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Role of Phosphodiesterase in Regulation of Calcium Current in Isolated Cardiac Myocytes

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SUMMARY

It has previously been shown that intracellular perfusion of isolated cardiac myocytes with cGMP reduces the amplitude of the trans-sarcolemmal calcium current (I_{Ca}) elevated by cAMP-dependent mechanisms. To test the hypothesis that cGMP acts by stimulating a cyclic nucleotide phosphodiesterase (PDE) activity, PDE activity and the effects of methylisobutylxanthine (MIX), a PDE inhibitor, on I_{Ca} were examined in cardiomyocytes dissociated from frog ventricle. PDE activity was determined by measuring hydrolysis of [^{33}P]cAMP in subcellular fractions. Using 100 μM cAMP as substrate, approximately 50% of the PDE activity was found in the 20,000 × *g* particulate fraction. Basal activity in this fraction had a V_{max} of 15.4 μmol of cAMP hydrolyzed/min/mg of protein and a K_m of 113 μM cAMP. The PDE activity of the particulate fraction was stimulated significantly by cGMP. Half-maximal stimulation was observed with 1.1 μM cGMP. This value is virtually identical to the value for the concentration of intracellular cGMP that produced a half-maximal reduction of cAMP-elevated I_{Ca} in electrophysiological experiments. The cGMP-stimulated PDE activity had a V_{max} of 9.5 μmol/min/mg and a K_m of 12.3 μM cAMP. MIX (100 μM) selectively inhibited the cGMP-stimulated PDE activity (IC_{50} = 20 μM). To determine whether PDEs modulate the amplitude of I_{Ca}, the effects of MIX were examined on basal I_{Ca} and cAMP-elevated I_{Ca}. MIX produced small increases in the basal I_{Ca} and increased I_{Ca} in the presence of 1 μM intracellular cAMP. MIX at 100 μM potentiated the effects of submaximal doses of isoproterenol and shifted the dose-response curve for cAMP to the left but did not affect the dose-response curve for 8-bromo-cAMP. MIX reversed the effect of cGMP on the cAMP-elevated I_{Ca}. We conclude that cyclic nucleotide PDEs play an important role in modulating the cardiac calcium current. The hypothesis that cGMP inhibits the cAMP-elevated I_{Ca} by activating a PDE is supported by the finding that MIX inhibited both the cGMP-stimulated PDE activity and the effect of cGMP on I_{Ca} at similar concentrations.

Calcium channels in cardiac muscle are responsible for carrying the slow inward calcium current (I_{Ca}) and participate in many aspects of cardiac function (1–4). I_{Ca} is involved in the development of the action potential (5, 6), in the generation of electrical pacemaking activity in nodal tissue (7), and in the initiation of contraction (8, 9). I_{Ca} is modulated by neurotransmitters and drugs that influence β-adrenergic and muscarinic cholinergic receptors on cardiac cells (3, 4, 10, 11). β-adrenergic agonists increase I_{Ca} by stimulating cAMP synthesis and cAMP-dependent phosphorylation of the calcium channel, thereby increasing the mean probability of channel opening (4, 11). Cholinergic agonists have no effect on basal I_{Ca} but decrease I_{Ca} that has been elevated by β-adrenergic agonists partly by inhibiting adenylate cyclase (12–14).

Although regulation of I_{Ca} through mechanisms that modulate cAMP synthesis is apparently well understood, the modulation of cAMP degradation is less well defined. cAMP is hydrolyzed to 5′-AMP by at least four classes of cyclic nucleotide PDE that have been described in cardiac tissue (15, 16). Type I PDE ('Ca-calmodulin-dependent cyclic nucleotide PDE') may be cGMP selective or nonselective for cGMP and cAMP depending upon animal species and heart chamber. Type II PDE ('cGMP-stimulated PDE') is nonselective for cGMP and cAMP and is characterized by the fact that the hydrolysis of cAMP is markedly stimulated by low concentrations of cGMP (17, 18). Type III PDEs are specific for cAMP. One subclass of Type III PDEs has received considerable attention recently because they are targets for 'cardiotonic' PDE inhibitors and are inhibited by low concentrations of cGMP (19).

Although PDEs are known to be regulated *in vitro* by various signals including Ca and cGMP, the physiological factors that regulate PDEs in the cell have not been clearly defined. It is not known whether different subtypes of PDE play different physiological roles in the cell, for example by regulating cyclic

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ABBREVIATIONS: PDE, phosphodiesterase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; TTX, tetrodotoxin; PMSF, phenylmethylsulfonyl fluoride; MIX, methylisobutylxanthine; ACh, acetylcholine.

nucleotide concentrations in different subcellular compartments or in regions of different effector systems (calcium channel, contractile proteins, sarcoplasmic reticulum, etc.). Nor is it known whether these PDEs are regulated differentially by extracellular signals.

Recently, we have suggested that cGMP-stimulated PDE may play a key role in regulation of the calcium channel (20). We showed that intracellular perfusion of cells with low concentrations of cGMP decreased I_{Ca} elevated by β-adrenergic agonists or cAMP (20, 21). A variety of different kinds of experiments, summarized in the Discussion, have provided strong but indirect support for the hypothesis that these effects of cGMP on I_{Ca} are mediated by a cGMP-stimulated PDE. Because cGMP levels change in cardiac cells in response to a variety of physiological and pathological conditions (22, 23), we think that cGMP-stimulated PDE may play a physiological role in regulation of I_{Ca} (20, 21). For example, cGMP-stimulated PDE could possibly be involved in mediating the effects on I_{Ca} of ACh (24), of nitroso compounds[2] that may be released endogenously (25), or of various neuropeptides such as atrionatriuretic peptide (26), all of which are known to elevate cGMP levels under certain circumstances (22, 24, 27, 28). To acquire more direct evidence in support of our hypothesis that type II PDE plays an important role in regulation of I_{Ca}, we have characterized the types of PDEs in our cells and attempted to determine the roles that these PDEs play in regulating I_{Ca}.

