) and concentrations of milrinone or MIX required for half-maximal stimulation of I_{Ca} (EC_{50}) were derived from this analysis. EC_{50} and E_{max} were, respectively, 1.4 μM and 94.8% for milrinone and 3.4 μM and 96.6% for MIX.

Site of action. Low-K_m cAMP PDE activities have been found in both soluble and particulate (membrane-associated) fractions of various cardiac tissues (15, 26–29). If MIX and milrinone affect I_{Ca} by inhibiting low-K_m PDEs competitively with cAMP (15, 17, 18), one would expect that the binding sites for MIX and milrinone would be intracellular. Because these drugs are lipophilic, extracellular milrinone and MIX could enter the cell by partitioning into the bilayer. If this is true, MIX and milrinone should produce similar effects when applied intracellularly. The following experiments were aimed at comparing the effects on I_{Ca} of extracellular and intracellular applications of milrinone and MIX.

Fig. 4 shows an experiment where 10 μM milrinone was perfused internally into a cell after I_{Ca} had been elevated with 2.5 μM cAMP. Milrinone produced a stimulation of I_{Ca} that resembled the effect produced by external milrinone. In seven experiments where 10 μM milrinone was perfused internally for an average of 7.9 ± 0.6 min in the presence of 2.5 μM cAMP, I_{Ca} increased by 52.8 ± 20.8% ($p < 0.05$). This effect was comparable in magnitude to that achieved with extracellular milrinone (Fig. 3). Thus, the site(s) of action of milrinone seem(s) to be equally accessible to the drug from both sides of the membranes.

Similar experiments were performed with MIX. Fig. 5 shows an experiment where the effects of internal and external applications of MIX on I_{Ca} elevated by 5 μM cAMP were compared in the same cell. Whereas 10 μM MIX produced a substantial and reversible increase in I_{Ca} when applied from outside the cell, a 10-fold higher concentration of MIX applied inside the cell did not modify the current. Intracellular MIX also did not modify the response of I_{Ca} to a second external application of 10 μM MIX. The absence of effect of intracellular MIX could not be attributed to a lack of diffusional access to the interior

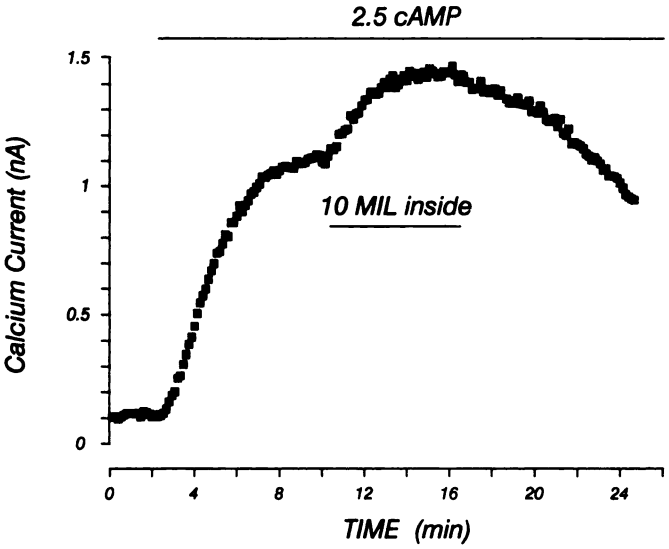

Fig. 4. Effect of internally perfused milrinone on I_{Ca} elevated by internal perfusion with cAMP. The cell was first perfused internally with 2.5 μM cAMP. When the current stabilized, 10 μM milrinone (*MIL*) was added to the internal perfusion during the period indicated.

