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Interaction between cyclic GMP protein kinase and cyclic AMP may be diminished in stunned cardiac myocytes

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Abstract

We tested the hypothesis that the importance of the negative functional effects of the cyclic GMP protein kinase would be reduced in stunned (simulated ischemia/reperfusion) cardiac myocytes. Ventricular cardiac myocytes were isolated from New Zealand white rabbits ($N = 7$). Myocytes were studied at baseline and after simulated ischemia (15 min of 95% N_2–5% CO_2 at 37 °C) followed by simulated reperfusion (reoxygenation). Cell shortening was studied with a video edge detector; O_2 consumption was measured using O_2 electrodes. Protein phosphorylation was measured autoradiographically after gel electrophoresis. Functional and metabolic data were acquired after: (1) 8-(4-chlorophenylthio)guanosine-3′,5′-monophosphate (PCPT, cGMP protein kinase agonist) 10^{-7} or 10^{-5} M; (2) 8-Br-cAMP 10^{-5} M followed by PCPT 10^{-7} or 10^{-5} M; (3) β-phenyl-1, N^2-etheno-8-bromoguanosine-3′,5′-monophosphorothioate, SP-isomer (SP, cGMP protein kinase agonist) 10^{-7} or 10^{-5} M; (2) 8-Br-cAMP 10^{-5} M followed by SP 10^{-7} or 10^{-5} M. At baseline, percent of shortening (Pcs) and maximal rate of shortening (Rs) were significantly lower in the stunned myocytes (Pcs: 5.0 ± 0.2% control vs. 3.8 ± 0.3 stunned; Rs: 64.8 ± 5.9 μm/s control vs. 46.9 ± 4.8 stunned). In both groups, PCPT and SP dose-dependently decreased Pcs and Rs. The effects were slightly, but not significantly, less in stunned myocytes. 8-Br-cyclic AMP significantly increased function in control, but not stunned myocytes (Pcs, 4.5 ± 0.5 to 6.2 ± 0.8 control vs. 3.1 ± 0.2 to 3.6 ± 0.2 stunned). The negative functional effects of PCPT and SP were diminished after 8-Br-cyclic AMP in control (from -39% to -29%) and diminished significantly more in the stunned myocytes (-19%). PCPT and cyclic AMP phosphorylated similar protein bands. In stunned myocytes, three (22, 31 and 53 kDa) bands were enhanced less by PCPT.

Keywords: cGMP; cGMP protein kinase; Ischemia/reperfusion; cAMP; (Rabbit)

1. Introduction

Cyclic guanosine monophosphate exerts negative metabolic and functional effects on isolated cardiac myocytes (Lohmann et al., 1991; Shah et al., 1994). It causes decreased oxygen consumption, percent shortening and maximum rate of shortening (Lohmann et al., 1991; Shah et al., 1994; Haddad et al., 1995). These effects are mediated by: (1) protein phosphorylation through cyclic GMP-dependent protein kinases; (2) direct effects on ion channels including inhibition of L-type calcium channel on cell membranes; (3) cyclic GMP-stimulated or inhibited cyclic AMP phosphodiesterases (Lohmann et al., 1991; Lincoln et al., 1994; Haddad et al., 1995). Cyclic GMP also antagonizes the positive inotropic effects of catecholamines (Lohmann et al., 1991; Corwell et al., 1994; Weiss et al., 1994). Several studies have suggested that blocking of cyclic GMP-dependent protein kinase pathway would significantly diminish the effects of cyclic GMP (Haddad et al., 1995; Mery et al., 1993). There are studies that suggest that the cyclic GMP-dependent protein kinase causes the cyclic GMP-induced inhibition of L-type calcium channels (Haddad et al., 1995; Mery et al., 1993; Wahler and Dollinger, 1995). Previous work from our laboratory also demonstrated that cyclic GMP-dependent protein kinase was a major pathway mediating the negative metabolic and functional effects of cyclic GMP (Straznicka et al., 1999). Cyclic AMP has positive effects on both

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myocardium and cardiac myocytes (Hove-Madsen et al., 1996; Sugden and Bogoyevitch, 1995). The cyclic AMP-dependent protein kinase is the key pathway for cyclic AMP (Hove-Madsen et al., 1996; Sugden and Bogoyevitch, 1995; Coppenoll et al., 1997). These two protein kinases are closely related and there may be crosstalk between cyclic AMP and cyclic GMP at the level of their protein kinases.

Myocardial stunning following even brief periods of ischemia/reperfusion is associated with a sharp decline in mechanical function, usually without a major change in myocardial metabolism (Birnbaum and Kloner, 1995; Chiu et al., 1994; Kusuoka and Marban, 1992; Naim et al., 1997; Schulz et al., 1995). These mechanical decrements were associated with a decline in shortening and changes in timing of contraction and relaxation. Inotropic agents have been shown to reduce myocardial stunning (Kusuoka and Marban, 1992; Schulz et al., 1995). However, previous work from our laboratory has shown that increasing levels of nitric oxide and the second messenger cyclic GMP reduce myocardial stunning (Padilla et al., 2000; Matoba et al., 1999), while reducing the level of cyclic GMP worsens stunning (Naim et al., 1997). We have also shown that cyclic GMP reduces ventricular myocytes stunning when administered during simulated ischemia/reperfusion and that these changes were associated with a reduction in the effects of the cyclic GMP protein kinase (Gandhi et al., 1999).

