Preservation of Base-line Hemodynamic Function and Loss of Inducible Cardioprotection in Adult Mice Lacking Protein Kinase Cε*

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Signaling pathways involving protein kinase C isozymes are modulators of cardiovascular development and response to injury. Protein kinase Cε activation in cardiac myocytes reduces necrosis caused by coronary artery disease. However, it is unclear whether protein kinase Cε function is required for normal cardiac development or inducible protection against oxidative stress. Protein kinase Cε activation is also observed during cardiac preconditioning. However, its role as a promoter or inhibitor of injury is controversial. We examined hearts from protein kinase Cε knock-out mice under physiological conditions and during acute ischemia reperfusion. Null-mutant and wild-type mice displayed equivalent base-line morphology and hemodynamic function. Targeted disruption of the protein kinase Cε gene blocked cardioprotection caused by ischemic preconditioning and α1-adrenergic receptor stimulation. Protein kinase Cζ activation increased in protein kinase Cε knock-out myocytes without altering resistance to injury. These observations support protein kinase Cε activation as an essential component of cardioprotective signaling. Our results favor protein kinase Cζ activation as a mediator of normal growth. This study advances the understanding of cellular mechanisms responsible for preservation of myocardial integrity as potential targets for prevention and treatment of ischemic heart disease.

Coronary artery disease and chronic heart failure are important causes of death worldwide. Cellular signaling pathways that regulate cardiovascular development and responses to injury, particularly those involving protein kinase C (PKC)1

isozymes, are under intense investigation. For example, Mochly-Rosen et al. (1) found that activation of PKCε translocation in transgenic mouse hearts caused physiological hypertrophy and reduced the size of individual myocytes. In contrast, postnatal inhibition of PKCε translocation produced lethal dilated cardiomyopathy and increased cardiac myocyte volumes (1). Wu et al. (2) later observed that activation of PKCε translocation converted the Goq transgenic hearts improved contractile function. Conversely, inhibition of PKCε translocation converted the Goq hypertrophic phenotype to a dilated cardiomyopathy (2). However, no previous investigations established whether PKCε activation was an absolute requirement for normal cardiac growth or whether compensatory changes in the expression of other myocardial proteins developed in the absence of PKCε-mediated signaling.

Two lines of evidence support the hypothesis that PKCε activation promotes myocardial resistance to injury during periods of oxidative stress. First, both ischemic preconditioning and pharmacological approaches that induce cardioprotection increase PKCε immunoreactivity and kinase function in cell particulate fractions (3). Second, transgenic expression of a constitutively active PKCε (4) or a peptide agonist of PKCε translocation (5) reduces cardiac myocyte necrosis during ischemia reperfusion. However, it is unclear whether PKCε activation represents a final common pathway for cardioprotection versus one branch of a network of cellular mechanisms that preserve myocardial integrity. Distinction between alternative models would establish the importance of this signaling molecule as a therapeutic target for prevention of ischemic heart disease. Previous efforts to test PKCε function as a requirement for cardioprotection were limited by toxicity and poor specificity of in vitro inhibitors of phosphorylation activity (6–8).

Ischemic preconditioning and pharmacological approaches that induce cardioprotection activate both PKCζ and PKCε in rat heart and cardiac myocyte models (9–12). The functional significance of PKCζ activation during myocardial ischemia reperfusion is controversial. For example, Chen et al. (13) found that inhibition of PKCζ translocation in rat hearts reduced myocyte injury measured as creatine kinase release during reperfusion. Hahn et al. (14) observed that transgenic expression of a peptide antagonist of PKCζ translocation in mouse hearts improved contractile recovery after prolonged ischemia. Contradicting these reports are investigations of pharmacologic-
Requirement for PKCe Activation in Acute Cardioprotection