Methods

Solutions. Cs-Ringer contained: 88.4 mM NaCl, 20 mM CsCl, 23.8 mM $NaHCO_3$, 0.6 mM $NaHPO_4$, 1.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM sodium pyruvate, 5 mM glucose, and 0.3 μM TTX, pH 7.4 with 5% CO_2–100% O_2. Control intracellular medium (MIG 13) contained: 120 mM CsCl, 5 mM K_2EGTA, 4 mM $MgCl_2$, 5 mM sodium creatine phosphate, 3 mM Na_2ATP, 10 mM HEPES, pH 7.1 with KOH. In some experiments, Na_2GTP (100–400 μM) was added. MIG 0 was a solution used for PDE assays (see below), which was MIG 13 without Na_2ATP or sodium creatine phosphate.

Electrophysiological recording. The methods for electrophysiological recording using the whole-cell patch clamp technique (29) have been described in detail elsewhere (14, 21, 30). Single myocytes were isolated from frog ventricle by enzymatic dissociation. I_{Ca} was recorded with patch pipets having resistances between 0.5 and 2.5 MΩ. The solution inside the pipet could be changed continuously by a perfusion system previously described (20, 21). I_{Ca} was elicited by voltage clamp pulses given at 8-sec intervals from a holding potential of −80 mV to 0 mV. Sodium current was blocked with TTX and potassium currents were blocked by the presence of Cs^+ in both the intracellular and extracellular solutions.

PDE assays. All preparative procedures were performed at 4°. Frog heart, free of blood vessels and connective tissue, was homogenized in 2 volumes of MIG 0 in a Polytron at setting 4.5 for two bursts of 10 sec. Immediately before homogenization, PMSF was added from a 100 mM stock solution in 2-propanol to the homogenization buffer to make 100 μM final PMSF concentration. Immediately after homogenization, another aliquot of PMSF was added.

The homogenate was centrifuged in a Sorvall RC-5B refrigerated centrifuge at 20,000 × *g* for 10 min. The supernate was removed and the pellet was washed three times by resuspension in the original volume of MIG 0, rehomogenization, and centrifugation.

PDE activity was assayed by the method of Walseth and Johnson (31) in a 100-μl reaction containing MIG 0, 5–20 μg of protein, and ~50,000 cpm of [^{32}P]cAMP or [^{32}P]cGMP. The reaction was initiated by addition of enzyme and incubated at 30° for 2–10 min. The reaction was terminated by addition of 50 μl of 1 N HCl and placed on ice. The HCl was neutralized by addition of 50 μl of 0.6 M HEPES/1.29 M NaOH. After neutralization, 0.1 units of 5′-nucleotidase was added and the reaction was incubated 15 min at 30°. The reaction was finally quenched by addition of 1 ml of 0.1 N H_3PO_4 containing 25 mg of charcoal (Sigma no. 5260; washed and equilibrated in 0.1 N H_3PO_4). After centrifugation for 1 min at 15,000 × *g* in a Microfuge, 0.75 ml of the supernatant was counted in 3 ml of water in a Beckmann LS7500 scintillation counter. Each point was performed in triplicate. Triplicates were within ±10% of each other. Controls were performed to show that MIX had no effect on 5′-nucleotidase activity. [^{32}P]cAMP and [^{32}P]cGMP were generously supplied to us by Dr. T. Walseth (University of Minnesota, Minneopolis, MN) and had been synthesized according to his method (31).

Results

Characterization of PDE Activity

In our previous studies (20, 21), we hypothesized that the effect of cGMP on I_{Ca} was mediated by a cGMP-stimulated PDE. A critical test of this hypothesis is the demonstration that such an enzyme is present in our cells and is regulated biochemically in a manner predicted from the electrophysiological experiments. Towards this goal, we characterized the PDE activity in crude fractions of heart prepared under conditions closely approximating those used for electrophysiological recording. Tissue was homogenized and assayed for PDE activity in a solution (MIG 0) containing 120 mM CsCl, 5 mM K_2EGTA, 4 mM $MgCl_2$, and 10 mM HEPES, pH 7.15 with KOH, which is very similar to that used to fill our patch pipets for voltage clamp recording of I_{Ca}. Because this solution contains EGTA, Ca-dependent PDEs are not considered in this study.

The 20,000 × *g* pellet and supernate from the homogenate were assayed for PDE activity in MIG 0 containing 100 μM cAMP as substrate. Approximately 50% of the PDE activity was contained in the 20,000 × *g* pellet, but because the protein concentration of the supernate was 6 times that of the pellet, the specific activity of the PDE was considerably greater in the pellet (Fig. 1). cAMP hydrolysis in both the supernate and pellet was stimulated by addition of 1 μM cGMP, but the stimulation of the particulate activity was substantially greater. For all subsequent studies, the washed 20,000 × *g* particulate

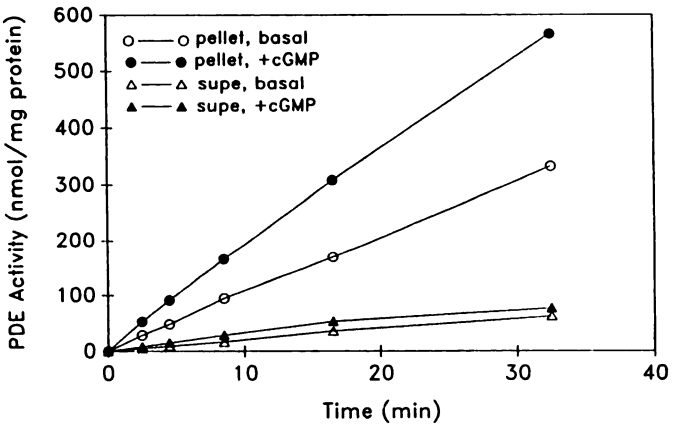

Fig. 1. Stimulation of PDE activity by cGMP. Frog ventricle was homogenized in MIG 0 and centrifuged at 20,000 × *g* for 10 min. The supernate and the washed pellet were assayed for PDE activity using 100 μM [^{32}P]cAMP as substrate. PDE activity was assayed in the absence or presence of 1 μM cGMP.

