

Infarct Size-limiting Effect of Calcium Preconditioning in Rabbit Hearts

Recent studies demonstrated that brief period of Ca^{2+} depletion and repletion (Ca^{2+} preconditioning, CPC) has strong protective effects against ischemia in a rat heart. CPC and classic preconditioning (IPC) were compared in relation with infarct size and protein kinase C (PKC) isozymes. Isolated Langendorff-perfused rabbit hearts were subjected to 45-min ischemia (Isc) followed by 120-min reperfusion (R) with or without IPC, induced by 5-min Isc and 10-min R. In the CPC hearts, 5-min Ca^{2+} depletion and 10-min repletion (CPC) were given before 45-min Isc, with or without concurrent PKC inhibition (calphostin C, 200 nmol/L). IPC enhanced recovery of LV function, while CPC did not. Infarct size was significantly reduced by both CPC and IPC ($p < 0.05$ vs. ischemic control). Membrane PKC was significantly increased from 2.53 ± 0.07 (baseline, nmol/g tissue) to 3.11 ± 0.07 , 3.34 ± 0.11 , 3.15 ± 0.09 , and 3.06 ± 0.08 by IPC, IPC and 45-min Isc, CPC and 45-min Isc, respectively ($p < 0.01$). Immunoblots of membrane PKC ϵ were increased by IPC, IPC and 45-min Isc, and CPC. These effects were abolished by PKC inhibition. Thus, activation of PKC ϵ may have trigger role in the mechanism of cardioprotective effect by CPC.

Key Words : Ischemic Preconditioning Myocardial; Myocardial Infarction; Reperfusion Injury; Protein Kinase C; Rabbits; Heart

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INTRODUCTION

A brief period of ischemia followed by reperfusion (ischemic preconditioning, IPC) can attenuate lethal injury to the heart as a result of prolonged period of ischemia and/or reperfusion (1-3). IPC has been shown to limit infarct size in ischemic myocardium to a remarkable degree in a variety of laboratory animals (4-9). Recent evidences suggest that this powerful endogenous cellular adaptation is conserved in a human heart (10). Activation of adenosine A_1 (11), α_1 adrenergic (12), bradykinin B_2 receptors (13), opening of K_{ATP} channels (14), preservation of myocardial high-energy phosphate (15), and attenuation of ischemia-induced acidosis (16) have been proposed as responsible mechanisms of IPC, but the exact mechanisms still remain inconclusive.

Ashraf et al. (17) have demonstrated that repetitive Ca^{2+} depletion and repletion for short duration (Ca^{2+} preconditioning, CPC) can protect the heart against ischemia/reperfusion injury via the activation of protein kinase C (PKC) in a rat heart. It has been well documented that the activation of PKC plays an important role in IPC-induced cardioprotection against the development of myocardial infarction and post-ischemic contractile dysfunction (3, 18, 19). However, the relationship between CPC and PKC and/or PKC isozymes in a rabbit heart remains unclear. In this respect, this study was performed to determine whether CPC is cardioprotective as IPC, and to assess whether PKCs are involved in Ca^{2+} -mediated preconditioning in rabbit hearts, if CPC is cardioprotective.

MATERIALS AND METHODS

Preparation of the heart

This study followed guidelines for the use of laboratory animals from the American Physiological Society. Male New Zealand White rabbits weighing 1.5 to 2.0 kg were used. They were fed ad libitum with balanced diet and water, and housed in an acclimated room throughout the experiments. The animal was stunned by a blow to the neck 30 min after intraperitoneal heparinization (300 IU/kg). The heart was rapidly isolated, transferred to a Langendorff perfusion apparatus (size 5, Hugo Sachs Elektronik, March-Hugstetten, Germany), and perfused with a constant flow rate (30 mL/min) of oxygenated Tyrode solution (containing in mmol/L: NaCl 140, KCl 4.4, CaCl_2 1.0, MgCl_2 1.0, HEPES buffer 3.0, and glucose 10.0) at 37 °C. The pH of the perfusate was equilibrated a physiological range (7.3-7.4). A latex balloon connected to a pressure transducer was inserted into the left ventricular cavity through the mitral opening. Eight to 10 mmHg of the initial left ventricular end-diastolic pressure (LVEDP) was loaded, and the heart was constantly paced at 150 beats/min with an electrical stimulator (Harvard Apparatus, Edenbridge, U.K.). Hemodynamic parameters including left ventricular developed pressure (LVDP) and differentiation of the left ventricular pressure were monitored throughout the experiment as previously described (5).

