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Implication of Protein Kinase C-α, δ, and ε Isoforms in Ischemic Preconditioning in Perfused Rat Hearts[1]

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Ischemic preconditioning is a phenomenon in which one or several cycle(s) of brief ischemia-reperfusion protects the myocardium against the cell injury caused by subsequent prolonged ischemia. Protein kinase C (PKC) inhibitors blunt the cardioprotection arising from ischemic preconditioning. To investigate which PKC isoform is involved in ischemic preconditioning, we identified the PKC isoform that translocates to the membrane fraction by means of immunoblotting with specific antibodies. PKC-α, δ, ε isoforms all increased in the membrane fraction after three cycles of 3 min ischemia and 5 min reperfusion (ischemic preconditioning) in the perfused rat heart. The ischemic preconditioning significantly improved the recovery of left ventricular developed pressure (LVDP) during reperfusion following 20 min of ischemia. A PKC specific inhibitor, chelerythrine (1.0 μM) blocked the effect of ischemic preconditioning on LVDP recovery and the translocation of PKC-α, δ, ε isoforms. These data suggest that one or more of these three isoforms of PKC is involved in ischemic preconditioning by phosphorylating membrane proteins.

Key words: ischemia-reperfusion, ischemic preconditioning, myocardium, PKC inhibitor, protein kinase C isoform.

Myocardial ischemic preconditioning is the phenomenon whereby a cycle(s) of brief ischemia followed by reperfusion protects the myocardium against subsequent prolonged ischemia (*1-8*). Several lines of evidence support the idea that protein kinase C (PKC) is involved in ischemic preconditioning; PKC activators such as phorbol esters or synthetic diacylglycerol analogues mimic the cardioprotection afforded by ischemic preconditioning, while various PKC inhibitors attenuate the cardioprotective effect of ischemic preconditioning on infarct size, contractile dysfunction, and arrhythmias during reperfusion (*2-8*).

Eleven PKC isoforms have been discovered and categorized into Ca^{2+}-dependent (conventional, c) PKC, Ca^{2+}-independent (novel, n) PKC, and diacylglycerol-independent (atypical, a) PKC isoforms (*9*). PKC translocates to the membrane fraction upon its activation. 5'-Nucleotidase (*8, 10*), ATP-sensitive K^+ channel (*11*), Na^+/H^+-exchanger (*12*), Ca^{2+}-channels (*13*), and other membrane proteins have been postulated to mediate the preconditioning effect through PKC activation. These findings suggest that PKC isoform(s) that translocate to the membrane fraction upon preconditioning play a pivotal role in the cardioprotection arising from ischemic preconditioning. We have recently detected the translocation of cPKC-α, nPKCs-δ, ε, and aPKC-ζ during global ischemia for 5-40 min in the perfused rat heart by immunoblotting and immunohistochemistry using isoform-specific antibodies (*14, 15*).

In the present study, we used immunoblotting to determine which PKC isoforms translocate to the membrane fraction in order to identify the isoforms involved in ischemic preconditioning.

MATERIALS AND METHODS

Materials—Antibodies to PKCs-δ,ε,ζ were obtained from Gibco. Anti-PKC-α antibody was raised in rabbits injected with a synthetic peptide (amino acids 666-672) and characterized as previously reported (*14*). In some experiments, anti-PKC-α monoclonal antibody (to amino acids 270-427) obtained from Transduction Laboratories was used with essentially the same results. Chelerythrine was purchased from LC Laboratories, and ECL Western blotting detection kit from Amersham.

Perfusion Procedure—Male Wistar rats weighing 300-400 g were anesthetized with sodium pentobarbital and the hearts were quickly excised. As previously reported (*14-16*), the hearts were perfused with Krebs-Henseleit (KH) solution gassed with 95% O_2-5% CO_2 at a constant pressure of 80 cmH_2O for about 10 min and then subjected to three cycles of 3 min of ischemia followed by 5 min of reperfusion (ischemic preconditioning) at 37°C. In the inhibitor group ($n=7$), a PKC inhibitor, chelerythrine [1.0 μM in 0.1% dimethyl sulfoxide (DMSO)], was perfused for 2 min prior to the initial ischemia for preconditioning. In the preconditioning ($n=13$) and control ($n=14$) groups, 0.1%

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DMSO was perfused for the same period. After the perfusion for preconditioning or for the control, the ventricles were quickly separated, frozen in liquid nitrogen, and stored at −70°C for later subfractionation study.