We tested the hypothesis that the importance of the negative functional effects of the cyclic GMP-dependent protein kinase would be reduced in stunned (simulated ischemia/reperfusion) cardiac myocytes as would its interaction with the cyclic AMP-dependent protein kinase. This hypothesis was tested in isolated ventricular myocytes from New Zealand white rabbits. Two specific cyclic GMP-dependent protein kinase activators and an exogenous membrane permeable analogue of cyclic AMP were used in this study. We determined changes in myocyte function and the degree of protein phosphorylation in myocytes. We found reduced protein phosphorylation with the cyclic GMP-dependent protein kinase and an increased ability of cyclic AMP to blunt its effects in stunned myocytes.

2. Materials and methods

New Zealand white rabbits ($n = 7$), weighing 2–3 kg, were used in this study. Experiments were performed on ventricular myocytes isolated from hearts of these rabbits. All experiments were conducted in accordance with Guide for the Care of Laboratory Animals (DHHS Publication No. 85-23, revised 1996) and were approved by our Institutional Animal Care and Use Committee.

2.1. Cell dissociation

Freshly isolated ventricular myocytes were prepared by a standard method as described previously (Gong et al., 1997). The rabbits were anesthetized and the heart was rapidly removed after an overdose of pentobarbital (100 mg/kg). Retrograde aortic perfusion of the heart was immediately begun at 70-mm Hg constant pressure with HEPES buffered minimal essential medium (MEM). This low-Ca^{2+} MEM solution had an osmolality of 296 mOsm, and the free Ca^{2+} activity was 2–5 μM. After 5 min of perfusion with low-Ca^{2+} MEM, the heart was perfused at 50 mm Hg with a 60 ml volume of low-Ca^{2+} MEM supplemented with 0.1% collagenase (Worthington type II). After 25 min of collagenase perfusion with recirculation, the heart was removed from the perfusion apparatus and cut into 8–10 pieces in MEM containing 1.0 mM $CaCl_2$ and 0.5% bovine serum albumin. This Ca^{2+}-MEM was supplemented with 0.1% collagenase. The tissue suspension was gently swirled by a wrist action shaker for 5 min. A slurry containing isolated heart cells was decanted from the tissue suspension. The isolated cells were washed three times. The combined, washed cells were then maintained at room temperature. The viability of the myocytes was about 55–70%. Yields were typically $10–14 \times 10^8$ rod-shaped cells per heart. Cells previously isolated in this fashion have been shown to have an intact cell coat as well as a functional sarcolemma and normal permeability barriers to extracellular ions, ADP and succinate.

2.2. Myocyte oxygen consumption measurement

Steady-state O_2 consumption was recorded continuously with a Clark-type oxygen electrode in a glass chamber using a two-channel oximeter (University of Pennsylvania, Philadelphia, PA) in seven rabbits. All experiments began at a PO_2 of 115 mm Hg and ended at no less than 25 mm Hg. Anaerobic metabolism occurs only at PO_2 levels below 5–10 mm Hg. Gradients in PO_2 were not likely in the chamber, since the cell suspension was well stirred. The chamber contained a small Teflon-coated stirring bar to maintain the cells in suspension by slow rotation. The recording chamber was bathed with 37 °C circulating water. The cuvette was mounted on a magnetic stirrer. A ground glass stopper was used to eliminate the gas phase. This stopper also provided access to the assay medium via a central hole (1.3-mm internal diameter). The volume of the recording chamber is 1.5 ml. Myocytes were added to the chamber and their number determined. The total volume of all agents added to the chamber were less than 100 μl so no significant dilution of the myocyte suspension occurred.

The Clark-type electrode was calibrated with solutions saturated with two known concentrations of oxygen. When calibrated, a 95% response could be obtained within 3–4 s.

Myocytes were paced with field electric stimulation (1 Hz, 5-ms duration, voltage at 10% above threshold, and the polarity alternated each pulse) by two platinum wires inserted into the center of the myocyte suspension. The rate of fall in oxygen tension within the chamber was used to determine O_2 consumption of the myocytes. Data were collected on a desktop computer and analyzed off-line. Oxygen consumption was expressed as nanoliters O_2/minute/100,000 myocytes. The sample was stirred at a rate sufficient to keep the cells suspended and yet not so rapidly as to compromise the viability of the cells. Inspection of cellular morphometry, made at random, indicated that at the completion of the experiment, the percentage of rod shaped cells was similar to the initial value.

2.3. Cell shortening measurement

Isolated cardiac myocytes were put into a open chamber (37 °C) on the stage of an inverted microscope (Zeiss Axiovert 125) with 2.0 mM Ca^{2+}-MEM solution. The volume of the chamber was 2.5 ml. At least 5 min was allowed to let the myocytes equilibrate. Myocytes were paced with extracellular field electrical stimulation (1 Hz, 5-ms duration, voltage at 10% above threshold, and the polarity alternated each pulse) by two platinum wires inserted into the center of the myocyte suspension. Unloaded cell shortening was measured on-line using a video-edge detector (Myotrack system, Crystal Biotech, Model VED-114) and a camera (Pulnix, TM-640), which detected the change of the position of both edges of the cell. The output of video-edge detector was fed into both a television monitor and a desktop computer. We determined percent shortening as (100 × (maximum length − minimum length)/maximum length) and maximal rate of shortening from the maximal first derivative of shortening. A 5-min stabilization was allowed after which contraction data for each myocytes were recorded from a minimum of 10 consecutive contractions. Observations were performed individually on at least three cells per heart. Cell viability at the conclusion of the experiment was assessed by maintenance of rod-shaped morphology and by continued responsiveness to electrical pacing.

