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Increase of L-type Calcium Current by cGMP-dependent Protein Kinase Regulates in Rabbit Ventricular Myocytes

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Background: We have previously reported that not only cGMP but also 8-Br-cGMP or 8-pCPT-cGMP, specific and potent stimulators of cGMP-dependent protein kinase (cGMP-PK), increased basal L-type calcium current (I_{Ca}) in rabbit ventricular myocytes. Our findings in rabbit ventricular myocytes were entirely different from the earlier findings in different species, suggesting that the activation of cGMP-PK is involved in the facilitation of I_{Ca} by cGMP. However, there is no direct evidence that cGMP-PK can stimulate I_{Ca} in rabbit ventricular myocytes. In this report, we focused on the direct effect of cGMP-PK on I_{Ca} in rabbit ventricular myocytes. **Methods and Results:** We isolated single ventricular myocytes of rabbit hearts by using enzymatic dissociation. Regulation of I_{Ca} by cGMP-PK was investigated in rabbit ventricular myocytes using whole-cell voltage clamp method. I_{Ca} was elicited by a depolarizing pulse to +10 mV from a holding potential of −40 mV. Extracellular 8-(4-Chlorophenylthio)-guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP), potent stimulator of cGMP-dependent protein kinase (cGMP-PK), increased basal I_{Ca}. cGMP-PK also increased basal I_{Ca}. The stimulation of basal I_{Ca} by cGMP-PK required both 8-Br-cGMP in low concentration and intracellular ATP to be present. The stimulation of basal I_{Ca} by cGMP-PK was blocked by heat inactivation of the cGMP-PK and by bath application of 8-(4-chlorophenylthio)-guanosine-3',5'-cyclic monophosphate, Rp-isomer (Rp-pCPT-cGMP), a phosphodiesterase-resistant cGMP-PK inhibitor. When I_{Ca} was increased by internal application of cGMP-PK, IBMX resulted in an additional stimulation of I_{Ca}. In the presence of cGMP-PK, already increased I_{Ca} was potentiated by bath application of isoprenaline or forskolin or intracellular application of cAMP. **Conclusions:** We present evidence that cGMP-PK stimulated basal I_{Ca} by a direct phosphorylation of L-type calcium channel or associated regulatory protein in rabbit ventricular myocytes.

Key Words: cGMP-dependent protein kinase (cGMP-PK), L-type calcium current (I_{Ca}), Rabbit ventricular myocytes, Whole-cell voltage clamp

INTRODUCTION

L-type calcium current (I_{Ca}) is fundamental in the generation of action potentials and neurohormonal regulation of cardiac electrical and contractile activity. It is well established that the cyclic nucleotide cAMP, increased by activation of the sympathetic nervous system or by -adrenergic agonists, plays an important role in regulation of I_{Ca} in the heart (Bean et al, 1984; Tsien et al, 1986; Harzell, 1988; Ochi et al, 1990; McDonald et al, 1994). The cyclic nucleotide cGMP is also known to be involved in regulation of cardiac function: (1) cGMP activates a specific protein kinase (cGMP-dependent protein kinase, cGMP-PK), which can phosphorylate a number of proteins (Levi et al, 1989; Wahler et al, 1990;

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Mery et al, 1991; Ono & Trautwein, 1991; Tohse et al, 1991; Bkaily et al, 1993; Han et al, 1998), (2) cGMP stimulates specific phosphodiesterase (cGMP-stimulated PDE) to produce antagonistic effects with cAMP-dependent pathway (Harzell & Fischmeister, 1986; Fischmeister & Harzell, 1987), or (3) cGMP inhibits specific PDE (cGMP-inhibited PDE) to produce synergistic effects with cAMP-dependent pathway (Ono & Trautwein, 1991; Kirstein et al, 1995).

Considering the involvement of cGMP in the modulation of basal I_{Ca} (not stimulated through the cAMP-dependent pathway), recent studies show that cGMP effects appears to be variant (Harzell & Fischmeister, 1986; Fischmeister & Harzell, 1987; Cuppoletti et al, 1989; Levi et al, 1989; Wahler et al, 1990; Mery et al, 1991; Ono & Trautwein, 1991; Tohse et al, 1991; Han et al, 1992, 1993b; Bkaily et al, 1993; Haddad et al, 1995). Although some investigators reported that there were no effects of cGMP on basal I_{Ca} (Harzell &Fischmeister, 1986; Fischmeister & Harzell, 1987; Cuppoletti et al, 1989; Ono & Trautwein, 1991; Mery et al, 1991), we have observed that intracellular application of cGMP increased basal I_{Ca} in rabbit ventricular myocytes (Han et al, 1995, 1998). In the following we found that 8-Br-cGMP or 8-pCPT-cGMP, specific and potent stimulators of cGMP-PK, increased basal I_{Ca} in rabbit ventricular myocytes (Han et al, 1998). Our findings in rabbit ventricular myocytes were entirely different from the earlier findings in different species (Ono & Trautwein, 1991; Mery et al, 1993; Kirstein et al, 1995). From our results, we suggested that the activation of cGMP-PK is involved in the facilitation of I_{Ca} by cGMP in rabbit ventricular myocytes. In this report, we confirmed this possibility directly by applying cGMP-PK into the cells in whole-cell voltage clamp. We present evidence that cGMP-PK increased basal I_{Ca} by a direct phosphorylation of L-type calcium channel or associated regulatory protein in rabbit ventricular myocytes.

