Activated Protein Kinase C Attenuates Ca^{2+} Overloading and Reoxygenation Hypercontracture in Isolated Rat Cardiomyocytes following Chemical Hypoxia

Shigeto Miyasaka, Takashi Ichiba, Hiroaki Kuroda and Shigetsugu Ohgi

Second Department of Surgery, Faculty of Medicine, Tottori University, Yonago 683-0826, Japan

The aims of this study were i) to test the effects of protein kinase C (PKC) activation on the intracellular Ca^{2+} concentration of rat cardiomyocytes during chemical hypoxia-reoxygenation, ii) to determine the contribution of the sarcoplasmic reticulum (SR) and the Na^{+}-Ca^{2+} exchange on the regulation of intracellular Ca^{2+} following PKC activation and iii) to test the role of PKC-dependent intracellular pH changes in intracellular Ca^{2+} regulation. We used the isolated adult rat cardiomyocyte perfusion model. Cardiomyocytes were loaded with the Ca^{2+}-fluorescent probe Fluo-3 and the pH-fluorescent probe SNARF1. Cells were subjected to 50 min of glucose-free and NaCN chemical hypoxia followed by 30 min of simulated reoxygenation. The activation of PKC significantly inhibited the hypoxia-induced increase of Fluo-3 fluorescent intensity (control; $692 \pm 100\%$ and activated PKC; $322 \pm 43\%$: $P < 0.05$). This inhibitory effect was not affected by the inhibition of the SR Ca^{2+} uptake induced by thapsigargin, but was cancelled by the inhibition of the Na^{+}-Ca^{2+} exchange with dichlorobenzamil (thapsigargin $337 \pm 47\%$; dichlorobenzamil $609 \pm 100\%$). PKC activation also attenuated the decrease in intracellular pH during chemical hypoxia, even in the presence of the Na^{+}-H^{+} exchange inhibitor amiloride (control; 6.54 ± 0.02, PKC; 6.72 ± 0.03, PKC + amiloride; 6.73 ± 0.03). We concluded that the PKC attenuation of Ca^{2+} overloading to rat cardiomyocytes during chemical hypoxia-reoxygenation does not depend on the Ca^{2+} uptake by the SR, but does require a Na^{+}-Ca^{2+} exchange. Since PKC attenuated the increasing intracellular H^{+} during chemical hypoxia, a low H^{+} concentration may be important for the maintenance of Ca^{2+} extrusion via the Na^{+}-Ca^{2+} exchange.

Key words: Ca^{2+} overload; intracellular pH; isolated rat cardiomyocytes; Na^{+}-Ca^{2+} exchanger; protein kinase C

Ischemic preconditioning reduces myocardial injury induced by sustained ischemia-reperfusion, with preservation of myocardial function and infarct size reduction. Previous findings suggest that the activation of protein kinase C (PKC) plays a role in the cardioprotective effect of ischemic preconditioning (Liu et al., 1994; Li and Kloner, 1995; Kitakaze et al., 1996). PKC activation by Ca^{2+} or pharmacologic agents mimics ischemic preconditioning (Miyawaki and Ashraf, 1977; Armstrong and Ganote, 1994; Tsuchida et al., 1994; Miyawaki et al., 1996). It has been proposed that PKC attenuates an increase in intracellular Ca^{2+} concentration during ischemia/reperfusion via i) intracellular alkalization (MacLeod and Harding, 1991), ii) activation of the sarcoplasmic reticulum (SR) calcium pump (Movesesian et al., 1984), iii) modification of the Na^{+}-Ca^{2+} exchange (Steenbergen et al., 1993) and iv) inhib-

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Abbreviations: amil, amiloride; [Ca^{2+}]$_i$, intracellular Ca^{2+} concentration; %[Ca^{2+}]$_i$, percentage of the pre-ischemic fluorescene intensity; DCB, dichlorobenzamil; Fluo-3, Fluo-3 acetoxymethylester; %L, percentage of the prehypoxic length; PKC, protein kinase C; pH$_{in}$, intracellular pH; PMA, phorbol 12-myristate 13-acetate; SNARF1, SNARF1 acetoxymethylester; SR, sarcoplasmic reticulum; thaps, thapsigargin

ition of the L-type Ca^{2+} channel (Leatherman et al., 1987; Zheng et al., 1992). However, the precise mechanism underlying the cardioprotection afforded by PKC activation is unknown.