2.4. In vitro phosphorylation reactions and phosphoprotein analysis

In vitro phosphorylation reactions and phosphoprotein analysis were performed five times. All reactions were carried out in microfuge tubes at room temperature. Myocytes were homogenized (Brinkmann Polytron homogenizer: 15 s at 49,000 × g) in buffer (5 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$, 0.25 M sucrose) and centrifuged at 25,000 × g for 20 min at 4 °C. The supernatant was aliquoted and used as the myocyte extract for all phosphorylation reactions. Activators, 8-bromo-cyclic AMP, 8-(4-chlorophenylthio) guanosine-3′,5′-monophosphate (PCPT) and a combination of these two reagents were added to 10 μl of extract (0.4 mg total protein/ml). Ten minutes were allowed for each reactant to equilibrate. After equilibration, each reaction was cooled on ice. Gamma-^{33}P-ATP (1 μl) at 10 μCi/μl was added to initiate the reaction. The reaction was terminated 15 min later by adding a volume of BioRad reducing sample buffer equal to the entire reaction volume. The samples were heated at 95 °C for 5 min and electrophoresed using miniature 12% sodium dodecyl sulfate (SDS) polyacrylamide slab gels. The gels were then stained with Coomassie Brilliant Blue, dried overnight using a Promega Gel Drying Kit and exposed to X-ray film at −20 °C for 24 h. The exposed X-ray film

Table 1
The effects of PCPT, SP and 8-Bromo-cAMP on the time to peak (s) and 90% relaxation time (s) in both control and stunned myocytes

	Control (time to peak)	Stunned (time to peak)	Control (90% relaxation time)	Stunned (90% relaxation time)
Base	0.24 ± 0.02	0.32 ± 0.05	0.26 ± 0.01	0.26 ± 0.02
PCPT 10^{-5} M	0.34 ± 0.03[a,b]	0.46 ± 0.04[a]	0.27 ± 0.02	0.33 ± 0.03
Base	0.44 ± 0.06	0.49 ± 0.03	0.28 ± 0.01[b]	0.38 ± 0.04
cAMP 10^{-5} M	0.41 ± 0.07	0.47 ± 0.06	0.28 ± 0.03	0.37 ± 0.04
PCPT 10^{-5} M	0.50 ± 0.04[c]	0.53 ± 0.04	0.34 ± 0.02[b]	0.46 ± 0.08
Base	0.26 ± 0.03	0.33 ± 0.04	0.26 ± 0.02	0.28 ± 0.02
SP 10^{-5} M	0.40 ± 0.05[a]	0.45 ± 0.05[a]	0.29 ± 0.04	0.29 ± 0.02
Base	0.47 ± 0.05	0.52 ± 0.05	0.29 ± 0.01	0.28 ± 0.01
cAMP 10^{-5} M	0.44 ± 0.05	0.50 ± 0.05	0.34 ± 0.02	0.36 ± 0.03
SP 10^{-5} M	0.53 ± 0.05[c]	0.52 ± 0.04[c]	0.42 ± 0.07[a]	0.38 ± 0.04[c]

[a] Significantly different from the base level.
[b] Significantly different from stunned myocytes.
[c] Significantly different from 8-Bromo-cyclic AMP.