METHODS

Preparation of single ventricular myocytes

Single ventricular myocytes were isolated from rabbit hearts by enzymatic dissociation, as discussed previously (Han et al, 1993a). Rabbits (400~600 g) of either sex were anaesthetized with intravenously administered sodium pentobarbitone (40~50 mg/kg) after heparin administration (300 IU/kg). The thorax and pericardium were opened under artificial respiration and the aorta was cannulated before removal of the heart. The excised heart was connected to a Langendorff perfusion apparatus and perfused with 80 cmH_2O pressure. Normal Tyrode solution was first perfused until the blood was washed out completely. And then Ca^{2+}-free Tyrode solution was perfused for 5 minutes and Ca^{2+}-free Tyrode solution containing 0.01% collagenase (5 mg/50 cc, Yakult, Japan) was perfused for 15~25 minutes. After enzymatic treatment, Krafts-Brhe (KB) solution (Isenberg & Klcknor, 1982) was perfused for washing out remaining enzyme in the heart. The KB solution contained (in mM): 50 glutamate, 20 HEPES, 20 taurine, 10 glucose, 3 $MgSO_4$, 0.5 EGTA, 30 KCl, 30 KH_2PO_4, the pH was adjusted to 7.4 with KOH. The temperature of the Langendorff column was kept at 37°C during all previous steps. The heart was then placed in 50 ml KB solution at room temperature. The ventricles were cut and small pieces of ventricular tissue were gently agitated. Isolated ventricular cells were equilibrated with 100% oxygen. The rod shape of the cell and the clear striations of sarcomere were important criteria used for selecting viable cells. The cells used for the experiments were cylindrical in shape and their striation was regular and fine. Such cells had membrane capacitance of 37.8 ± 6.9 pF (n=15) and displayed resting potentials of -77.4 ± 2.8 mV (n=15).

Solutions and drugs

The bath solution used to superfuse was normal Tyrode solution, the composition of which was (in mM): 143 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5.5 glucose, 5-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), the pH was adjusted to 7.4 with NaOH. The composition of the standard internal solution in the patch electrode was (in mM): 110 CsCl, 5 Mg-ATP, 2.5 di-Tris creatine phosphate, 2.5 disodium creatine phosphate, 1 $MgCl_2$, 5 ethyleneglycol-bis-(b-aminoethylether)-N,N,N',N',-tetraacetic acid (EGTA), 5 HEPES, 20 tetraethylammonium cholide (TEA-Cl), the pH was adjusted to 7.4 with CsOH. Isoprenaline, forskolin, cAMP, cGMP and 8-Br-cGMP were obtained from Sigma chemical Co. (St.Louis, MO, U.S.A.). cGMP-dependent protein kinase (cGMP-PK) was obtained from Promega (Medison, WI, U.S.A.). 8-(4-Chlorophenylthio)-guano-

sine-3',5'-cyclic monophosphate (8-pCPT-cGMP) and its Rp-isomer (Rp-pCPT-cGMP) were obtained from Biolog Life Science Institute (Bremen, FRG). Another drugs and chemicals were obtained from Sigma. Isoprenaline and Rp-8-pCPT-cGMP were dissolved in distilled water to provide a stock solution (5 mM). IBMX was dissolved in DMSO to provide a 100 mM stock solution. It was confirmed that the solvent used, DMSO (up to 0.1%), did not affect L-type calcium current (I_{Ca}). cGMP-PK was directly dissolved in the internal solution at a final concentration of 5 U/l. cGMP-PK was stored at 4°C and used within 7 days. The bath solution could be replaced within 10 seconds by switching from one solution to another. Experiments were performed at room temperature (25 2°C).

Electrophysiological measurements (Whole-cell voltage clamp recording)

L-type calcium current (I_{Ca}) was obtained through the whole-cell patch method (Hamill et al, 1981) by using a patch-clamp amplifier (Axoclamp-1D, Axon Instrument, Inc., CA, U.S.A.). Patch electrodes were pulled with a vertical puller (PP-83, Narishige, Tokyo, Japan or L/M-3P-A, List Medical Electronics, Darmstadt, Germany) from borosilicate capillary tubes (Mercer glass NO. MX-999, N.Y., U.S.A.) and had tip resistance of 2~3 MΩ when filled with internal solution. Neither the capacitance current nor series resistance with the cell membrane were compensated. Membrane current signals were digitized at a sampling rate of 48 kHz, and recorded on a chart recorder (Recorder 220, Gould, U.S.A.) or digital tape recorder (DTR-1200, Biologic, Grenoble, France) for later analysis. We applied the depolarizing step pulse from the holding potential of −40 mV to various potentials for 200 ms to inactivate the fast sodium current. Potassium currents were blocked with intracellular cesium (110 mM). Our prior experiments (Han et al, 1993b) showed that various calcium channel blockers abolished the current activated by this protocol, and that any remaining time-dependent current was negligible. This indicated that our protocol activated I_{Ca} with little contamination from other currents, so that it could be used to investigate changes of I_{Ca}. Amplitude of I_{Ca} was measured as the difference between peak current and holding current measured at the start of 200 ms pulse. The current amplitude was then standardized with membrane capacitance, 100 pF. In the text, the "basal" I_{Ca} refers to the current which does not involve the cAMP/cAMP-dependent protein kinase (cAMP-PK) pathway. Cells which showed rundown were excluded from the analysis by only accepting the data from cells in which I_{Ca} was stable in the control condition and reached a stable effect with the tested drugs.

Statistics

The values were expressed as the mean plus or minus the standard deviation to the mean (S.D.). Statistical significance of paired and unpaired data was determined by student's *t* test. A *P* value less than 0.05 was assumed significant.