The present study i) examined how PKC affects the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and cell length of isolated rat cardiomyocytes during chemical hypoxia-reoxygenation, ii) tested whether the SR and Na^+-Ca^{2+} exchanger on the sarcolemma contribute to the attenuation of Ca^{2+} overloading during ischemia under the activation of PKC and iii) examined how PKC affects intracellular pH (pH_{in}) and the regulation of the intracellular Ca^{2+} during chemical hypoxia-reoxygenation.

Materials and Methods

Isolation of rat cardiomyocytes

Ventricular myocytes were isolated from adult rat hearts by enzymatic dissociation (Satoh et al., 1994; Siegmund et al., 1994). In brief, 8-week-old male Wistar rats weighing 200 to 250 g were anesthetized by inhalation of diethyl ether. After an injection of 50 U of heparin into the inferior vena cava, the hearts were quickly excised and attached to a Langendorff apparatus. Each heart was perfused through the coronary artery with i) Ca^{2+}-free Tyrode solution (136.5 mmol/L NaCl, 5.4 mmol/L KCl, 0.53 mmol/L $MgCl_2$, 5.5 mmol/L HEPES, 5.5 mmol/L glucose, adjusted to pH 7.4 with NaOH) at 37˚C for 3 min, ii) Tyrode solution containing collagenase type I (0.04 g/100 mL, Sigma Chemical Co, St. Louis, MO) for 20 min and iii) modified Kraftbruhe (KB) solution (70 mmol/L KOH, 40 mmol/L KCL, 20 mmol/L KH_2PO_4, 3 mmol/L $MgCl_2$, 50 mmol/L glutamic acid, 10 mmol/L glucose, 10 mmol/L HEPES, 0.5 mmol/L EGTA, titrated to pH 7.4 with KOH). The ventricles were excised and minced in modified KB solution. The cells were filtered through a stainless steel mesh (150 μm) and incubated for 2 h.

The Guidelines for Animal Experimentation at the Faculty of Medicine, Tottori University, were followed.

Loading of Fluo-3 and SNARF1

The culture medium was changed from KB solution to Medium 199 (GIBCO, Grand Island, NY) containing 1% bovine serum albumin (wt/vol), the Ca^{2+}-selective fluorescence probe 10 μmol/L Fluo-3 acetoxymethylester (Fluo-3) (Dojindo, Kumamoto, Japan). Pluronic F127 [0.075% (wt/vol)] (Molecular Probes, Inc., Evgene, OR) was used as a detergent for cell permeation of the dye (Satoh et al., 1994; Lopez et al., 1996). Cells were loaded for 30 min at 37˚C, followed by incubation for 30 min with Tyrode solution to allow hydrolysis of the Fluo-3. The fluorescent pH indicator SNARF1 acetoxymethylester (SNARF1) (Molecular Probes) at 10 μmol/L was loaded in the same way as Fluo-3 (Seksek et al., 1991; Zaguilan et al., 1991).

Apparatus and measurement of $[Ca^{2+}]_i$, cell length and pH_{in}

Cardiomyocytes attached to a glass-bottomed dish (content volume 1 mL) on the stage of an inverted conforcal laser microscope (InSIGHT, Meridian Instruments Far East, Tokyo, Japan) were perfused (0.6 mL/min) with Tyrode solution. The temperature of the dish was maintained at 37˚C by a temperature controller (Medical Systems Corp, NY).