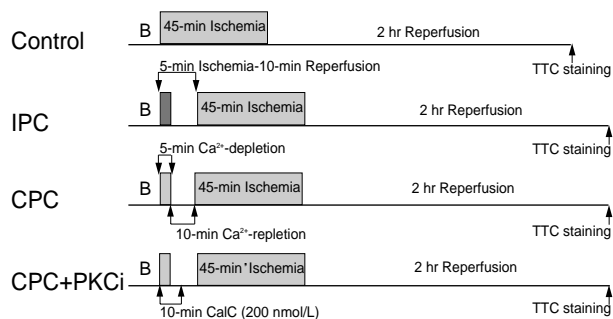


Fig. 1. Schematic illustration of the experimental protocol. Hearts were subjected to 5-min global ischemia and 10-min reperfusion in the ischemic preconditioned (IPC) group and to 5-min Ca²⁺-depletion and 10-min reperfusion in the Ca²⁺-preconditioned (CPC) group, before 45-min ischemia. In the CPC+PKCi (PKC inhibitor) group, calphostin C (CalC) was given for 10 min and washed out for 5 min during CPC. B, baseline. TTC: triphenyltetrazolium.

Experimental protocol

Experimental protocols are shown in Fig. 1. After commencing retrograde aortic perfusion, hearts were allowed to stabilize for 50 min, and baseline hemodynamics were measured. Coronary flow was measured by timed collection of coronary venous effluent as previously described (5). The hearts were randomly assigned to the following experimental groups: 1) in the non-preconditioned ischemic control ($n=7$), hearts were subjected to 45-min global ischemia followed by 120-min reperfusion; 2) in the ischemic preconditioning (IPC) group ($n=8$), hearts were preconditioned with 5-min global ischemia followed by 10-min reperfusion, and then subjected to 45-min global ischemia and 120-min reperfusion; 3) in the Ca²⁺ preconditioning (CPC) group ($n=10$), hearts were preconditioned with 5-min Ca²⁺-free perfusion followed by 10-min perfusion with Tyrode solution (containing 1.0 mmol/L CaCl₂), and then subjected to 45-min global ischemia and 120-min reperfusion; 4) in the CPC and PKC inhibitor-treated (CPC+PKCi) group ($n=8$), the experimental method was as similar as in the CPC group, except calphostin C (200 nmol/L) was given for 10 min during CPC regimen. We did not determine the effect of calphostin C in the non-preconditioned heart in which 45-min ischemia was given as in this study, since 200 nmol/L of calphostin C did not affect post-ischemic functional and biochemical data in those hearts of our previous study (20).

To determine the changes of PKC activity, supplement experiments ($n=5$ each) were added: experiments stopped just after baseline stabilization (base), 45-min global ischemia (Isc), IPC regimen (IPC), IPC regimen and 45-min global ischemia (IPC+Isc), CPC regimen (CPC), CPC regimen and 45-min global ischemia (CPC+Isc), CPC regimen and PKCi treatment (CPC+PKCi) and CPC regimen, PKCi treatment and 45-min global ischemia (CPC+PKCi+Isc). All heart specimens were frozen in liquid nitrogen and stored at -80 °C before biochemical and molecular assays.

Any heart without established baseline for prolonged perfusion for more than 50 min with poor contractility and persistent arrhythmia (ventricular tachycardia or fibrillation persisting for more than 20 min) on initial perfusion or reperfusion were excluded from the further study.

Determination of infarct size and lactate dehydrogenase (LDH) leakage

Infarct area was determined by the method previously described (5). In brief, after the experiment, the heart was perfused with 1% triphenyltetrazolium chloride (in phosphate buffer) for 20 min at room temperature and immersed overnight in 10% neutral formalin. Heart slices were photographed and the infarct area was determined by computerized morphometry.

LDH leakage was determined by the measurement of its activity in the timely collected coronary effluent with a diagnostic kit (Sigma).