Table I

|                         | Wild-type mice | PKCe KO mice |
|-------------------------|----------------|--------------|
| **Morphometric assessment** |                |              |
| Body weight (g)         | 38 ± 5         | 36 ± 2       |
| Heart weight (mg)       | 167 ± 15       | 152 ± 30     |
| **Non-invasive hemodynamics (no anesthesia)** |              |              |
| Heart rate (beats/min)  | 679 ± 28       | 655 ± 43     |
| Systolic blood pressure (mm Hg) | 109 ± 6       | 100 ± 11     |
| **Echocardiographic analysis (no anesthesia)** |              |              |
| Heart rate (beats/min)  | 620 ± 107      | 689 ± 56     |
| Left ventricular ejection fraction (%) | 81 ± 2        | 79 ± 3       |
| Left ventricular mass (mg) | 100 ± 18      | 91 ± 14      |
| **Diastole**            |                |              |
| Left ventricular internal dimension (mm) | 3.15 ± 0.37   | 2.87 ± 0.12  |
| Interventricular septum thickness (mm) | 1.20 ± 0.24   | 1.15 ± 0.04  |
| Posterior wall thickness (mm) | 1.23 ± 0.09   | 1.20 ± 0.22  |
| **Systole**             |                |              |
| Left ventricular internal dimension (mm) | 1.17 ± 0.25   | 0.99 ± 0.13  |
| Interventricular septum thickness (mm) | 1.88 ± 0.10   | 1.93 ± 0.07  |
| Posterior wall thickness (mm) | 1.69 ± 0.15   | 1.60 ± 0.24  |
| **Invasive hemodynamics (ketamine anesthesia)** |              |              |
| Heart rate (beats/min)  | 368 ± 72       | 383 ± 81     |
| Mean arterial pressure (mmHg) | 78 ± 14       | 69 ± 16      |
| LV systolic pressure (mm Hg) | 90 ± 12        | 87 ± 15      |
| +dP/dt (mm Hg/s)        | 9,138 ± 2,810  | 8,844 ± 3,005|
| LV end-diastolic pressure (mm Hg) | 5.4 ± 1.4   | 4.9 ± 2.0   |

In this study, we examined hearts from PKCe knock-out (KO) mice under physiological conditions and during ischemia reperfusion. PKCe expression was clearly not a requirement for normal development because null-mutant mice could not be distinguished from their wild-type littermates by general appearance or cardiovascular function. However, acute cardioprotection induced by ischemic preconditioning or α1-adrenergic receptor stimulation was blocked in PKCe KO hearts. Targeted disruption of the PKCe gene increased expression and activation of PKCe in cardiac myocytes. However, PKCe activation in the absence of PKCe expression did not alter resistance to ischemia-reperfusion injury. These results support PKCe activation as an essential component of cardioprotection and an important target for prevention of the pathophysiology associated with coronary artery disease. Our data favor PKCe function as a mediator of normal cardiovascular development, particularly in the context of compromised PKCe signaling.

**EXPERIMENTAL PROCEDURES**

**Animals**—Mutant mice lacking PKCe were originally derived by homologous recombination in J1 embryonic stem cells (16). F1 generation hybrid C57BL/6J129SvJae heterozygous progeny were then intercrossed to generate F2 generation hybrid wild-type and PKCe-null littermates for study. Male mice 12–16 weeks of age were used for experiments. Animal care and handling procedures were in accordance with established institutional and National Institutes of Health guidelines.

**Cardiac Myocyte Culture Model**—Left ventricular myocytes were cultured using modification of the method described by Zhou et al. (17). Excised hearts were cannulated via the aorta for retrograde perfusion with Ca2+-free buffer containing (in mmol/liter) NaCl 120, KC1 5.4, MgSO4 1.2, NaH2PO4 1.2, glucose 5.6, NaHCO3 20, 2,3-butanedione monoxime 10, taurine 5, and type B collagenase 1.5 mg/ml (Worthington, Lakewood, NJ). Myocytes were resuspended as described previously (17) and plated on glass chamber slides at 5,000 cells/cm2.