The ion-selective fluorescent probes were excited by an argon laser, at wave lengths of 488 nm for Fluo-3 and 514 nm for SNARF1. The light emitted from the cells was monitored by a CCD camera (Meridian Instruments) using interference filters (530 nm for Fluo-3 and 580 nm and 644 nm for SNARF1). Images were recorded by a video cassette recorder (SONY, Tokyo) at 30 frames per second.

The fluorescence intensity of Fluo-3 was analyzed by the InSIGHT PLUS-IQ analysis system (Meridian Instruments). Because Fluo-3 has no spectral shift on binding Ca^{2+}, the absolute value of $[Ca^{2+}]_i$ can not be measured (Minta et al., 1989; Hayashi et al., 1994). Changes in $[Ca^{2+}]_i$ are expressed as the percentage of the preischemic fluorescence intensity ($\%[Ca^{2+}]_i$).
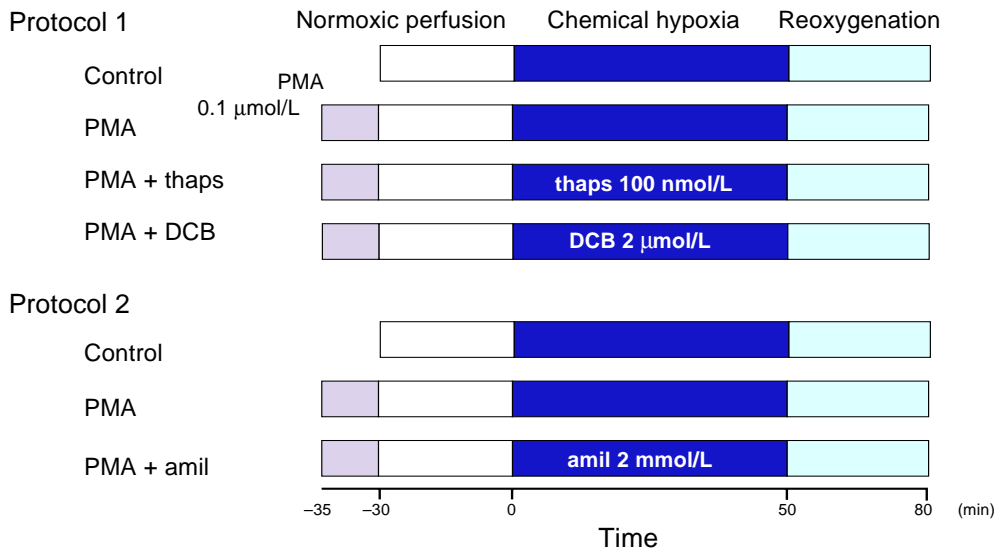
Fig. 1. Schematic representation of the 2 cardiomyocyte treatment protocols. Cells were perfused with 100% oxygenated Tyrode solution during normoxic perfusion and reoxygenation periods. Chemical hypoxia was achieved by the perfusion of Tyrode solution with 2 mmol/L NaCN without glucose at pH 6.7. In the activated PKC group, cells were perfused with Tyrode solution with 0.1 μmol/L PMA for 5 min from –35 to –30 min. amil, amiloride; DCB, dichlorobenzamil; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; thaps, thapsigargin.

The fluorescence intensity of SNARF1 was analyzed by the InSIGHT PLUS-IQ analysis system with a ratio of 580/644 nm. The in situ calibration of SNARF1 was conducted by the nigericin-K⁺ method (Seksek et al., 1991; Zaguilan et al., 1991). The fluorescence intensity ratio of 580/644 nm was converted to pH_{in} using a published calibration curve.

Cell length was measured from the Fluo-3 fluorescence image by the InSIGHT PLUS-IQ analysis system, and changes in cell length are expressed as the percentage of the prehypoxic cell length (%L).

The $\%[Ca^{2+}]_i$, pH_{in} and %L were calculated from the mean of 30 frames (1 s) at each experimental point.