PKC assay and Western blot analysis of PKC isozymes

The left ventricle including interventricular septum was homogenized in 3 vols of buffer solution (containing in mmol/L: Tris-HCl 20.0, sucrose 250.0, iodoacetic acid 1.0, phenylmethylsulfonyl fluoride 1.0, EDTA 1.0, EGTA 1.0, and β -mercaptoethanol 10.0; pH 7.4 at 4 °C) using Ultra-Turrax homogenizer (10,000 rpm, 3 times for 30 sec each). Crude homogenate was centrifuged (360 g at 4 °C for 10 min). The supernatant was centrifuged at 100,000 g at 4 °C for 1 hr and the resulting supernatant was used for the measurement of cytosol PKC activity. Pellet was resuspended in 2 vols of the same buffer containing 0.3 vol% of Triton X-100 and gently stirred at 4 °C for 1 hr, centrifuged at 100,000 g for 1 hr, and the resulting supernatant was used for measurement of membrane PKC activity. PKC activity was determined by using a commercial kit (PKC Assay System, RPN 77A, Amersham) as previously described (22, 23), and the protein concentration in each fraction was determined by using DC Protein Assay (Bio-Rad).

Cytosol and membrane PKC samples (each sample containing 50 μ g of protein) were processed by SDS-PAGE (7.5% separating gel) at 25 mA constant current for 5 hrs. Bio-Rad Kaleidoscope prestained standards were used to determine molecular masses. Proteins were transferred from gels to PVDF membrane (Hoefer Scientific). The PVDF membrane was incubated in blocking buffer (pH 7.6) containing 5% skim milk, 25 mmol Tris-HCl, 100 mmol NaCl and 0.1% Tween 20 for 1 hr at room temperature, and then incubated for 1 hr at room temperature with primary monoclonal antibodies (α , β , δ , ϵ , and ζ , Transduction Laboratory) at indicated concentrations (1:1,000). After intensive washing with 5% skim milk-free blocking buffer, blots were incubated for 1 hr in buffer con-

taining goat anti-mouse peroxidase conjugate (1:1,000, Bio-Rad). Finally, immunoreactive bands were visualized by enhanced chemiluminescence method (Amersham).

Statistics

Data were presented as mean \pm SEM; they were analyzed by paired t-test within the same experimental group and by one-way ANOVA for repeated measures within different experimental groups. When significant differences were observed, the mean values were evaluated with Tukey's post-hoc test. A *p* value less than 0.05 was considered significant.

RESULTS

Functional parameters

The baseline values of LVDP, +dP/dt, LVEDP and coronary flow were not different between experimental groups. Ischemia caused a rapid drop in LVDP, +dP/dt and coronary flow; these parameters stayed at zero point during ischemia for 45-min. On reperfusion, IPC enhanced LVDP and +dP/dt recovery, but CPC did not. LVDP and dP/dt recovery on reperfusion were greater in the IPC group (>90% of pre-

ischemic value) than in the ischemic control group (<70% of preischemic value), however, these parameters in the CPC and CPC+PKCi groups were less than 50% of preischemic values (Fig. 2). Coronary flow recovery in the IPC group was greater than in the ischemic control group. It reached near to preischemic value and maintained approximately at >90% of preischemic value in the IPC group, but difference between the IPC and ischemic control groups (80-90% of preischemic value) was not evident. In contrast, CPC and CPC+PKCi depressed the coronary flow recovery. Coronary flow recovery was smaller in the CPC group (<60% of preischemic value) than that in the ischemic control group (Fig. 2), but the difference was not evident between the CPC and CPC+PKCi groups. In all experimental groups, LVEDP was increased by 35 min after ischemia and further increased immediately after the onset of reperfusion. The increased levels of LVEDP lasted throughout reperfusion both in the ischemic control and IPC group, however, the increase was less prominent in the IPC group (*p*<0.05). This result means that IPC attenuated myocardial contracture during reperfusion. In the CPC group, LVEDP reached a plateau (>100 mmHg, *p*<0.01 vs ischemic control group) in the earlier period of reperfusion and progressively decreased afterwards (approximately 50 mmHg at the end of reperfusion).

