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Ca^{2+} current is regulated by cyclic GMP-dependent protein kinase in mammalian cardiac myocytes

(L-type Ca^{2+}-channel current/whole-cell patch clamp/internal perfusion/isolated heart cells/Western blot)

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ABSTRACT **Regulation of cardiac contraction by neurotransmitters and hormones is often correlated with regulation of the L-type Ca^{2+}-channel current (I_{Ca}) through the opposite actions of two second messengers, cyclic AMP and cyclic GMP. While cyclic AMP stimulation of I_{Ca} is mediated by the activation of cyclic AMP-dependent protein kinase, inhibition of I_{Ca} by cyclic GMP in frog heart is largely mediated by activation of cyclic AMP phosphodiesterase. The present patch-clamp study reveals that, in rat ventricular cells, cyclic GMP can also regulate I_{Ca} via activation of endogenous cyclic GMP-dependent protein kinase (cGMP-PK). Indeed, the effect of cyclic GMP on I_{Ca} was mimicked by intracellular perfusion with the proteolytic active fragment of purified cGMP-PK. Moreover, cGMP-PK immunoreactivity was detected in pure rat ventricular myocytes by using a specific polyclonal antibody. These results demonstrate a dual mechanism for the inhibitory action of cyclic GMP in heart, as well as a physiological role for cGMP-PK in the control of mammalian heart function.**

It was in the heart that cyclic GMP levels were first discovered to be physiologically regulated (1). Subsequently, cyclic GMP was shown to exert various inhibitory actions on cardiac cells, including a negative inotropic effect (2, 3), a reduction of gap junctional conductance (4), and an inhibition of the L-type Ca^{2+} current (I_{Ca}) (5–8). The once prevalent idea that cyclic GMP may serve as the second messenger for acetylcholine in regulation of cardiac function (2, 3, 9) has been continually challenged, however, because of difficulties demonstrating the presence of cyclic GMP-dependent protein kinase (cGMP-PK) in cardiac myocytes (10, 11). Other cyclic GMP binding proteins, such as the cyclic GMP-activated cyclic AMP phosphodiesterase (12), have alternatively been proposed to mediate some of the inhibitory effects of cyclic GMP (3, 5, 6, 10).

In the present studies, we have performed patch-clamp measurements of I_{Ca} on purified rat myocytes internally perfused with a catalytically active fragment of cGMP-PK, which was prepared by limited trypsin proteolysis of the holoenzyme. This proteolysis removes the amino-terminal end of cGMP-PK, which has been shown to contain both the dimerization and regulatory domains of cGMP-PK, the latter of which inhibits enzyme activity in the absence of cyclic GMP (13). Use of this fragment permitted us to examine the direct effect of cGMP-PK on I_{Ca} in the absence of added cyclic GMP to distinguish this mechanism of cyclic GMP action from alternative ones. The results indicated that cGMP-PK could mimic the actions of cyclic GMP in inhibiting I_{Ca} that had been elevated by cyclic AMP. Furthermore, additional experiments with nonhydrolyzable analogs of cyclic AMP and cyclic GMP indicated that, unlike in frog heart (5, 6), cyclic GMP did not affect I_{Ca} via stimulation of a cyclic AMP phosphodiesterase. Some of these results have appeared in abstract form (14).

MATERIALS AND METHODS

Materials. Chemicals used in patch-clamp experiments, including cyclic AMP, cyclic GMP, 8-bromo-cyclic AMP (8Br-cAMP), 8-bromo-cyclic GMP (8Br-cGMP), 3-isobutyl-1-methylxanthine (IBMX), adenosine 5'-[γ-thio]triphosphate (ATP[γS]), and tetrodotoxin were purchased from Sigma. cGMP-PK was purified (15) and an antibody was prepared against it (15, 16) as described. Bovine pancreas trypsin L-1-tosylamido-2-phenylethyl chloromethyl ketone (235 units per mg of protein) was from Worthington, phenylmethylsulfonyl fluoride was from Sigma, Kemptide was from Peninsula Laboratories, and ^{125}I-labeled protein A was from Amersham.