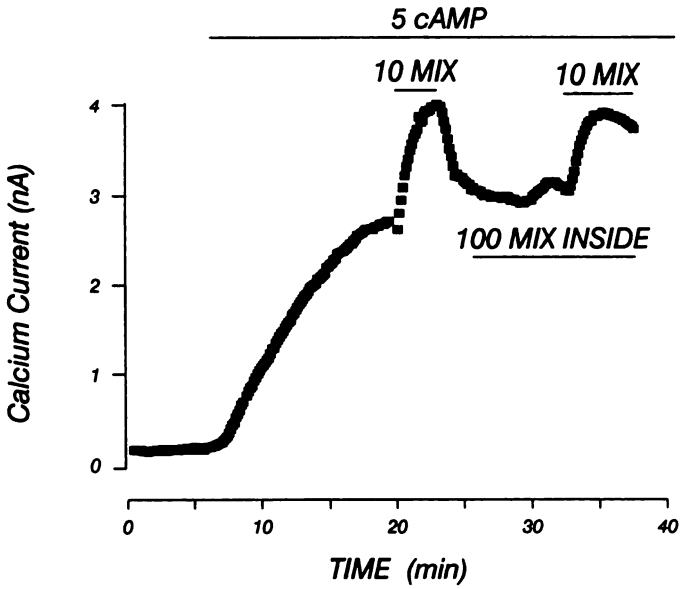

Fig. 5. Lack of effect of internal MIX on I_{Ca} elevated by 5 μM cAMP. I_{Ca} was first elevated by internal perfusion with 5 μM cAMP and then 100 μM MIX was perfused internally or 10 μM MIX was applied extracellularly, as indicated. Similar absence of effect was observed with lower internal MIX concentrations as well.

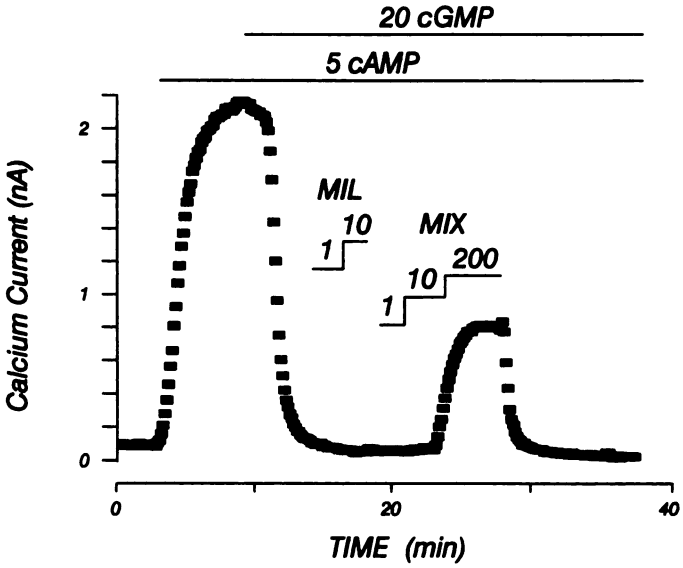

Fig. 6. Effect of milrinone and MIX on I_{Ca} in the presence of cAMP and cGMP. I_{Ca} was increased by internal perfusion with 5 μM cAMP and then reduced by addition of 20 μM cGMP to the internal perfusion solution, as indicated. The cell was then exposed to 1 or 10 μM milrinone (*MIL*) or 1, 10, or 200 μM MIX.

of the cell, because intracellular perfusion with 5 μM cAMP produced a large stimulation of I_{Ca}. In four cells where I_{Ca} was previously enhanced by 1–5 μM cAMP and internal perfusion was judged to be working properly by normal responses to cAMP, I_{Ca} was 98.7 ± 3.6% of its cAMP-elevated value (mean ± SE) after 6.8 ± 0.6 min of intracellular perfusion with 100 μM MIX. A similar absence of effect was seen with 10 μM internal MIX. These results confirm our previous findings (12, 19) that MIX is able to stimulate I_{Ca} only when applied from outside the cell.