Experimental protocol 1

Effects of PKC activation on Ca^{2+} over-loading and cell length during chemical hypoxia-reoxygenation (Fig. 1).

**Control:** Cardiomyocytes loaded with Fluo-3 were perfused with Tyrode solution equilibrated with 100% O_2 for 30 min. The cells were then subjected to chemical hypoxia for 50 min and reoxygenation for 30 min. The chemical hypoxic solution was based on Tyrode solution but contained 2 mmol/L NaCN, no glucose and a pH of 6.7.

PMA: Cells were exposed to 0.1 μmol/L phorbol 12-myristate 13-acetate (PMA) (Wako, Osaka, Japan) for 5 min before normoxic perfusion. The cells were subsequently perfused with oxygenated Tyrode solution and subjected to chemical hypoxia-reoxygenation in the same way as the controls.

PMA + thaps: The procedure was similar to the PKC group except that 100 nmol/L thapsigargin (thaps) (Funakoshi, Tokyo) was included in the perfusion solution administered during chemical hypoxia (Wu and Feher, 1995; Meldrum et al., 1996).

PMA + DCB: The procedure was similar to the PKC group except that 20 μmol/L 2-4-dichlorobenzamil (DCB) (Molecular Probes) was included in the perfusion solution administered during chemical hypoxia (Schiffman et al., 1990; Kawada et al., 1992).

Fluo-3 signals were obtained 1 min before
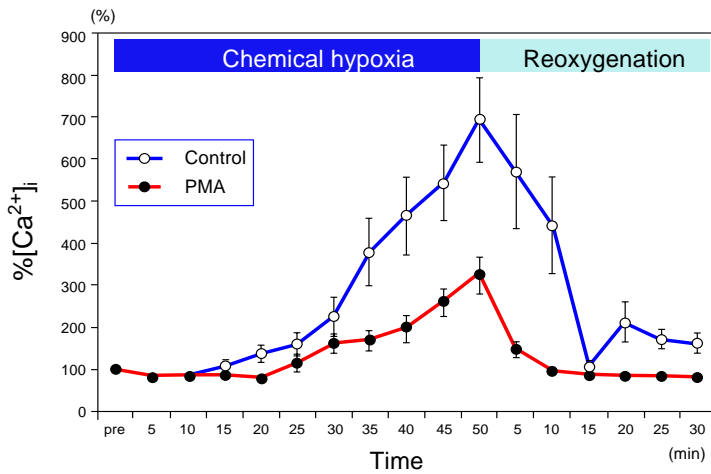
Fig. 2. The time course of changes in %[Ca^{2+}]$_i$ during chemical hypoxia-reoxygenation in untreated (open circles, $n = 11$) and activated PKC-treated (closed circles, $n = 11$) rat cardiomyocytes. Data are mean ± SEM of control and PMA-treated cells. PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

chemical hypoxia as prehypoxic data and for every 5-min period during chemical hypoxia-reoxygenation.

Experimental protocol 2

Effects of PKC activation on intracellular acidosis during chemical hypoxia-reoxygenation (Fig. 1).

Control: Cells loaded with SNARF1 underwent the same procedure as the control group in protocol 1.

PMA: Cells loaded with SNARF1 underwent the same procedure as the PMA group in protocol 1.

PMA + amil: This procedure was similar to that used for PMA, except that 2 mmol/L amiloride (amil) (an inhibitor of the Na$^+$-H$^+$ exchanger) was administered during chemical hypoxia.

The data were collected at the same point as those for protocol 1.