Patch-Clamp Studies with Rat Ventricular Myocytes. Ventricular cells were enzymatically dispersed from hearts of male Wistar rats (200–250 g) as described (17, 18). All K^+ currents were blocked with intracellular and extracellular Cs^+. The fast Na^+ current was blocked with 50 μM tetrodotoxin. In most experiments, the cell was routinely depolarized every 8 s from −80 mV to −50 mV during 50 ms and subsequently to 0 mV for 200 ms (Fig. 1). In <10% of the cells, the prepulse to −50 mV allowed the elimination of a small contaminating Na^+ current that remained despite the high concentration of tetrodotoxin used. In all other cells (>90% of total), the current elicited by the test pulse to 0 mV was identical whether the −50-mV prepulse was applied or not. Under these conditions, the current elicited by a depolarizing test pulse to greater than −40 mV was composed of the transsarcolemmal I_{Ca} that was blocked completely with Cd^{2+} (20) and a small time-independent leak current. All experiments were done at room temperature (21°C–24°C).

Solutions and Drugs. Control external solution contained the following: 127.1 mM NaCl, 20 mM CsCl, 4 mM $NaHCO_3$, 0.8 mM NaH_2PO_4, 1.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM D-glucose, 5 mM sodium pyruvate, 50 μM tetrodotoxin, and 10 mM Hepes, adjusted to pH 7.4 with NaOH. Control or IBMX-containing solutions were applied to the exterior of the cell by placing the cell at the opening of a 250-μm inner diameter capillary from which the external solution was flowing at a rate of ≈10 μl/min (19). Patch electrodes (0.8–1.6 MΩ) were filled with control internal solution containing the following: 139.8 mM CsCl, 5 mM EGTA, 4 mM $MgCl_2$, 5 mM disodium phosphocreatine, 3.1 mM Na_2ATP, 0.42 mM Na_2GTP, 0.062 mM $CaCl_2$ (pCa 8.5), and 10 mM Hepes,

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Abbreviations: I_{Ca}, L-type Ca^{2+}-channel current; cGMP-PK, cyclic GMP-dependent protein kinase; 8Br-cAMP, 8-bromo cyclic AMP; 8Br-cGMP, 8-bromo cyclic GMP; IBMX, 3-isobutyl-1-methylxanthine; ATP[γS], adenosine 5'-[γ-thio]triphosphate.

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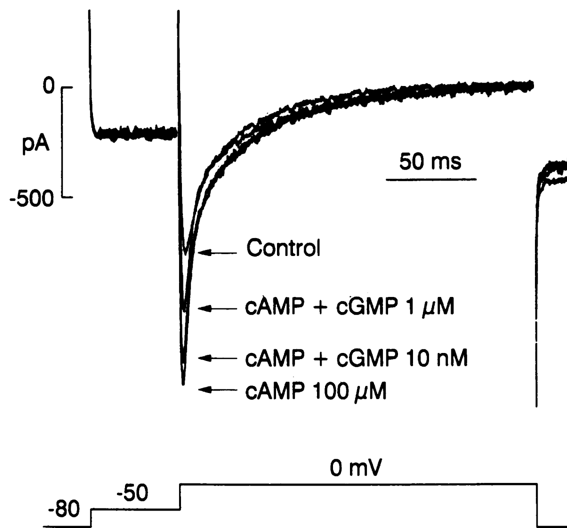

FIG. 1. Effect of cyclic AMP and cyclic GMP on rat ventricular cell I_{Ca} recorded by the whole-cell patch-clamp technique (19). The individual current traces were obtained after 5–6 min of perfusion with control internal solution, or an internal solution containing either 100 μM cyclic AMP alone, or cyclic AMP in the presence of 10 nM or 1 μM cyclic GMP.

adjusted to pH 7.1 with KOH. Drug (cyclic AMP, 8Br-cAMP, cyclic GMP, 8Br-cGMP, etc.)-containing or protein (cGMP-PK)-containing solutions were then delivered to the interior of the cell by perfusion of the patch electrode (5, 6).

