Journal of Cardiovascular Pharmacology
Issue: Volume 28(5), November 1996, pp 723-731

Publication Type: [Article]
ISSN: 0160-2446
Accession: 00005344-199611000-00016
Keywords: Protein kinase C, Ischemia/reperfusion, Arrhythmias, Cardiac function, Preconditioning, Rat, Isolated heart

The Role of Protein Kinase C in Ischemic/Reperfused Preconditioned Isolated Rat Hearts

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Abstract

Summary: Protein kinase C (PKC) has been implicated in the preconditioning-induced cardiac protection in ischemic/reperfused myocardium. We studied the effect of PKC inhibition with calphostin C (25, 50, 100, 200, 400, and 800 nM), a potent and specific inhibitor of PKC, in isolated working nonpreconditioned and preconditioned ischemic/reperfused hearts. In the nonpreconditioned groups, all hearts underwent 30 min of nonmothermic global ischemia followed by 30 min of reperfusion. In the preconditioned groups, hearts were subjected to four cycles of ischemic preconditioning by using 5 min of ischemia followed by 10 min reperfusion, before the induction of 30 min ischemia and reperfusion. At low concentrations of calphostin C (25, 50, and 100 nM), the PKC inhibitor had no effect on the incidence of arrhythmias or postischemic cardiac function in the nonpreconditioned ischemic/reperfused groups. With 200 and 400 nM of calphostin C, a significant increase in postischemic function and a reduction in the incidence of arrhythmias were observed in the nonpreconditioned ischemic/reperfused groups. Increasing the concentration of calphostin C to 800 nM, the recovery of postischemic cardiac function was similar to that of the drug-free control group. In preconditioned hearts, lower concentrations (<100 nM) of calphostin C did not change the response of the myocardium to ischemia and reperfusion in comparison to the preconditioned drug-free myocardium. Two hundred and 400 nM of the PKC inhibitor further reduced the incidence of ventricular fibrillation (VF) from the preconditioned drug-free value of 50% to 0 ($p < 0.05$) and 0 ($p < 0.05$), respectively, indicating that the combination of the two, preconditioning and calphostin C, affords significant additional protection. Increasing the concentration of calphostin C to 800 nM blocked the cardioprotective effect of preconditioning (100% incidence of VF). The recovery of cardiac function was similarly improved at calphostin C doses of 200 and 400 nM and was reduced at 800 nM ($p < 0.05$). With 200 and 400 nM of calphostin C, both cytosolic and particulate PKC activity were reduced by [almost equal to]40 and 60%, respectively, in both preconditioned and

preconditioned/ischemic/reperfused hearts. The highest concentration of calphostin C (800 nM) resulted in almost a complete inhibition of cytosolic (100%) and particulate (85%) PKC activity correlated with the abolition of preconditioning-induced cardiac protection. In conclusion, calphostin C protects the ischemic myocardium obtained from intact animals, provides significant additional protection to preconditioning at moderate doses, and blocks the protective effect of preconditioning at high concentrations. The dual effects of calphostin C appear to be strictly dose and "enzyme inhibition" related.

Protein kinase C (PKC) was first identified by Takai et al. (1) as a proteolytically activated protein kinase, and is now known to be ubiquitous in many different organs. PKC is activated by receptor-mediated hydrolysis of inositol phospholipids, whereby it relays information via the cell membrane to regulate many calcium-dependent processes. PKC isoforms ([alpha], [beta]I, [beta]II, [gamma], [delta], [varepsilon], and [zeta]) are important regulators of cellular functions, including signal transduction, proliferation, and differentiation (2,3). Its activity is dependent on intracellular calcium and discylglycerol contents, both of which are regulated by phosphatidylinositol hydrolysis. In the initial phase of cellular responses, PKC has a dual effect, providing both positive and negative feedback controls over various steps of its own and other signaling pathways, including receptors coupled to inositol phospholipid hydrolysis and growth factors. Specific inhibitors of PKC may therefore have therapeutic value. Recently the activation and inhibition of PKC has been implicated in the mechanisms of ischemia/reperfusion-induced injury and preconditioning (PC) (4,5). Although staurosporine and other derivatives are potent inhibitors of PKC, these compounds also inhibit other protein kinases including cyclic adenosine monophosphate (cAMP)-dependent protein kinase (6). Calphostin C, a protein kinase inhibitor from Cladosporium cladosporioides, is the first specific inhibitor of PKC with high potency and negligible activity for other protein kinases (6). In this study, we tested whether the inhibition of PKC by calphostin C can reduce the incidence of reperfusion-induced arrhythmias and improve postischemic cardiac function. Furthermore we studied the effect of calphostin C in preconditioned ischemic/reperfused myocardium obtained from intact animals.

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METHODS

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Animals

Male Sprague-Dawley rats (320-350 g body weight) were used for all studies. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health (NIH Publication No. 86-23, revised 1985).

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Isolated working heart preparation

Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg body weight) and then given intravenous heparin (500 IU/kg). After thoracotomy, the heart was excised and placed in ice-cold perfusion buffer. Immediately after preparation, the aorta was cannulated, and the heart was perfused (at 37°C) according to the Langendorff method for a 5-min washout period at a constant perfusion pressure equivalent to 100 cm of

water (10 kPa). The perfusion medium consisted of a modified Krebs-Henseleit bicarbonate buffer ([millimolar concentration] sodium chloride, 118; potassium chloride, 4.7; calcium chloride, 1.7; sodium bicarbonate, 25; potassium biphosphate, 0.36; magnesium sulfate, 1.2; and glucose, 10). The Langendorff preparation was switched to the working mode after the washout period, as previously described in detail by Tosaki and Braquet (7) and Tosaki and Hellegouarch (8). Aortic flow was measured by an in-line calibrated rotameter. Coronary flow rate was measured by a timed collection of the coronary effluent that dripped from the heart.