Medium was changed to minimal essential medium with Hanks’ buffered salt solution containing transferrin (10 ng/ml) and insulin (10 ng/ml). This protocol yielded 80% rod-shaped myocytes with defined sarcomeric striations viable at pH 7.2 for 48 h.

**In Vivo Hemodynamic Measurements—**Systolic blood pressure and heart rate were measured in conscious mice after training using a non-invasive computerized tail cuff system (VisiTech Systems, Apex, NC). Results are expressed as the means of three independent daily measurements with at least 15 of 20 successful analyses each (18). Echocardiographic studies were performed on conscious mice after training using a 15-MHz linear array transducer and commercially available imaging system (Acuson Sequoia c256, Mountain View, CA). Cardiac catheterization was performed in closed-chest mice after the administration of ketamine. A Mikrotip™ pressure-transducing catheter (Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced retrograde into the left ventricle. Heart rate, aortic pressure, and left ventricular pressures were monitored continuously using a chart recorder with signal conditioning units (Gould Electronics, Hayward, CA).

**Ex Vivo Ischemia-reperfusion Model of Myocardial Infarction—**Excised hearts were cannulated via the aorta and perfused on a Langendorff apparatus using Krebs-Henseleit solution containing (in mmol/liter) NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 24, glucose 5.5, sodium pyruvate 5.0, and EDTA 0.5. Hearts were paced at 6 Hz, and left ventricular (LV) developed pressure (LVDP = LV systolic pressure – LV end-diastolic pressure (LVEDP)) was measured using a micromanometer (Millar Instruments) passed into a polyvinyl chloride balloon in the LV cavity (19). Base-line hemodynamic parameters were recorded during 20-min equilibration before hearts were subjected to 20-min global ischemia and 30-min reperfusion.

Wild-type and PKCe KO hearts were pretreated with 2-min global ischemia (transient ischemic preconditioning, TIP) or 2-min phenylephrine (PHE) infusion (20 μmol/liter) followed by a 5-min washout prior to index ischemia reperfusion. In separate experiments, wild-type and PKCe KO hearts were preconditioned with four cycles of 4-min global ischemia and 6-min reperfusion (4-CYCLE IP). Hearts isolated from C57BL/6J mice were pretreated with PKCe agonist peptide (YGRKKRRQRRR-HDAPIGYD), antagonist (YGRKKRRQRRR-EAVSG-LKPT), or scrambled antagonist (YGRKKRRQRRR-LSETKPAV) in infusion (1 μmol/liter) with no washout period before ischemia reperfusion. Peptides were synthesized at the University of California San Francisco Biomolecular Resource Center using N-(9-fluorenylethoxycarbonyl) (Fmoc) chemistry. All of the peptides were purified (>95%) by preparative reversed-phase high performance liquid chromatography (19). Creatine kinase (CK) activity in coronary effluent collected during reperfusion was measured using a commercial kit (Sigma) and corrected for flow rate and wet heart weight. After reperfusion, hearts were perfused with 1% triphenyltetrazolium chloride solution, fixed in 10% neutral buffered formalin, and sectioned (20). Planimetry of viable (stained) and necrotic (unstained) tissue was performed using NIH
Image software. Infarction size was corrected for the weight of each heart section.

Protein Kinase C Assays—Western blot analysis of PKC isozyme expression was performed as described previously (19). Left ventricular tissue was homogenized, and samples of the 100,000 × g supernatant and Triton X-100-extracted pellet fractions were subjected to SDS-PAGE and then transferred to nitrocellulose membrane. PKC distribution was determined using isozyme-selective primary antibodies (Transduction Laboratories, Lexington, KY) and enhanced chemiluminescence detection (Amersham Biosciences). In separate experiments, intact mitochondria were isolated from individual mouse hearts using differential centrifugation protocols (21) and subjected to Western analysis. Immunofluorescence microscopy was performed as described previously (5, 11). Myocytes were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cells were incubated with PKC isozyme-selective primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and fluorescein isothiocyanate-conjugated secondary antibodies. In selected experiments, cells were stained with Mitotracker Red CMXRos (Molecular Probes, Eugene, OR) prior to fixation. Fluorescent images of labeled myocytes were acquired using a Leica TCS confocal laser-scanning microscope system (Leica Lasertechik, Heidelberg, Germany).