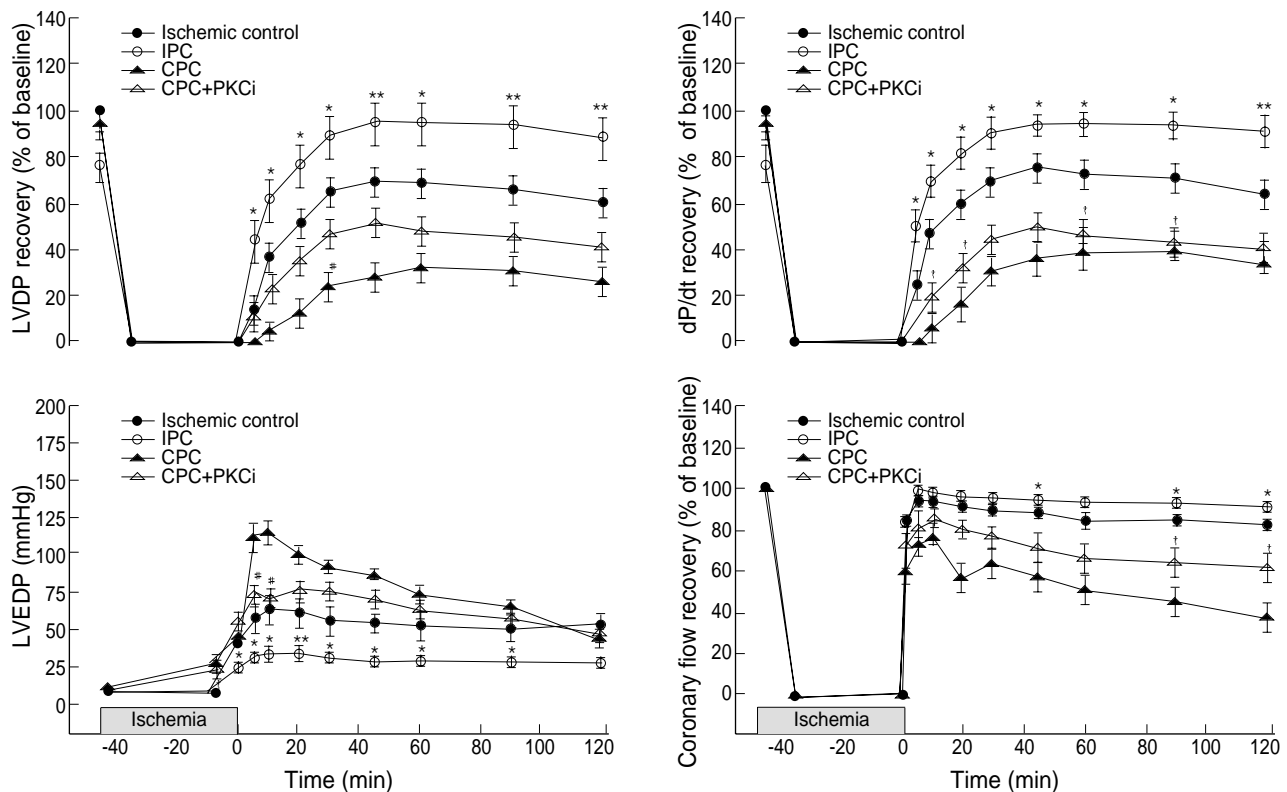


Fig. 2. Changes of the recovery rate of left ventricular developed pressure (LVDP), contractility (+dP/dt), left ventricular end-diastolic pressure (LVEDP), and coronary flow during ischemia and reperfusion. **p*<0.05, ***p*<0.01, ischemic control vs. IPC; †*p*<0.05, CPC+PKCi vs. CPC; ‡*p*<0.05, ischemic control vs. CPC+PKCi.

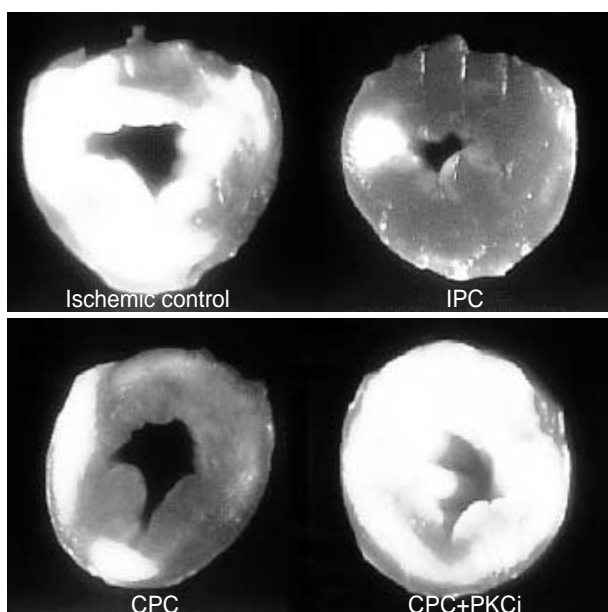


Fig. 3. Heart slices stained with triphenyltetrazolium chloride (TTC). Infarct (white area) was limited by IPC and CPC but infarct size-limiting effect is abolished by PKC inhibition with calphostin C (CPC+PKCi).