In the physiology study, the hearts of the four groups (control $n=7$, preconditioning $n=6$, preconditioning plus chelerythrine $n=5$, double cycles of ischemic preconditioning $n=6$) were subjected to global ischemia for 20 min followed by 30 min of reperfusion after the perfusion for preconditioning or for the controls. The heart rate, left ventricular developed pressure (LVDP), and its first derivative (dP/dt) were continuously monitored through a latex balloon connected to a pressure transducer attached to a Macintosh computer loaded with an analysis system (MacLab, AD Instruments).

Subcellular Fractionation—As previously reported (*14, 15*), the frozen hearts were minced and homogenized in 5 volumes of buffer containing 320 mM sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 5 mM NaN_3, 10 mM β-mercaptoethanol, 20 μM leupeptin, 0.15 μM pepstatin A, 0.2 mM phenylmethanesulfonyl fluoride, and 50 mM NaF (STE buffer) with a Polytron homogenizer at the maximum speed (PT1200, Kinematica AG), three times for 30 s each. The homogenates were mixed with an equal volume of STE buffer and centrifuged at $1,000\times g$ for 10 min, and then the supernatant was centrifuged at $100,000\times g$ for 60 min. The $1,000\times g$ pellet and $100,000\times g$ pellet were designated P1 and P2, respectively, while the $100,000\times g$ supernatant was referred to as S. Protein concentrations were determined by the method of Lowry *et al.* (*17*) using bovine serum albumin as a standard.

Immunoblotting and Quantification of PKC—The fractions were subjected to SDS-polyacrylamide gel electrophoresis in 7.5% polyacrylamide gels by the method of Laemmli (*18*) and then immunoblotted according to Towbin *et al.* (*19*) with some modifications (*14*). The blots were blocked with 5% skim milk in buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 0.05% Tween-20 for at least 1 h, then incubated with one of the 2,000- to 3,000-fold diluted antibodies against PKC isoforms for 1 h at room temperature, and the PKC isoforms were visualized by the use of an ECL Western blotting detection kit. The amounts of PKC isoforms on the immunoblots were measured with an image analyzer (Densitograph AE-6900, Atto) as described previously (*14*). The amount of protein applied to the gel was varied for each isoform and fraction so as to maintain linearity of the intensity × area of the band on the immunoblot.

RESULTS

The P1 fraction contained most of the histone H1 and troponin-T, nuclear and myofibril markers, while a membrane marker ATP-sensitive K^+-channel was enriched in the P2 fraction (data not shown). Thus, the P1 and P2 fractions represent the nucleus-myofibril fraction and the membrane fraction, respectively, as confirmed previously by electron microscopy (*14*). Protein recovery in the three