Data Analysis. Currents were not compensated for capacitive and leak currents. On-line analysis of the recordings was made possible by programming a Compaq 386/25 Desk-pro computer in Pascal language to determine, for each membrane depolarization, peak and steady-state current values (19). I_{Ca} was determined as the difference between peak current and the leak current amplitude (I_{200}) measured at the end of the 200-ms pulse (5, 6, 19, 21). As described earlier in our laboratory (20), this determination gave similar current amplitudes and voltage dependence as when measuring by subtraction the characteristics of the cadmium-sensitive current. Current–voltage relationships for I_{Ca} and I_{200} (Fig. 2*B*) and I_{Ca} inactivation curves (Fig. 2*C*) were obtained by voltage clamp protocols as described (19).

In the text, "basal" condition refers to the absence of either cyclic AMP, 8Br-cAMP, or IBMX—i.e., to the absence of stimulation of cyclic AMP-dependent phosphorylation of Ca^{2+} channels. Average values are expressed as means ± SEM.

Preparation of a Catalytically Active Fragment of cGMP-PK. Bovine lung cGMP-PK holoenzyme (150 kDa) was purified by affinity chromatography using 8-(6-aminohexylamino) cyclic AMP Sepharose (15). The kinase was eluted from the column with cyclic AMP, which was subsequently removed by dialysis. Use of this purification procedure makes it highly unlikely that the purified cGMP-PK was contaminated by any cyclic GMP or cyclic GMP-regulated phosphodiesterases. A proteolytic active fragment (monomer, 65 kDa) was prepared from the holoenzyme as described (13). After optimal proteolysis was achieved, further action of trypsin was inhibited by the addition of a 10-fold excess of an irreversible inhibitor, phenylmethylsulfonyl fluoride. The 65-kDa fragment was cyclic GMP independent in an *in vitro* Kemptide substrate protein kinase assay performed as described (22). The active cGMP-PK was prepared at 1–3 μM concentration in 2–3 ml of PEMT buffer (10 mM potassium phosphate/1 mM EDTA/15 mM 2-mercaptoethanol/50 units of Trasylol per ml). Immediately before use, cGMP-PK was dialyzed against control internal solution for 14 hr at 4°C. A