Statistical Analysis—Results are reported as the means ± S.E. Comparisons between groups were made using one-way ANOVA or repeated measures ANOVA as indicated. Differences were confirmed using a Bonferroni post hoc test. *p < 0.05 was considered significant.

RESULTS

Normal Base-line Morphology and in Vivo Cardiac Function in PKCε KO Mice—Because PKCε activation is important for physiological myocardial development in other mouse models (1, 2), we were surprised to find that hearts from PKCε KO mice were normal in weight and appearance (Table I). Furthermore, cellular volumes of myocytes isolated from PKCε KO hearts were no different from wild-type cardiac myocytes (22,275 ± 958 versus 24,565 ± 918 μm²; n = 4, p = not significant) as measured electronically with a Coulter counter (22). Therefore, targeted disruption of the PKCε gene had no obvious effect on overall heart morphology or individual cardiac myocyte size.

PKC activation also regulates myocardial contractility through direct phosphorylation of myofilament proteins (23). However, we found no difference in base-line hemodynamic function between wild-type and PKCε KO mice during in vivo catheterization (Table I). Non-invasive techniques performed in conscious mice validated results obtained using invasive approaches. First, there were no differences in left ventricular mass or contractile function between groups measured using two-dimensional and M-mode echocardiography (Table I). Second, tail cuff measurements of systolic blood pressure and heart rate were normal in PKCε KO mice (Table I).

Loss of Cardioprotection in PKCε KO Mice—PKCε activation in cardiac myocytes induces resistance against myocardial ischemia-reperfusion injury (4, 5). However, it is unclear whether PKCε activation is required for the beneficial effects of acute ischemic or pharmacological preconditioning. Here we investigated cardioprotective signaling using buffer-perfused hearts from wild-type and PKCε KO mice to eliminate potential confounding effects of PKCε gene disruption on pulmonary, vascular (24), and central nervous system (25) functions. We observed no differences in LVDP between wild-type and PKCε KO hearts (84 ± 3 versus 86 ± 3 mm Hg; n = 18, p = not significant), confirming measurements made during in vivo catheterization.

Prolonged ischemia reperfusion severely impaired LVDP recovery (Fig. 1A) in both groups of hearts (20 ± 4 versus 19 ± 3 mm Hg; n = 6, p = not significant). Independent laboratories have shown that pretreatment with brief ischemia, here termed TIP, prevents cardiac injury during prolonged ischemia reperfusion in association with PKCε activation (9, 10). In this study, we found that TIP improved LVDP recovery during reperfusion (Fig. 1B) in wild-type hearts (61 ± 3 versus 20 ± 4 mm Hg for controls; n = 6, p < 0.05). However, the beneficial effects of TIP on contractility did not develop in PKCε KO hearts (Fig. 1B). Cardioprotection in association with PKCε activation has also been observed after stimulation with pharmacological agents such as adenosine A1 receptor agonists (15), δ-opioid receptor agonists (12, 26), ethanol (19, 27), sphingosine-1-phosphate (20), and α1-adrenergic receptor agonists (9, 28). Here we pretreated with PHE at concentrations selective for α1-adrenergic receptor stimulation and improved LVDP recovery during reperfusion in wild-type but not PKCε KO hearts (Fig. 1C). Similarly, TIP and PHE prevented pathological elevation of LVEDP after 30-min reperfusion only in WT hearts; *p < 0.05 versus WT control (CON).

