Downregulation of Protein Kinase C Inhibits Activation of Mitochondrial $K_{\text{ATP}}$ Channels by Diazoxide

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Background—The mitochondrial $K_{\text{ATP}}$ (mito$K_{\text{ATP}}$) channel has been shown to confer short- and long-term cardioprotection against prolonged ischemia via protein kinase C (PKC) signaling pathways. However, the exact association between PKC or its isoforms and mito$K_{\text{ATP}}$ channels has not yet been clarified. The present study tested the hypothesis that the activity and translocation of PKC to the mitochondria are important for cardiac protection elicited by mito$K_{\text{ATP}}$ channels.

Methods and Results—PKC was downregulated by prolonged (24-hour) treatment with phorbol 12-myristate 13-acetate (4 $\mu$g/kg body weight) before subsequent experiments in rats. Langendorff-perfused rat hearts were subjected to 40 minutes of ischemia followed by 30 minutes of reperfusion. Effects of PKC downregulation on the activation of mito$K_{\text{ATP}}$ channels and other interventions on hemodynamic, biochemical, and pathological changes were assessed. Subcellular localization of PKC isoforms by Western blot analysis and immunocytochemistry demonstrated that PKC-$\alpha$ and PKC-$\delta$ were translocated to the sarcolemma and that PKC-$\delta$ was translocated to the mitochondria after diazoxide treatment. In hearts treated with diazoxide (80 $\mu$mol/L), a significant improvement in cardiac function and an attenuation of cell injury were observed. In PKC-downregulated hearts, protection was abolished because mito$K_{\text{ATP}}$ channels could not be activated by diazoxide.

Conclusions—These data suggest that PKC activation is required for the opening of mito$K_{\text{ATP}}$ channels during protection against ischemia and that this effect is linked to isoform-specific translocation of PKC-$\delta$ to the mitochondria. (Circulation. 2001;104:85-90.)

Key Words: ischemia ■ ion channels ■ protein kinase C ■ myocardium

Recently we reported that activation of mitochondrial $K_{\text{ATP}}$ (mito$K_{\text{ATP}}$) channels elicits cardiac protection against ischemia via protein kinase C (PKC) signaling pathways. In that study, we demonstrated by immunocytochemistry that PKC-$\delta$ was translocated to the mitochondria and sarcolemma after treatment with diazoxide (DZX), a relatively specific opener of mito$K_{\text{ATP}}$ channels. PKC is also known to accelerate the activity of mito$K_{\text{ATP}}$ channels, as reported by Sato et al. In the latter study, however, the effect of PKC inhibition during DZX-induced activation of mito$K_{\text{ATP}}$ channels was not studied. PKC is believed to be an important intracellular regulatory enzyme of the myocardium under both normal and abnormal conditions. However, the pathways by which DZX activates PKC and mito$K_{\text{ATP}}$ channels are not clear. It has also been reported that PKC activates sarcolemmal $K_{\text{ATP}}$ channels and plays a significant role in ischemic preconditioning. Because inhibition of PKC abolishes the ischemic protection mediated by mito$K_{\text{ATP}}$ channels, it appears that the effect of mito$K_{\text{ATP}}$ channels and PKC is interdependent. Most previous studies on the role of PKC in signaling pathways have been done with pharmacological inhibitors. Approaches to create PKC-knockout mice to study the importance of PKC in signaling pathways have not yet been successful. This problem is further complicated by the presence of several PKC isoforms that have been implicated in different functions of cardiac cells. In light of these difficulties, studies in chemically PKC–downregulated rats may provide some insight into the responses of mito$K_{\text{ATP}}$ channel activation in cardiac protection. In the current study, we determined whether downregulation of PKC renders the mito$K_{\text{ATP}}$ channels ineffective for cardiac protection. The data in this investigation suggest that PKC translocation to the mitochondria is required for activation of mito$K_{\text{ATP}}$ channels.

Methods

Chemicals

DZX and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. Tetramethylrhodamine ethyl ester (TMRE) was obtained from Molecular Probes. Optimum cutting temperature (OCT, Tissue-Tek) compound was obtained from Miles Inc. Rabbit polyclonal isoform-specific anti-PKC antibodies were purchased from Santa Cruz Biotechnology Inc. Indocarbocyanine-conjugated anti-rabbit IgG antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. Reagents for Western blot detection by enhanced chemiluminescence were purchased from...
Amersham Pharmacia Biotech. Peroxidase-labeled, affinity-purified antibody was purchased from Kirkegaard & Perry Laboratories.