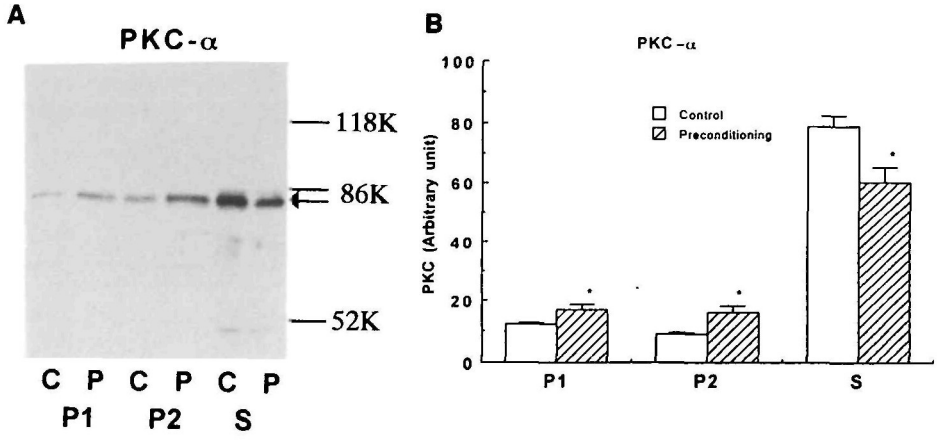

Fig. 1. **Ischemic preconditioning induces the translocation of PKC-α in myocardial subfractions as shown by representative immunoblots (panel A) and quantitation of the blots (means±SE) (panel B).** The amounts of P1, P2, and S fractions applied were 20, 20, and 10 μg protein, respectively. PKC-α increased in the P1 and P2 fractions after preconditioning, while it decreased in the S fraction ($^*p<0.05$ *vs.* control). The molecular masses of the protein standards are indicated to the right.

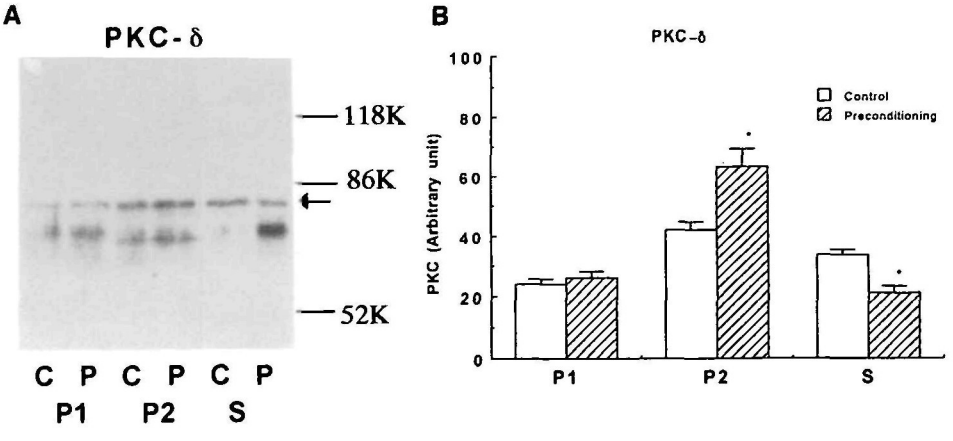

Fig. 2. **Ischemic preconditioning induces the translocation of PKC-δ in myocardial subfractions as shown by representative immunoblots (panel A) and the quantitation of the blots (means±SE) (panel B).** The amounts of P1, P2, and S fractions applied were 30, 15, and 20 μg protein, respectively. PKC-δ increased in the P2 fraction after preconditioning, while it decreased in the S fraction ($^*p<0.05$ *vs.* control).

fractions was not different between control and preconditioned hearts (data not shown).

For the immunoblots shown in Figs. 1-3 (panels A), equal amounts of protein for each fraction (indicated in the figure legends) were applied and the amount of a PKC isoform in a fraction was expressed in arbitrary units. The sum of each PKC isoform in the three fractions showed no difference between the control and the preconditioning groups. We previously confirmed the specificity of the antibodies used (*14*). As shown in Fig. 1, PKC-α (80K) translocated significantly from the S to the P1 and P2 fractions after ischemic preconditioning consisting of three cycles of 3 min ischemia followed by 5 min reperfusion. Figures 2 and 3 show that after the preconditioning, both PKC-δ (78K) and

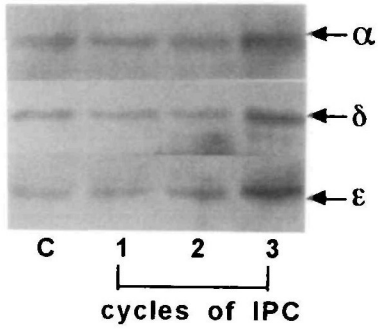

Fig. 4. **Comparison of one, two, and three cycles of brief ischemia (3 min) and reperfusion (5 min) on the translocation of PKC isoforms.** Only three cycles of brief ischemia-reperfusion induced the translocation of PKC-α, δ, and ε. See quantification in Table I.

TABLE I. **Comparison of one, two, and three cycles of brief ischemia (3 min) and reperfusion (5 min) on the translocation of PKC isoforms.** The amount of PKC isoforms, expressed as an arbitrary unit (mean value of control: 100), was determined from the immunoblots as demonstrated in Figs. 1-3 (II) and Fig. 4 (I) by image analyzer. $^*p<0.05$ *vs.* control.