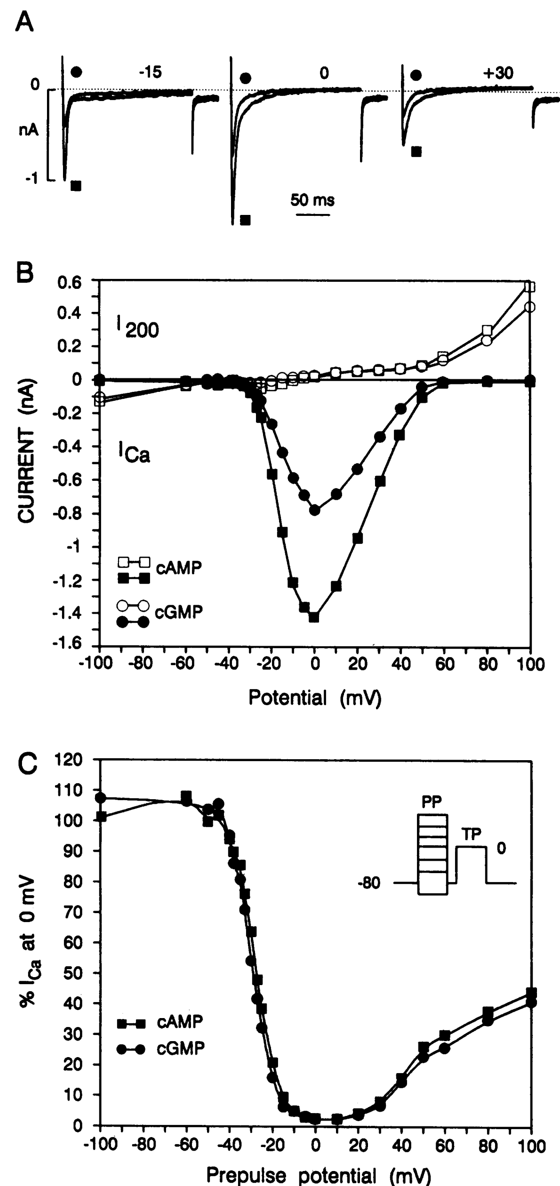

FIG. 2. Voltage dependence of the inhibitory effect of cyclic GMP on I_{Ca}. (*A*) Individual current traces obtained at three different potentials (−15, 0, and +30 mV) from −80 mV holding potential. The lower traces (squares) were recorded in the presence of 100 μm cyclic AMP; the upper traces (circles) were recorded 10 min after 1 μM cyclic GMP was added to the cyclic AMP-containing solution. The dotted line indicates the zero-current level. (*B*) Current–voltage relationship of I_{Ca} (solid symbols) and steady-state leak current (I_{200}, open symbols) measured during 200-ms depolarizations to various potentials from −80 mV holding potential in a cell intracellularly perfused with 100 μM cyclic AMP alone (squares) and after 10 min of intracellular perfusion with 1 μM cyclic GMP added to the cyclic AMP-containing solution (circles). I_{Ca} and I_{200} were measured as described (ref. 19; see also *Materials and Methods*). (*C*) Inactivation curve of I_{Ca}. A double-pulse protocol was used as indicated (*Inset*). I_{Ca}, measured during a 200-ms test pulse (TP) at 0 mV, is plotted as a function of prepulse (PP) potential (200 ms duration) and is expressed as percentage of I_{Ca} at 0 mV in the absence of prepulse (for more details, see ref. 19). Squares, 100 μM cyclic AMP alone; circles, 100 μM cyclic AMP + 1 μM cyclic GMP. All experiments were done on the same cell.

mock trypsin assay was performed in the absence of cGMP-PK, dialyzed as described above, and used as a control. This control buffer alone had no effect on I_{Ca}.

Preparation of Tissue and Cell Extracts. Tissue homogenates were prepared by homogenizing rat tissues in 5 vol of

Table 1. Effects of guanine nucleotides and cGMP-PK on basal and stimulated I_{Ca}

Initial stimulus	% increase over control	Test compound (M)	% change	n
None		Cyclic GMP (10^{-6})	−0.5 ± 4.5	4
		Cyclic GMP (10^{-4})	0.2 ± 2.9	4
		cGMP-PK (10^{-6})	−5.0 ± 3.2	4
Cyclic AMP (10^{-4} M)	144.9 ± 38.3	Cyclic GMP (10^{-7})	−29.4 ± 4.8*	5
	126.4 ± 27.0	Cyclic GMP (10^{-6})	−50.0 ± 9.2*	8
	99.5 ± 26.9	Cyclic GMP (10^{-5})	−71.0 ± 8.7*	5
	80.5 ± 11.4	8Br-cGMP (10^{-7})	−16.7 ± 8.6	5
	99.3 ± 20.6	8Br-cGMP (10^{-6})	−27.1 ± 18.6	5
	86.4 ± 21.2	8Br-cGMP (10^{-5})	−38.4 ± 9.8*	6
	92.8 ± 25.3	cGMP-PK (10^{-8})	−29.5 ± 7.2†	4
	77.9 ± 12.4	cGMP-PK (10^{-7})	−36.1 ± 8.2*	9
8Br-cAMP (10^{-4} M)	125.6 ± 28.7	8Br-cGMP (10^{-7})	−27.9 ± 18.2	5
	124.2 ± 29.5	8Br-cGMP (10^{-6})	−33.1 ± 19.2†	5
	136.6 ± 34.5	8Br-cGMP (10^{-5})	−55.1 ± 18.2†	4
	90.8 ± 14.7	cGMP-PK (10^{-7})	−52.4 ± 6.4†	3
IBMX (10^{-4} M)	70.4 ± 8.7	Cyclic GMP (10^{-6})	−61.7 ± 16.1†	3
	62.8 ± 11.7	Cyclic GMP (10^{-5})	−78.7 ± 6.6*	3

Cyclic nucleotides and cGMP-PK were applied intracellularly (5, 6), and IBMX was applied externally. Data are means ± SEM. The effects of test compounds are expressed as % change with respect to either basal I_{Ca} (in the absence of initial stimulus) or with respect to the enhancement of I_{Ca} induced by the initial stimulus. Statistical significance was determined by a one-sample analysis t test when comparing the mean % change values to zero. n, Number of cells.
*$P < 0.01$.
†$P < 0.05$.