**Heart Preparation**

Hearts from anesthetized male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, Ind) were removed and retrogradely perfused in a noncirculating Langendorff apparatus with Krebs-Henseleit buffer, which consisted of the following (in mmol/L): NaCl 118, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, CaCl2 1.8, NaHCO3 25, and glucose 11. The buffer was saturated with 95% O2/5% CO2 (pH 7.4, 37°C) for 50 minutes. Hearts were perfused at a constant pressure of 80 mm Hg. A water-filled latex balloon–tipped catheter was inserted into the left ventricle through the left atrium and was adjusted to a left ventricular end-diastolic pressure (LVEDP) of 4 to 6 mm Hg during initial equilibration. Thereafter, the balloon volume was not changed. The distal end of the catheter was connected to a Digi-Med heart performance analyzer (model 210, version 1.01, Micro-Med) by way of a pressure transducer (Case). Hearts were paced at 350 beats/min except during ischemia. Pacing was reinitiated after 3 minutes of reperfusion in all groups. The indexes of myocardial function were determined as previously described. The present study conformed to the protocols approved by the institutional Animal Care Committee.

**Experimental Protocol**

After equilibration, hearts were randomly divided into the following experimental groups.

**Group 1: Normal Control and Vehicle Control**

Hearts (n=6) were perfused for 95 minutes with Krebs-Henseleit buffer as a normal control for the different experimental groups. Hearts were treated for 6 minutes with vehicle (0.04% dimethyl sulfoxide, n=6), after which they were subjected to ischemia/reperfusion. The amount of dimethyl sulfoxide used in the vehicle control experiments did not affect any hemodynamic parameters, lactate dehydrogenase (LDH) release, ATP content, or cell morphology.

**Group 2: Ischemia/Reperfusion**

After equilibration, hearts (n=6) were subjected to ischemia for 40 minutes followed by reperfusion for 30 minutes.

**Group 3: Role of MitoKATP Channels in Cardiac Protection**

A potent activator of mitoKATP channels, DZX was used before ischemia. DZX opens sarcomemal KATP channels at higher concentration (855 μmol/L=K1/2), whereas it opens mitoKATP channels at very low concentration (0.4 μmol/L=K1/2). Activation of this channel is blocked by a specific inhibitor, 5-hydroxydecanoic acid, as previously reported. This group tested the hypothesis that direct activation of mitoKATP channels induces cardiac protection. For this purpose, hearts (n=6) were perfused with Krebs-Henseleit buffer containing DZX (80 μmol/L) for 6 minutes. Then the hearts were subjected to ischemia/reperfusion.

**Group 4: Effect of PKC Downregulation on MitoKATP Channel Activation by DZX**

The purpose of these experiments was to determine whether activation of mitoKATP channels after PKC downregulation could protect the heart against ischemia. PKC was downregulated by prolonged treatment with 4 μg/kg body weight PMA injected into the tail vein of rats every 4 to 6 hours for 24 hours, a procedure known to downregulate PKC activity. After 24 hours, hearts (n=6) were perfused in a manner similar to that for group 3.

**Group 5: Direct Influence of PKC on Cardiac Protection**

To determine whether direct activation of PKC protects the hearts in a manner similar to that of mitoKATP channel activation, hearts were treated for 6 minutes with PMA (100 nmol/L) before ischemia/reperfusion.

**Measurement of LDH and Tissue ATP**

LDH, an indicator of myocardial tissue injury, was determined in the coronary effluent by a coupled enzyme-spectrometric technique, and tissue ATP was analyzed by a spectrophotometric method as described earlier.

**Separation of Membrane, Cytosolic, and Mitochondrial Fractions for PKC Localization**

Membrane and cytosolic fractions were prepared as previously described. The purity of the cytosolic extracts was examined by using LDH as a marker. The sarcomemal fraction was confirmed with a 5’-nucleotidase assay as described. Mitochondria were isolated by differential centrifugation. Succinate cytochrome c reductase activity was used to determine the purity of the mitochondrial pellet as described previously.