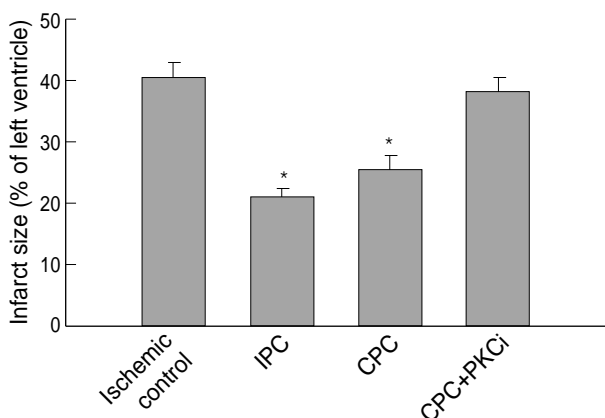


Fig. 4. Infarct size as percentage of the left ventricle. Infarct size was reduced by IPC and CPC but reduction of infarct size is abolished by PKC inhibition with calphostin C (CPC+PKCi). * $p < 0.05$, vs. ischemic control.

Infarct size and LDH leakage

Infarct size (Fig. 3, 4) was expressed as a percentage of the left ventricle including the interventricular septum. We did not assume risk area since the entire myocardium might be at risk in global ischemia. Compared with the ischemic control ($45.2 \pm 2.4\%$), the infarct size was reduced by IPC ($21.0 \pm 1.3\%$, $p < 0.05$) and CPC ($25.5 \pm 2.2\%$, $p < 0.05$); however, infarct size was not reduced or limited by PKCi treatment (CPC+PKCi, $37.9 \pm 2.5\%$).

LDH leakage at the baseline in the coronary effluent of the

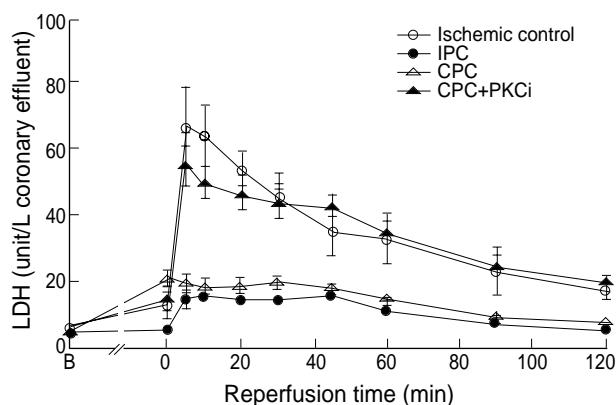


Fig. 5. Lactate dehydrogenase (LDH) leakage in the coronary effluent. During early period on reperfusion, LDH leakage is significantly increased in the ischemic control and CPC+PKCi. B, baseline. * $p < 0.05$, vs. CPC+PKCi.

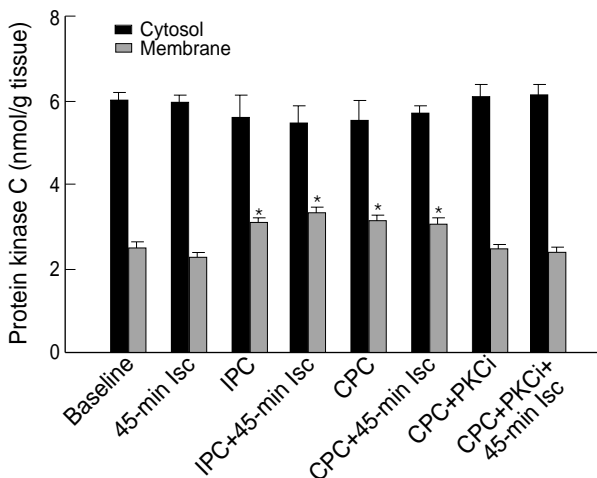


Fig. 6. Protein kinase C (PKC) activity. PKC activities of the cytosol fractions are not significantly different between the experimental groups; however, those of the membrane fractions increase by ischemic preconditioning (IPC), IPC and 45-min ischemia (IPC+45-min Isc), Ca^{2+} preconditioning (CPC), and CPC and 45-min ischemia (CPC+45-min Isc). * $p < 0.01$, vs. baseline.