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Induction of ischemia and reperfusion

After a 10-min aerobic perfusion of the heart, the left atrial inflow and aortic outflow lines were clamped at a point close to their origin. Reperfusion was initiated by unclamping the atrial inflow and aortic outflow lines. To prevent the myocardium from drying out during normothermic global ischemia, the thermostated glassware (in which hearts were suspended) was covered, and the vapor content was kept at a constant level (90-100%).

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Experimental time course

Ischemia and reperfusion. Hearts (n = 12 in each group) were subjected to 30 min of normothermic global ischemia followed by 30 min of reperfusion. Myocardial function (heart rate [HR], coronary flow [CF], aortic flow [AF], left ventricular pressure [LVP], and the first derative of LVP [LVPdp/dt]) was measured before ischemia and after 30 min of reperfusion. In calphostin C-treated groups, the drug (25, 50, 100, 200, 400, or 800 nM) was perfused for 10 min before the induction of ischemia, and pre- and postischemic cardiac function was recorded.

Ischemic preconditioning. In the drug-free control group, four cycles of PC, each consisting of 5-min normothermic global ischemia followed by 10-min reperfusion, were carried out before the onset of 30 min of normothermic global ischemia and 30 min of reperfusion. Four cycles of ischemia PC were selected because by using this protocol in previous studies, a significant reduction in the incidence of reperfusion-induced arrhythmias was observed (9,10). In the drug-treated groups, calphostin C (100, 200, 400, or 800 nM) was perfused before the onset of PC and ischemia/reperfusion for 10 min.

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Indices measured

An epicardial electrocardiogram (ECG) was recorded by a polygraph throughout the experimental period by two silver electrodes attached directly to the heart. The ECGs were analyzed to determine the incidence of ventricular fibrillation (VF) and ventricular tachycardia (VT) and whether VF was nonsustained (spontaneously reverting to regular rhythm) or sustained (persisting through the first 3 min of reperfusion). After 3 min of sustained VF, hearts were defibrillated, and myocardial function was recorded. In additional experiments, preconditioned hearts were defibrillated (if it was necessary) after 15 s of reperfusion to avoid the fibrillation-induced stress on the myocardium, and cardiac function was registered. The heart was considered to be in VF if an irregular undulating baseline was apparent on the ECG. VT was defined as five or more consecutive premature ventricular complexes, and this classification included repetitive monomorphic VT, which is difficult to dissociate from rapid VT. In each instance, VT switched spontaneously to sinus rhythm or VF; therefore VT was considered nonsustained. The heart was considered to be in sinus rhythm if normal sinus complexes occurring in a regular rhythm were apparent on the ECG. Before ischemia and during reperfusion, HR, CF, and AF rates were registered. LVP and

LVPdp/dt were also recorded by the insertion of a Millar catheter into the left ventricle via the left atrium and mitral valve. The hemodynamic parameters were registered by a Cordat II acquisition system.

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Measurement of cytosolic and particulate PKC activity

In preliminary experiments, to study the translocation of PKC to the particulate fractions, we directly measured the enzyme activity after four cycles of PC, each consisting of 5 min of ischemia followed by 10 min of reperfusion, just before the onset of 30 min of normothermic global ischemia in drug-free and calphostin C-treated hearts.

PKC activity was also directly measured in the preconditioned ischemic/reperfused drug-free and calphostin C-treated (200, 400, and 800 nM) ischemic/reperfused myocardium. Approximately 0.2 g tissue was homogenized in 2 ml of a buffer consisting of 50 mM Tris/HCl (pH = 7.5), 5 mM EDTA, 10 mM EGTA, 0.3% wt/vol [beta]-mercaptoethanol, 10 mM benzamidine, and 50 mg/ml phenylmethyl-sulphonyl fluoride with a polytron homogenizer. The homogenate was centrifuged at 100,000 g for 60 min, and the supernatant was used to measure the cytosolic PKC activity. The pellet was resuspended in 0.5 ml of homogenization buffer in the presence of 0.2% Triton X-100. After 60 min of incubation, the suspension was centrifuged at 100,000 g for 20 min, and the supernatant, fraction I, was obtained. The pellet was reextracted, recentrifuged, and additional supernatant was obtained (fraction II). Fractions I and II were combined and used for the measurement of particulate PKC activity (11,12) by using Amersham PKC assay kit. The substrate was Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu, which is a highly specific peptide for PKC. The total protein concentration in cytosolic and in particulate fractions was determined by using BCA (bicinchoninic acid) protein assay kit (Pierce, Rockville, IL, U.S.A.). PKC activity was determined in reaction mixtures (0.1 ml at 25°C) containing 12 mM Ca-acetate in a buffer consisting of 50 mM Tris/HCl, 0.03% wt/vol Na-azide, 8 mol% L-[alpha]-phosphatidyl-L-serine, and 24 mg/ml phorbol 12-myristate 13-acetate in 50 mM Tris/HCl containing 0.05% wt/vol Na-azide (pH = 7.5), 30 mM dithiothreitol in 50 mM Tris/HCl, and 0.05% wt/vol Na-azide (pH = 7.4), 150 µM adenosine triphosphate (ATP), and 45 mM Mg-acetate in 50 mM Tris/HCl with 0.05% wt/vol sodium azide (pH = 7.5). To this reaction mixture, 10 µCi/ml of [[gamma]-^{32}P]-ATP (Amresham) and 25 µg cytosolic or solubilized particulate protein were added. Triton X-100 was added to the supernatant fractions such that the concentration of Triton X-100 in both the cytosolic and particulate fractions was 0.04% in the final reaction mixtures. The reaction was started by adding 25 µl of Mg-[^{32}P]-ATP buffer. The reaction was terminated with 100 ml of stop-reagent. Thus 125 µl of the each terminated reaction mixture was pipetted onto a numbered square of binding paper, and the solution was allowed to soak into the paper. The binding paper was placed in 5% vol/vol acetic acid, and then the paper was carefully removed from its container by using suitable forceps and placed in an individual scintillation vial. Finally, 10 ml of liquid scintillation solution was added into each vial, and the radioactivity was measured by a liquid scintillation counter.