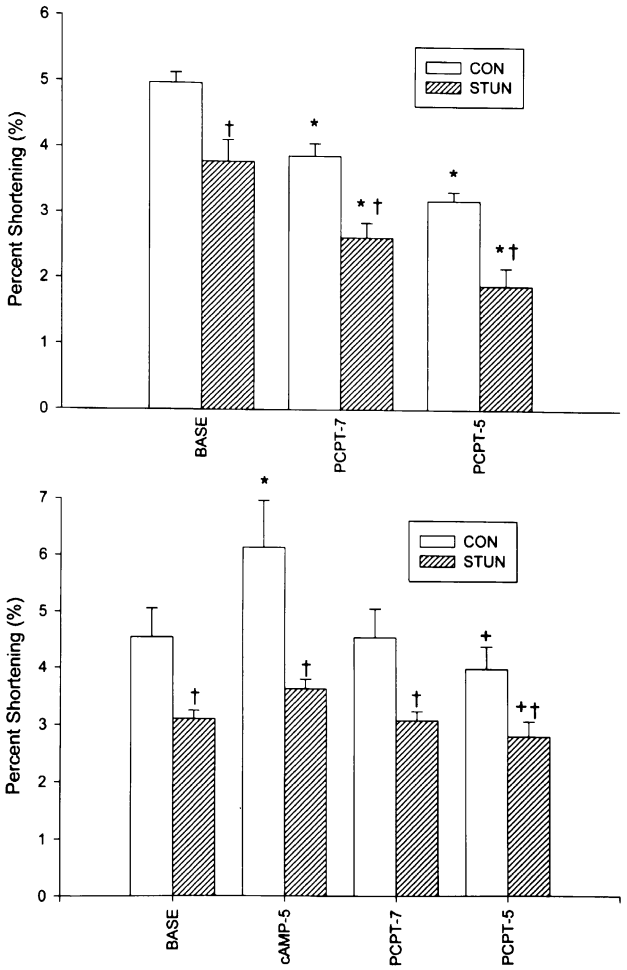

Fig. 1. The effects of PCPT $10^{-7,-5}$ M on percent shortening of both control and stunned myocytes are shown (top). The percent shortening of stunned myocytes was significantly lower than in the control group, but the responses to PCPT were similar. The effects of PCPT $10^{-7,-5}$ M following the addition of 8-Bromo-cyclic AMP are shown (bottom). Note the reduced response to 8-Bromo-cyclic AMP in the stunned myocytes. The responses to PCPT were slightly but not significantly altered by 8-Bromo-cyclic AMP. * Significantly different from the base level. †Significantly different from similar control group value. + Significantly different from 8-Bromo-cyclic AMP.

demonstrated phosphate-labeled proteins that were then sized by comparison to molecular weight standard markers. The exposed X-ray films were analyzed by using an Imaging Densitometer (BioRad, Model GS-670). The analysis of the image was performed using Molecular Analyst Software (BioRad, Version 1.5). We obtained the mean density of each band in each film.

2.5. Experimental protocol

Two groups of myocytes were used in the following protocol for cell functional and O_2 consumption measurements. The first untreated group served as a control group. In the simulated ischemia/reperfusion (stunned) group, all myocytes were subjected to simulated ischemia (15 min of 95% N_2–5% CO_2 at 37 °C), which reduced the PO_2 to no higher than 6 mm Hg. They were then subjected to 30 min of reoxygenation. In both groups used in the O_2 consumption measurements, aliquots of myocytes were suspended in a well oxygenated chamber with 2 mM Ca^{2+}-MEM at an appropriate concentration and the cells were allowed to stabilize for 10 min. The myocytes were paced with electrical field stimulation. A 5-min interval was allowed between the addition of reagents during which cell contractility was measured. The following protocol was used for the O_2 consumption measurements. A 5-min recording was obtained as baseline. The O_2 consumption was measured after addition of PCPT 10^{-5} M or after the addition of 8-Br-cAMP 10^{-5} M followed by PCPT 10^{-5} M. Other aliquots of control and stunned myocytes were administered β-phenyl-1, N^2-etheno-8-bromoguanosine-3′,5′-

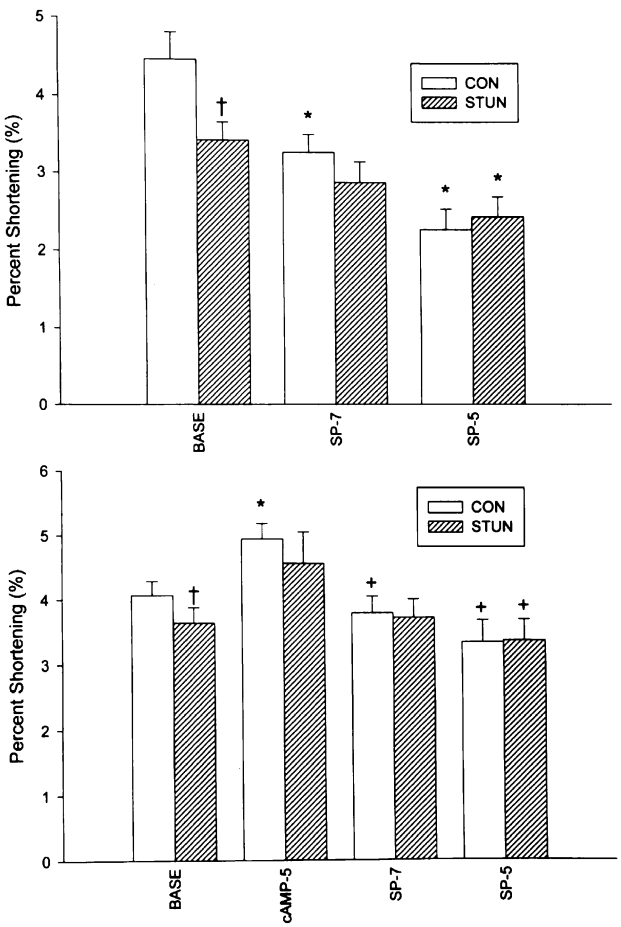

Fig. 2. The effects of SP $10^{-7,-5}$ M on percent shortening of both control and stunned myocytes are shown (top). The base percent shortening of stunned myocytes was significantly lower than in the control group, but the responses to SP were not significantly smaller. The effects of SP $10^{-7,-5}$ M following the addition of 8-Bromo-cyclic AMP are shown (bottom). The response to 8-Bromo-cyclic AMP was not significant in the stunned cells and the effect of SP was slightly but not significantly altered by 8-Bromo-cyclic AMP. * Significantly different from the base level. †Significantly different from similar control group value. + Significantly different from 8-Bromo-cyclic AMP.