Effects of milrinone and MIX on cGMP-stimulated PDE. A cGMP-stimulated PDE has been characterized in frog ventricular cells by both electrophysiological and biochemical methods (11, 12, 19). The main characteristic of this PDE is that hydrolysis of cAMP is stimulated by micromolar concentrations of cGMP (15, 16). cGMP-stimulated PDE plays a major role in the regulation of I_{Ca} by cGMP (11, 12). As shown earlier (11), intracellular perfusion of a frog ventricular cell with 20 μM cGMP completely antagonizes the stimulatory action of 5 μM cAMP (Fig. 6). Fig. 6 shows, in addition, that, in the presence of a maximal concentration of cGMP, 1–10 μM milrinone or MIX was completely ineffective in stimulating I_{Ca}. In this concentration range, milrinone and MIX inhibit low-K_m cAMP PDEs and have no effect on cGMP-stimulated PDE, which is inhibited with a K_i of 150 μM milrinone (15, 18) and 30 μM MIX (19). When the concentration of MIX was increased to 200 μM, a concentration expected to inhibit cGMP-stimulated PDE (15, 19), I_{Ca} increased to about 40% of its cAMP-elevated amplitude (11, 12). In five similar experiments, 1 and 10 μM levels of either milrinone or MIX had no detectable effect on I_{Ca} in the presence of 5 μM cAMP plus 20 μM cGMP (Fig. 7). Increasing the concentration of MIX to 100 and 200 μM rescued I_{Ca} to, respectively, 38.2 ± 14.1% (n = 2) and 68.8 ± 7.3% (n = 3) of its value in cAMP alone (Figs. 6 and 7). After cGMP had been washed out of the intracellular perfusion system and I_{Ca} had returned to a normal level of stimulation

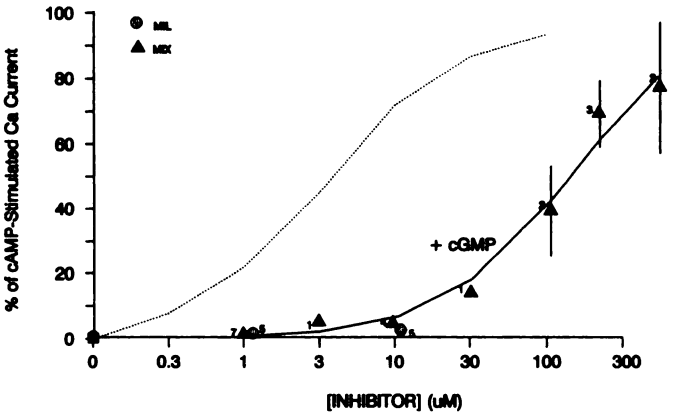

Fig. 7. Concentration-response curve for the effect of MIX and milrinone on I_{Ca} in the presence of cGMP and cAMP. I_{Ca} was elevated by internal perfusion with 5 μM cAMP and then decreased with 20 μM cGMP, as in Fig. 6. The effect of exposure to MIX or milrinone (*MIL*) was then measured and is expressed as the percentage of the cAMP-elevated current before drug addition. Thus, 100% represents complete reversal of the cGMP-induced inhibition of the cAMP-elevated current. ——, Least-mean-squares fit of the data for MIX to the Michaelis equation. *Numbers beside the symbols*, number of cells. *Bars* standard error. · · · · ·, Concentration-response curve for MIX in the absence of cGMP, taken from Fig. 3. The *y*-axis for this line is percentage of increase in Ca current, as in Fig. 3.

by cAMP, low concentrations (1–10 μM) of milrinone and MIX stimulated I_{Ca} normally (not shown).