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Exclusion criteria

Preselected exclusion criteria for our studies demanded that hearts were excluded if (a) ventricular arrhythmias occurred during the period before the induction of global ischemia, and (b) coronary flow and aortic flow were <19 ml/min and 35 ml/min, respectively, before the initiation of ischemia.

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Statistics

The data for myocardial function and cytosolic and particulate PKC activity were expressed as the mean ± SEM. One-way analysis of variance was first carried out to test for any differences between the mean values of all groups. If differences were established, the values of the calphostin C-treated groups were compared with those of the drug-free control group by a modified t test. An analog procedure was followed for distribution of discrete variables such as the incidence of VF and VT. An overall $[chi]^2$ test for a $2 \times n$ table was constructed, followed by a sequence of 2×2 $[chi]^2$ tests to compare individual groups. A change of $p < 0.05$ was considered significant.

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RESULTS

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Arrhythmias in ischemic/reperfused hearts

It has been previously reported (13) that in the rat heart, the vulnerability to reperfusion-induced arrhythmias is determined by the duration of the preceding ischemic period and that a complex bell-shaped time-response relation exists. In our studies, we required that the control group exhibit a high vulnerability to reperfusion-induced arrhythmias to demonstrate any antiarrhythmic effects. To ensure this within the experimental time course and condition defined for this study, 30 min of normothermic global ischemia followed by 30 min reperfusion was selected. The results demonstrate (Fig. 1A) that in rats subjected to ischemia/reperfusion protocol, the incidence of reperfusion-induced VF was reduced from its control value of 92 to 42% ($p < 0.05$) and 25% ($p < 0.05$), respectively, with the concentrations of 200 and 400 nM calphostin C. With a higher dose of calphostin C (800 nM), this protection was abolished. The incidence of reperfusion-induced VT showed the same pattern (Fig. 1B). The loss of cardiac protection could be explained by drug toxicity or other nonspecific effects by using the highest concentration of calphostin C.

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Arrhythmias in preconditioned hearts

Hearts were subjected to four cycles of PC, each consisting of 5 min of global ischemia followed by 10 min of reperfusion, before the induction of 30 min of ischemia and reperfusion. Figure 2 shows that four cycles of PC ($4 \times PC$) significantly reduced the incidence of VF (Fig. 2A) and VT (Fig. 2B) in comparison with that of the nonpreconditioned group. Hearts treated with calphostin C of 100, 200, or 400 nM demonstrated a further reduction in the incidence of arrhythmias (Fig. 2). This demonstrates that a combination of PC and PKC inhibition affords additional protection. At a high concentration (800 nM) of calphostin C, the PC-induced cardiac protection was abolished, indicating the importance of the concentration of PKC blocker and enzyme inhibition in our model system.

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Effects of calphostin C on cardiac function in ischemic and reperfused hearts

Table 1 shows the absolute values for HR, CF, AF, LVP, and LVPdp/dt before the induction of ischemia; no statistically significant difference was found between the drug-free and calphostin-treated groups. Table 2 shows the postischemic recovery of cardiac function after 30 min of ischemia followed by 30 min of reperfusion in the drug-free and calphostin C-perfused groups. Thus in hearts perfused with 200 or 400 nM of calphostin C, a significant recovery in AF, LVP, and LVPdp/dt was observed in comparison with that of the drug-free control group. At a high concentration (800 nM), as well as at low

concentrations (25, 50, or 100 nM), calphostin C failed to improve the recovery of cardiac function in ischemic/reperfused hearts (Table 2).

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Calphostin C and cardiac function in preconditioned hearts

Table 3 shows the absolute values for postischemic myocardial function in the drug-free control and drug-treated four-cycle preconditioned groups. Four cycles of PC followed by 30 min of ischemia and 30 min of reperfusion (Table 3) resulted in a nonsignificant recovery of contractile function in preconditioned hearts. The perfusion of 200 and 400 nM calphostin C significantly improved the postischemic recovery of AF, LVP, and LVPdp/dt in the four-cycle preconditioned groups (Table 3). At the highest concentration of calphostin C (800 nM), a significant reduction in postischemic function was observed in preconditioned hearts (Table 3).

To determine whether the duration of ventricular fibrillation (3 min) was the reason for the lack of PC-induced functional recovery during reperfusion, in additional studies, preconditioned hearts were allowed to fibrillate for only 15 s before defibrillation (if it was necessary) and measurements of cardiac function were done. The results show (Table 4) that the duration of VF may determine cardiac recovery after a period of VF, but these results (after 15 s or 3 min of VF) did not show a significant change in cardiac function in our model system. In another words, cardiac function was not significantly improved after four cycles of PC in hearts fibrillated for 15 s in comparison with those fibrillated for 3 min during the reperfusion period. These results indicate that the duration of VF <=3 min does not change significantly the extent of the recovery in cardiac function after four cycles of ischemic PC in our model.

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Effects of calphostin C on cytosolic and particulate PKC activity

After four PC cycles (Fig. 3), before the initiation of the 30-min ischemia and reperfusion protocol, cytosolic and particulate PKC activity was measured. Thus after four PC cycles, particulate PKC activity was significantly increased from its nonpreconditioned and nonischemic/reperfused value of 16 ± 2.5 pmol/min to 37 ± 4.1 pmol/min in the four-cycle preconditioned hearts obtained from intact animals. Cytosolic PKC activity was also significantly increased from its nonpreconditioned and nonischemic control value of 33 ± 5 pmol/min to 102 ± 14 pmol/min in the four-cycle preconditioned group. Calphostin C reduced, in a dose-dependent manner, both the cytosolic and particulate PKC activity in the preconditioned myocardium (Fig. 3).