RESULTS

We have observed that intracellular application of cGMP increases the basal I_{Ca} in rabbit ventricular myocytes without any previous stimulation (Han et al, 1993b, 1995, 1998). One possible interpretation of our results was that cGMP increases the basal I_{Ca} in rabbit ventricular myocytes through stimulation of the cGMP-dependent protein kinase (cGMP-PK), and subsequent phosphorylation of some protein involved in L-type calcium channel function. We first examined the effect of a potent activator of cGMP-PK, 8-(4-chlorophenylthio)-guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP), on the basal I_{Ca}. 8-pCPT-cGMP is an analogue of the natural signal molecule cGMP in which the hydrogen in position 8 of the heterocyclic base is replaced by the lipophilic para-chlorophenylthio moiety. It is a selective stimulator of cGMP-PK with increased activation potential compared to its parent compound cGMP. It has higher lipophilicity so that it should permeate the cell membrane at higher rates. It is not degraded by cyclic nucleotide phosphodiesterases (PDE) in long lasting effects thus avoiding toxic metabolites and their side effects. It has these advantages over other membrane-permeable analogues of cGMP. In the experiment shown in Fig. 1, 400 μM 8-pCPT-cGMP, when added to the bath solution, increased basal I_{Ca}. Average basal I_{Ca} was increased from 0.55±0.07 to 0.96± 0.11 nA/100 pF ($P<0.05$, $n=6$, 400 μM 8-pCPT-cGMP) at +10 mV. Fig.1A shows a representative set of membrane currents obtained with a holding potential of −40 mV in control (*left traces*) and at

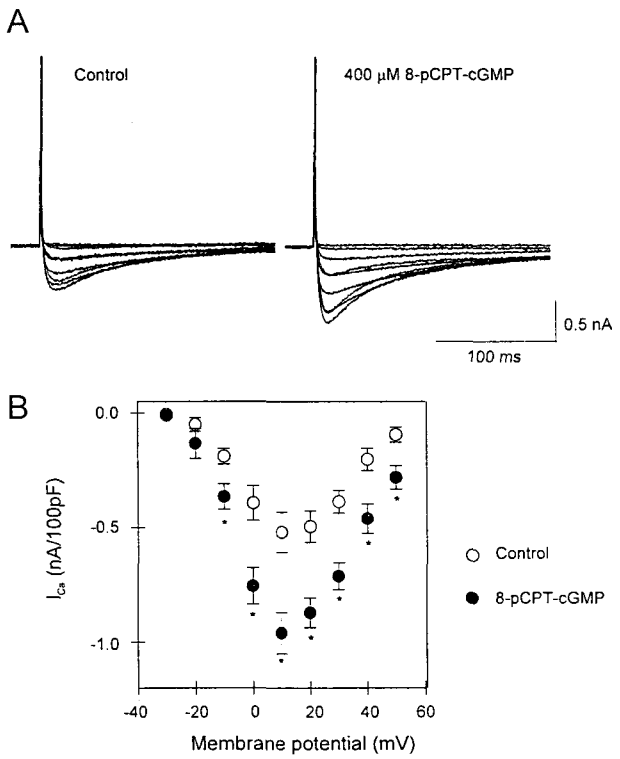

Fig. 1. Effect of 8-(4-Chlorophenyl-thio)-guanosine 3',5'-cyclic monophosphate (8-pCPT-cGMP) on basal L-type calcium current (I_{Ca}) in rabbit ventricular myocytes. A. Superimposed current traces elicited by voltage pulses from −30 to +50 mV in 10-mV steps from a holding potential of −40 mV in control conditions (left traces), with 400 μM 8-pCPT-cGMP (right traces) in the bath solution. Superimposed current traces from the same ventricular cells (membrane capacitance = 40.1 pF). B. Graph showing current-voltage relationship for I_{Ca} (nA/100 pF) before treatment (Control, open circles) and after extracellular application of 400 μM 8-pCPT-cGMP (closed circles). * $P<0.05$.

the time of maximal effect of 8-pCPT-cGMP (*right traces*). Fig. 1B shows the current-voltage relationship obtained before (Control, marked with open circle) and after 400 μM 8-pCPT-cGMP treatment (8-pCPT-cGMP, marked with closed circle). As evident from the figure, the shape of current-voltage relationship for I_{Ca} was not significantly affected by 400 μM 8-pCPT-cGMP, with a threshold potential at about −30 mV, a maximum at +10 mV and an apparent reversal potential at about +60 mV.

The finding that both 100 μM 8-Br-cGMP and 400 μM 8-pCPT-cGMP, which are known to be more powerful than cGMP as a stimulator of cGMP-PK, increase the basal I_{Ca} (Han et al, 1998; Fig. 1)