10 mM potassium phosphate (pH 7.4) containing 1 mM EGTA, 1 mM EDTA, and 50 units of Trasylol per ml. Homogenates were diluted 2:1 (vol/vol) with Laemmli gel $NaDodSO_4$-containing stop solution and boiled immediately before $NaDodSO_4$/PAGE as described (23). A cell suspension of rat myocytes was added directly to stop solution and boiled. Other samples were analyzed for protein according to the method of Lowry (24).

The Lowry method indicated that the myocytes contained 20 ng of protein per cell. Microscopic examination of cells suggested that the purity of the myocyte preparation was >99%. The dimensions of rat myocytes (25) were used to calculate an approximate cell volume of 20 pl. These data were combined with that from Western blot analysis (see below) of the amount of cGMP-PK per μg of protein in rat myocytes to estimate the intracellular concentration of cGMP-PK.

Western Blot. Proteins separated by $NaDodSO_4$/PAGE were transferred to nitrocellulose sheets and radioimmunolabeled as described (23) except that the blocking medium contained phosphate-buffered saline, 1% hemoglobin, and 0.01% NaN_3. cGMP-PK was labeled with an antibody made against bovine lung cGMP-PK (dilution, 1:400) and 0.1 μCi of ^{125}I-labeled protein A per ml (1 Ci = 37 GBq).

RESULTS

The effect of intracellular cyclic GMP on I_{Ca} was examined in isolated internally perfused rat ventricular myocytes by the whole-cell patch-clamp technique (26). Table 1 shows that cyclic GMP, up to 100 μM, exerted no significant effect on rat heart basal I_{Ca}. However, when added after I_{Ca} stimulation by intracellular perfusion of cyclic AMP, cyclic GMP concentrations >10 nM exerted a strong dose-dependent inhibitory effect on I_{Ca} (Fig. 1; Table 1). As shown in Fig. 2, cyclic GMP inhibited cyclic AMP-elevated I_{Ca} at all potentials (Fig. 2*A*) without causing a significant change in the shape of the current–voltage relationship (Fig. 2*B*) or inactivation curve (Fig. 2*C*) of I_{Ca}. Besides, cyclic GMP did not modify the leak current, I_{200} (Fig. 2*B*). These results indicate that cyclic GMP inhibits I_{Ca} without modifying the voltage dependence of peak current and inactivation.

Studies of the interaction of hydrolysis-resistant analogs of cyclic GMP and cyclic AMP on I_{Ca} (Table 1; Fig. 3) provided initial evidence that the mechanism of action of cyclic GMP in rat heart differed from that previously characterized in frog heart (5, 6). For example, 8Br-cGMP produced a dose-dependent inhibition of I_{Ca} elevated by 8Br-cAMP (100 μM) in rat cells, whereas 8Br-cGMP had no effect on I_{Ca} in frog cells (5, 6). In addition, I_{Ca} elevated by extracellularly applied IBMX (100 μM), a nonspecific phosphodiesterase inhibitor, was also strongly inhibited by cyclic GMP (Table 1). IBMX is a universal phosphodiesterase inhibitor and inhibits all the known cyclic GMP-regulated phosphodiesterases likely to be present in the heart—most relevant in this case, the cyclic