In calphostin C-treated preconditioned ischemic/reperfused hearts, the activity of cytosolic and particulate PKC was measured after 30 min of ischemia followed by 30 min of reperfusion. Figure 4 shows that both cytosolic (Fig. 4A) and particulate (Fig. 4B) PKC activity was increased after ischemia and reperfusion in preconditioned drug-free hearts, and this activity was reduced by a different extent in hearts treated with various doses of calphostin C. Thus the activation of PKC in the preconditioned myocardium indicates the importance of PKC in PC, and its inhibition (%) reflected in a reduction or aggravation, depending on the inhibition of the enzyme or its isoforms, in the incidence of VF and VT (Fig. 4). It is of interest that an increase in cytosolic and particulate PKC activity was observed in preconditioned/ischemic/reperfused hearts and in preconditioned myocardium (Fig. 3).

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DISCUSSION

The importance of protein kinase C has recently been implicated in the mechanism of cardiac PC and ischemia/reperfusion-induced injury (4,5). Protein phosphorylation catalyzed by PKC may exert profound modulation of various Ca^{2+}-mediated processes, such as release reactions and exocytosis, cell proliferation and differentiation, membrane conductance and transport, potentiation and desensitization of other receptor systems, smooth muscle contraction, and other metabolic processes (3). Different lines of evidence suggest that PKC modulates ion conductance by phosphorylating membrane proteins such as pumps, channels, and ion-exchange proteins. It has been suggested that PKC may play a role in Ca extrusion into the cytosol immediately after its mobilization, and that Ca-transport ATPase is a possible target of this protein kinase. Studies with myocardial sarcoplasmic reticulum suggest that Ca-transport ATPase is activated by the application of PKC (14).

In our experiments, we studied whether the specific inhibition of PKC activity by calphostin C would affect ischemia/reperfusion-induced injury in our model system. Calphostin C acts on the regulatory domain of PKC and induces a more specific inhibition of PKC than do other PKC inhibitors (6). Furthermore, we studied the importance of the enzyme inhibition in the myocardium subjected to PC. Our data show that ischemia/reperfusion-induced injury can be attenuated if PKC is inhibited with a selective antagonist (calphostin C), with reperfusion-induced arrhythmias and cardiac function used as end points in an in vitro rat heart preparation. The results also demonstrate that this protection window is within a very narrow range (200 to 400 nM) of calphostin C, where the inhibition of cytosolic and particulate PKC enzyme activity is between 40 and 60% in preconditioned and in preconditioned and ischemic/reperfused hearts. Lower concentrations of calphostin C (25 and 50 nM), as well as highest concentration (800 nM; cytosolic and particulate PKC inhibition [almost equal to]99 and 85%, respectively), failed to reduce the incidence of reperfusion-induced arrhythmias and improve postischemic cardiac function in isolated working rat heart. The reduction in the incidence of reperfusion-induced VF and VT was reflected in the significant improvement of postischemic cardiac function with the concentrations of 200 and 400 nM calphostin C. At a high concentration of calphostin C (800 nM), a significant reduction in postischemic cardiac function was observed in comparison with the drug-free ischemic/reperfused control group.

It has also been proposed that PKC plays a central role in ischemic PC. It has been proposed that an endogenous ligand binds to the adenosine $[alpha]_1$ receptor on the surface of myocytes, which then activates phospholipase C via a G protein, which then activates phospholipase C, causing the breakdown of phosphatidylinositol 4,5-diphosphate and phosphatidylcholine to produce diacylglycerol and inositol-1,4,5-trisphosphate. Increased concentration of diacylglycerol then activates PKC in the membrane and causes translocation and activation of cytosolic PKC, and the activated PKC phosphorylates a secondary effector, which may or may not induce the protective effect of PC (15,16). Evidence for the involvement of PKC is provided by Ytrehus et al. (17), who studied the effects of PKC inhibitors, staurosporine and polymyxin B, to block PC in a rabbit in vivo model in which infarct size was measured.

Although PKC activation or inhibition may play an important role in preconditioned ischemic/reperfused hearts (4,5,15), the aforementioned studies have been carried out with a single dose of PKC inhibitors, without a dose-response study or measuring the inhibition of cytosolic or particulate PKC activity. We have found that a high concentration of the PKC blocker (800 nM; cytosolic and particulate enzyme inhibition was 99 and 85%) increased the incidence of reperfusion-induced arrhythmias and reduced the recovery of postischemic cardiac function in comparison with the lower concentrations of the drug. In

addition, high-dose PKC blocker inhibited PC-induced cardioprotection. Surprisingly, lower concentrations of calphostin C (200 nM and 400 nM, enzyme inhibition 40 and 60%, respectively), afforded an additional protection to the precondition-mediated effect. Further reduction in the concentrations of calphostin C failed to reduce the incidence of arrhythmias and improve postischemic cardiac function. These results indicate that the inhibition of PC-induced cardiac protection by a PKC blocker is dependent on the concentration and percentage inhibition of cytosolic and particulate enzyme activity. Precondition-mediated cardiac protection could be blocked (at high concentrations) by an inhibitor of PKC or could afford further protection in a dose-response manner (at low concentrations) to preconditioned ischemic/reperfused hearts. Our results suggest that the inhibition of enzyme activity at various degree by calphostin C may inhibit the function of PKC isoforms ([alpha], [beta]I, [beta]II, [gamma], [delta], [varepsilon], and [zeta]) in various extents, and the inhibition of different isoforms may regulate the various phosphorylation mechanisms changing the function of the myocardium. Thus the inhibition of some isoforms of PKC may be responsible for the regulation and manifestation of arrhythmogenesis and others for the regulation of cardiac function or infarct size (18,19). Although PKC activity was not directly measured in their studies, Hu and Nattel (19) concluded, by using pharmacologic approaches in their PC studies, that ischemic PC in rat hearts is caused by stimulation of $[alpha]_{1B}$-adrenoceptors by endogenous catecholamines through the activation of PKC through a pertussis toxin-sensitive G protein.