Our study next focused on whether improvement of LVDP recovery was associated with reduction of myocardial necrosis (29, 30). We observed that 20-min ischemia and 30-min reperfusion produced extensive infarction in both wild-type and PKCε KO hearts (Fig. 2A). TIP reduced infarction size (Fig. 2B) in wild-type hearts (27 ± 3 versus 47 ± 3% LV mass for WT
controls; \(n = 6, p < 0.05\)). However, effects of TIP on infarction size were blocked in PKCε KO hearts (Fig. 2B). Similarly, PHE reduced infarction in wild-type hearts to \(29 \pm 3\%\) LV mass (\(n = 6, p < 0.05\) versus WT controls) but did not induce protection in PKCε KO hearts (Fig. 2C). Creatine kinase activity in coronary effluent validated measurements of infarction size (Fig. 2). PKCε Activation Required for Acute Cardioprotection in C57BL/6 Mice—Although the PKCε KO mouse model is a powerful tool for investigation of cardioprotective signaling, we sought alternative approaches for inhibition of PKCε function. Chen et al. (13) previously used modulators of PKCε isoform translocation in \textit{ex vivo} rat hearts to examine the effects of PKCδ and PKCε activation on creatine kinase release during ischemia reperfusion. Guffa et al. (31) recently confirmed that trans-activating protein-mediated protein transduction delivered cardioprotective peptides into buffer-perfused rat hearts, including cytosolic and mitochondrial fractions.

In this study, we added a peptide agonist of PKCε translocation (5, 32) linked to amino acids derived from the protein transduction domain of human immunodeficiency virus transactivating protein protein (33) to perfusate for 20 min before global ischemia. PKCε agonist pretreatment did not affect the subcellular distributions of PKCα and PKCδ in left ventricular lysates (Fig. 3A). However, agonist infusion increased the proportion of PKCε localized to 100,000 \(\times g\) particulate fractions (0.63 \(\pm 0.02\) versus 0.46 \(\pm 0.02\) for controls; \(n = 3, p < 0.01\)), indicating activation (3, 34). In independent experiments, we found that TIP did not alter the subcellular distribution of PKCα but increased the proportion of PKCδ (0.54 \(\pm 0.02\) versus 0.40 \(\pm 0.02\) for controls; \(n = 3, p < 0.01\)) and PKCε (0.57 \(\pm 0.06\) versus 0.37 \(\pm 0.03\) for controls; \(n = 3, p < 0.05\)) in particulate fractions (Fig. 3B). PKCε antagonist had no effect on TIP-induced translocation of PKCδ (Fig. 3C). However, the agonist blocked translocation of PKCε to particulate fractions (64 \(\pm 2\) versus 117 \(\pm 12\) density units for scrambled antagonist; \(n = 3, p < 0.05\)), indicating degradation of activated isozyme not bound by its anchoring proteins or receptors for activated C-kinase (3). Scrambled PKCε antagonist had no effect on the subcellular distributions of the PKC ε isozymes examined. Therefore, trans-activating protein-mediated protein transduction delivered selective activators and inhibitors of PKCε function into mouse hearts.

Ping et al. (4) developed transgenic mice in which cardiac expression of constitutively active PKCε induced resistance to ischemia-reperfusion injury. Dorn et al. (5) established that PKCε activation caused by transgenic expression of PKCε agonist peptide in mouse hearts also caused sustained cardioprotection. Here we found that pretreatment with PKCε agonist improved LVDP recovery (Fig. 4A) in normal hearts from C57BL/6 mice (66 \(\pm 4\) versus 28 \(\pm 4\) mm Hg for controls; \(n = 6, p < 0.05\)). PKCε agonist pretreatment also reduced infarction and creatine kinase release caused by ischemia reperfusion (Fig. 4A). Thus, direct activation of PKCε translocation through acute modulation of interactions with anchoring proteins mimicked the cardioprotective effects of ischemic and \(\alpha_{1}\)-adrenergic receptor-mediated preconditioning.