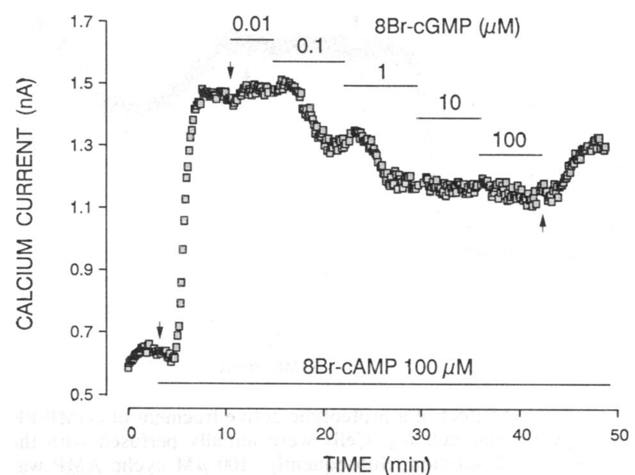

FIG. 3. Effect of increasing concentration of 8Br-cGMP on I_{Ca}. The cell was initially perfused with control internal solution until 100 μM 8Br-cAMP was added to the patch-electrode internal perfusion solution (first arrow). In the continued presence of 8Br-cAMP, 8Br-cGMP was added to the internal solution (second arrow) in consecutively increasing concentrations (10 nM to 100 μM), each for the duration indicated. At the third arrow, the internal perfusion solution was changed back to 8Br-cAMP alone.

GMP-stimulated phosphodiesterase (12). Therefore, cyclic GMP inhibition of rat heart I_{Ca} could not be mediated by cyclic GMP activation of a cyclic AMP phosphodiesterase as had been shown in frog. An identical conclusion concerning the mechanism of cyclic GMP action in guinea pig (7) and embryonic chicken (8) ventricular cells was drawn from recent experiments, stimulating us to examine the possibility that cyclic GMP inhibits mammalian Ca^{2+} channels by activation of a cGMP-PK.

That a phosphorylation-dependent mechanism mediated the inhibitory effect of cyclic GMP on I_{Ca} was suggested by comparing the reversibility of the effect of cyclic GMP in cells perfused with ATP-containing intracellular solution or with ATP[γS] substituting for ATP. While the inhibitory effect of 1 μM cyclic GMP on I_{Ca} elevated by 100 μM cyclic AMP was reversed after wash-out of cyclic GMP in three of four cells within 4–6 min (by 65%, 85%, and 100%, respectively), the effect of cyclic GMP was irreversible in each of three cells perfused with ATP[γS]. Further evidence for a phosphorylation-dependent mechanism was demonstrated by the finding that intracellular perfusion with the catalytically active fragment of purified cGMP-PK inhibited cyclic AMP-elevated I_{Ca} in a manner similar to that of cyclic GMP (Fig. 4*A*; Table 1). Heat-inactivated (95°C for 30 min) cGMP-PK (10 nM) had no effect, while the same concentration of active cGMP-PK induced a substantial reduction of I_{Ca} (Fig. 4*B*; Table 1).

For cyclic GMP to exert its inhibitory action on I_{Ca} via cyclic GMP-dependent phosphorylation, rat ventricular cells must possess endogenous cGMP-PK. Immunocytochemical methods have revealed that the major concentration of cGMP-PK in heart resides in the smooth muscle of cardiac vessels (10, 27, 28). However, cardiac myocytes may possess a very low concentration of cGMP-PK that is difficult to detect with anti-cGMP-PK antibodies by immunocytochemistry. To examine this hypothesis, we used an alternative immunological method (Western blot) to identify the presence of cGMP-PK in a highly concentrated and purified suspension of rat ventricular myocytes. Fig. 5 shows that the polyclonal antibody directed against bovine lung cGMP-PK (16), which recognizes both cGMP-PK isoforms I_α and I_β (29), immunoreacted with the preparation of cardiac myocytes. The labeled protein had an apparent molecular mass of 74 kDa, identical to that of purified cGMP-PK and cGMP-PK in whole rat heart and cerebellum homogenates (Fig. 5). The concentration of cGMP-PK holoenzyme in rat ventricular myocytes was estimated to be ≈30 nM, in comparison to a ≈10 times higher estimated concentration (0.36 μM) of kinase in smooth muscle (30).