Statistical analysis

All data are expressed as mean ± SEM. The significance of differences among groups was determined using an analysis of variance with Fisher's PLSD post hoc test. Values of $P < 0.05$ were considered significant. All statistical analysis was performed using the software program StatView version J-4.5 on a Macintosh computer.
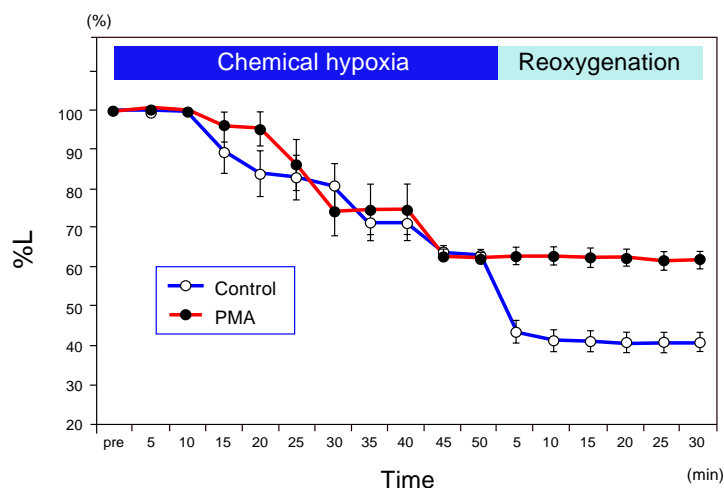
Fig. 3. The time course of % changes in initial cell length during chemical hypoxia-reoxygenation in untreated (open circles, $n = 11$) and activated PKC (closed circles, $n = 11$) rat cardiomyocytes. PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.
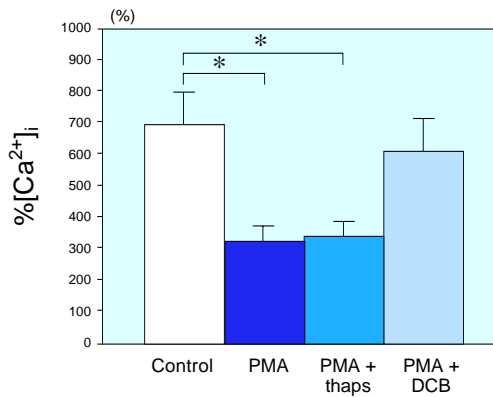
Fig. 4. Intracellular Ca^{2+} concentration changes in isolated rat cardiomyocytes treated with PMA, thaps or DCB. The PMA group and the PMA + thaps group showed lesser increases of $\%[Ca^{2+}]_i$ than the controls, but the PMA + DCB group showed no significant difference from the controls at the end of chemical hypoxia. $*P < 0.05$. DCB, dichlorobenzamil; PMA, phorbol 12-myristate 13-acetate; thaps, thapsigargin.
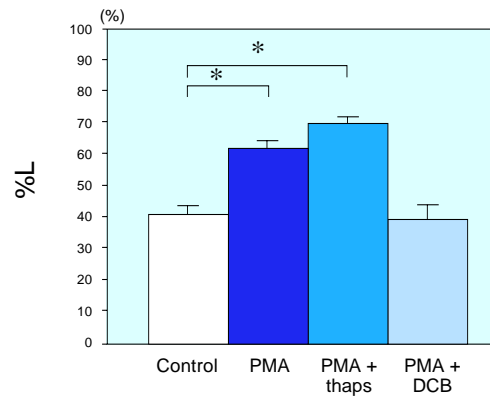
Fig. 5. Cell length changes in isolated rat cardiomyocytes treated with PMA, thaps or DCB. In the PMA group and the PMA + thaps group, cell lengths were maintained compared with the controls, but in the PMA + DCB group, the cell lengths were not significantly different from those of the controls at the end of reoxygenation. $*P < 0.05$. DCB, dichlorobenzamil; PMA, phorbol 12-myristate 13-acetate; thaps, thapsigargin.