We previously used inhibitors of PKCε translocation in a neonatal rat cardiac myocyte model of hypoxic preconditioning to show that PKCε activation was necessary for acute protection against hypoxia-induced cell death (11). Ping et al. (35) later observed that adenovirus-mediated expression of dominant-negative mutant PKCε in isolated rabbit cardiac myo-
cytes also blocked protection during simulated ischemia. In this study, pretreatment of intact heart with PKCe antagonist had no effect on baseline contractile function. However, inhibition of PKCe translocation blocked improvement of LVDP recovery after preconditioning (Fig. 4, B and C). Selective PKCe inhibition also prevented TIP- and PHE-mediated reduction of infarction size and creatine kinase release. Scrambled PKCe antagonist had no effect on cardiac contractility, infarction size, or creatine kinase release. Thus, PKCe activation was necessary for the acute protection of intact myocardium against ischemia-reperfusion injury in C57BL/6 mice.

Contractile Recovery and Infarction after Pretreatment with Four Cycles of Transient Ischemia—In an earlier investigation, Saurin et al. (36) observed that four cycles of 4-min ischemia and 6-min reperfusion prior to prolonged ischemia reperfusion also reduced infarction size in ex vivo hearts from mice expressing PKCe but not in PKCe KO hearts. In contrast to our current study, those investigators demonstrated that four-cycle ischemic preconditioning improved contractile recovery during reperfusion in both control and PKCe KO hearts. Differences in results from the two laboratories raised the possibility that PKCe-independent myocardial signaling sufficient to reduce oxidant stress and storing during reperfusion may develop after repeated exposure to brief ischemia (36). We tested this hypothesis by subjecting wild-type and PKCe KO hearts to four cycles of 4-min ischemia and 6-min reperfusion prior to prolonged ischemia reperfusion.

4-CYCLE IP improved LVDP recovery at 30-min reperfusion in wild-type hearts (59 ± 8 versus 20 ± 4 mm Hg for controls; n = 6, p < 0.05) but not in PKCe KO hearts (Fig. 5A). Similarly, 4-CYCLE IP prevented pathological elevation of LVEDP after ischemia reperfusion only in wild-type hearts (Fig. 5A). In agreement with the results of Saurin et al. (36), we found that 4-CYCLE IP reduced infarction after prolonged ischemia reperfusion in wild-type hearts (28 ± 3 versus 47 ± 3% LV mass for controls; n = 6, p < 0.05) but not in PKCe KO hearts (Fig. 5B). Creatine kinase activity in coronary effluent validated measurements of infarction size. Therefore, improved contractile recovery caused by four-cycle preconditioning was associated with reduced tissue necrosis in wild-type hearts but was blocked in PKCe KO hearts.

Compensatory Increases in PKCδ Expression and Activation in PKCe KO Hearts—Hypoxic preconditioning activates both PKCδ and PKCe in neonatal rat ventricular myocyte models (11). Ischemic preconditioning is also known to activate PKCδ and PKCe in ex vivo rat hearts (9, 10). However, PKCδ-mediated signaling during ischemia reperfusion has not been fully explored. Here we confirmed that targeted disruption of the PKCe gene blocked the expression of PKCe protein (Fig. 6A). We also found that PKCe protein expression increased substantially in hearts from mice lacking PKCe (166 ± 14 versus 93 ± 8 density units for wild type; n = 4, p < 0.01).

Because Western analysis provides crude localization of signaling molecules, we adapted an adult mouse cardiac myocyte
model suitable for confocal microscopy techniques (17). We next observed that PKCδ localized to nuclei of all wild-type cardiac myocytes (Fig. 6B). In contrast, PKCδ localized to perinuclear sites in all of the myocytes prepared from PKCe KO hearts (Fig. 6B). Importantly, PKCδ translocated acutely to identical perinuclear sites in wild-type cells after treatment with the PKC activator 4β-phorbol 12-myristate 13-acetate (Fig. 6B). These results suggested that PKCδ was chronically activated in cardiac myocytes from PKCe KO hearts.