	PKC isoform			
	α	δ	ε	n
(I)				
Control	100±5.8	100±13.1	100±1.5	3
One	93.2±4.5	102.1±15.5	90.3±6.4	3
Two	89.4±23.9	104.0±15.3	90.9±13.8	3
Three	162.8	151.2	141.1	1
(II)				
Control	100±4.8	100±6.5	100±4.7	14
Three	177.5±23.3*	174.0±14.7*	156.9±14.8*	13

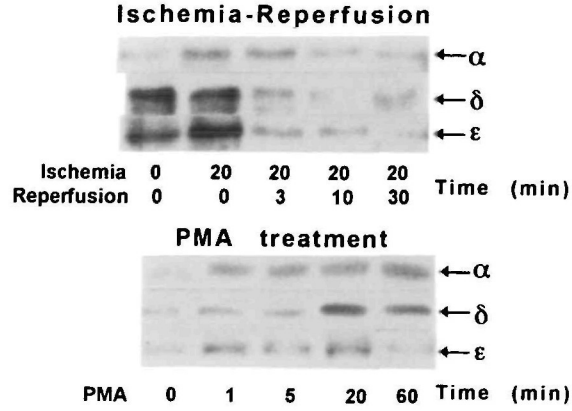

Fig. 5. **Membrane translocation of PKC isoforms after 20 min of ischemia and 3-30 min of reperfusion (upper panel) or after PMA (1 μM) treatment for 1-60 min (lower panel).** Ischemia induced the translocation of PKC-α and ε to the P2 fraction and dissociation of PKC-δ from the fraction. Reperfusion induced the dissociation of PKC-α, δ, and ε. PMA increased the P2 association of PKC-α, δ, and ε.

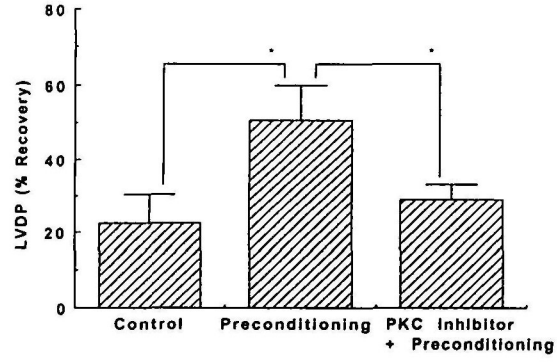

Fig. 6. **Effect of a PKC inhibitor on the recovery of left ventricular developed pressure (LVDP) after 30 min reperfusion following 20 min ischemia.** The beneficial effect of ischemic preconditioning was prevented by chelerythrine (1.0 μM) perfusion (2 min) before the preconditioning procedure ($^*p<0.05$).

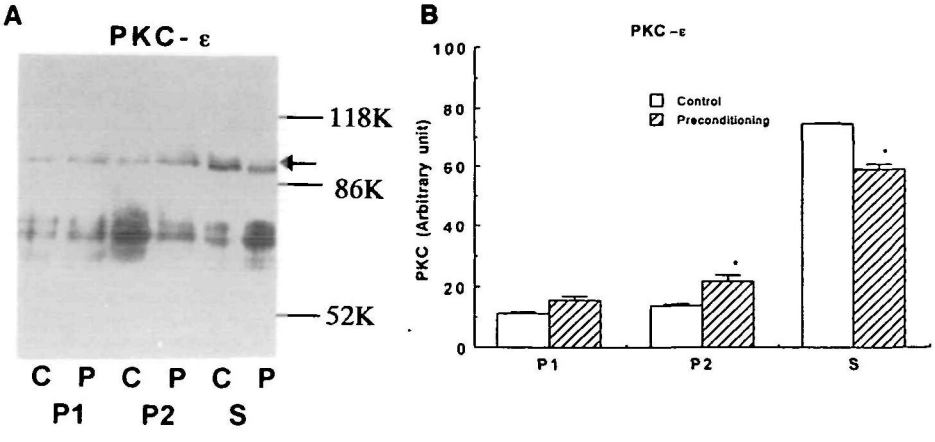

Fig. 3. **Ischemic preconditioning induces the translocation of PKC-ε in myocardial subfractions as shown by representative immunoblots (panel A) and the quantitation of the blots (means ± SE) (panel B).** The amounts of P1, P2, and S fractions applied were 20, 20, and 10 μg protein, respectively. PKC-ε increased in the P2 fractions after preconditioning, while it decreased in the S fraction ($^*p<0.05$ *vs.* control).