Results
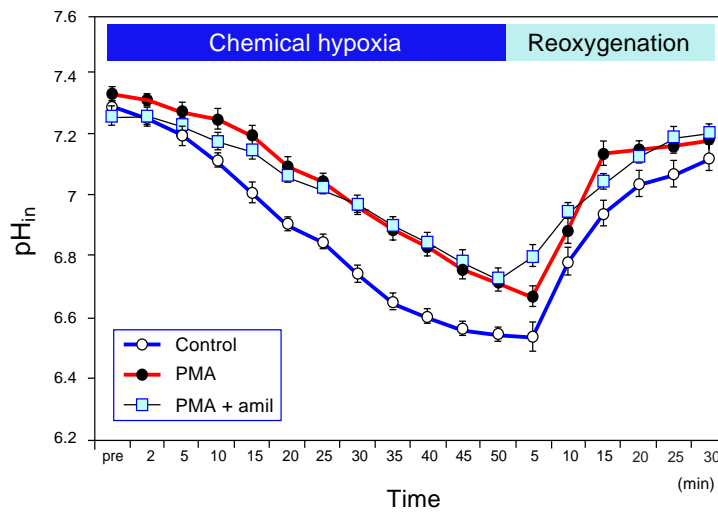
Isolated cardiomyocytes

We selected the following cells, each loaded with fluorescent dye to use as material in the experiment: i) cells that were rod-shaped, ii) those with clear cross-striations and iii) quiescent cells. The mean initial cell length was 106 ± 3 μm ($n = 42$), and there were no significant differences in cell length among these 4 groups. The cells which had entirely balled up into blebbed forms with an absent sarcomere pattern were defined as hypercontractured cells.

Effects of PKC activation on $[Ca^{2+}]_i$ and cell length

In the control and PMA groups, $[Ca^{2+}]_i$ increased significantly 25 min after the induction of chemical hypoxia (Fig. 2). The $\%[Ca^{2+}]_i$ rose continuously to the end of chemical hypoxia in both groups. However, the increase of $[Ca^{2+}]_i$ in the PMA group was considerably attenuated from 35 to 50 min of chemical hypoxia compared with controls. Finally, the $\%[Ca^{2+}]_i$ of the control and PMA groups reached $692 \pm 100\%$ ($n = 11$) and $322 \pm 43\%$ ($n = 11$) respectively in chemical hypoxia ($P < 0.05$) (Fig. 4). After reoxygenation, $\%[Ca^{2+}]_i$ decreased in both groups and reestablished preischemic levels within 15 min.

Ischemic cell shortening (rigor shortening) was observed 15 min after the initiation of chemical hypoxia in both groups (Fig. 3). The cell shapes changed rapidly from rod to square with a length reduction to about 62% of the initial length. At the end of chemical hypoxia, the %L values of the control and PMA groups were reduced to $62.9 \pm 1.7\%$ and $62.5 \pm 1.5\%$, respectively. In the control cells, hypercontraction occurred at the initiation of reoxygenation. In contrast, hypercontraction was not observed in the PMA group, and some cells slightly recovered their length. At 30 min after reoxygenation, the %L values of the control and PMA groups were $40.8 \pm 2.6\%$ and $61.8 \pm 2.1\%$ ($P < 0.05$), respectively (Fig. 5).

Fig. 6. The time course of changes in pH_{in} during chemical hypoxia-reoxygenation in untreated rat cardiomyocytes (open circles, $n = 10$), those with activated PKC (closed circles, $n = 9$), and those with activated PKC and inhibited Na^+-H^- exchanger (squares, $n = 9$). amil, amiloride; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

Contribution of SR and the Na^+-Ca^{2+} exchanger

In the PMA + thaps group, the increase of $\%[Ca^{2+}]_i$ during chemical hypoxia was attenuated. The PMA + thaps value of $\%[Ca^{2+}]_i$ at 50 min of hypoxia was $337 \pm 47\%$ ($n = 10$), with no significant difference between the PMA and PMA + thaps group (Fig. 4).

The alteration of %L in the PMA + thaps group was similar to that of the PMA group; hypercontraction was not observed after reoxygenation, and the final %L was $69.4 \pm 1.78\%$, not significantly different from the PMA group value (Fig. 5). These results indicate that the effects of PKC activation were not abolished despite the inhibition of SR Ca^{2+} uptake.