The presence of different isoforms of PKC in the heart might be expected to impart a high degree of specificity and flexibility to the signal-transduction mechanism. Although a recent study reported the translocation of PKC-[zeta], the major PKC isoform, to the particulate fraction of ventricular myocytes by epinephrine and endothelin-1 (20), it is possible that different PKC isoforms could be activated selectively by different receptor agonists or PC. Such a mechanism could involve receptor-activated phosphatidylcholine hydrolysis, which is stimulated by agonists that promote phosphoinositide hydrolysis and results in the selective and sustained accumulation of diacylglycerol without the calcium-mobilizing second messenger inositol-1,4,5-triphosphate (IP_3). Moreover, because phosphoinositides and phosphatidylcholine characteristically have different fatty acyl moieties, phosphatidylcholine hydrolysis would be predicted to lead to the production of a distinct pool of diacylglycerol, which may differentially activate different isoforms of PKC (21). The expression of various PKC isoforms and the different numbers of PC cycles may be responsible for the cardiac protection observed in cardiac function, infarct size, or arrhythmias. PKC isoform-specific translocation to distinct intracellular structures could lead to differences in substrate accessibility and thereby lead to PKC isoform-specific actions. Thus an isoform putatively identified as PKC-[beta] has been reported to translocate to the sarcolemma and perinuclear area an exposure to phorbol esters, whereas a different and as yet unidentified PKC isoform translocates to the myofibrils under the same experimental conditions (22). Taken together, these studies raise the possibility that, on activation, distinct isoforms of PKC translocate to the distinct intracellular sites of their protein substrates and that isoform-dependent differences in substrate specificity and isoform-dependent differences in intracellular compartmentalization might determine functional responsiveness.

Our studies provide a basis for inquiry but do not dissociate the role of different PKC isoforms in protein phosphorylation, as was stressed by Mitchell et al. (23), in preconditioned ischemic/reperfused hearts. This could be explained by the fact that PC under certain conditions, may attenuate the incidence of arrhythmias but may not improve postischemic cardiac function. Besides many positive results, PC failed to improve cardiac function (24,25) but reduced infarct size (24) in ischemic and reperfused hearts. These and

other conflicting results may possibly be explained on the basis of experimental conditions including the different species and the numbers of PC cycles. Interpretation of the various results is further complicated by the fact that cardiac function, arrhythmias, and infarct size were studied as end points in many experiments, but the measurement of cytosolic and particulate PKC activity was not carried out with different doses of PKC inhibitors in preconditioned ischemic/reperfused hearts or myocytes (4,5,26). However, our studies show absence of additional deterioration of cardiac function after prolonged myocardial ischemia in the presence of previous ischemic events in the drug-free preconditioned hearts. Therefore it is safe to assume that brief ischemic episodes do not cause additive myocardial injury when isolated hearts are subjected to subsequent prolonged periods of ischemia and reperfusion. Our results show that arrhythmogenesis and cardiac function may be regulated by different isoforms of PKC (e.g., [alpha], [beta]I, [beta]II, [gamma], [delta], [varepsilon], and [zeta]), which may be activated or blocked at different degree in preconditioned ischemic/reperfused hearts, leading to cardiac protection [e.g., via the synthesis of heat-shock proteins (27,28)] or leading to the upregulation of protooncogenes (29,30), aggravating the postischemic cardiac function (31,32). Immunofluorescence microscopic studies show (33) the translocation and activation of specific PKC isoforms in ischemic/preconditioned hearts via the bradykinin-mediated mechanism that (bradykinin) activates myocardial [beta]$_2$-receptors to initiate a series of intracellular events leading to the activation of PKC and its isoforms.

In conclusion, our results demonstrate the activation (increased particulate activity) of PKC in preconditioned hearts obtained from intact animals, and this increase in PC-induced PKC activity is sustained and can be seen after the subsequent prolonged ischemic/reperfused period. Furthermore, calphostin C, a PKC inhibitor, dose and “enzyme inhibition” dependently could block the protective effect of PC or provide significant additional protection to PC in ischemic/reperfused hearts.

Our model has several limitations. The relaxation of cardiac muscle during normothermic global ischemia is independent of load and is determined primarily by the intracellular processes that influence the rate and extent of force inactivation, which can include the activation or inactivation of different enzymes including PKC isoforms, the ATP-dependent rate, and calcium ion sequestration capacity of the sarcoplasmic reticulum. The interpretation of the results must be limited by our observations in the isolated working rat heart and by the fact that we used an isolated preparation. The use of an isolated perfused heart, although permitting study of direct cardiovascular responses independent of various peripheral factors, has the disadvantage of being denervated and perfused with asanguineous solutions. The immediate extrapolation of our results, obtained in isolated rat hearts, to an actual clinical situation should be viewed with some caution because of the absence of blood and its elements (e.g., platelets and leukocytes) in our model system and possible interspecies differences in myocardial metabolism.

Acknowledgment: This work was supported by USPHS grants NIH HL 22559, HL 34360, and the Grant-in-Aid from the American Heart Association.

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Key Words: Protein kinase C; Ischemia/reperfusion; Arrhythmias; Cardiac function; Preconditioning; Rat; Isolated heart