[2] H. C. Hartzell, unpublished data.

fraction was used. The PDE activity in the absence of cGMP was termed 'basal' activity. The cGMP-stimulated activity was defined as the difference between the total activity in the presence of cGMP and the basal activity.

cGMP stimulation. In the experiment of Fig. 2A, basal PDE activity was 4.68 nmol/min/mg. Addition of 10 μM cGMP to the assay stimulated PDE activity 2-fold over basal (Fig. 2A). A maximal effect of cGMP occurred with concentrations of 10 μM. At higher concentrations, the stimulation was less (see Fig. 2B) probably because cGMP competed for cAMP hydrolysis (see below). The apparent K_{50} for cGMP stimulation determined by fitting the data in Fig. 2A to the Michaelis equation was 1.1 μM. In other experiments, values ranged from 0.4 μM to 1.2 μM. These values are remarkably similar to the value of 0.7 μM we have previously reported for the half-maximal effect of cGMP on I_{Ca} (Fig. 2B). In Fig. 2B, the dose-response curve for the stimulation of PDE activity by cGMP (data from Fig. 2A) is superimposed on data we have previously published for the effect of cGMP on I_{Ca}. Considering the very different techniques utilized for these two determinations, the dose-response curves are remarkably similar. We previously reported that 8-bromo-cGMP had no effect on I_{Ca}. Likewise, 8-bromo-cGMP at concentrations between 1 and 20 μM had no effect on PDE activity (data not shown). In the same experiments, 10 μM cGMP stimulated PDE activity approximately 2-fold. These experiments demonstrated a remarkable similarity between the properties of the cGMP-stimulated PDE activity and the effect of cGMP and cGMP analogs on I_{Ca} and strongly support the conclusion that cGMP regulates I_{Ca} via a cGMP-stimulated PDE.

Kinetic constants. Basal and cGMP-stimulated activities exhibited different kinetic constants for cAMP hydrolysis (Fig. 3A; Table I). The V_{max} for the basal activity was somewhat greater than for the cGMP-stimulated activity, but the K_m for the basal activity was about 10-fold greater than the K_m for the cGMP-stimulated activity. The data points for both the basal and cGMP-stimulated activities were well fit by the Michaelis

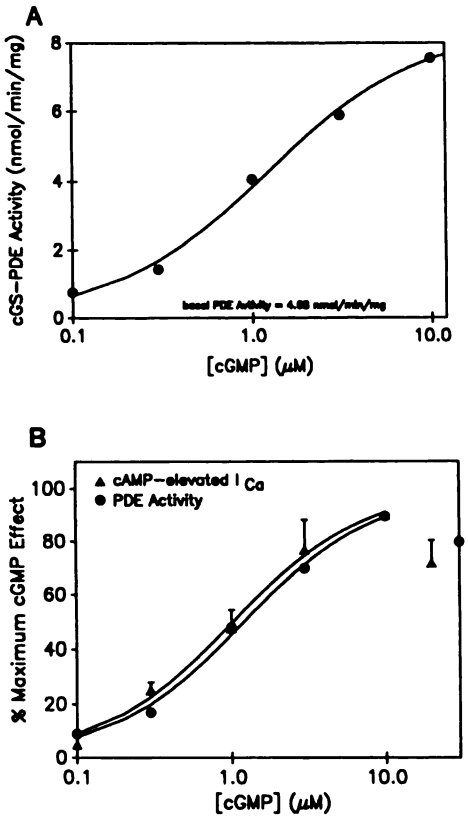

Fig. 2. Dose-response curve for stimulation of PDE activity by cGMP. A, The cGMP-stimulated PDE activity [defined as the difference between basal activity (*open symbols* in Fig. 1) and total activity in the presence of cGMP (*closed symbols* in Fig. 1)] is plotted as a function of cGMP added to the assay. The basal PDE activity in this preparation was 4.68 nmol/min/mg of protein. The *solid line* is the best fit to the Michaelis equation with K_{50} = 1.1 μM and V_{max} = 8.4 nmol/min/mg. *B*, Comparison of the dose-response curves for the effect of cGMP on PDE activity and on I_{Ca}. ●, Data redrawn from Fig. 2A with 8.4 nmol/min/mg set to 100%; ▲, data from Ref. 21. The values are the per cent inhibition of I_{Ca} elevated by 5 μM cAMP by different concentrations of cGMP. The *solid lines* are the best fits to the Michaelis equation for data between 0.1 and 10 μM cGMP. For the I_{Ca} data, K_{50} was 1.0 μM. In our previous studies, we fit the I_{Ca} data between 0.1 and 30 μM cGMP and arrived at a K_{50} of 0.7 μM for the cGMP effect on I_{Ca}.