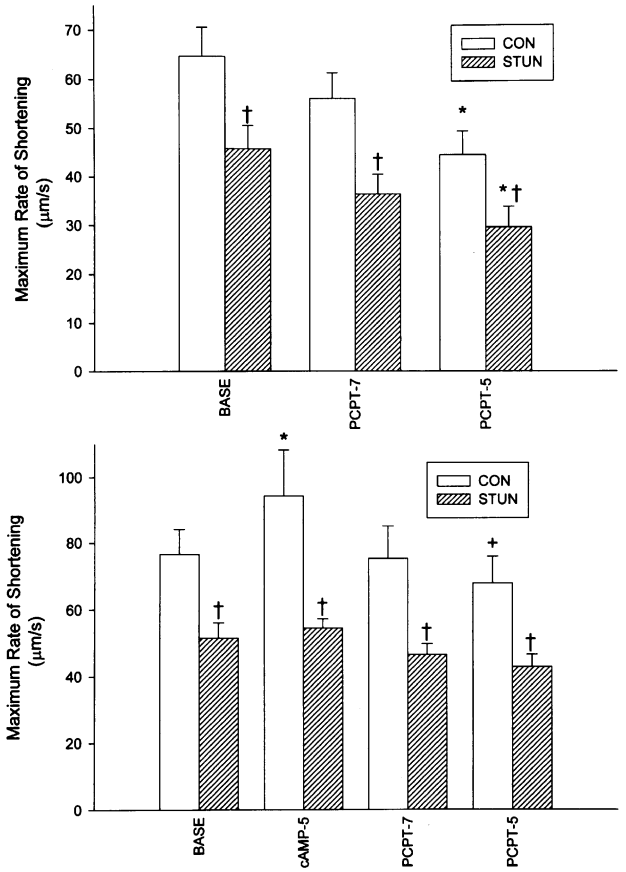

Fig. 3. The effects of PCPT $10^{-7,-5}$ M on maximal rate of shortening of both control and stunned myocytes are shown (top). The maximal rate of shortening of stunned myocytes was significantly lower than in the control group, but the responses to PCPT were similar. The effects of PCPT $10^{-7,-5}$ M following the addition of 8-Bromo-cyclic AMP are shown (bottom). Note the reduced response to both PCPT and 8-Bromo-cyclic AMP in the stunned myocytes. * Significantly different from the base level. †Significantly different from similar control group value. + Significantly different from 8-Bromo-cyclic AMP.

monophosphorothioate, SP-isomer (SP) 10^{-5} M or 8-Br-cAMP 10^{-5} M followed by SP 10^{-5} M. For the functional measurements, additional doses of 10^{-7} M PCPT or 10^{-7} M SP were used. These doses were selected after preliminary experiments indicated that they produced good functional changes without cell damage. There were no significant alterations in the number of rod shaped cells at the end of O_2 consumption experiments.

2.6. Statistics

All results are expressed as means ± S.E.M, reporting between heart variation. A factorial analysis of variance (ANOVA) was used to compare variables measured in the experimental and control conditions. Duncan's multiple range test was used to compare the differences post hoc. The logit transformation was performed on the percent shortening data prior to analysis. This analysis was used to determine differences between groups and treatments for both cardiac myocyte function and O_2 consumption. In all cases, $P < 0.05$ was accepted as significant. For densitometry data, we used Wilcoxon Rank-Sum test.

3. Results

3.1. Functional data

We report four parameters of myocyte function, percent shortening (Pcs, %), maximal rate of shortening (Rs, μm/s), time to peak contraction (s) and 90% relaxation time (s) in contracting control cells and cells that have undergone simulated ischemia/reperfusion. The effects of different reagents on percent shortening and maximal rate of shortening are depicted in Figs. 1–4. Simulated ischemia/reperfusion (stunning) significantly decreased the baseline levels of functional parameters such as percent of

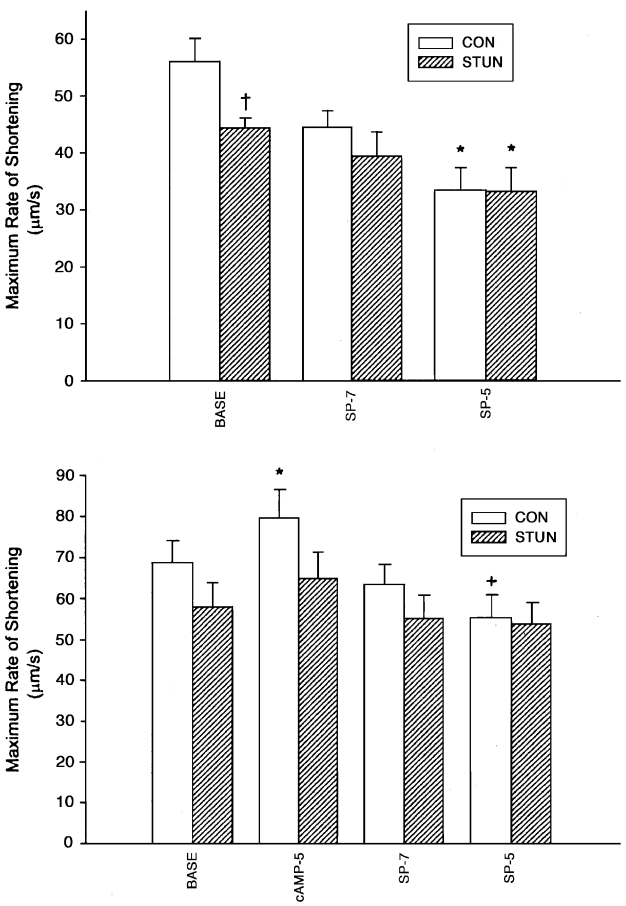

Fig. 4. The effects of SP $10^{-7,-5}$ M on maximal rate of shortening of both control and stunned myocytes are shown (top). The base maximal rate of shortening of stunned myocytes was significantly lower than in the control group, but the responses to SP were not significantly smaller. The effects of SP $10^{-7,-5}$ M following the addition of 8-Bromo-cyclic AMP are shown (bottom). Note the reduced response to both SP and 8-Bromo-cyclic AMP in the stunned myocytes. * Significantly different from the base level. †Significantly different from similar control group value. + Significantly different from 8-Bromo-cyclic AMP.