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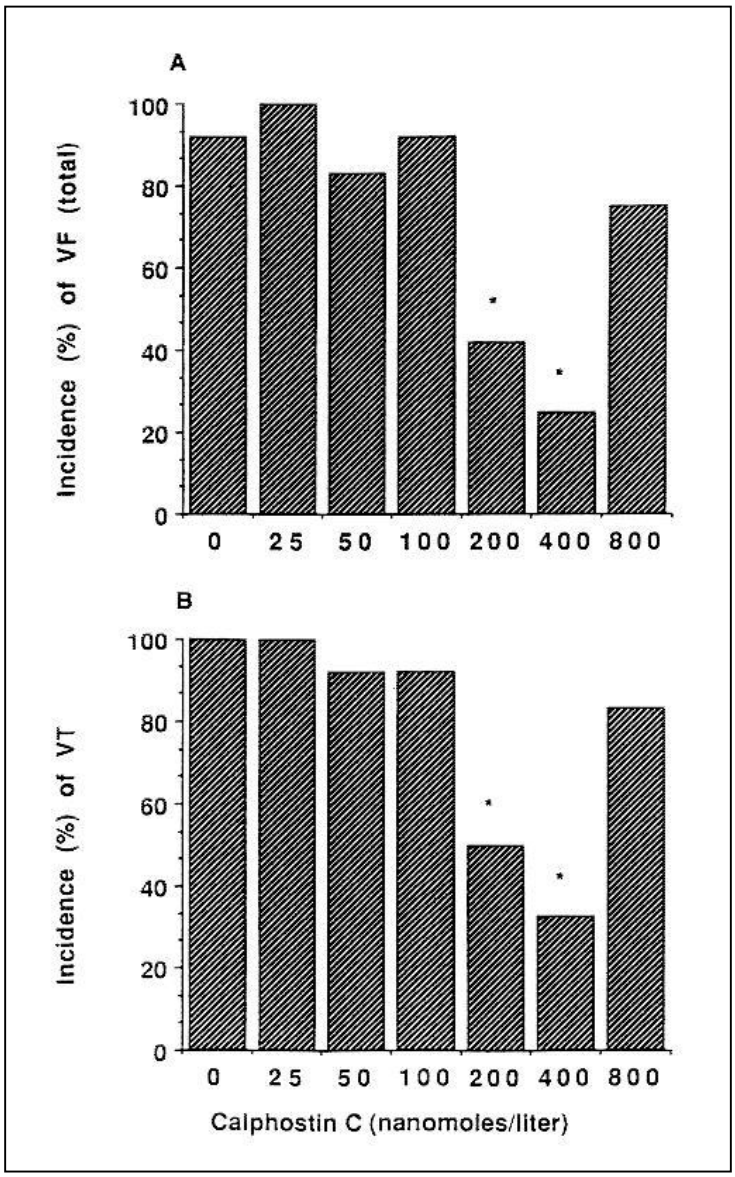

FIG. 1. Dose-response studies for the ability of calphostin C to reduce reperfusion-induced arrhythmias. Hearts (n = 12 in each group) were subjected to 30-min global ischemia followed by 30-min reperfusion. Various concentrations of calphostin C were included in the perfusion buffer, and hearts were perfused with the drug before the induction of ischemia for 10 min. The incidence (%) of ventricular fibrillation (A) and ventricular tachycardia (B) is shown. Statistical comparisons have been made for each concentration group against the drug-free control group. *$p < 0.05$.

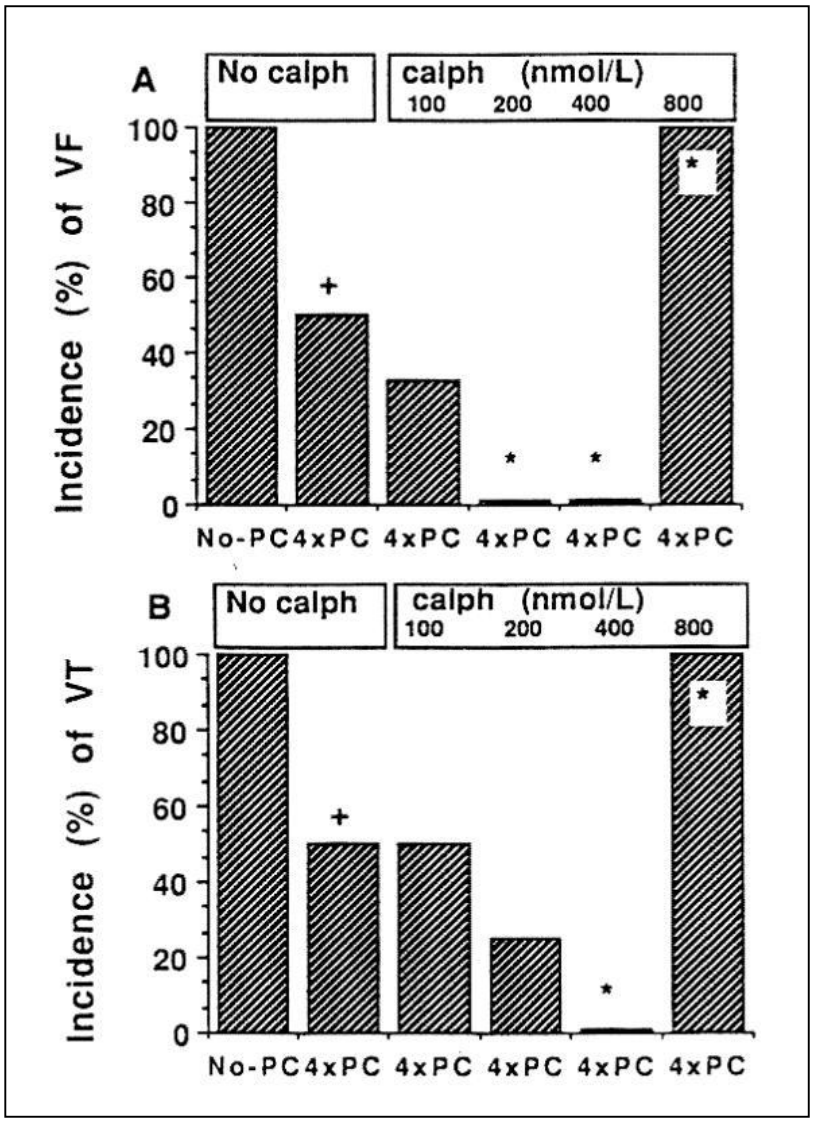

FIG. 2. The effect of calphostin C on the incidence of reperfusion-induced ventricular fibrillation (A) and ventricular tachycardia (B) in hearts subjected to four cycles [4 x preconditioning (PC)] of ischemic preconditioning before the induction of 30-min global ischemia followed by reperfusion. n = 12 in each group; +comparison was made to the nonpreconditioned (no-PC) drug-free group (p *comparisons were made to the 4 x PC drug-free group ($p < 0.05$).