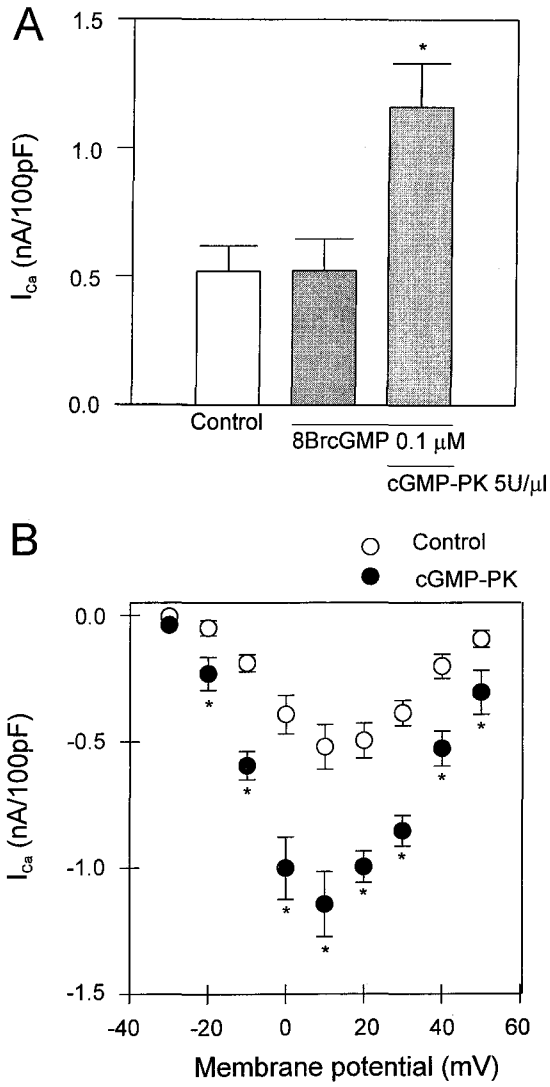

Fig. 2. cGMP-dependent protein kinase (cGMP-PK) stimulates I_{Ca} in rabbit ventricular myocytes. A. Effect of low doses of 8-Br-cGMP (0.1 μM) and cGMP-PK (5 U/μl) on basal I_{Ca}. 8-Br-cGMP and cGMP-PK were applied from an internal solution of the patch pipette. The peak I_{Ca} was evoked by depolarizing pulse of +10 mV from a holding potential −40 mV. As shown, 8-Br-cGMP alone and cGMP-PK alone (data not shown) had only little effect. It can be seen that 8-Br-cGMP and cGMP-PK were required together to produce a stimulatory effect. B. The stimulatory effect of cGMP-PK on the current-voltage relationship for basal I_{Ca} in rabbit ventricular myocytes. Current-voltage curves of peak I_{Ca} from 6 cells for the control condition (open circle) and after application of cGMP-PK (closed circle). Currents are normalized with membrane capacitance of 100 pF. Data points are shown as mean±S.D.. As can be seen, the threshold voltage was about −30 mV, and the voltage for peak activation occurred at about +10 mV. The reversal potential was +60 mV both in control and in the presence of cGMP-PK. * $P<0.05$.

supports the idea that cGMP can increase the basal I_{Ca} through cGMP-PK. In order to test this possibility, we directly applied cGMP-PK into the cell in whole-cell patch configuration (Fig. 2). A low concentration of 8-Br-cGMP (0.1 μM) always accompanied the cGMP-PK in order to activate its enzymatic activity since there is no catalytic subunit. Since a low concentration (0.1 μM) of the potent and unhydrolyzable cGMP-PK activator, namely 8-Br-cGMP, was present to activate the enzymatic activity, we first tested its effect (Fig. 2A). 8-Br-cGMP (0.1 μM) itself had little effect on basal I_{Ca} (from 0.52 ± 0.10 to 0.54 ± 0.12 nA/100 pF; n=5; $P>0.05$). However, the intracellular application of 0.1 μM 8-Br-cGMP and 5 U/μl cGMP-PK together increased basal I_{Ca}. Average basal I_{Ca} was increased from 0.52 ± 0.10 to 1.16 ± 0.17 nA/100 pF at +10 mV ($P<0.05$, n=6) by 5 U/μl cGMP-PK and 0.1 μM 8-Br-cGMP together. As can be seen in our reports (Han et al, 1995, 1998), higher concentration of 8-Br-cGMP (100 μM) increased basal I_{Ca}. cGMP-PK (5 U/μl) alone did not increase the amplitude of I_{Ca} (data not shown; 0.57 ± 0.10 nA/100 pF; n=5; $P>0.05$). The current-voltage relationship for basal I_{Ca} before (Control, marked with open circle) and after application of 5 U/μl cGMP-PK (cGMP-PK, marked with closed circle) are summarized in Fig. 2B. As evident from the figure, the shape of current-voltage relationship for I_{Ca} was not significantly affected by 5 U/μl cGMP-PK, with a threshold potential at about −30 mV, a maximum at +10 mV and an apparent reversal potential at about +60 mV.

As can be seen in Methods section, 5 mM ATP is present in the standard internal solution. In order to test whether the effect of cGMP-PK on basal I_{Ca} was due to the activation of L-type calcium channel by phosphorylation, we omitted 5 mM ATP from the standard internal solution, and this modified solution was used for the internal solution. We performed the experiments by using the protocol of Fig. 2A. As can be seen in Fig. 3, 0.5 μM 8-Br-cGMP alone ($0.52\pm$ 0.12 nA/100 pF; n=3; $P>0.05$), 5 U/μl cGMP-PK alone (data not shown; 0.54 ± 0.12 nA/100 pF; n=3; $P>0.05$), and 0.5 μM 8-Br-cGMP and 5 U/μl cGMP-PK together (0.65 ± 0.20 nA/100 pF; n=3; $P>0.05$) had little effect on basal I_{Ca} in the absence of ATP in the patch pipette. These results would predict that cGMP-PK is effective only in the presence of cGMP and ATP, suggesting that cGMP-PK acts through phosphorylation of L-type calcium channel or an associated protein.