Table 2
The effects of PCPT, SP and 8-Bromo-cyclic AMP on oxygen consumption (VO_2, nl O_2/min/100,000 myocytes) in both control and stunned myocytes

	Control (VO_2)	Stunned (VO_2)
Base	2313±449	2531±379
PCPT 10^{-5} M	1186±121[a]	1439±289
Base	2007±393	2393±347
cAMP 10^{-5} M	2159±323	2039±245
PCPT 10^{-5} M	839±132[a,b]	821±144[a,b]
Base	2459±513	2734±392
SP 10^{-5} M	1097±199[a]	1326±209[a]
Base	2324±511	2492±514
cAMP 10^{-5} M	2220±326	2595±482
SP 10^{-5} M	898±100[a,b]	930±196[a,b]

[a]Significantly different from the base level.
[b]Significantly different from 8-Bromo-cyclic AMP.

shortening (Pcs) by 24%, and maximal rate of shortening (Rs) by 29%. The time to peak and 90% relaxation time data are summarized in Table 1. These parameters were not significantly altered by ischemia/reperfusion at baseline.

Both PCPT and SP significantly decreased percent shortening in a dose dependent manner (Figs. 1 and 2). Basal percent shortening values were lower in the stunned cells. The responses to the cyclic GMP protein kinase activators were slightly, but not significantly, diminished in the stunned cells. Similar results were obtained for the maximal rate of shortening (Figs. 3 and 4). There was a dose dependent decrease in maximal rate of shortening with these cyclic GMP protein kinase activators. This response was slightly, but not significantly, diminished in the stunned ventricular myocytes.

The addition of 8-Br-cyclic AMP 10^{-5} M significantly increased both percent shortening and maximal rate of shortening in the control ventricular myocytes (Figs. 1–4). In the stunned myocytes, the addition of 8-Br-cyclic AMP 10^{-5} M had no statistically significant effects on percent shortening or maximum rate of shortening. In the control myocytes, the addition of 8-Br-cyclic AMP 10^{-5} M prior to PCPT or SP significantly diminished their negative functional responses. The average decline in function was 39% with PCPT or SP, but after the addition of 8-Br-cyclic AMP, this decrement was reduced to 29%. In the simulated ischemia/reperfusion group, the effect of these activators was greatly reduced after the addition of 8-Br-cyclic AMP. The average decline in function was 35% with

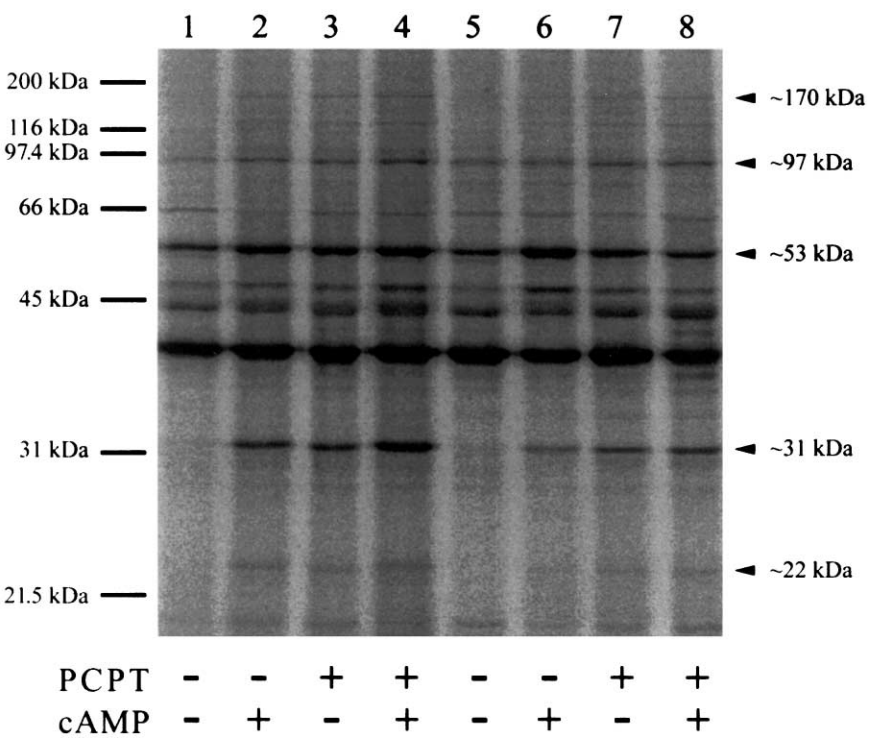

Fig. 5. The effects of PCPT and 8-Bromo-cyclic AMP on protein phosphorylation of both control and stunned rabbit ventricular myocytes. Lanes 1–4 are from control myocytes and lanes 5–8 are from stunned myocytes. Basal phosphorylation is shown in lanes 1 (control) and 5 (Stun). The effects of 8-Br-cAMP on both groups of myocytes are shown in lanes 2 (control) and 6 (Stun). The effects of PCPT were shown in lanes 3 (control) and 7 (Stun). The effects of addition of both PCPT and 8-Br-cGMP are shown in lanes 4 (control) and 8 (Stun), respectively. Molecular weight standards are shown at the left. Note that in the control group, both PCPT and 8-Br-cAMP increased phosphorylation of five protein bands at 170, 97, 53, 31 and 22 kDa and their combination increased more. The effects of PCPT were reduced in the stunned myocytes. Proteins at approximately 22, 31 and 53 kDa were phosphorylated less by PCPT and 8-Br-cAMP effects at 22 and 31 kDa were reduced in stunned myocytes.