ischemic control, IPC, CPC, and CPC+PKCi groups were 6.00 ± 0.62 , 5.29 ± 0.50 , 5.50 ± 0.54 , 5.81 ± 0.60 unit/mL, respectively. It was significantly increased in the ischemic control and CPC+PKCi groups on reperfusion ($p < 0.05$ vs CPC group, 5–20 min), however, no significant difference was found between the IPC and CPC groups (Fig. 5).

PKC activity and Western blot analysis of PKC isozymes

Total PKC activity in the baseline, after 45-min ischemia, IPC, IPC and 45-min ischemia, CPC, CPC and 45-min ischemia, CPC+PKCi, and CPC+PKCi and 45-min ischemia were 8.57 ± 0.06 , 8.31 ± 0.04 , 8.75 ± 0.12 , 8.81 ± 0.10 ,

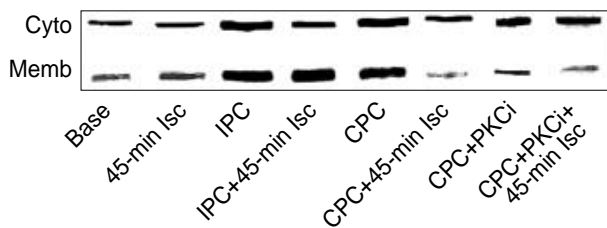


Fig. 7. Western blot analyses of PKC ϵ . Membrane (Memb) PKC ϵ is increased by IPC, IPC and 45-min ischemia (IPC+45-min Isc), and by CPC, while cytosol fraction between groups is not significantly different. Base, baseline; PKCi, PKC inhibition with calphostin C.

8.68 \pm 0.14, 8.76 \pm 0.06, 8.61 \pm 0.05, 8.52 \pm 0.12 nmol/g tissue, respectively. It was slightly increased after the IPC and CPC regimen, however, there was no significant difference. As shown in the Fig. 6, cytosol PKC activity was not different between groups and membrane PKC activity was not changed after 45-min ischemia, CPC +PKCi, and CPC +PKCi and 45-min ischemia; however, membrane PKC activity was significantly increased after IPC, IPC and 45-min ischemia, CPC, and CPC and 45-min ischemia ($p < 0.01$), from 2.53 \pm 0.07 nmol/g tissue (baseline) to 3.11 \pm 0.07 (IPC), 3.34 \pm 0.11 (IPC+45-min Isc), 3.15 \pm 0.09 (CPC), and 3.06 \pm 0.08 nmol/g tissue (CPC+45-min Isc). These results indicate that PKC was activated and translocated from the cytosol to the cell membrane by IPC or CPC.

Five PKC isozymes (α , β , δ , ϵ , and ζ) were analyzed by Western blot with corresponding monoclonal antibodies against each isozymes. Distribution and intensity of PKC ϵ isozymes except PKC ϵ were not different between experimental groups (data not shown). In comparison with the baseline, the membrane fraction of PKC ϵ increased after IPC, IPC and 45-min ischemia, and CPC (Fig. 7).

DISCUSSION

As already established, the myocardial cell uses Ca²⁺ as the essential final step in excitation-contraction coupling, the process by which depolarization of the cell surface membrane initiates the interactions between the contractile proteins that lead to tension development and shortening in the wall of the heart. Ca²⁺ also mediates stimulus-secretion coupling in a variety of non-motile cells. As indicated, Ca²⁺ ions, by carrying signals generated at the cell surface to a variety of intracellular organelles and proteins, can be viewed as the most important of the intracellular messengers (24). It is generally believed that a high Ca²⁺ influx during ischemia/reperfusion (Ca²⁺ overload) causes pathological changes in the ischemia/reperfusion injury; however, in a rat heart, Ashraf et al. (17) have demonstrated that repetitive Ca²⁺ depletion and repletion for a short duration (Ca²⁺ preconditioning, CPC) had induced a significant functional recovery and remarkable preservation of cell