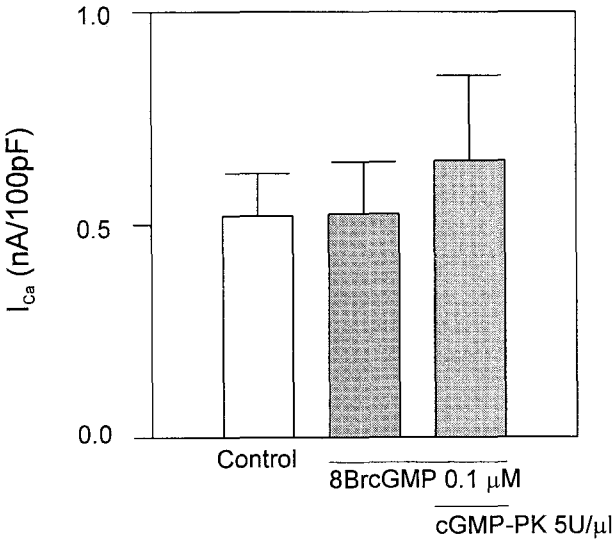

Fig. 3. Effect of low doses of 8-Br-cGMP (0.1 μM) and cGMP-PK (5 U/μl) on basal I_{Ca} in the absence of internal ATP. 8-Br-cGMP and cGMP-PK were applied from an internal solution of the patch pipette. The peak I_{Ca} was evoked by depolarizing pulse of +10 mV from a holding potential −40 mV. As shown, 8-Br-cGMP alone, cGMP-PK alone (data not shown) and 8-Br-cGMP and cGMP-PK together had only little effect. It can be seen that ATP, 8-Br-cGMP and cGMP-PK were all required together to produce a stimulatory effect.

In order to check whether the effect of cGMP-PK on basal I_{Ca} was due to its enzymatic activity, a stock solution containing only cGMP-PK was incubated in a hot water bath (100°C) for 20 minutes, and this heated cGMP-PK was used for the internal solution. As can be seen in Fig. 4A, the heat-inactivated cGMP-PK had little effect (0.51 ± 0.11 nA/100 pF; n=3; $P>0.05$), which is almost equal to the effect of the low concentration of 8-Br-cGMP alone ($0.52\pm$ 0.12 nA/100 pF; n=3; $P>0.05$). In addition, we examined the effect of 8-(4-Chlorophenylthio)-guanosine-3',5'-cyclic monophosphate, Rp-isomer (Rp-pCPT-cGMP), a selective and membrane-permeant inhibitor of cGMP-PK (Fig. 4B). With I_{Ca} already increased by cGMP-PK (1.16 ± 0.17 nA/100 pF; n=6), bath application of 400 μM Rp-pCPT-cGMP partially reversed the effect of cGMP-PK (0.78 ± 0.19 nA/100 pF; n=4; $P<0.05$). These results suggest that the increase of I_{Ca} by cGMP-PK is due to its enzymatic activity.

In order to rule out the possible involvement of PDE's in the stimulatory effect of cGMP-PK, we used a non-specific PDE inhibitor, IBMX near the