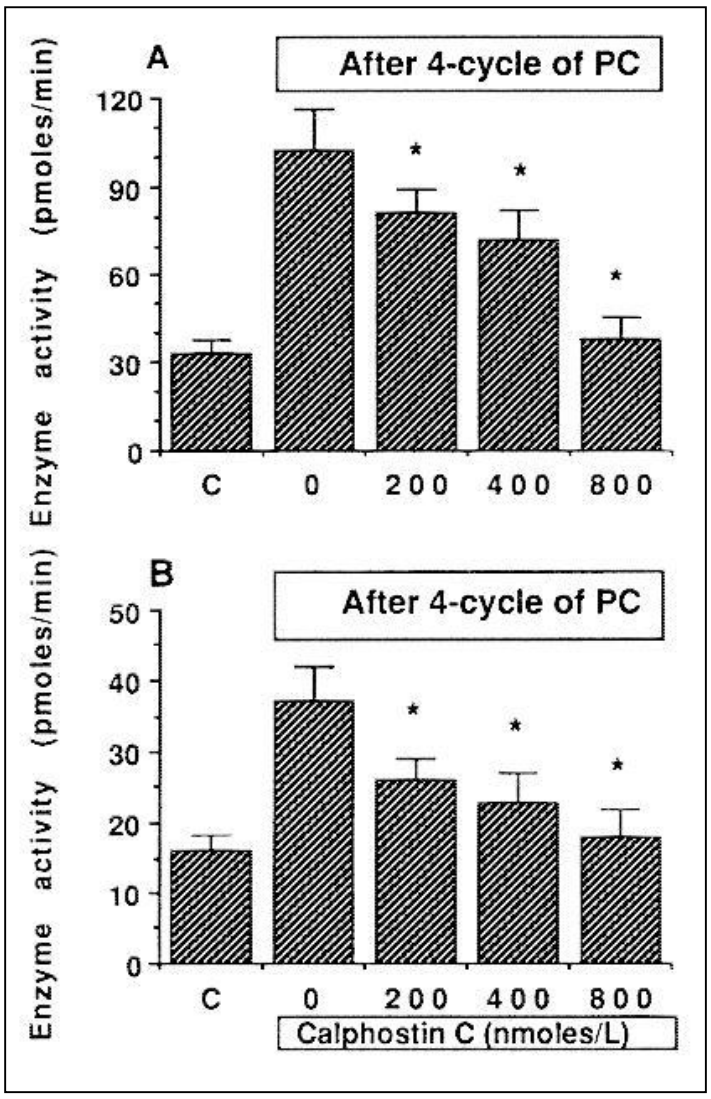

FIG. 3. Protein kinase C inhibition measured by a direct radioactive method after four-cycle of preconditioning (PC). Hearts were subjected to 0, 200, 400, or 800 nM of calphostin C and four cycles of PC. Both cytosolic (A) and particulate (B) PKC activity were monitored. Comparisons were made to the PC drug-free group. C, Nonpreconditioned control; n = 6 in each group; mean +/- SEM; *$p < 0.05$.

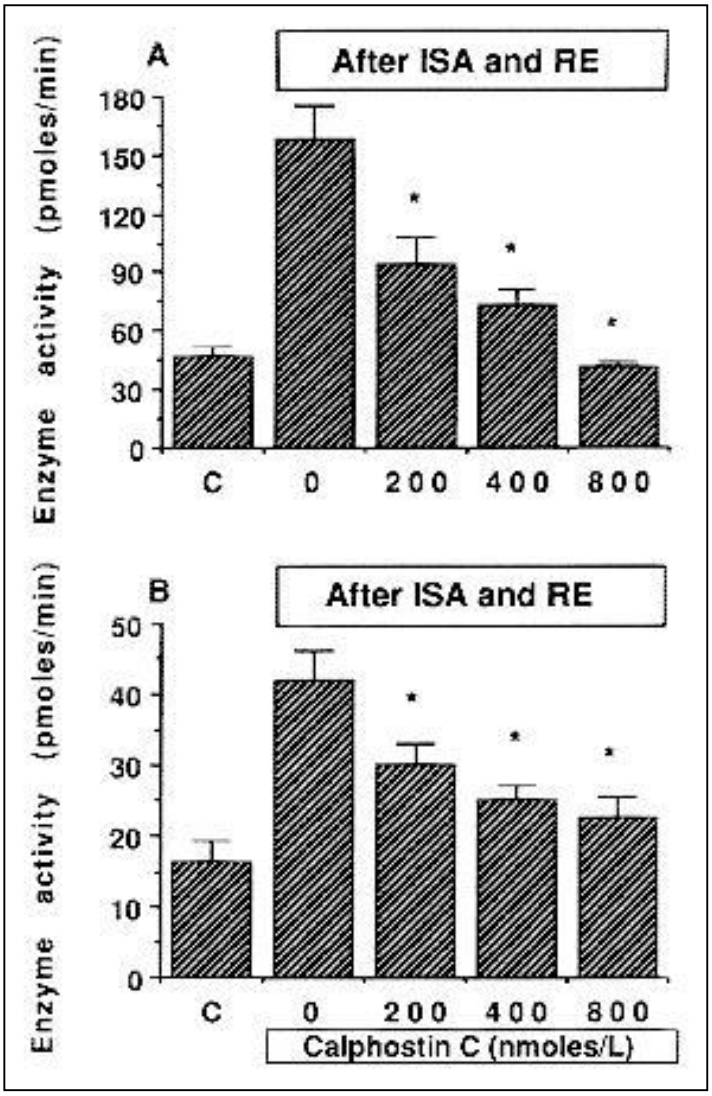

FIG. 4. Protein kinase C inhibition measured by a direct radioactive method in the isolated preconditioned/ischemic/reperfused myocardium subjected to 200, 400, and 800 nM of calphostin C. Both cytosolic (A) and particulate (B) PKC activity were monitored. Comparisons were made to the preconditioned drug-free ischemic/reperfused hearts. C, Nonpreconditioned and nonischemic control; n = 6 in each group; mean +/- SEM; *$p < 0.05$.