Effects of other PDE inhibitors on I_{Ca}. Three other inhibitors of the low-K_m cAMP PDEs have been tested for their effects on I_{Ca}, rolipram and Ro 20-1724 (15, 26, 27), which inhibit the rolipram-sensitive subclass of low-K_m PDEs, and indolidan, which inhibits the cGMP-inhibited PDE (29–31). As illustrated in Fig. 8, all of these compounds increased cAMP-elevated I_{Ca} at 1 μM. Table 1 summarizes experiments where these compounds were applied at 1 or 10 μM to cells internally perfused with cAMP concentrations ranging from 1.5 to 5 μM. It appears that, for all three compounds, 1 μM concentrations

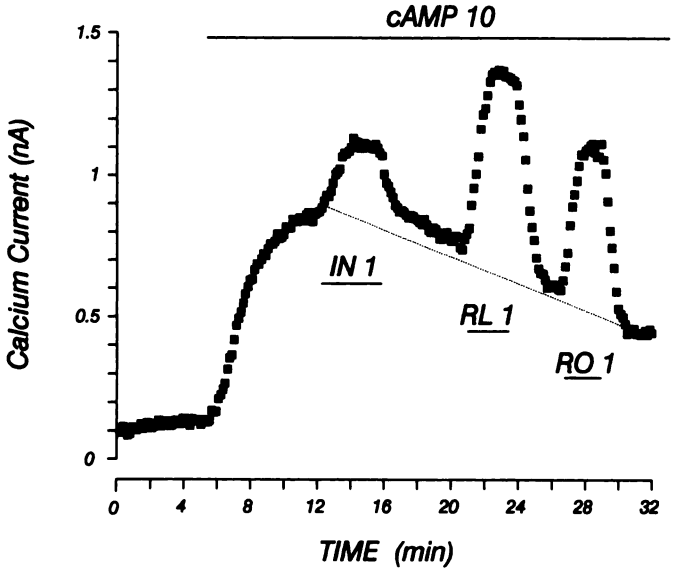

Fig. 8. Effect of several low-K_m cAMP PDE inhibitors on cAMP-elevated I_{Ca}. I_{Ca} was elevated by internal perfusion with 10 μM cAMP. The cell was then exposed to 1 μM indolidan (*IN*), rolipram (*RL*), or Ro 20-1724 (*RO*), as indicated. During the course of this experiment, I_{Ca} ran down somewhat with time. · · · · ·, Projected run-down.

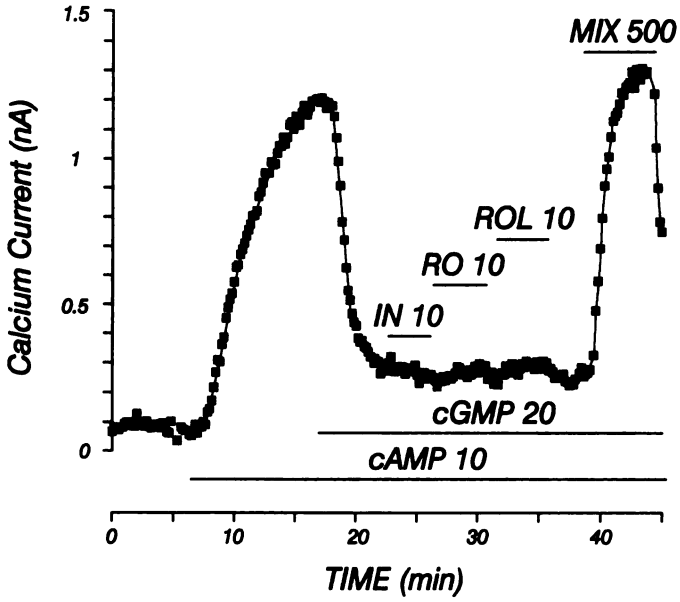

Fig. 9. Effect of several low-K_m cAMP PDE inhibitors on I_{Ca} in the presence of both cAMP and cGMP. I_{Ca} was elevated by 10 μM cAMP and then decreased by addition of 20 μM cGMP to the internal perfusion solution. Then, 10 μM indolidan (*IN*), Ro 20-1724 (*RO*), or rolipram (*RL*) or 500 μM MIX was applied extracellularly, as indicated.

TABLE 1

Percentage increase in I_{Ca}

Results are mean ± standard error.