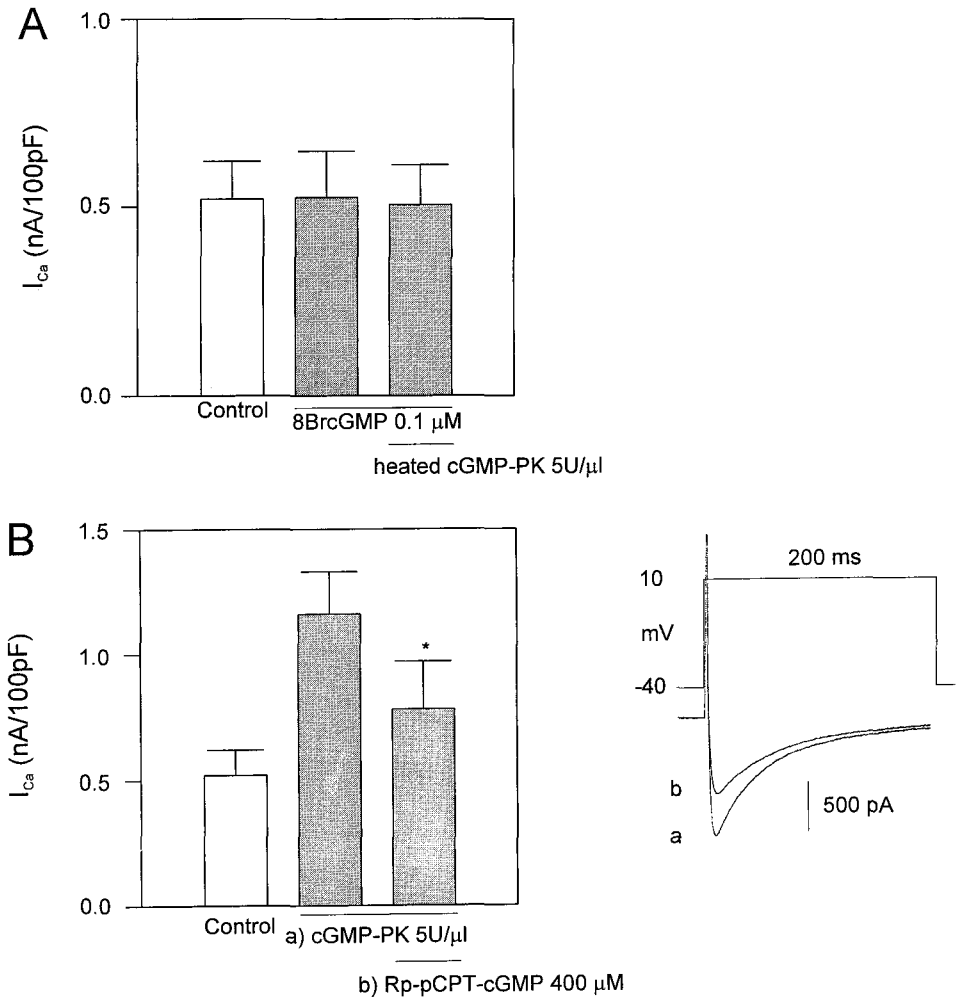

Fig. 4. Stimulatory effect of cGMP-PK on basal I_{Ca} results from its enzymatic activity. A. Heat-inactivated cGMP-PK showed only little effect on basal I_{Ca} in rabbit ventricular myocytes. 8-Br-cGMP and heated cGMP-PK were applied from an internal solution of the patch pipette. The peak I_{Ca} was evoked by depolarizing pulse of +10 mV from a holding potential −40 mV. As shown, 8-Br-cGMP alone, heated cGMP-PK alone (data not shown) and 8-Br-cGMP and heated cGMP-PK together had only little effect. B. Prevention of the effect of cGMP-PK on basal I_{Ca} in rabbit ventricular myocytes by Rp-pCPT-cGMP, a phosphodiesterase-resistant cGMP- PK inhibitor. The cGMP-PK was always accompanied by 8-Br-cGMP (0.1 μM). cGMP-PK increased the basal I_{Ca}, and Rp-pCPT-cGMP inhibited the current prestimulated by cGMP-PK. Right panel shows the superimposed current traces at the condition indicated by 'a' (cGMP-PK) and 'b' (additional application of 400 μM Rp-pCPT-cGMP). The two current traces were obtained from the same myocyte (membrane capacitance = 25.9 pF) and evoked by depolarizing pulse of +10 mV from a holding potential −40 mV. * $P<0.05$.

concentration required for half-maximal stimulation of I_{Ca} (EC_{50}=64±20 μM) in rabbit ventricular myocytes, as Akita et al (1994) reported earlier. We first internally dialyzed the cells to 5 U/μL cGMP-PK (with 0.1 μM 8-Br-cGMP), and then added 50 μM IBMX. In the experiment shown in Fig. 5A, when I_{Ca} was increased by the internal application of cGMP-PK (1.14 nA/100 pF), this current was further increased by the external application of 50 μM IBMX to 1.53 nA/100 pF. Fig. 5B summarizes the effect of 5 U/μl cGMP-PK alone, 50 μM IBMX alone, and 5 U/μl cGMP-PK and 50 μM IBMX together. cGMP-