PCPT or SP, but after the addition of 8-Br-cyclic AMP, this decrement was significantly reduced to 19%.

The data for time to peak shortening and 90% relaxation time are summarized in Table 1. Both PCPT and SP significantly increased time to peak in both control and stunned myocytes. These activators had no significant effect on the 90% relaxation time. There were no significant effects of 8-Br-cyclic AMP, but both PCPT and SP lengthened time to peak and 90% relaxation time after its addition of 8-Br-cyclic AMP.

3.2. Metabolic data

As shown in Table 2, PCPT $10^{-7,-5}$ M and SP $10^{-7,-5}$ M induced statistically significant decrements in cardiac myocyte O_2 consumption in both the control and simulated ischemia/reperfusion groups. 8-Br-cyclic AMP did not increase myocyte O_2 consumption significantly in either group. However, 8-Br-cyclic GMP also significantly decreased O_2 consumption after 8-Br-cyclic AMP. There were no differences between the effects of the two specific activators with or without 8-Br-cyclic AMP. There were also no significant differences of the effects of the cyclic GMP-dependent protein kinase activators between the two groups of ventricular myocytes.

3.3. Phosphoprotein analysis

The baseline protein phosphorylation pattern was similar between two groups. In Fig. 5, the first four lanes were from control myocyte extracts and lanes 5–8 were from simulated ischemia/reperfusion myocyte extracts. As indicated in the figure legend, we observed the protein phosphorylation pattern at baseline, with the addition of PCPT alone, 8-Bromo-cyclic AMP alone, and the combination of 8-Bromo-cyclic AMP and PCPT. In the cell extracts from both control and simulated ischemia/reperfusion rabbit ventricular myocytes, the addition of PCPT or 8-Br-cyclic AMP significantly enhanced the labeling of the same five specific protein bands at approximate molecular weight of 170, 97, 53, 31, and 22 kDa (Fig. 5). The combination of PCPT and cyclic AMP resulted in a significantly greater enhancement of these five protein bands compared to either agent alone. In the simulated ischemia/reperfusion myocytes, three bands at approximately 22, 31 and 53 kDa were phosphorylated significantly less by PCPT than in control myocytes. The effect of 8-Bromo-cyclic AMP was reduced in the 22 and 31 kDa bands in the stunned myocyte extract. Similar results were obtained in extracts prepared from four rabbits.

4. Discussion

Simulated ischemia/reperfusion led to a reduction in ventricular myocyte function. In control myocytes, activation of the cyclic GMP-dependent protein kinase led to a reduction in function, while increases in cyclic AMP increased function. Cyclic AMP reduced the effects of the cyclic GMP protein kinase in the control myocytes. The major finding of this study was that in stunned (simulated ischemia/reperfusion) myocytes, these effects were blunted, especially the interaction between the cyclic GMP-dependent protein kinase and cyclic AMP. The degree of protein phosphorylation caused by the cyclic GMP protein kinase was also reduced in the stunned myocytes.

The use of isolated rabbit ventricular myocytes in this study obviated concerns arising from the use of heart tissues containing heterogeneous cell types, which could act as confounding sources of the oxygen consumption measured. The yields were high with 55–70% of rod-shaped healthy cells. The yields after 15 min of simulated ischemia and 30 min of simulated reperfusion were similar. This protocol has been used by others to cause myocyte stunning and cell damage (Dougherty et al., 1998; Gandhi et al., 1999; Liang and Gross, 1999). The viability of the cells was confirmed by rechecking the percentage of rod-shaped cells at the end of each experiment. Measurement errors in oxygen consumption data due to damaged cells should be small. Although these cells may still metabolize to an unknown extent, this would have led to a shift in the absolute value of these parameters, without altering our conclusion. The cells used to determine the functional parameters were rod shaped and could react to different reagents throughout the course of the experiment. Untreated cells continued to contract with a constant shortening over the time course of the experiment. Cells from all hearts were studied for the functional and O_2 consumption measurements. For the phosphoprotein analysis, frozen cells from each experimental animal were kept in -70 °C freezer until used. Two specific activators of cyclic GMP-dependent protein kinase with high specificity were used in this study. A membrane permeable analogue of cyclic AMP, 8-Bromo-cyclic AMP, was also used. The variability of our functional measurements was similar to other studies (Gandhi et al., 1999; Shah et al., 1994; Tajima et al., 1998).