Agent	Concentration	*n*	[cAMP]	I_{Ca} increase
	μM		μM	%
Indolidan	1	5	2	42.5 ± 8.4
	10	4	1.5, 2, 5	45.5 ± 9.0
Rolipram	1	4	2	57.6 ± 11.3
	10	1	2	46.9
Ro 20-1724	1	1	2	49.0
	10	4	1.5, 2, 5	42.9 ± 7.1

produced nearly maximal stimulation of I_{Ca}. All compounds seem to have a similar efficacy in stimulating I_{Ca}. In the presence of intracellular cGMP, however, none of these compounds had any significant effect on I_{Ca}, even at 10 μM concentration, whereas 500 μM MIX did stimulate the current (Fig. 9).

Discussion

Although the effects of PDE inhibitors on myocardial contraction (20–22, 32, 33), action potentials (6, 22, 23, 32, 34), Ca transients (20), and cyclic nucleotide metabolism (Refs. 14, 17–20, 28, 29, 33, and 35–37; see Ref. 15 for review) are well studied in cardiac preparations, relatively little is known of their effects on I_{Ca}. Indeed, most earlier voltage-clamp experiments were performed in multicellular preparations and/or at concentrations of PDE inhibitors too high to permit a separation of the effects on the various classes of cAMP PDEs (21–23). Therefore, our study provides the first quantitative analysis at the single-cell level of the regulation of cardiac I_{Ca} by inhibitors of the low-K_m cAMP PDEs.

Does I_{Ca} reflect cAMP levels? In the present study, as in most of our studies over the last 5 years, we have used the I_{Ca} as an index of cAMP levels inside frog ventricular cells. Although this approach has become routine in many laboratories, one must be aware of the fact that this approach has a shortcoming that resides in the nonlinearity of the response of I_{Ca} to cAMP. First, I_{Ca} saturates at high concentrations of cAMP (9, 12); therefore, when maximally stimulated, I_{Ca} becomes inefficient in detecting a further increase in cAMP levels. Likewise, a threshold concentration of cAMP may be required for activation of I_{Ca}, such that the current will not respond to changes in low concentrations of cAMP. In this regard, acetylcholine, which reduces both basal and stimulated (by β-adrenergic agonists or forskolin) adenylyl cyclase activity in frog ventricular cells (38), does not inhibit basal I_{Ca} in these cells (3). This might suggest that the basal level of cAMP is below the threshold for activation of Ca channel phosphorylation. Finally, I_{Ca} may not necessarily detect a change in cAMP taking place in the core of the cell and cAMP could possibly regulate other systems that modulate Ca channels. Taken together, these considerations imply that a variation of cAMP may occur inside the cell without necessarily reflecting a variation in I_{Ca}, which sets a limitation on the use of I_{Ca} as a probe of cAMP concentration.

Inhibition of PDE activity? Of all the PDE inhibitors (milrinone, MIX, Ro 20-1724, rolipram, and indolidan) that we have tested for their effects on I_{Ca}, none increased I_{Ca} in the absence of elevated cAMP. The absence of effects on basal I_{Ca} suggests that these compounds do not directly activate Ca channels in frog ventricular cells. Recently, a stimulatory effect of PDE inhibitors on adenylyl cyclase activity has been reported in a different tissue (39). The absence of a stimulatory effect of PDE inhibitors on basal I_{Ca} (Figs. 1 and 2) and the fact that the effects of I_{Ca} were similar in the presence of exogenous cAMP and in the presence of activators of adenylyl cyclase, such as forskolin or isoprenaline (Fig. 2), suggest that these drugs do not activate adenylyl cyclase in our cardiac preparation.

If these compounds act primarily as PDE inhibitors, it is

understandable that these drugs do not affect basal I_{Ca} if the basal adenylyl cyclase activity is very low. The absence of effect on basal I_{Ca}, however, is at variance with most electrophysiological and contractile studies on multicellular preparations, which show that specific PDE inhibitors, namely milrinone, increase slow action potential duration (32), Ca transients (20), myocardial contraction (20, 32), and I_{Ca} (22, 23) in the absence of any prior stimulation of these parameters over their basal levels. The most likely reason for this discrepancy is a difference in basal cAMP levels, which may be due to differences in animal species (frog versus mammals) and/or in the experimental procedures used (dialyzed single cell versus multicellular preparations).