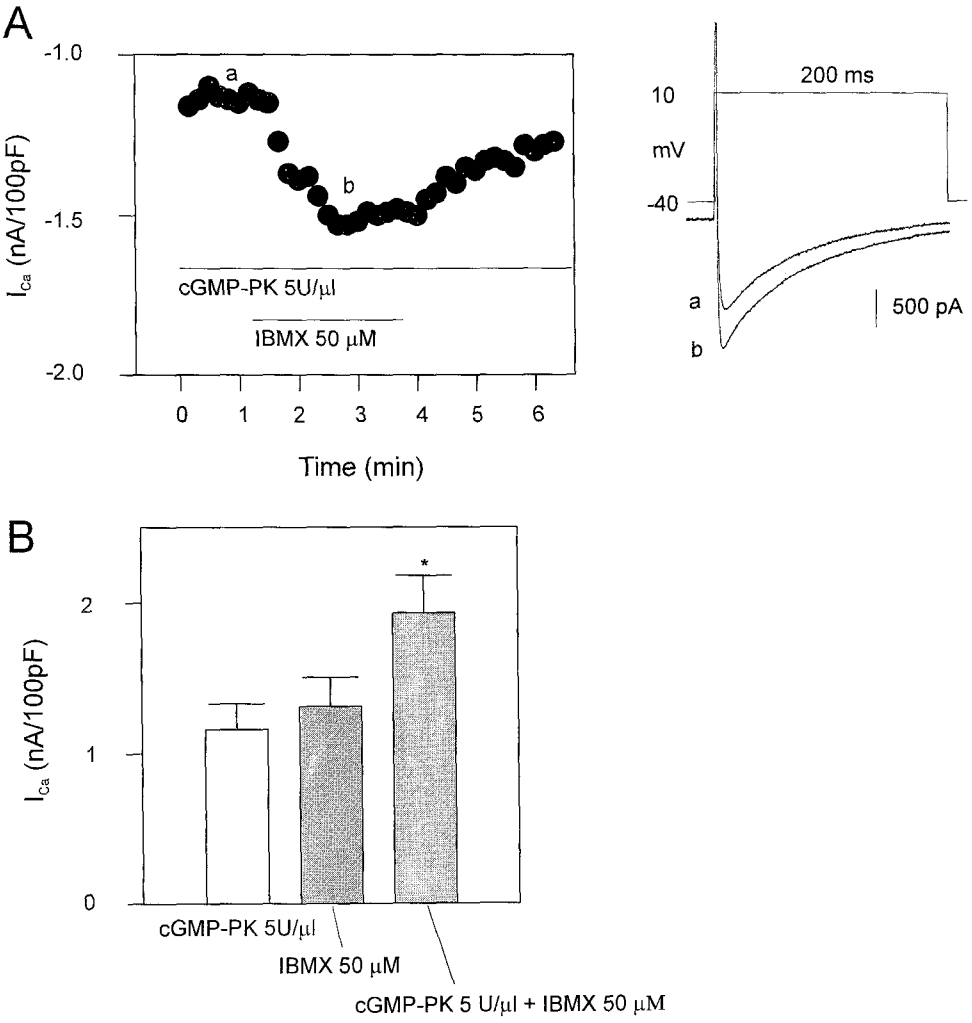

Fig. 5. Effect of bath application of 3-isobutyl-1-methylxanthine (IBMX) on I_{Ca} stimulated by cGMP-PK. A. Time course of the effect of external application of 50 μM IBMX on I_{Ca} with 5 u/μl cGMP-PK. cGMP-PK increased the basal I_{Ca}, and IBMX further increased I_{Ca}. Right panel, Selected current traces of I_{Ca} (*a*, *b*) at points denoted on the time-course curve. The superimposed current traces were obtained from the same ventricular cell (membrane capacitance = 30.8 pF) and elicited by 200 ms depolarizing step pulses from −40 mV to +10 mV. B. Bar graph showing average mean values of I_{Ca} by treatment with 5 U/μl cGMP-PK (n=6), 50 μM IBMX (n=5), or 5 U/μl cGMP-PK plus 50 μM IBMX (n=3). Error bars indicate S.D.. The increase in I_{Ca} induced by 5 U/μl cGMP-PK plus 50 μM IBMX is significantly different from that in I_{Ca} induced by 5 U/μl cGMP-PK, 50 μM IBMX alone. * $P<0.05$.

PK alone, as we presented above for a separate group of cells, increased basal I_{Ca} (1.16±0.17 nA/100 pF; n=6; $P<0.05$). Extracellular application of 50 μM IBMX increased basal I_{Ca} (1.31±0.20 nA/100 pF; n=5; $P<0.05$). When we applied 50 μM IBMX in the presence of 5 U/μl cGMP-PK, the average I_{Ca} was further increased from 1.16±0.17 to 1.93±0.25 nA/100 pF (n=3; $P<0.05$). These results indicate that the action of cGMP-PK was not mediated through PDE activation.

In order to determine whether cGMP-PK and cAMP-PK are acting at the same site, we examined the effect of isoprenaline, forskolin or cAMP on I_{Ca} already enhanced by cGMP-PK (Fig. 6). When I_{Ca} was increased by intracellular application of 5 U/μl cGMP-PK (n=6), bath application of isoprenaline (1

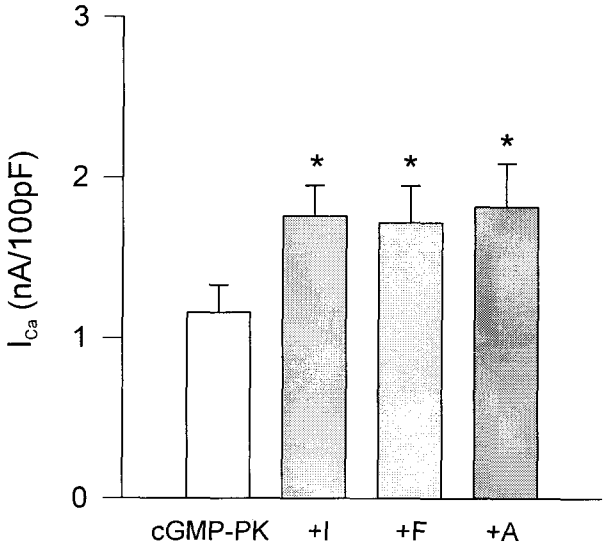

Fig. 6. Bar graph showing average mean values of I_{Ca} by treatment with 5 U/μl cGMP-PK alone (n=6), 5 U/μl cGMP-PK plus 2 μM isoprenaline (marked with $+I$, n=5), 5 U/μl cGMP-PK plus 2 M forskolin (marked with $+F$, n=5), or 5 U/μl cGMP-PK plus 50 μM cAMP (marked with $+A$, n=3). Error bars indicate S.D.. The increase in I_{Ca} induced by the additional treatment of 2 μM isoprenaline, 2 μM forskolin, or 50 μM cAMP is significantly different from that in I_{Ca} induced by 5 U/μl cGMP-PK alone. Peak inward currents (mean ± S.D.) were elicited by 200 ms depolarizing step pulses from −40 mV to +10 mV. * $P<0.05$.

M), forskolin (1 μM) or intracellular application of cAMP (100 μM) resulted in an additional increase of I_{Ca}. Increases in I_{Ca} by isoprenaline (n=5), forskolin (n=5) or cAMP (n=3), were significant in all cases ($P<0.05$). These results suggest that the target site for cGMP-PK is different from that for cAMP-PK.

DISCUSSION

In the present study, we examined the stimulatory effect of cGMP-PK on L-type calcium current (I_{Ca}) in rabbit ventricular myocytes. The major findings of this study are summarized as follows:

1) 8-(4-Chlorophenylthio)-guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP), potent stimulator of cGMP-dependent protein kinase (cGMP-PK), increases basal I_{Ca}.

2) cGMP-PK increased basal I_{Ca}. The stimulation of basal I_{Ca} by cGMP-PK required both 8-Br-cGMP in low concentration of and intracellular ATP to be present.

3) The stimulation of basal I_{Ca} by cGMP-PK was blocked by heat inactivation of the cGMP-PK and by 8-(4-chlorophenylthio)-guanosine-3',5'-cyclic monophosphate, Rp-isomer (Rp-pCPT-cGMP), a phosphodiesterase-resistant cGMP-PK inhibitor.

4) When I_{Ca} was increased by internal application of cGMP-PK, IBMX resulted in an additional stimulation of I_{Ca}.

5) In the presence of cGMP-PK, already increased I_{Ca} was potentiated by bath application of isoprenaline or forskolin or intracellular application of cAMP.