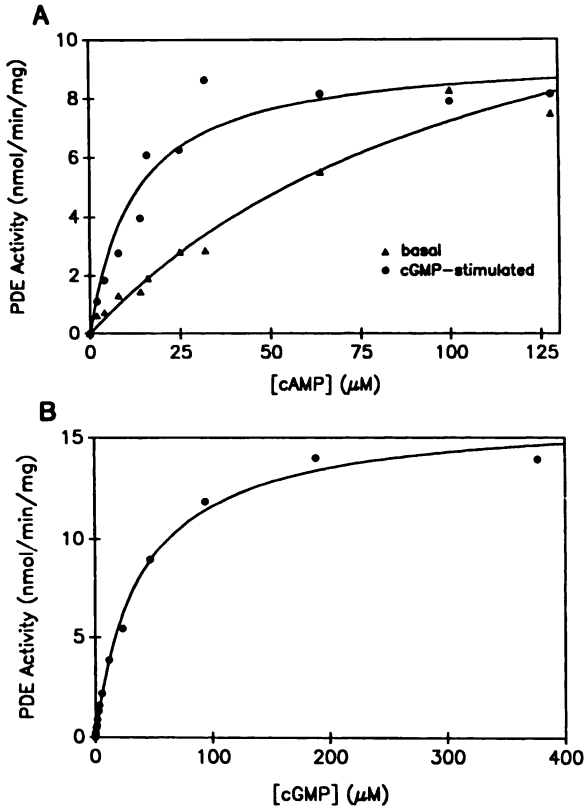

Fig. 3. Kinetic analysis of PDEs. Ventricular tissue was homogenized in MIG 0 and the washed 20,000 × *g* particulate fraction assayed for PDE activity with varying (A) cAMP or (B) cGMP as substrate. A, cAMP hydrolysis. cGMP-stimulated activity was determined by subtracting activity in the absence of cGMP from total activity in the presence of 1 μM cGMP. *Lines* are best fits to the Michaelis equation. *B*, Hydrolysis of cGMP. Kinetic constants obtained by best fits are shown in Table 1.

TABLE 1

Kinetic constants of PDE activities

Kinetic constants were determined as described in Fig. 3. IC_{50} for MIX was determined as shown in Fig. 4.

Activity	K_m	V_{max}	IC_{50} (MIX)	K_m	V_{max}
	μM cAMP	μmol/min/mg	μM MIX	μM cGMP	μmol/min/mg
Basal	113.0	15.4	355.0		
cGMP-stimulated	12.3	9.5	20		
Pellet				35	15

equation, suggesting that one enzyme was primarily responsible for each activity. The washed pellet can also utilize cGMP as a substrate (Fig. 3B; Table I), but the K_m for cGMP was severalfold greater than the K_m of the cGMP-stimulated activity for cAMP.

Effects of MIX. The cGMP-stimulated PDE activity was selectively inhibited by the PDE inhibitor MIX (Fig. 4). The IC_{50} for MIX inhibition of the basal activity was 18-fold greater than the IC_{50} for the cGMP-stimulated PDE. At 100 μM MIX, cGMP-stimulated PDE is inhibited selectively compared with the basal activity.

Effects of MIX on I_{Ca}

Basal I_{Ca}. To determine whether PDEs have any role in determining the amplitude of basal I_{Ca}, we examined the effects of MIX on basal I_{Ca} recorded by voltage clamp. A typical experiment is shown in Fig. 5A. In this experiment, I_{Ca} was elicited every 8 sec by a 400-msec voltage-clamp pulse from −80 mV to 0 mV (see *inset*, Fig. 5B). The net calcium current (I_{Ca}) was plotted versus time. When this cell was superfused with 100 μM MIX, I_{Ca} increased a small amount. The effect was reversed upon washing out MIX. The effect of MIX on basal I_{Ca} was small; I_{Ca} was increased only 53 ± 22% (n = 22). Also, an increase in I_{Ca} was not observed in every cell; MIX increased in I_{Ca} in only 14 of the 22 cells tested.

Isoproterenol-stimulated I_{Ca}. The small effect of PDE inhibition on basal I_{Ca} is probably due to the fact that adenylate cyclase is catalytically inactive under these conditions. If this is indeed true, we would expect a PDE inhibitor to have a greater effect if adenylate cyclase were active. To test this prediction, the effect of MIX on cells exposed to various concentrations of isoproterenol was examined. In the cell shown in Fig. 5B, superfusion with either 100 μM MIX alone or 5 nM isoproterenol alone for the periods indicated had only very small effects on I_{Ca}. However, when MIX and isoproterenol were applied together, I_{Ca} increased about 10-fold from 150 pA to 1.5 nA.

Data from similar experiments on four cells are shown in Fig. 5C. Two doses of isoproterenol were chosen, a submaximal concentration (100 nM) and a supramaximal concentration (1 μM) (20). The low dose of isoproterenol (100 nM) had a small stimulatory effect on I_{Ca}, increasing it from 3.3 ± 0.3 pA/pF to 6.1 ± 1.9 pA/pF. Superfusion of the cell with MIX (100 μM) plus isoproterenol (100 nM) resulted in a greater than 3-fold

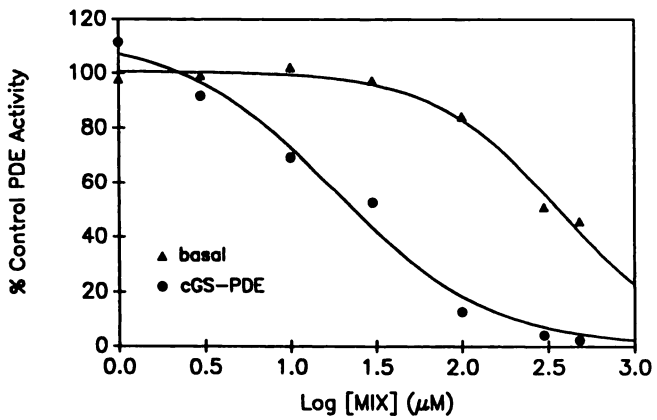

Fig. 4. Inhibition of PDE activity by MIX. The effect of differing concentrations of MIX on basal PDE activity or on cGMP-stimulated activity (cGS-PDE) was measured using 100 μM cAMP as substrate.