All these PDE inhibitors exerted stimulatory effects on I_{Ca} only when the current had been previously enhanced by cAMP-dependent pathways. However, their maximal stimulatory effects were reduced as the cAMP concentration was increased. These two findings strongly suggest that cAMP and these compounds function through the same final pathway. Unless PDE inhibitors directly stimulate cAMP-dependent protein kinase (Refs. 28 and 40, but see Ref. 33), it is most likely that these drugs act primarily by increasing the concentration of cAMP available for activation of cAMP-dependent protein kinase and phosphorylation of Ca channels. Because the increase in cAMP concentration is unlikely to be due to a stimulation of adenylyl cyclase, the stimulatory effects on I_{Ca} of all compounds tested here take place in a way that is consistent with an inhibition of PDE activity.

What type of PDE? Our experiments provide support for the suggestion that inhibition of rolipram-sensitive and cGMP-inhibited PDE activity increases I_{Ca}. The concentrations of milrinone, rolipram, Ro 20-1724, or indolidan required to stimulate I_{Ca} are compatible with the concentrations of these drugs necessary to inhibit low-K_m cAMP PDE activity (15, 17, 27–29, 36). The nonspecific PDE inhibitor MIX produced an increase in I_{Ca} at concentrations that inhibit rolipram-sensitive [K_i = 15 μM (18)] and cGMP-inhibited PDEs [K_i = 2 μM (15, 18)] and possibly the Ca^{2+}-calmodulin-dependent type I PDE [K_i = 2.5 μM (18)]. However, because our intracellular solution contained 5 mM EGTA (pCa = 8.5–10), it was unlikely that Ca-calmodulin-dependent PDE would play a major role under our experimental conditions (35). None of the selective low-K_m cAMP PDE inhibitors, at low concentrations, had any effect on I_{Ca} in the presence of cGMP. As shown earlier (11, 12), the strong reduction of I_{Ca} induced by cGMP is primarily due to a stimulation of the cGMP-stimulated PDE (Ref. 16; see Ref. 15 for Refs.). Thus, the low-K_m cAMP PDE inhibitors do not significantly inhibit cGMP-stimulated PDE. Finally, it should be added that recent biochemical experiments suggest the presence of both cGMP-inhibited and rolipram-sensitive low-K_m cAMP PDEs in our preparation, because cAMP hydrolysis is inhibited ~60% by Ro 20-1724 (20 μM) and ~40% by milrinone (10 μM) in a frog ventricular membrane fraction (37).

Because these drugs are potent cardiotonic agents (15, 20), it is most likely that elevation of I_{Ca} plays a major role in their positive inotropic action. Moreover, the fact that all these compounds induced a rather large stimulatory effect on I_{Ca} elevated by either isoprenaline, forskolin or cAMP indicates that the degree of any hormonal or nonhormonal stimulation of I_{Ca} depends to a large extent on the basal activity of low-K_m cAMP PDEs. In several cell types, low-K_m cAMP PDEs are regulated by hormones, and there is evidence that glucagon regulates low-K_m cAMP PDEs in the heart (37). It is likely, therefore, that these PDEs might play an important role in the regulation of cardiac excitability and contractility. However, one proviso should be considered. In these studies, we examined the effect of PDE inhibitors on I_{Ca} elevated by internally perfused cAMP, which could exist in different compartments than the cAMP generated by β-adrenergic receptor stimulation. Thus, the possibility remains to be explored that the cAMP generated by adenylyl cyclase is not equally accessible to these PDEs as is internally perfused cAMP.