Intracellular cGMP has been known to increase I_{Ca} in the frog (Mery et al, 1993), guinea pig (Ono & Trautwein, 1991) heart cells, and human atrial myocytes (Kirstein et al, 1995) via inhibition of the cGMP-inhibited PDE. In human atrial myocytes, cGMP-inhibited PDE controls the amplitude of basal I_{Ca}, i.e., in the absence of a previous stimulation of I_{Ca} by cAMP-elevating agents. However, in the frog and guinea pig ventricular myocytes, the stimulatory effect of cGMP on I_{Ca} was only observed after a previous stimulation of cAMP-dependent phosphorylation. In our initial experiments (Han et al, 1993), we reported that intracellular application of cGMP prolonged the duration of action potential and increased its plateau level in rabbit ventricular myocytes. The alteration of action potential configuration was also observed with the application of isoprenaline or cAMP (Han et al, 1993). These results would predict that cGMP regulates I_{Ca} in rabbit ventricular myocytes. In the following, we mainly focused our attention to the modulation by cGMP of I_{Ca} in rabbit ventricular myocytes. It was reported that in rabbit ventricular myocytes cGMP by other means increased I_{Ca} even in the absence of a previous stimulation of cAMP/cAMP-PK pathway (Han et al, 1992, 1994). This was also an observation made in human atrial myocytes (Kirstein et al, 1995). However, our findings in rabbit ventricular myocytes in which 8-Br-cGMP or 8-pCPT-cGMP, specific and potent stimulators of cGMP-PK, increased basal I_{Ca} (Han et al, 1998) were entirely different from the earlier findings in the frog (Harzell & Fischmeister, 1986; Fischmeister & Harzell, 1987), guinea pig (Ono & Trautwein, 1991) and human cardiac myocytes (Kirstein et al, 1995). Our results suggest that cGMP increases I_{Ca} through the activation of cGMP-PK in rabbit ventricular myocytes.

The present study was focused on the cGMP-PK

regulation of I_{Ca} in rabbit ventricular myocytes. We used a low concentration of 8-Br-cGMP (0.1 μM) in order to activate the enzymatic activity of cGMP-PK since 0.1 μM 8-Br-cGMP was known to give maximum activation of cGMP-PK in vitro (Lincoln & Corbin, 1983). In the present study, 0.1 μM 8-Br-cGMP itself had little effect on basal I_{Ca}, whereas 0.1 μM 8-Br-cGMP and 5 U/μl cGMP-PK together increased I_{Ca}. On the other hand, we have already reported that higher concentration (100 μM) of 8-Br-cGMP increased basal I_{Ca} (Han et al, 1998). These results would predict that 0.1 μM 8-Br-cGMP is sufficient to maximally activate the exogenous cGMP-PK in the pipette but activate the endogenous cGMP-PK to only a small extent. From the results we provide the direct evidence that I_{Ca} in rabbit ventricular myocytes can be stimulated through activation of cGMP-PK.

As indicated in Fig. 3, the increase in I_{Ca} by cGMP-PK did not occur in the absence of exogenous ATP in the pipette. These data are in contrast to the experiment (Fig. 2) in the presence of exogenous ATP (5 mM) in the pipette. In all cases of the present study 5 mM ATP is present in the standard internal solution except for the experiment of Fig. 3. cGMP-PK activation of I_{Ca} required both a low concentration of 8-Br-cGMP and ATP, suggesting that cGMP-PK acts through a phosphorylation-dependent mechanism. In summary, the cGMP is stimulating the cGMP-PK, which in turn is increasing I_{Ca} by phosphorylation of the L-type calcium channel or an associated regulatory protein.

In order to determine the mechanism of stimulation by cGMP-PK on I_{Ca} we performed two types of experiments. Heat-inactivated cGMP-PK had little effect on I_{Ca} (Fig. 4A). We also found that the stimulatory effect of cGMP-PK was suppressed by Rp-pCPT-cGMP, a phosphodiesterase-resistant cGMP-PK inhibitor (Fig. 4B). Therefore, our data indicate that the increase of I_{Ca} by cGMP-PK is due to its enzymatic activity in rabbit ventricular myocytes.

The mechanism of the effect of cGMP-PK may be explained by the following two possibilities: (1) direct phosphorylation of the channel or an associated protein (Han et al, 1998), (2) activation of cGMP-inhibited PDE. It has been reported that, in human atrial myocytes, cGMP increases basal I_{Ca} via cGMP-inhibited PDE (Kirstein et al, 1995). The finding that bath application of 8-pCPT-cGMP further increases I_{Ca} prestimulated by IBMX, a non-specific phosphodiesterase inhibitor, excluded the involvement of cGMP-inhibited PDE in the stimulatory action of cGMP (Han et al, 1998). We found here that IBMX resulted in an additional increase of I_{Ca}, previously increased by cGMP-PK. The results support that the action of cGMP-PK is not mediated through PDE activation.

We have already shown that after previous stimulation of I_{Ca} by cGMP, 8-Br-cGMP or 8-pCPT-cGMP, intracellular cAMP-elevating agents (isoprenaline, forskolin or cAMP itself) potentiate I_{Ca} (Han et al, 1993, 1998). We also found here that intracellular cAMP-elevating agents had a similar effect on I_{Ca} already increased by cGMP-PK. These results suggest that the target phosphorylation site for cGMP-PK is a different site from that is phosphorylated by cAMP-PK.

In conclusion, our results suggest that the stimulatory effect of cGMP-PK on I_{Ca} is mediated by a direct phosphorylation of the L-type calcium channel or an associated regulatory protein, providing supporting evidence for the modulation of I_{Ca} by cGMP.

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