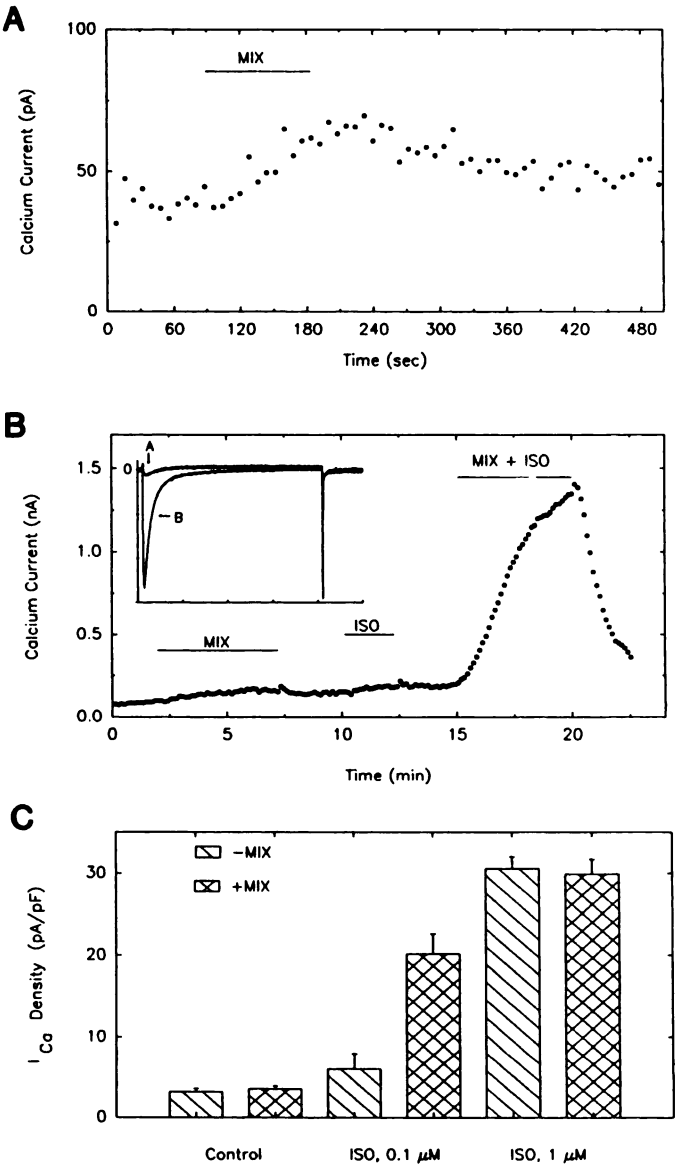

Fig. 5. Effects of MIX on I_{Ca}. A, Basal I_{Ca}. I_{Ca} was elicited every 8 sec by a 400-msec voltage clamp pulse from −80 mV to 0 mV. The net calcium current is plotted as a function of time. Superfusion of the cell with 100 μM MIX increased I_{Ca} a small amount. The effect was reversible. B, Isoproterenol-elevated I_{Ca}. The cell was superfused with 100 μM MIX alone, or 5 nM isoproterenol (*ISO*) alone, or both of the drugs together for the periods marked. *Inset*, sample tracings of the calcium current obtained under control conditions (*trace A*) and in the presence of MIX and isoproterenol (*trace B*). *Ticks* = 100 msec, 500 pA. C, Summary of data from four cells showing effect of 100 μM MIX on basal I_{Ca} and I_{Ca} elevated by either 100 nM isoproterenol or 1 μM isoproterenol.

increase in I_{Ca} over that observed with isoproterenol alone (I_{Ca} = 20.2 ± 2.4 pA/pF). Application of a supramaximal concentration of isoproterenol (1 μM) increased I_{Ca} to 30.6 ± 1.4 pA/pF. Addition of MIX (100 μM) to this dose of isoproterenol did not affect I_{Ca} (I_{Ca} = 29.9 ± 1.8 pA/pF). This is consistent with the hypothesis that MIX produces its effects on I_{Ca} by inhibition of PDE activity. In the presence of a concentration of isoproterenol that effectively saturates the cAMP-dependent phosphorylation of the Ca channel, a further increase in cAMP levels via PDE inhibition would not be expected to affect I_{Ca}.

cAMP-stimulated I_{Ca}. To gain additional insight into the mechanism of action of the PDE inhibitor on I_{Ca}, we examined

the effects of MIX on the dose-response curve for intracellular cAMP on I_{Ca} (fig. 6). In the experiment of Fig. 6A, the cell was perfused internally with increasing concentrations of cAMP from 0.3 to 30 μM. Before perfusion with cAMP was begun, MIX had no effect on basal I_{Ca}. In contrast, superfusion with 100 μM MIX produced large increases in I_{Ca} when the cell was internally perfused with submaximal concentrations of cAMP but had no effect on I_{Ca} in the presence of a saturating concentration of cAMP (30 μM). Interestingly, the lowest concentrations of cAMP produced little or no effect by themselves but produced large increases in I_{Ca} when MIX was added. The average dose-response curve from five to six cells for the effect of cAMP on I_{Ca} in the presence and absence of MIX is shown in Fig. 6B. The dose-response curves were fit to the following equation:

$$I_{Ca}\ \text{density} = (I_{max} \cdot [\text{cAMP}])/(ED_{50} + [\text{cAMP}]) + c$$

to obtain the maximal effect (I_{max}) and the concentration of cAMP that elicited 50% of the maximum response (ED_{50}). In the absence of MIX, the ED_{50} for cAMP was 16.9 μM, whereas in the presence of MIX it was 0.5 μM. Thus, MIX decreased the effective concentration required for a half-maximal effect of cAMP approximately 34-fold.

If this effect of MIX is related to inhibition of cAMP hydrolysis, one would predict that MIX would have no effect on the dose-response curve for intracellular 8-bromo-cAMP, which is a poor substrate for PDEs. Fig. 6C shows that this is indeed the case. In the absence of MIX, the calculated maximum I_{Ca} was 26.7 pA/pF and the ED_{50} was 0.8 μM. Superfusion with MIX resulted in a slight but statistically insignificant shift of the dose-response curve for 8-bromo-cAMP (I_{max} = 26.4 pA/pF, ED_{50} = 0.5 μM). These results support the conclusion that MIX potentiates the effects of low concentrations of cAMP by blocking cAMP hydrolysis by PDEs.