The second messenger cyclic GMP has negative metabolic and functional effects on both the myocardium and isolated cardiac myocytes (Lohmann et al., 1991; Shah et al., 1994). There was, at least, one study that suggested that the cyclic GMP protein kinase was not important for the negative inotropic effects of cyclic GMP in heart (MacDonell and Diamond, 1997). However, it had been suggested that activation of cyclic GMP-dependent protein kinase might be an essential step in the course of inhibition of contractility by elevating cyclic GMP level (Lincoln et al., 1994). Others have also suggested an important role for protein phosphorylation in the action of cyclic GMP in myocytes (Haddad et al., 1995; Mery et al., 1993; Wahler and Dollinger, 1995). Previous work from our laboratory using cardiac myocytes (Straznicka et al., 1999) demonstrated that specific inhibitors of cyclic GMP-dependent

protein kinase reduced the metabolic and functional effects of cyclic GMP. In current study, we use two specific activators of cyclic GMP-dependent protein kinase and obtained dose-dependent decrements in both function and metabolism. This indicated that this protein kinase is an important regulator of cardiac myocyte function.

There is a significant interaction between the second messengers cyclic GMP and cyclic AMP in cardiac myocytes. Cyclic AMP exerts positive effects on isolated cardiac myocytes. Cyclic GMP can change cyclic AMP level through cyclic GMP-regulated cyclic AMP phosphodiesterases. This is through the action of two phosphodiesterases, a cyclic GMP-stimulated- and a cyclic GMP-inhibited cyclic AMP phosphodiesterase (Lohmann et al., 1991). This effect of cyclic GMP on cyclic AMP levels should not be important in the current study because of the excess of the exogenous cyclic AMP analog. It has been demonstrated that cyclic AMP operates primarily through the cyclic AMP-dependent protein kinase (Sugden and Bogoyevitch, 1995; Coppenoll et al., 1997). Thus, it is likely that cyclic AMP and cyclic GMP can also interact at the level of their respective protein kinases. There is evidence that the cyclic AMP protein kinase and cyclic GMP protein kinase phosphorylate different specific sites on proteins, such as L-type calcium channels (Lincoln et al., 1994; Haddad et al., 1995; Sumii and Sperelakis, 1995). We have also reported that cyclic AMP and cyclic GMP have crosstalk at the level of their respective protein kinase (Yan et al., 2000). In the current study, both protein kinases phosphorylated similar proteins, which suggested that they might target on different subunits. The combination of two reagents resulted in stronger enhancement of the five bands, which was consistent with our previous study (Yan et al., 2000).

After even a brief period of ischemia/reperfusion, there is a decline in mechanical function, usually without a change in myocardial metabolism (Vanden Hoek et al., 1996; Birnbaum and Kloner, 1995; Chiu et al., 1994). In vivo studies have demonstrated a similar functional decrement without change in myocardial O_2 consumption (Chiu et al., 1994; Kusuoka and Marban, 1992; Naim et al., 1997). In the current study, stunned cardiac myocytes were obtained by subjecting myocytes to 15 min of simulated ischemia (95% N_2, 5% CO_2) followed by 30 min of reperfusion (reoxygenation). Both in vivo and isolated heart studies have shown that stunned myocytes have decreased baseline functional measurements compared to control myocytes (Sato et al., 1995). We also obtained similar results. The basal percent shortening and maximal rate of shortening in stunned (simulated ischemia/reperfusion) myocytes were significantly lower than those in control myocytes. Oxygen consumption was not altered by this simulated ischemia/reperfusion.

In this study, we observed that cyclic GMP-dependent protein kinase activators had only slightly reduced negative effects on function and metabolism in stunned (simulated ischemia/reperfusion) myocytes. However, from the protein phosphorylation analysis, we found that PCPT phosphorylated three bands (22, 31 and 53 kDa) to a significantly lesser extent in the stunned myocytes. The functional effects of cyclic AMP were significantly reduced in the stunned myocytes and protein phosphorylation at 22 and 31 kDa was reduced compared to control myocytes. This suggested that one of these proteins might be the active site for the functional effects of cyclic AMP and cyclic GMP. In control myocytes, cyclic AMP significantly reduced the effect of the cyclic GMP protein kinase. In stunned myocytes, the negative functional effects of the cyclic GMP protein kinase were reduced by approximately one-third after cyclic AMP. The reduced protein phosphorylation and functional effects after cyclic AMP suggest some change or damage to the cyclic GMP dependent protein kinase during myocardial stunning. Since nitric oxide and cyclic GMP may be protective against myocardial stunning (Naim et al., 1997; Padilla et al., 2000; Gandhi et al., 1999; Matoba et al., 1999), there may be a shift in the relative importance of the mechanisms through which cyclic GMP operates to affect cardiac myocytes away from the cyclic GMP protein kinase.

In summary, we found that cyclic GMP-dependent protein kinase activators dose-dependently decreased metabolism and function in control myocytes. 8-Bromo-cyclic AMP significantly increased the functional parameters of control myocytes. After the addition of 8-Bromo-cyclic AMP, the effects of the cyclic GMP protein kinase activators were blunted. Both the cyclic GMP-dependent protein kinase activator and 8-Bromo-cyclic AMP phosphorylated similar proteins and the combination of both reagents led to enhanced phosphorylation. In stunned myocytes, the effect of the cyclic GMP-dependent protein kinase was slightly blunted. The effects of 8-Bromo-cyclic AMP and the interaction between cyclic AMP and cyclic GMP-dependent protein kinase were significantly reduced. Several protein bands were phosphorylated less by these agents. This suggests that these target proteins might be important in exerting the negative effects of cyclic GMP-dependent protein kinase.

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