structure against ischemia/reperfusion injury. Our results are partly consistent with Ashraf et al. (17). However, functional recovery by CPC was not seen, although infarct size was significantly reduced by IPC and CPC; LVDP and dP/dt recovered only 25-30% of the baseline values, compared with those in the IPC group (80-90% of baseline values) or even in the ischemic control group (60-70% of baseline values). The reason of depressed functional recovery by CPC in this study is unclear. Differences in species and experimental methods could be causing factors, but they are not satisfactory explanations. Since the early increase of LVEDP on reperfusion after 45-min ischemia in the CPC group was evident, it was doubtful whether the CPC regimen in this study would cause Ca²⁺ paradox injury, in which necrosis of the cell is a common feature, or not. However, functional parameters after Ca²⁺ depletion and repletion in the CPC group were not significantly different from the baseline values, and LDH leakage was not different between the IPC and CPC groups after 45-min ischemia. Moreover, infarct size was reduced or limited by CPC as seen in the IPC group. From these results, it could be concluded that 5-min Ca²⁺ depletion and 10-min Ca²⁺ repletion did not cause Ca²⁺ paradox injury. Reduced response or sensitivity of contractile proteins to normal Ca²⁺ concentration or reduced energy production on reperfusion due to myocardial stunning by CPC could be another possible factor, although intracellular Ca²⁺ and energy metabolism were not considered. They can cause a retarded functional recovery with diastolic abnormalities.

The physiological function of protein kinases is not yet fully understood, but PKC is activated upon external stimulation of the myocyte by various ligands including hormones, neurotransmitters, and growth factors. Many lines of evidence indicate that PKC is involved in IPC of the heart. In this study, membrane PKC activity was increased by IPC and CPC, while PKC inhibition did not increase the activity. These results indicate that PKC was activated by IPC and CPC, and translocated from the cytosol to the cell membrane. It has been suggested that the elevation of [Ca²⁺]_i during IPC, in part, resulted from Ca²⁺ entry via voltage-dependent Ca²⁺ channel (8, 25) triggers activation of PKC. However, cytosol PKC was not significantly decreased in both IPC and CPC in this study as in a rat heart (19), in contrast to other studies (8, 26). The reason for this is unclear, however, there is a possibility that a small change in the cytosol PKC by CPC or IPC did not affect the total amount of cytosol PKC, since the amount of cytosol PKC is much greater than that of membrane PKC.

In addition, in this study, immunoblots of the membrane PKC ϵ were evidently increased by IPC, IPC and 45-min ischemia and by CPC, while concurrent PKC inhibition abolished the increase of membrane PKC ϵ . These results strongly suggest that the activation of membrane PKC ϵ is closely related with the infarct size-limiting effects of IPC and CPC. There are some evidences that specific PKC isozymes were responsible for the mechanism of IPC or CPC. Ping et al. (27) described that IPC caused selective translocation of PKC ϵ and

PKC γ without demonstrable changes in total myocardial PKC activity in the conscious rabbit heart, and our previous study (20) demonstrated that PKC ϵ has a trigger role for cardioprotective effect of IPC in the isolated rabbit heart. In contrast, Miyawaki et al. (19) demonstrated that immunolocalization of PKC α and PKC δ increased in the cell membrane of rat hearts. However, there is a controversy over the different PKC-coupled receptor systems, which might be involved in IPC. PKC activator did not reduce infarct size in canine hearts (22), and in porcine hearts, even the infarct size reduced by PKC inhibitor (28). These inconsistent results might have been derived from the following possibilities: 1) activation of PKC was not sufficient to phosphorylate certain key proteins; 2) expression of specific PKC isozymes was not significant to participate in the protection; or 3) mechanical factors of IPC or CPC protocol itself caused an aggravation of myocardial stunning. These experimental data provide that the activation of PKC may not be an universally applicable mechanism for the cardioprotective effect of IPC or CPC in common laboratory animals, however, from this study, it is conceivable that a single dose of CPC could not enhance functional recovery but could protect the heart from infarction. Activation of membrane PKC ϵ may be responsible for trigger role in the cardioprotection by both IPC and CPC.

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