Effect of MIX on the cGMP inhibition of cAMP-elevated I_{Ca}. It has recently been shown (20, 21) that the elevation of I_{Ca} following intracellular perfusion with cAMP can be antagonized by addition of cGMP intracellularly. We hypothesize that this effect of cGMP was due to cGMP-stimulation of a PDE. One argument supporting this hypothesis is that MIX blocks the effects of cGMP. An example of such an experiment is shown in Fig. 7A. In this experiment, the cell was first perfused internally with a saturating concentration of cAMP (30 μM), which increased I_{Ca} 18-fold. This was followed by the addition of 10 μM cGMP to the internal perfusion, which caused I_{Ca} to decrease substantially. Subsequent superfusion of the cell with 100 μM MIX blocked the effects of cGMP almost entirely even though this concentration of MIX had no effect on the cAMP-elevated current in the absence of cGMP (c.f. 30 μM cAMP in Fig. 6). On average, MIX (100 μM) resulted in a 62 ± 4% reversal of the cGMP effect (n = 30). Sulfophenyltheophylline (100 μM), a potent adenosine receptor antagonist that does not inhibit PDE, was ineffective in reversing the cGMP effect (sulfophenyltheophylline actually increased cAMP-elevated I_{Ca} 4 ± 3%, n = 3). This indicates that the effects of MIX were not a result of an action on purinergic receptors.

Role of cGMP-stimulated PDE in mediating the effects of ACh. Because MIX appears to be a relatively selective inhibitor of the cGMP-stimulated PDE, we felt that it would be a useful tool for investigating the role of this enzyme in mediating the effects of ACh. The experiment of Fig. 7B shows the effects of MIX on the effect of ACh on I_{Ca}. I_{Ca} was first elevated by exposure of the cell to 1 μM isoproterenol. When ACh and MIX were added simultaneously, there was no significant reduction in I_{Ca}. Removal of MIX from the superfusion medium resulted in a large decrease in I_{Ca} due to ACh. Readdition of MIX then partially blocked the effects of ACh. We do

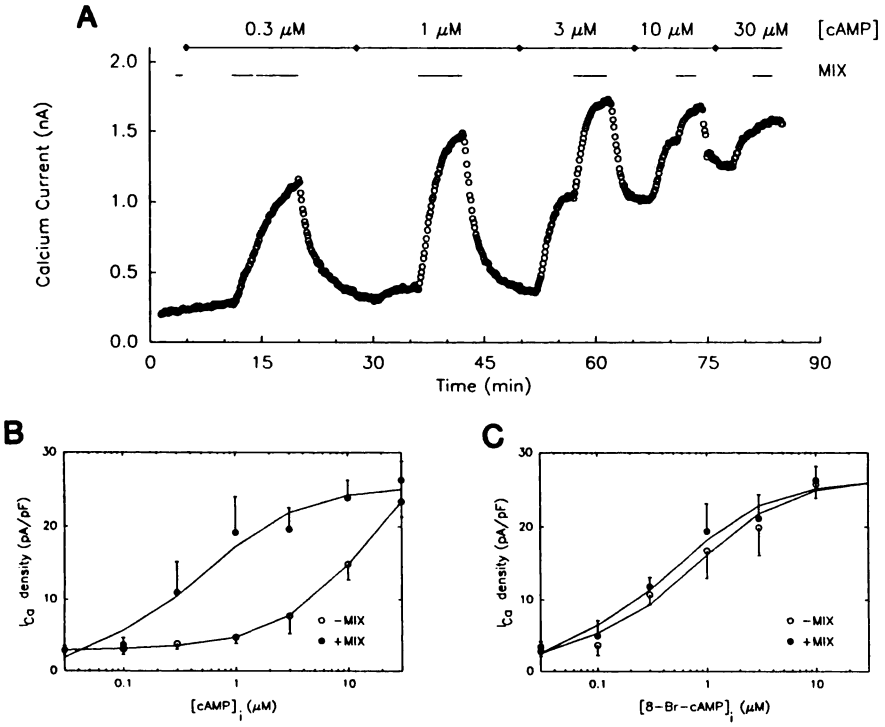

Fig. 6. Effects of MIX on I_{Ca} elevated by various concentrations of cAMP. A, The cell was perfused internally with increasing concentrations of cAMP as shown on the *top line*. At various times during this perfusion, the cell was exposed to 100 μM MIX extracellularly (indicated by the *bars*). MIX produced no effect on basal I_{Ca} or I_{Ca} elevated by concentrations of cAMP that produced maximal increases in I_{Ca} (30 μM cAMP). In contrast, MIX produced large increases in I_{Ca} when the cell was perfused with low concentrations of cAMP. B, Dose-response curves for effects of intracellular cAMP on I_{Ca} in the absence and presence of 100 μM MIX. C, Dose-response curves for effects of intracellular 8-bromo-cAMP in the absence and presence of 100 μM MIX.