In the PMA + DCB group, the $\%[Ca^{2+}]_i$ at 50 min of hypoxia and final %L were $609 \pm 100\%$ and $39.4 \pm 4.6\%$ ($n = 10$), respectively. These values were not significantly different from those of the controls (Figs. 4 and 5). These results suggest that the effects of PKC activation were abolished by inhibition of the Na^+-Ca^{2+} exchanger.

Effects of PKC activation on pH_{in} during chemical hypoxia

The pH_{in} values before chemical hypoxia in the control, PMA and PMA + amil groups were 7.30 ± 0.03 ($n = 10$), 7.33 ± 0.02 ($n = 9$) and 7.25 ± 0.03 ($n = 9$), respectively. In all groups, a decrease of pH_{in} was observed during chemical hypoxia (Fig. 6).

In the PMA and PMA + amil groups, intracellular acidification was significantly reduced compared with the control group at 25 min before reoxygenation. The final pH_{in} values during chemical hypoxia of the control, PMA and PMA + amil groups were 6.54 ± 0.02, 6.72 ± 0.03 and 6.73 ± 0.03, respectively (Fig. 6).

In the PMA + amil group, the effect of PKC activation was observed despite inhibition of the Na^+-H^+ exchanger.

Discussion

This study demonstrated that PKC activation by PMA affects the $[Ca^{2+}]_i$, L and pH_{in} of isolated rat cardiomyocytes during chemical hypoxia. Intracellular Ca^{2+} is a very important factor in cell damage during ischemia-reperfusion (Smith and Allen, 1988). A Ca^{2+} overloading of cardiomyocytes accelerates ATP depletion and activates Ca^{2+}-dependent enzymes, leading to irreversible changes including hypercontraction at reperfusion (Nicotera et al., 1989; Ladilov et al., 1997). The reduction of a Ca^{2+} overload may thus reduce reperfusion injury, preserve cardiac function and limit infarction size.

The results of the present study indicate that the activation of PKC reduced the increase of $[Ca^{2+}]_i$ during chemical hypoxia. This suggests

that activating PKC might protect the myocardium from ischemia and reperfusion injury, as previously reported (Miyawaki and Ashraf, 1977; Armstrong and Ganote, 1994; Tsuchida et al., 1994; Miyawaki et al., 1996).

Cell-length changes of isolated cardiomyocytes may be useful as an index for systolic and diastolic function of the myocardium. We observed that the rat cardiomyocytes shortened to 65% of their initial length during chemical hypoxia. This hypoxic shortening is reversible, and myocytes have the ability to undergo twitch contractions if the cells have suffered no irreversible ischemic damage. When the ischemic injury was more severe, reoxygenation caused a sudden further contraction of the cells at reperfusion, to 36% of their initial length. Hyper-contracted cells never recover their length and have no contraction ability (Stern et al., 1985). Pathologically, hypercontraction may prevent recovery of cardiac function. The lengths of the present PKC-activated cells were maintained after reoxygenation compared with the controls, which suggests that activated PKC may protect myocardial function. Meldrum and colleagues (1996) demonstrated the effects of activated PKC on cardiac function and the leakage of creatinine kinase during ischemia-reperfusion, using the Langendorff model. Our results support their study; we examined the same phenomena at the cellular level.

Several mechanisms for the attenuation of Ca^{2+} overload during ischemia by activated PKC have been proposed. Movsesian and coworkers (1984) reported that PKC stimulates Ca^{2+} uptake from cytosol by the SR. Steenbergen and coworkers (1993) suggested that PKC activation modifies the Na$^+$-Ca^{2+} exchanger. Lacarda and colleagues (1988) proposed that activated PKC inhibits the L-type Ca^{2+} channel. However, the precise mechanism of how PKC attenuates a Ca^{2+} overload remains uncertain. In the present study, we tested the contribution of Ca^{2+} uptake by SR and Ca^{2+} extrusion by the Na$^+$-Ca^{2+} exchanger under the activation of PKC.