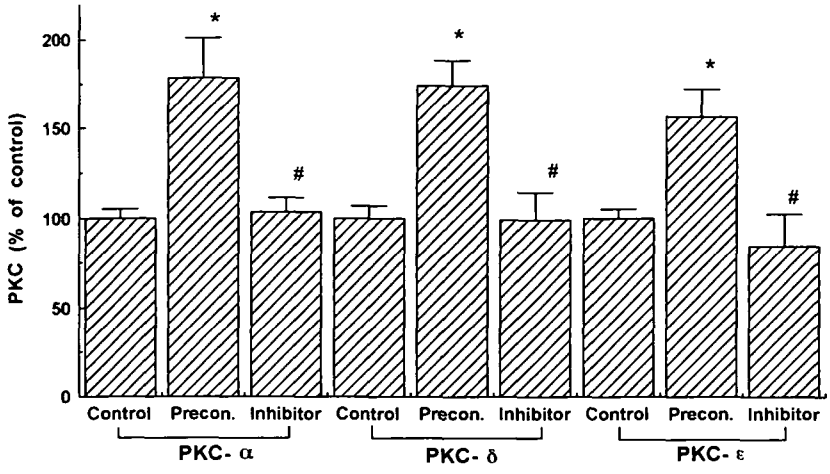

Fig. 7. **Chelerythrine inhibits the translocation of PKC isoforms after ischemic preconditioning.** The translocation was quantified by densitometric scans of the immunoblots ($^*p<0.05$ *vs.* control, $^\#p<0.05$ *vs.* preconditioning).

PKC-ε (97K) translocated from the S to the P2 fraction. About 40% of PKC-δ was associated with the P2 fraction in control heart. PKC-ζ, predominantly cytosolic, did not translocate after ischemic preconditioning (data not shown), while rat heart does not express other PKC isoforms, as we previously reported (*14, 15*). The single or double cycles of brief ischemia-reperfusion did not increase the association of PKC-α, δ, and ε isoforms with the membrane fraction, as shown in Fig. 4 and Table I.

PKC translocation after 20 min of ischemia followed by reperfusion or that after PMA treatment was examined, as shown in Fig. 5. Ischemia alone induced translocation of PKC-α and ε to the membrane fraction and dissociation of PKC-δ from membrane as we previously reported (*14*). Reperfusion for 3–30 min following 20 min of ischemia dissociated PKC-α, δ, and ε isoforms from the membrane fraction. On the other hand, phorbol-12,13-myristate acetate (PMA, 1 μM) induced membrane translocation of PKC-α, δ, and ε isoforms.

The influence of a PKC inhibitor on the effect of ischemic preconditioning afforded by three cycles of ischemia (3 min)-reperfusion (5 min) on the recovery of left ventricular developed pressure (LVDP) after 30 min of reperfusion following 20 min of ischemia is shown in Fig. 6. The improvement of the LVDP recovery after the reperfusion were observed only after three cycles of the brief ischemia-reperfusion (59.6±10.9% *vs.* control 27.0±9.0%) but not after two cycles (25.9±6.8%). A highly specific PKC inhibitor, chelerythrine (1.0 μM) that was perfused only before the preconditioning, blunted the effect of preconditioning on the contractile recovery during reperfusion. This finding, showing the involvement of PKC in ischemic preconditioning, led us to examine the effect of chelerythrine on the PKC translocation. Figure 7 shows that chelerythrine (1.0 μM) suppressed the membrane translocation of PKCs-α, -δ, and -ε.