Group	HR (beats/min)	CF (ml/min)	AF (ml/min)	LVP (kPa)	LVP*dp/dt* (kPa/s)
Control	319 ± 8	26.9 ± 0.9	51.4 ± 1.5	17.9 ± 0.3	795 ± 26
25 n*M*	325 ± 7	28.0 ± 1.1	49.6 ± 1.2	18.4 ± 0.5	817 ± 31
50 n*M*	316 ± 7	27.3 ± 1.3	50.8 ± 1.7	17.4 ± 0.4	769 ± 24
100 n*M*	330 ± 9	26.2 ± 0.9	51.6 ± 2.0	18.7 ± 0.5	791 ± 32
200 n*M*	322 ± 7	26.8 ± 0.7	49.9 ± 1.8	17.3 ± 0.5	763 ± 18
400 n*M*	313 ± 8	28.0 ± 1.4	51.8 ± 2.0	17.7 ± 0.4	776 ± 23

n = 12 in each group; values reported as mean ± SEM.
HR, heart rate; CF, coronary flow; AF, aortic flow; LVP, left ventricular pressure; LVP*dp/dt*, first derivative of LVP.

TABLE 1. Effects of calphostin C on preischemic cardiac function

Group	HR (beats/min)	CF (ml/min)	AF (ml/min)	LVP (kPa)	LVP*dp/dt* (kPa/s)
Control	298 ± 8	17.0 ± 0.6	10.9 ± 0.9	11.2 ± 0.5	458 ± 19
25 n*M*	307 ± 9	16.4 ± 0.5	9.9 ± 0.6	10.7 ± 0.6	500 ± 38
50 n*M*	288 ± 6	18.0 ± 0.7	11.0 ± 0.8	11.8 ± 0.5	467 ± 24
100 n*M*	292 ± 5	17.3 ± 0.8	11.9 ± 1.1	12.1 ± 0.6	493 ± 32
200 n*M*	309 ± 9	16.2 ± 0.7	15.8 ± 1.3[b]	14.4 ± 0.5[b]	587 ± 28[b]
400 n*M*	287 ± 8	18.3 ± 1.0	20.7 ± 1.3[b]	15.8 ± 0.4[b]	608 ± 30[b]
800 n*M*	296 ± 9	17.2 ± 0.6	11.6 ± 1.1	12.0 ± 0.5	487 ± 27

n = 12 in each group; values reported as mean ± SEM.
HR, heart rate; CF, coronary flow; AF, aortic flow; LVP, left ventricular pressure; LVP *dp/dt*, first derivative of LVP.
[a]After 30 min of ischemia followed by 30 min of reperfusion.
[b]Comparisons were made to the drug-free group ($p < 0.05$).

TABLE 2. Effects of calphostin C on postischemic cardiac function

Group	HR (beats/min)	CF (ml/min)	AF (ml/min)	LVP (kPa)	LVP *dp/dt* (kPa/s)
Control (no PC)	293 ± 8	16.9 ± 0.6	9.5 ± 0.6	10.9 ± 0.5	448 ± 24
Control (4 × PC)	287 ± 9	17.4 ± 0.5	10.6 ± 0.5	11.1 ± 0.5	462 ± 19
4 × PC + 100 n*M* calph	300 ± 7	18.0 ± 0.6	11.8 ± 0.7	11.6 ± 0.6	486 ± 27
4 × PC + 200 n*M* calph	279 ± 9	16.5 ± 0.7	17.4 ± 0.9[b]	15.8 ± 0.5[b]	598 ± 31[b]
4 × PC + 400 n*M* calph	284 ± 8	17.3 ± 0.8	19.8 ± 1.0[b]	16.4 ± 0.5[b]	647 ± 28[b]
4 × PC + 800 n*M* calph	297 ± 8	16.0 ± 0.7	4.5 ± 0.5[b]	6.3 ± 0.4[b]	331 ± 17[b]

n = 12 in each group; values reported as mean ± SEM.

PC, preconditioning; calph, calphostin C; HR, heart rate; CF, coronary flow; AF, aortic flow; LVP, left ventricular pressure; LVP*dp/dt*, first derivative of LVP.

[a]After 30 min of ischemia followed by 30 min of reperfusion.

[b]Comparisons were made to the control four-times preconditioned (4 × PC) group ($p < 0.05$).

TABLE 3. Effects of calphostin C on postischemic cardiac function in preconditioned hearts

Group	HR (beats/min)	CF (ml/min)	AF (ml/min)	LVP (kPa)	LVP *dp/dt* (kPa/s)
Control (no PC)	288 ± 9	17.2 ± 0.7	10.6 ± 0.6	11.2 ± 0.5	439 ± 18
Control (4 × PC)	300 ± 7	16.6 ± 0.8	11.5 ± 0.8	12.1 ± 0.5	470 ± 29
4 × PC + 100 n*M* calph	279 ± 9	17.6 ± 0.6	18.2 ± 1[b]	13.8 ± 0.6[b]	579 ± 22[b]
4 × PC + 200 n*M* calph	294 ± 6	18.1 ± 0.9	21.4 ± 0.8[b]	16.1 ± 0.6[b]	621 ± 24[b]
4 × PC + 400 n*M* calph	290 ± 8	16.8 ± 0.5	22.5 ± 1.1[b]	17.0 ± 0.5[b]	660 ± 30[b]
4 × PC + 800 n*M* calph	280 ± 7	18.0 ± 0.7	5.2 ± 0.4[b]	7.0 ± 0.3[b]	324 ± 16[b]

n = 12 in each group; values reported as mean ± SEM.

PC, preconditioning; calph, calphostin C; HR, heart rate; CF, coronary flow; AF, aortic flow; LVP, left ventricular pressure; LVP *dp/dt*, first derivative of LVP.

[a]After 30 min of ischemia followed by 30 min of reperfusion.

[b]Comparisons were made to the control four-times preconditioned (4 × PC) group ($p < 0.05$).

TABLE 4. Effects of calphostin C on postischemic cardiac functiona in preconditioned hearts allowed to fibrillate for 15 S at beginning of reperfusion