**Western Blots**

The subcellular localization of PKC isoforms was examined by quantitative immunoblotting. Equal amounts of proteins were loaded on each lane of a 10% SDS-polyacrylamide gel. Proteins were separated by electrophoresis and transferred from the gel to nitrocellulose membranes (Bio-Rad catalog No. 162-0009) by using an electroblotting apparatus. The membrane was incubated for 60 minutes in 5% dry milk and Tris-buffered saline (20 mmol/L Tris HCl [pH 7.4] and 137 mmol/L NaCl) to block nonspecific binding sites. Western blots were probed with affinity-purified, PKC isoform–specific primary antibodies at dilutions of 1:500 to 1:1000 for 1 hour. After being washed, blots were incubated with a 1:10 000 dilution of horseradish peroxidase–labeled anti-rabbit IgG for 1 hour at room temperature. PKC isoforms were detected by the enhanced chemiluminescence method. The amounts of PKC isoforms on the immunoblots were quantified by use of a computer program (Image-Quant Solution).

**Subcellular Localization of PKC Isoforms by Immunocytochemistry**

Immunocytochemical localization of PKC isoforms after various interventions was performed as previously described. Sections (5 μm thick) were fixed for 10 minutes in 70% acetone/30% methanol mixture at −20°C, incubated in 10% normal goat serum in phosphate-buffered saline for 30 minutes to block nonspecific binding, and then incubated with primary antibodies. Confocal images were also obtained with a Leitz DMRBE fluorescence microscope equipped with a TCS 4D confocal scanning attachment (Leica, Inc.). Fluorescence was excited by the 568-nm wavelength from a krypton/argon laser, and emission at 568 to 580 nm was recorded. For mitochondrial imaging, additional hearts were perfused with either 200 nmol/L TMRE, a mitochondrial marker alone, or with 200 nmol/L TMRE plus 80 μmol/L DZX for 6 minutes. Tissue was frozen and sectioned for microscopy. TMRE excitation was induced by using the 488-nm wavelength of a krypton/argon laser and was recorded at >530 nm with a fluorescein isothiocyanate filter.

**Statistical Analysis**

All values are expressed as mean±SEM. Group comparisons were analyzed by 1-way ANOVA (Statview 4.0). All groups were analyzed simultaneously with a Bonferroni/Dunn test. A difference of P<0.05 was considered significant.

**Results**

**Ischemic Control Hearts**

Heart function was significantly decreased after ischemia. At the end of reperfusion, left ventricular developed pressure (LVDP) and coronary flow (CF) were decreased, whereas LVEDP was increased (Figures 1A through 1C). ATP levels were reduced to 4.62±0.9 μmol/g dry weight compared with
the normal control value of $22.3 \pm 0.8 \ \mu\text{mol/g dry weight}$ (Figure 2), and LDH leakage was increased on reperfusion compared with the control value (Figure 2).

Effect of DZX and PMA on Ischemic Injury

A significant increase in LVDP and CF or a decrease in LVEDP was observed on reperfusion of hearts pretreated with DZX and PMA (Figures 1A through 1C). ATP contents were markedly preserved after ischemia/reperfusion in hearts pretreated with both DZX and PMA, and LDH release was also significantly decreased (Figure 2).

Effect of DZX on the Heart After PKC Downregulation

In hearts subjected to repetitive injection of PMA, DZX was totally ineffective. LVDP, LVEDP, and CF values were similar to those for ischemia/reperfusion (Figure 1). No significant differences in ATP content and LDH release were observed in ischemic control and treated hearts (Figure 2).