There is considerable evidence for the involvement of intracellular Ca^{2+} overloading in myocardial injury due to ischemia or reperfusion (*12, 13, 16, 20*). We examined the development of diastolic tension during ischemia (this is supposed to indicate Ca^{2+} overloading). As shown in Fig. 8, ischemia for 20 min produced a remarkable increase in the development of diastolic tension, while ischemic preconditioning alleviated this increase. However, chelerythrine

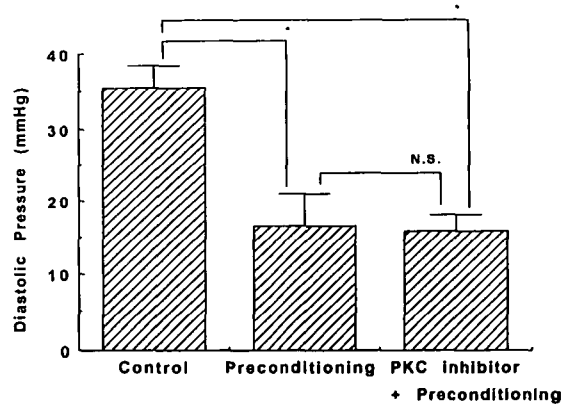

Fig. 8. **Ischemic preconditioning improves the diastolic tension development after 20 min ischemia ($^*p<0.05$) but chelerythrine (1 μM) does not block the effect (N.S.: not significant).**

did not attenuate the beneficial effect of preconditioning.

DISCUSSION

This study provides the first demonstration of the translocation of PKC-α,δ,ε isoforms to the membrane fraction during ischemic preconditioning, as determined by immunoblotting of the subcellular fractions (Figs. 1-3). A highly specific PKC inhibitor, chelerythrine, inhibits both the improvement of contractile dysfunction during reperfusion by ischemic preconditioning (Fig. 6) and the translocation of the PKC-α, δ, and ε isoforms (Fig. 7). In addition, three cycles, but not two cycles of brief ischemia-reperfusion translocated the PKC-α, δ, ε isoforms and improved the functional recovery during reperfusion (Fig. 4, Table I). These observations suggest that any of the three PKC isoforms mediates the preconditioning effect on the recovery of contractile dysfunction during reperfusion.

Recently, Mitchell *et al.* (*7*) reported that PKCs-δ and -ε translocate to the membrane and the nucleus, respectively, from the cytosol after ischemic preconditioning in perfused rat hearts, as detected by immunohistochemistry. In their study, PKC-δ was found mainly in the cytosol in non-preconditioned hearts, but we showed that about 40% of the PKC-δ resides in the membrane fraction (Fig. 2) (*14*). This

constitutive association of PKC-δ with the membrane is also documented in fibroblasts (*21*). Recently, it was reported that ceramide induces dissociation of membrane-bound PKC-δ in response to tumor necrosis factor-α and anti-Fas antibody, while PMA blocked the dissociation (*22*). In the present study, PKC-δ translocated to the membrane fraction after ischemic preconditioning (Fig. 2) and PMA treatment, but global ischemia induces its dissociation, and subsequent reperfusion greatly enhanced the dissociation (Fig. 5). Although it remains unclear whether membrane translocation of PKC-δ implies its activation, these observations appear to support the idea of Mitchell *et al.* that PKC-δ mediates the protective effect of ischemic preconditioning (*7*), because ischemia *per se* can induce translocation of PKC-α and ε, but not PKC-δ (Fig. 5). Alternatively, synergistic translocation of PKC-α, δ, and ε isoforms may be required for the protective effect of ischemic preconditioning.

Although there has been controversy concerning the presence of PKC-α in adult rat heart (*23, 24*), in a recent study we have shown that adult heart expresses PKC-α (*14*) by immunoblotting, immunohistochemistry and measurement of the activity. This study shows that PKC-α and ε isoforms translocated to the membrane fraction after ischemic preconditioning (Figs. 1-3), after PMA treatment and in globally ischemic heart, while postischemic reperfusion reversed the membrane translocation of PKC-α and ε (Fig. 5). In accordance with our observation, Miyawaki *et al.* recently reported that the preconditioning effect of three cycles of Ca^{2+} depletion and Ca^{2+} repletion induced the membrane translocation of PKC-α and PKC-δ in the perfused rat heart as demonstrated by immunofluorescence study (*25*).