The inhibition of Ca^{2+} uptake from the cytosol into the SR by thapsigargin did not block the effects of activated PKC during chemical hypoxia. This result suggests that the SR did not play an important role in the effect of PKC. Since the SR Ca^{2+} pump requires ATP to function, its activation by PKC might increase ATP consumption, and the pump would thus not function during severe ischemic ATP depletion.

However, when the Na$^+$-Ca^{2+} exchanger was inhibited, the effects of PKC activation were abolished. This indicates that the attenuation of a Ca^{2+} overload by PKC depends on the Na$^+$-Ca^{2+} exchanger. The Na$^+$-Ca^{2+} exchanger is a passive Ca^{2+} transport system that is dependent on the electrical membrane potential and the intra- and/or extra-cellular concentrations of Ca^{2+} and Na$^+$. The Na$^+$-Ca^{2+} exchanger is thus a potentially important mechanism in the regulation of Ca^{2+} under the condition of severe ATP depletion.

The activation of PKC also reduced intracellular acidification during chemical hypoxia. Meldrum and colleages (1997) and Rehring and colleagues (1996) reported that the activation of PKC by PMA or α_1 adrenoreceptor stimulation limits ischemic acidosis in rat hearts. This effect was independent of the Na$^+$-H$^+$ exchanger, because it occurred despite Na$^+$-H$^+$ exchanger inhibition. Macleod and Harding (1991) proposed that PKC does not activate the Na$^+$-H$^+$ exchanger directly but rather the Na$^+$-H$^+$ exchanger function was modified by the change in pH$_{in}$. The mechanisms underlying the reduction of intracellular acidification following PKC activation are unknown, although Murry and coworkers (1990) showed that preconditioning reduced the production of lactate, resulting in a drop in cytosolic pH during ischemia.

Kohmoto and colleagues (1990) and Coravoeuf and coworkers (1981) suggested that intracellular acidosis impairs Ca^{2+} extrusion via the Na$^+$-Ca^{2+} exchanger. Intracellular H$^+$ may directly inhibit the Na$^+$- Ca^{2+} exchanger (Philipson et al., 1982), and may also indirectly inhibit it by increasing the Na$^+$ influx via the Na$^+$-H$^+$ exchanger (Baker and McNaughton, 1976). If activated PKC attenuates intracellular acidosis by stimulation of the Na$^+$-H$^+$ exchanger, the intracellular Na$^+$ would increase and inhibit the Na$^+$-Ca^{2+} exchanger. Thus, PKC may not directly stimulate the Na$^+$-Ca^{2+} exchange but rather may inhibit it via a reduction of H$^+$

production. Our present results suggest that a reduced pH_{in} leads to a reduced Na^+ influx via the Na^+-H^+ exchanger and limits increases in $[Ca^{2+}]_i$ by maintaining the function of the Na^+-Ca^{2+} exchanger. This hypothesis is the same as that of Steenbergen (1993), who reported similar ionic changes using a nuclear magnetic resonance (NMR) analysis of ischemically preconditioned guinea pig hearts.

Since the signal transduction pathways following PKC activation are complex, there are other possible mechanisms for cardioprotection from Ca^{2+} overload and reoxygenation hypercontracture. Ladilov and co-workers (1997) suggested that the prevention of the ischemic dephosphorylation of contractile proteins reduces the susceptibility of cells to reoxygenation hypercontracture. The reduction of myofilament sensitivity to calcium may be mediated by PKC-dependent pathways and contribute to the reduction of the susceptibility of isolated cardiomyocytes to reoxygenation hypercontracture.

In summary, the activation of PKC attenuates the Ca^{2+} overload of rat cardiomyocytes and facilitates the maintenance of cell length during chemical hypoxia-reoxygenation. These effects do not depend on the Na^+-H^+ exchanger or SR, but require the Na^+-Ca^{2+} exchanger function and a reduction of intracellular acidosis.

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