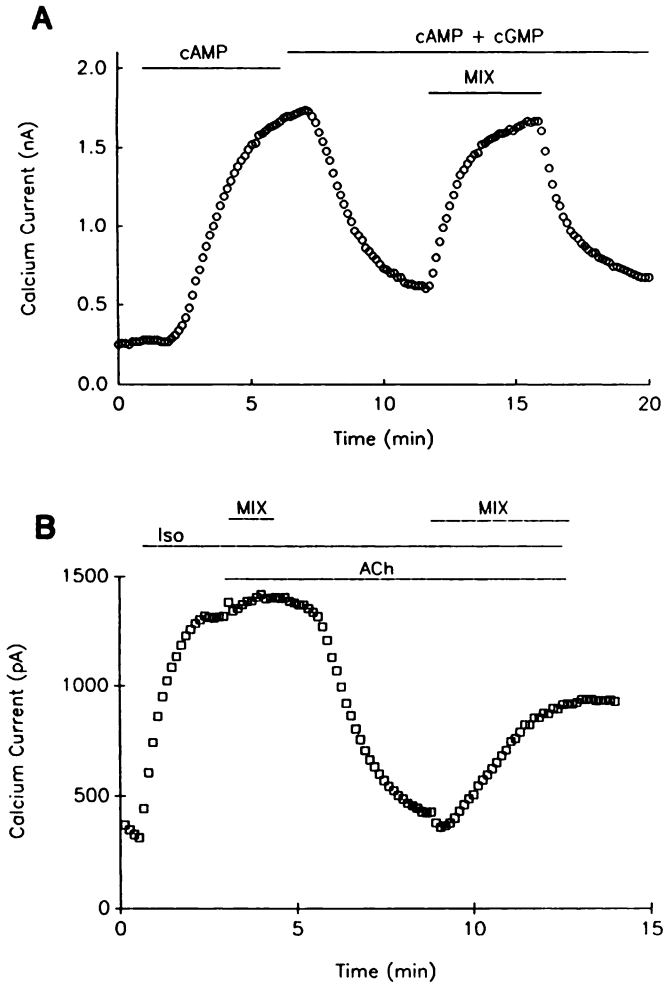

Fig. 7. MIX blocks effects of cGMP and ACh on I_{Ca}. A, cGMP. The cell was first perfused with 30 μM cAMP, which caused a 16-fold increase in I_{Ca}. This was followed by addition of 10 μM cGMP, which caused I_{Ca} to decrease substantially. Superfusion of the cell with 100 μM MIX reversed the effect of cGMP about 60%. B, ACh. The cell was exposed to a maximal concentration of isoproterenol (2 μM) (*ISO*). Subsequent addition of 1 μM ACh and 100 μM MIX to the superfusion had little effect on I_{Ca}. Removal of MIX resulted in a large decrease in I_{Ca} that was partially blocked by subsequent addition of MIX.

not understand why MIX was less effective when applied after ACh than when they were applied simultaneously, but this was a common finding.

Discussion

Role of cGMP-stimulate PDE in regulation of I_{Ca}. In our earlier studies (20, 21), we demonstrated that intracellular perfusion with cGMP caused a reduction in I_{Ca} elevated by β-adrenergic agonists. A variety of indirect evidence was presented to support the suggestion that the mechanism of action of cGMP is due to stimulation of cGMP-stimulated PDE. 1) cGMP has an effect only on I_{Ca} elevated by cAMP-dependent mechanisms. cGMP has no effect on basal I_{Ca} or on I_{Ca} elevated by dihydropyridine calcium channel agonists. 2) cGMP reduces I_{Ca} elevated by intracellular perfusion with cAMP but not I_{Ca} elevated by intracellular perfusion with nonhydrolyzable derivatives of cAMP, such as 8-bromo-cAMP, or with the catalytic subunit of cAMP-dependent protein kinase. These results demonstrate that there must be a hydrolyzable form of cAMP present in the cell for cGMP to produce an effect. 3) The effects of cGMP are blocked by MIX. 4) The effects of cGMP are not mimicked by 8-bromo-cGMP, which does not activate the PDE but which is a potent activator of cGMP-dependent protein kinase.

The present studies provide additional support for the hypothesis that cGMP acts by stimulation of cGMP-stimulated PDE. First, we show that an enzyme that hydrolyzes cAMP and is stimulated by cGMP is present in these cells under the conditions of our intracellular perfusion. The quantity of enzyme activity seems sufficient to account for the electrophysiological results. The specific activity of the cGMP-stimulated enzyme activity in the whole homogenate is approximately 6 nmol/min/mg of protein (Fig. 3A) with 30 μM cAMP as substrate (the condition of Fig. 6). If 1 mg of protein is equivalent to 20 mg of tissue wet weight, 1 liter of cells could hydrolyze 300 μmol of cAMP/min. Thus, this quantity of enzyme activity is sufficient to hydrolyze the cellular contents of cAMP in a fraction of a minute. Basal activity under the same conditions is about 50% of the cGMP-stimulated activity. Thus, even basal activity is sufficient to hydrolyze cAMP rapidly. However, it is possible that the enzyme responsible for the basal activity is compartmentalized in the cell or is regulated by factors not active in the homogenate. Second, 8-bromo-cGMP, which has no effect on I_{Ca}, also has no effect on PDE activity. Third, the dose-response curves for the effect of cGMP on I_{Ca} and on the cGMP-stimulated PDE activity are remarkably similar. These similarities between the biochemical and electrophysiological effects of cGMP argue strongly that a cGMP-stimulated PDE is involved in both effects.

Because our preparation hydrolyzes both cGMP and cAMP, it must be recognized that the kinetic constants for cAMP hydrolysis by the cGMP-stimulated enzyme may be complicated by the fact that, especially at low cAMP concentration, cGMP may compete for cAMP hydrolysis. Thus, at low cAMP concentrations, PDE activity may be underestimated by virtue of competition between cGMP and cAMP for the hydrolytic site and also by virtue of the fact that cGMP concentration may be falling during the assay. Thus, the K_m of the cGMP-stimulated activity for cAMP may be smaller than we have estimated. However, it seems that these factors are unlikely to contribute a significant error. With 1 μM cGMP at the lowest cAMP concentration used (2 μM), the ratio of the velocity of cAMP hydrolysis to cGMP hydrolysis is 3:1, calculated from the kinetic constants in Table I ignoring any competition. At cAMP concentrations greater than 4 μM, cGMP hydrolysis would constitute less than 10% of the cAMP hydrolysis. Furthermore, because less than 25% of the cAMP was hydrolyzed during the assay, cGMP concentration was unlikely to have changed more than 10%. The V_{max} is not subject to such an error because at 100 μM cAMP, 1 μM cGMP would be hydrolyzed only very slowly.