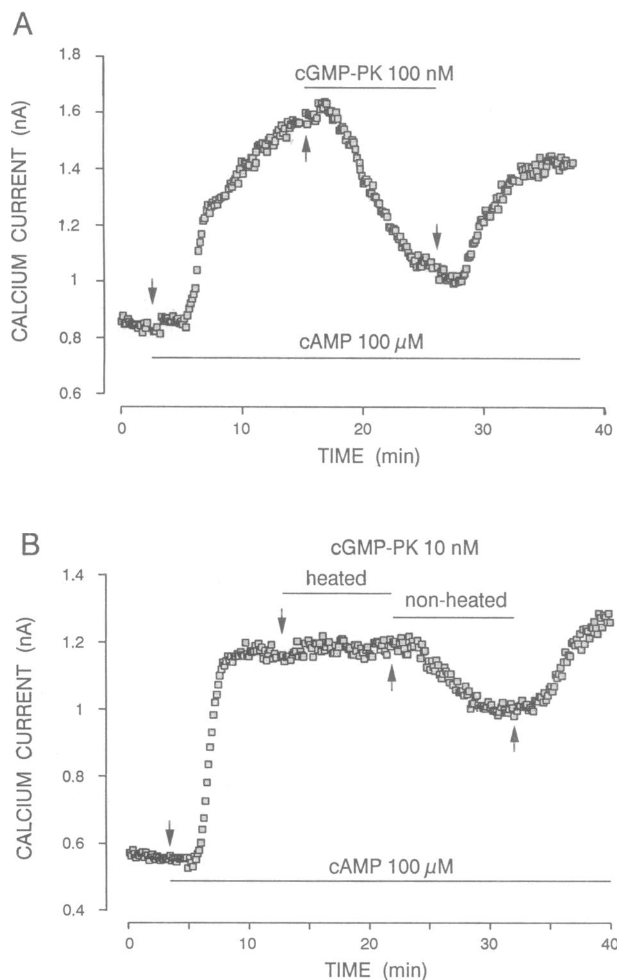

FIG. 4. (*A*) Effect of a proteolytic active fragment of cGMP-PK on rat ventricular cell I_{Ca}. Cells were initially perfused with the control internal solution. Subsequently, 100 μM cyclic AMP was added to the internal perfusion solution (first arrow) and the length of its presence is indicated by a duration line in both *A* and *B*. (*A*) The addition (second arrow) and duration of perfusion of the proteolytic active fragment of cGMP-PK (100 nM) in the cell are indicated. (*B*) The addition of 10 nM heat-inactivated (95°C, 30 min; second arrow) or active cGMP-PK (third arrow) to the internal perfusion solution, and the length of their respective durations, are indicated. In both *A* and *B*, the change of the internal solution back to one containing cyclic AMP alone is indicated by the last arrow.

DISCUSSION

The present study demonstrates that cyclic GMP inhibits I_{Ca} in mammalian cardiac cells by a newly discovered mechanism. The similarity between the effects of cyclic GMP, 8Br-cGMP, and the active fragment of cGMP-PK on I_{Ca}, as well as the identification of endogenous cGMP-PK in rat ventricular cells, strongly suggest that I_{Ca} regulation by cyclic GMP involves the activation of cyclic GMP-dependent phosphorylation.

When comparing the present results to our earlier ones (5, 6), one can conclude that there is a major difference between the effect of cyclic GMP in amphibian and mammalian ventricular cells. In frog heart, the use of nonhydrolyzable (8Br) analogs indicated that cyclic GMP decreased I_{Ca} by stimulation of cyclic AMP hydrolysis because (*i*) cyclic GMP was without effect on I_{Ca} that had been elevated by 8Br-cAMP, (*ii*) 8Br-cGMP (which is a much better stimulator of