Subcellular Distribution of PKC Isoforms

Figure 3 shows representative Western blots and their quantitative estimates of PKC isoforms in membrane and mitochondrial fractions after various treatments. No significant differences in the distribution of PKC isoforms were observed in cytosolic fractions (data not shown). DZX treatment before ischemia significantly increased the content of PKC-\(\alpha\), \(\varepsilon\), and \(\delta\) isoforms in the membrane fractions, but only PKC-\(\delta\) was increased in the mitochondria compared with the other isoforms (Figure 3). PKC-\(\alpha\), \(\delta\), and \(\varepsilon\) were not activated after DZX treatment of PKC-downregulated hearts, and similarly, translocation of PKC-\(\delta\) to the mitochondria did not occur in downregulated hearts.
Immunohistochemical Localization of PKC Isoforms in Myocytes

Representative photomicrographs from the immunohistochemical study are shown in Figure 4. PKC-α, which was diffusely distributed in the cytoplasm of control cells, was distinctly localized in the sarcolemma of DZX-pretreated hearts. DZX pretreatment further resulted in translocation of PKC-ε to intercalated discs and mitochondria; however, its translocation to the mitochondria was weaker than that of PKC-δ, of PKC-β, to the nuclear region, and of PKC-δ to the mitochondria. No specific distribution of PKC-β1, -ε, and -δ isoforms was observed in PKC-downregulated hearts after DZX treatment. Staining with PKC-ζ was less intense and weaker than with PKC-α, -β1, -ε, and -δ isoforms after DZX treatment (data not shown).

Discussion

In the current study, we have demonstrated that PKC is an integral component of the mitoKATP channel–induced protection against ischemic injury. First, isoform PKC-δ is translocated to the mitochondria at the time of mitoKATP channel activation. Second, mitoKATP channels could not be opened by DZX in PKC-downregulated hearts, resulting in no protection against ischemia. These results suggest that PKC activity is required for the mitoKATP channels to be effective against ischemia. Previous studies have shown that PKC is an important component of signal transduction pathways leading to protection by ischemic preconditioning. How PKC regulates mitoKATP channels is not yet known. PKC is known to upregulate KATP channels at physiological levels of ATP. Recently Sato et al have demonstrated that mitochondrial flavoprotein oxidation by a selective mitoKATP channel opener, DZX, was potentiated by PMA, suggesting that the activity of mitoKATP channels is upregulated by PKC in isolated myocytes. PKC inhibition before activation of mitoKATP channels by DZX resulted in the loss of protection against ischemic injury in the rat myocardium. Thus, it
Figure 4. Fluorescence microscopy of PKC isoforms. A, Negative control (no PKC antibody). B, PKC-α in control hearts. Diffuse cytoplasmic distribution of α isoform was observed. PKC-δ, -ε, -β, or others in control hearts were similar to that shown in B (data not shown). C, PKC-α in DZX-treated heart. Immunofluorescence was observed in sarcolemma (arrow). D, PKC-β staining was observed in nucleus (arrow). E, PKC-ε in DZX-treated heart. PKC-ε was prominently distributed in intercalated disc (arrow). F, In DZX-treated heart, PKC-δ was positively localized in mitochondrial sites between myofibrils (arrow). G, Hearts were perfused with TMRE and DZX. Green areas represent mitochondria lying between myofibrils preferentially stained with TMRE, which had beaded appearance (arrow). H, Same section as in G, except stained with PKC-δ antibody. In this photomicrograph, antibody staining was carried out with TMRE to colocalize mitochondria by confocal microscopy. Yellowish-orange areas represent immunostain for PKC-δ, which was seen over mitochondria (arrow). No specific distribution of PKC-β, -ε, and -δ was observed in hearts treated with DZX after PKC downregulation. All original magnifications ×400 except confocal image, which is 630×1.4.

appears that PKC-catalyzed phosphorylation of K$_{ATP}$ channels is vital in preconditioning-induced protection.

PKC regulates a number of functions, and because of the lack of a PKC-knockout animal model and the complexity of PKC isoforms, it is rather difficult to assess the exact role of PKC in ischemic preconditioning. Long-term exposure of cells to phorbol esters is known to cause downregulation of PKC and loss of responsiveness to phorbol esters. Young et al reported that downregulation of PKC by phorbol ester is a consequence of either an increased rate of degradation of the polypeptide or multiple proteases may be involved in PKC downregulation. The mechanism whereby phorbol esters induce downregulation of PKC is still unclear. In this study, downregulation of PKC totally blocked the protection by DZX, suggesting a crucial role for PKC in the regulation of mitoK$_{ATP}$ channels. This study also strongly showed that PKC translocation to the mitochondria was essential for activation of mitoK$_{ATP}$ channels. The importance of PKC was further emphasized by Sato et al, who suggested that PKC primes the mitoK$_{ATP}$ channel to open earlier and more intensely during prolonged ischemia. It is well established that DZX selectively opens mitoK$_{ATP}$ channels and is responsible for both early and late preconditioning.