A common finding of the present study was that none of the specific inhibitors of the low-K_m cAMP PDEs increased I_{Ca} in the presence of cGMP. Because low concentrations of cGMP (<1 μM) would inhibit cGMP-inhibited PDE (15, 17), one would expect not to see any additional effect of milrinone or indolidan under these conditions. However, the absence of effect of rolipram and Ro 20-1724 was more surprising, because cGMP should not affect rolipram-sensitive PDE and, therefore, these agents should be able to inhibit cAMP hydrolysis and stimulate I_{Ca} even in the presence of intracellular cGMP. The fact that this was not the case suggests that rolipram-sensitive PDE activity is small relative to cGMP-stimulated PDE activity. The additional observation that intracellular cGMP only reduces I_{Ca} (Fig. 7; Refs. 11 and 12) suggests that cGMP-stimulated PDE also dominates over low-K_m cAMP PDE. Possible explanations for these observations are 1) that cGMP-stimulated PDEs are in large concentration or have a much larger capacity to hydrolyze cAMP than the low-K_m cAMP PDEs or 2) that cGMP-stimulated PDE is more efficiently coupled to Ca channels than other PDEs. Further experiments are required to examine these hypotheses.

These conclusions depend on a number of assumptions. We have assumed that frog PDEs exhibit similar pharmacological profiles as do mammalian PDEs and that the pharmacological properties of PDEs in intact cells are similar to the properties of solubilized enzymes or enzymes in subcellular fractions. There are very few data available, however, to evaluate the validity of this assumption. Furthermore, as discussed above, we assume that these inhibitors produce their effects via inhibition of PDEs. Because milrinone and MIX have been shown to affect G_i function, one might hypothesize that the effects we have observed are due to effects of the inhibitors on direct GTP-binding protein regulation of Ca^{2+} channels. In our preparation, however, we find no evidence for direct GTP-binding protein modulation of Ca^{2+} channels (5, 41). It should also be noted that we have only examined the effects of these inhibitors on the steady state I_{Ca}. It is conceivable that, because the K_m values of various PDEs for cAMP differ, certain species of PDE may contribute differentially to the rate of increase or decrease in I_{Ca} in response to inotropic agents.

Sidedness of action. Both rolipram-sensitive and cGMP-inhibited subclasses of the low-K_m cAMP PDEs have been found to be present in soluble and particulate fractions of cardiac tissue (26–31, 36). However, the use of PDE inhibitors has shown that the milrinone-sensitive, cGMP-inhibited, type IV low-K_m cAMP PDE is essentially a particulate enzyme, most likely bound to the membrane of sarcoplasmic reticulum (28–31, 36), whereas the rolipram-sensitive low-K_m cAMP PDE is essentially a soluble enzyme (26, 27, 29). Assuming that milrinone readily enters the cell when applied from outside, the

observation that milrinone was equally potent in stimulating I_{Ca} when applied either from outside or from inside the cells (Fig. 4) is in agreement with the action of the drug taking place at some internal site, such as sarcoplasmic reticulum membranes. It is surprising, however, that MIX, at concentrations up to 100 μM, did not stimulate I_{Ca} when applied internally but was effective only when applied outside the cell (Fig. 5). We cannot explain this result, but two hypotheses can be considered. 1) MIX acts on a PDE subunit that is located on the plasma membrane and whose MIX binding sites are only accessible from the outside. 2) For some unknown reason, MIX is unable to diffuse in the cytoplasm to the PDE, although milrinone and cAMP are. It is worth mentioning that we also found recently that the dihydropyridine Bay K 8644 increased I_{Ca} when applied externally but had no effect when perfused inside the cells (see Ref. 42).[2]

In addition to its ability to inhibit PDE activity, MIX also is a potent adenosine receptor blocker. One could imagine that the reason MIX only works from the exterior is related to its adenosine receptor antagonist properties. This seems unlikely, because the amount of adenosine a single cell could produce is unlikely to be sufficiently high to activate adenosine receptors, especially because the cells are constantly superfused at a relatively rapid rate. Further experiments are required to identify the sidedness of action of pharmacological substances.

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