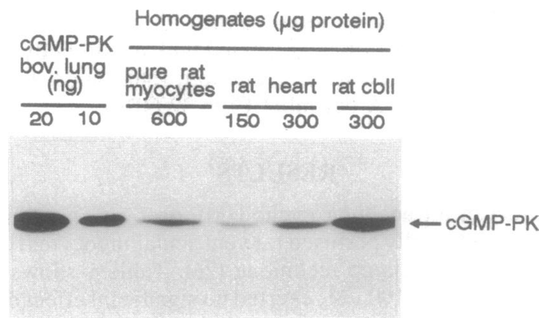

FIG. 5. Autoradiogram demonstrating Western blot detection of cGMP-PK in rat cardiac myocytes using a specific antibody and ^{125}I-labeled protein A. The amount of cGMP-PK contained in homogenates from pure isolated rat ventricular myocytes, entire rat heart, or rat cerebellum (cbll) was compared with purified standards of bovine lung cGMP-PK (20 and 10 ng) as shown. The content of cGMP-PK in purified rat myocytes was estimated to be 5 ng per 600 μg of homogenate protein. Control preimmune serum did not recognize any protein in these tissues.

cGMP-PK than is cyclic GMP, and a much worse stimulator of cyclic GMP-stimulated cyclic AMP phosphodiesterase than is cyclic GMP) had no effect on I_{Ca}, and (*iii*) the inhibitory effect of cyclic GMP on cyclic AMP-stimulated I_{Ca} was largely reversed by IBMX, a general phosphodiesterase inhibitor (5, 6). The present study performed in rat cells, as well as a preliminary study performed in guinea pig cells (7), demonstrated that none of these findings was valid in mammalian myocytes. It is likely, therefore, that mammalian myocytes, in comparison to amphibian ones (31), contain a cyclic GMP-stimulated cyclic AMP phosphodiesterase that either has a lower capacity to hydrolyze cyclic AMP or is less efficiently coupled to Ca^{2+} channels than are other effector systems such as cGMP-PK. Further experiments designed to analyze the effect of cGMP-PK and its presence in frog cardiac myocytes are required to clarify these issues and whether the cyclic GMP-dependent decrease in I_{Ca} in the different species is really due to the presence of different regulatory systems or their amounts.

Demonstrated effects of cGMP-PK on Ca^{2+} and other ion channels are rather limited. Cyclic GMP-PK has been shown to interact with Ca^{2+} channels in snail neurons (32) and with a cation channel in the kidney apical membrane (33). An interesting difference between these studies and ours is that we find that the inhibitory effects of both cyclic GMP and cGMP-PK on heart I_{Ca} occur only after I_{Ca} has been elevated by cyclic AMP-dependent phosphorylation (Table 1). This may indicate that cGMP-PK does not directly interact with the Ca^{2+}-channel protein, but rather phosphorylates a regulatory protein involved in the cascade leading to cyclic AMP-stimulated phosphorylation of Ca^{2+} channels. However, the fact that inhibitory effects of cyclic GMP still occurred after I_{Ca} had been elevated by cyclic AMP in the presence of ATP[γS] precludes the possibility of cyclic GMP activation of a phosphatase or inhibition of cyclic AMP-dependent protein kinase (cAMP-PK). An alternative hypothesis is that cyclic GMP-dependent phosphorylation can modify Ca^{2+} channel activity only after prior phosphorylation of one of the channel subunits by cAMP-PK. This hypothesis would be supported by the identification of specific phosphorylation sites for both the cAMP-PK and the cGMP-PK on α_1 and β subunits of the purified Ca^{2+} channel from skeletal muscle (34, 35). Our results of course do not exclude that cyclic GMP regulation of I_{Ca} in mammalian heart may involve additional mechanisms as well.

Submicromolar concentrations of cyclic GMP or cGMP-PK produced substantial reductions of rat heart I_{Ca}. This was consistent with Western blot estimation of 30 nM endogenous cGMP-PK, equivalent to 120 nM cyclic GMP-binding sites. Because cyclic GMP levels are elevated in cardiac cells by various hormones known to inhibit I_{Ca}, notably acetylcholine (1, 3, 36) and atrial natriuretic factor (37–40), our results strengthen the idea that cyclic GMP serves a second messenger role in the negative cardiac inotropic effect of these hormones (2, 3, 37, 38).

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