There are several differences between our results and those of other laboratories. First, we find that the cGMP-stimulated PDE is largely a particulate enzyme under our conditions. In preliminary experiments we have found that this enzyme is in purified canine sarcolemma. Thus, it seems likely that at least a fraction of this enzyme is associated with the plasma membrane and resides near the calcium channel and adenylate cyclase. These findings differ from those of Martins *et al.* (18), who find most of the cGMP-stimulated activity in a soluble

fraction in heart tissue. However, Pyne *et al.* (32) have recently reported that cGMP-stimulated PDE is found both in the soluble and plasma membrane fractions of rat liver.

Role of PDE activity in regulating I_{Ca}. MIX increased basal I_{Ca} a small amount but produced a large increase in I_{Ca} elevated by 1 μM cAMP. The small and variable effect of MIX on basal I_{Ca} probably reflects the very low levels of intracellular cAMP under these conditions. The finding that MIX at 100 μM dramatically potentiated the effects of low concentrations of cAMP on I_{Ca} was surprising in view of the biochemical experiments (Fig. 4) showing that this concentration of MIX had very little effect on basal PDE activity. This observation raises the possibility that PDE activity may be altered either during the cell preparation or during homogenization.

The possibility that enzymatic dissociation of the cells alters PDE activity is a particularly disturbing one. Our concern is increased by the observation (33) that collagenase treatment inhibits plasma membrane PDE activity in fat cells. It should be noted that the dose-response curve for cAMP (Fig. 6B) in the present study was shifted approximately one log unit to the right compared with other published experiments from our laboratory (20, 34). We interpret this difference to be due to different basal levels of PDE activity in these cells because the dose-response curve in the presence of MIX in Fig. 6B is very similar to that in the absence of MIX in our other experiments. During the same period when the dose-response curve was shifted to the right, the cells were also very unresponsive to isoproterenol. Extreme unresponsiveness to isoproterenol was observed during 2 weeks in March 1987, when our cells did not respond *at all* to isoproterenol at any concentration up to 20 μM. However, the isoproterenol dose-response curve in the presence of 100 μM MIX, which by itself had no effect on basal I_{Ca}, was similar to that previously reported in the absence of MIX (20, 34). These observations show that under certain conditions PDE activity can be sufficiently high to hydrolyze all the cAMP synthesized by hormone-stimulated adenylate cyclase. The reason for this variability is at present unknown.

Physiological role of cGMP-stimulated PDE. Although these experiments have provided a clearer understanding of the mechanism of action of cGMP on I_{Ca}, these experiments do not provide an unambiguous demonstration of the physiological role of this enzyme. Fig. 7B shows that MIX, which we have shown is a selective inhibitor of the cGMP-stimulated PDE at the concentration used, blocks the effects of ACh on isoproterenol-elevated I_{Ca}. These results suggest that ACh may act partially by activation of the cGMP-stimulated PDE. However, MIX also can potentiate the effects of low concentrations of cAMP or isoproterenol on I_{Ca}, suggesting that in the intact cell MIX can inhibit other PDE activities than just the cGMP-stimulated PDE. Inhibition of any PDE activity should blunt the effects of ACh regardless of whether ACh stimulates PDE activity, because block of cAMP hydrolysis will effectively neutralize the effects of adenylate cyclase inhibition by ACh.

The ability of MIX to block the effect of ACh is somewhat unexpected because other investigators have reported that ACh can antagonize the effects of MIX (35–37). There are several possible explanations for this difference. 1) The conditions of whole-cell recording may inhibit or wash out a critical factor important in a mechanism of action of ACh that is separate from the adenylate cyclase system. 2) MIX may have effects on other ionic currents in addition to I_{Ca}. Previous studies that did not directly measure I_{Ca} may have been complicated by this other ionic current.

Specificity and mechanisms of PDE inhibitors. Although MIX has been shown to have a variety of effects in addition to inhibition of PDE activity, several experiments reported here suggest that the effects of MIX on I_{Ca} can be explained entirely by inhibition of PDE. MIX had no effect on I_{Ca} elevated with maximal or supramaximal concentrations of isoproterenol or cAMP. Furthermore, MIX had no effect on I_{Ca} elevated by any concentration of 8-bromo-cAMP. The fact that MIX had little effect on basal I_{Ca} suggests that adenylate cyclase activity is very low in these cells; thus, PDE inhibition would not be expected to alter cAMP levels. In some cells, MIX did stimulate basal I_{Ca}. This result was most frequently seen when the intracellular solution contained 0.4 mM GTP, which may have stimulated adenylate cyclase activity. In cells perfused with 0.4 mM GTP, I_{Ca} slowly increased with time after patch-break, presumably due to a gradual buildup of cAMP in the cell.

We have previously noted (21) that MIX has no effect on I_{Ca} when applied by internal perfusion but only acts when applied extracellularly. This observation is both interesting and troubling. There are several possible explanations. The most interesting possibility is that the cGMP-stimulated PDE has a subunit that is an integral membrane protein and that MIX inhibits enzyme activity by binding to this subunit from the extracellular surface of the cell. This possibility can be tested experimentally. Another possibility is that the PDE is in a subcellular compartment that is isolated from the rest of the cell and is inaccessible to internal MIX. Because MIX is hydrophobic, it could presumably penetrate the plasma membrane and enter this compartment. This possibility seems unlikely inasmuch as internally perfused cAMP and cGMP apparently have access to this compartment. On the other hand, because MIX is hydrophobic, MIX may diffuse out of the cell more rapidly than it enters the cell from the pipet or it may be bound to sites in the cell before it reaches the PDE. The final possibility is that MIX acts by other mechanisms, for example by blocking adenosine receptors or releasing internal Ca. Both these possibilities seem unlikely because internal Ca is buffered with EGTA and sulfophenyltheophylline (an adenosine receptor antagonist) has no effect.

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