Activation of mitoK$_{ATP}$ channels retards calcium accumulation by the mitochondria and either preserves ATP or increases ATP synthesis. Szewczyk pointed out that the opening of mitoK$_{ATP}$ channels partially compensates the membrane potential, thereby enabling additional protons to be pumped out to form a substantial proton electrochemical gradient for both ATP synthesis and Ca$^{2+}$ transport. Jancewski et al reported that the Ca$^{2+}$ transient is important for ATP synthesis. Therefore, the increase in ATP retention or synthesis as a result of the opening of mitoK$_{ATP}$ channels is important for improving postischemic cardiac functional recovery and maintaining Ca$^{2+}$ homeostasis. The loss of mitoK$_{ATP}$ channel–mediated effects by downregulation of PKC further reinforces the notion that signal transduction cascades of a large number of receptors are coupled to PKC activation, which may phosphorylate different channels leading to preconditioning.

It has previously been reported that in rat myocardial cells, PKC-δ, -α, -ε, and -β are the dominant isoforms expressed. These isoforms have specific functions. For example, activation of PKC-β and -ε in hypertrophied hearts and translocation to cell junctions of cardiac myocytes have been reported. PKC-α is growth inhibitor. Mitchell et al reported translocation of PKC-δ, -ε, or both to the membrane after preconditioning ischemia. However, in the former study, PKC-ε was translocated in the perinuclear zone. In other studies, it has been shown that PKC-ε but not -δ is associated with the early phase of ischemic preconditioning in rabbit cardiomyocytes and reduction of ischemic injury in rat myocytes. Our present study demonstrates that DZX pretreatment caused translocation of PKC-δ to the mitochondria, which may phosphorylate mitoK$_{ATP}$ channels or accelerate their opening. On the other hand, overexpression of PKC-ε could confer beneficial effects against ischemic injury. It is also noteworthy that DZX pretreatment, besides PKC-δ, also caused the translocation of PKC-α to the sarclemma, of -β, to the nucleus, and of -ε to intercalated discs. PKC-α may have a role in phosphorylating membrane proteins, which have been implicated in ischemic preconditioning. Translocation of PKC-ε to the intercalated disc of myocytes may facilitate intercellular communication through nexuses located in the intercalated disc, or PKC-ε may participate in late preconditioning. Translocation of PKC isoforms to the nucleus may have implications for late preconditioning induced by DZX treatment. It is still a matter of debate which PKC isoform mediates the effect of preconditioning. To further implicate that downregulation of PKC is responsible for the loss of protection, DZX was unable to open the channels in PKC-downregulated hearts, as evidenced by the extent of cell injury. Two approaches were
taken to assess the modulation of mitoK$_{ATP}$ channels by PKC. First, PKC was downregulated in the rat heart by prolonged treatment with PMA, and second, the mitoK$_{ATP}$ channel was activated pharmacologically by a selective opener, DZX. To assess the effectiveness of PKC downregulation, Western blot analysis and immunocytochemistry of PKC isoforms were carried out. As expected, no isoforms could be detected in PKC-downregulated hearts after 24 hours. DZX was totally ineffective on the mitoK$_{ATP}$ channels in PKC-downregulated hearts.

This is the first study to demonstrate a PKC isoform–specific role in cardiac protection. PKC-δ was significantly translocated to the mitochondrial fraction after DZX treatment, and no other isoforms were observed in the mitochondria, suggesting a role for PKC-δ in mitoK$_{ATP}$ channel–mediated protection. Thus, it appears that PKC-dependent phosphorylation of mitoK$_{ATP}$ channels may indeed be an important link in ischemic preconditioning. In summary, these data demonstrate that PKC downregulation renders the mitoK$_{ATP}$ channel ineffective, resulting in increased ischemic injury. The data further suggest that PKC-δ may be important for mitoK$_{ATP}$ channel–mediated protection.

Acknowledgment

This study was supported by grants HL 23597 and HL 55678 from the National Institutes of Health, Bethesda, Md.

References


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Circulation. 2001;104:85-90
doi: 10.1161/01.CIR.104.1.85

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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