It is noteworthy that the PKC inhibitor, given only before the ischemic preconditioning, blocked the cardioprotection afforded by the preconditioning (Fig. 6) and the translocation of PKC (Fig. 7), as we previously reported for preconditioning in the dog model (*8*). Although PKC-α and ε isoforms translocated to the membrane during global ischemia (Fig. 5), there is no evidence that the protective effect of PKC is activated during postischemic reperfusion. The apparent lack of effect of PKC activation during global ischemia can be explained on the basis of the fact that PKC activation before the onset of global ischemia is an essential requirement for the protection (*1-8*). Enhanced activity of 5′-nucleotidase, ATP-sensitive K^+ channel or other proteins *via* PKC activation that precedes sustained ischemia may be required for the protection during ischemia-reperfusion (*8, 10, 11*). In further support of the importance of the timing of PKC activation on its outcome, we previously showed that PMA administration just before thrombin treatment inhibits platelet activation through suppression of intracellular Ca^{2+} mobilization (*26, 27*), although Kaibuchi *et al.* found that PKC activation and Ca^{2+} mobilization synergistically activates platelets (*28*). On the other hand, sustained ischemia induces irreversible changes in the myocardium through activation of Ca^{2+}-dependent protease, calpain (*16*), or other processes that cannot be overcome by PKC activation. Thus, the PKC translocation during global ischemia may not be able to protect against the irreversible change after sustained ischemia. At present, we cannot conclude which of the three PKC isoforms plays a key role in the ischemic preconditioning. To determine the responsible PKC isoform(s), the use of isoform-specific PKC inhibitors or a molecular biological approach in cultured cells is required.

In a canine model, one of us demonstrated that ischemic preconditioning induces the translocation of Ca^{2+}-dependent PKC activity to the membrane, where PKC activates 5′-nucleotidase that produces adenosine through the α_1 adrenergic receptor (*8, 10*). It is known that there are species variations in the involvement of PKC and in mechanisms of ischemic preconditioning (*29, 30*). In fact, preliminary experiments in our laboratory showed no significant activation of 5′-nucleotidase after ischemic preconditioning in the perfused rat heart (data not shown). There is also no substantial evidence for the involvement of PKC in the activation of the K^+-channel during ischemic preconditioning. On the other hand, there are two lines of evidence for the implication of Ca^{2+} overloading in preconditioning in perfused rat heart. Steenbergen *et al.* reported that preconditioning attenuates the increase in intracellular Ca^{2+}, Na^+, and H^+ during ischemia, most likely due to a reduced stimulation of Na^+/H^+ and Na^+/Ca^{2+}-exchangers, thereby improving functional recovery during reperfusion (*12*). Zucchi *et al.* reported that preconditioning reduces the ryanodine binding of the sarcoplasmic reticulum Ca^{2+} release channel, thereby attenuating Ca^{2+} release and subsequent Ca^{2+} overloading during ischemia and reperfusion (*13*). The present study confirmed that preconditioning alleviates the increase in diastolic tension development during ischemia that is thought to represent Ca^{2+} overloading (Fig. 8). However, chelerythrine does not block the preconditioning effect on the ischemia-induced increase in diastolic pressure (Fig. 8), in contrast to its effect on the recovery of LVDP during postischemic reperfusion (Fig. 6). Thus, PKC may not mediate the effect of ischemic preconditioning through the alleviation of Ca^{2+} overloading during ischemia. It remains to be determined which membrane protein among the candidate proteins mediates the preconditioning effect by PKC-α, δ, and ε isoforms in rat heart.

Recently, we reported that mitogen-activated protein kinase (MAPK) translocates from the cytosol to the nucleus during ischemia for 20 min and is activated during reperfusion (*31*). The present study showed that PKC-α is translocated to the nucleus-myofibril as well as to the membrane fraction after ischemic preconditioning (Fig. 1). Since MAPK activation is thought to induce *c-fos* and *c-jun* mRNA and activate transcription (*32*) and PKC was shown to mediate MAPK redistribution in smooth muscle (*32*), ischemic preconditioning may up-regulate these processes that lead to the later phase of protection (*29*) through PKC activation.

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