Mitochondrial localization of activated PKCδ may be required for physiological function. For example, Caruso et al. (37) established that mitochondrial translocation of PKCδ was necessary for insulin stimulation of pyruvate dehydrogenase complex activity in muscle and liver cells. Fryer et al. (12) found mitochondrial translocation of PKCδ important for the cardioprotective effects of δ1-opioid receptor stimulation in a rat model of acute myocardial infarction. In this study, incubation of wild-type myocytes with fluorescent probe MitoTracker Red labeled mitochondria in longitudinal arrays between myofibrils (Fig. 6C). In contrast, staining with selective PKCδ antibodies revealed predominant localization to transverse tubules (38).

Mitochondrion-selective labeling of PKCe KO cardiac myocytes also revealed longitudinal arrays between myofibrils (Fig. 6C). Importantly, targeted disruption of the PKCe gene did not increase localization of PKCe to mitochondria, demonstrated by confocal images simultaneously acquired from myocytes dual-labeled with PKCe antibodies and MitoTracker Red (Fig. 6C). In separate experiments, we subjected mitochondrial fractions from wild-type and PKCe KO hearts to Western analysis and found no differences in PKCe content between groups (Fig. 6D). Thus, PKCe expression and perinuclear localization increased in PKCe KO hearts without changes in distribution to mitochondria or base line resistance to ischemia-reperfusion injury.

DISCUSSION

Our echocardiographic analyses and miniaturized catheterization studies yielded the first published in vivo measurements of hemodynamic function in PKCe KO mice and demonstrated normal cardiac morphology and contractility. In an early investigation of ex vivo mouse heart function, Saurin et al. (36) observed that ischemic preconditioning reduced infarction size during ischemia reperfusion in hearts expressing PKCe but not in PKCe KO hearts (38). In contrast to our study, those investigators demonstrated that ischemic preconditioning improved contractile recovery during reperfusion in both control and PKCe KO hearts. At least three differences in experimental conditions used by each laboratory may provide important insights regarding PKCe function in cardioprotective signaling. First, although similar strategies were used to target the PKCe gene, knock-out mice were independently derived in different genetic backgrounds (16, 39). Second, Saurin et al. (36) paced hearts at 10 Hz to mimic rates observed in intact mice and generated LV developed pressures below physiologi cal levels (36). We paced all of the hearts at 6 Hz to remain within the oxygen and substrate transfer limits of the buffer perfusion system and generated LV developed pressures equal to those measured during in vivo cardiac catheterization. Finally, the hearts in the previous study were preconditioned with four cycles of transient ischemia, whereas our work focused on cardioprotection conferred by a single cycle of transient ischemia.

We tested the hypothesis that PKCe-independent cardioprotective signaling develops after repeated exposure to brief ischemia by subjecting hearts from wild-type and PKCe KO mice to four-cycle ischemic preconditioning. We found that 4-CYCLE IP reduced subsequent infarction in control hearts but not in PKCe KO hearts. In contrast to the results of Saurin et al. (36), we found that 4-CYCLE IP improved contractile recovery in control hearts but not in PKCe KO hearts. Thus, PKCe activation was required for reduction of tissue necrosis and improvement of function caused by four-cycle ischemic preconditioning. There was no dichotomy of infarction size and contractile recovery (36) in any combination of animals and preconditioning strategies studied. Our investigation was not designed to identify cellular mediators of cardioprotection downstream of PKCe. However, the clear distinction of preconditioning effects on contractility between PKCe KO mice derived in different backgrounds suggests that these animals will be useful for future exploration of myocardial stunning using genomic and proteomic approaches. Alternatively, more exhaustive examination of pacing rate, oxygenation, and substrate utilization effects on PKCe KO heart function may yield novel insights regarding metabolic pathways that are responsible for preservation of myocardial integrity during periods of oxidative stress.

Results from experiments using null-mutant animals supported the hypothesis that PKCe activation is required for reduction of infarction size during prolonged ischemia reperfusion. However, we recognized that the loss of inducible cardioprotection in PKCe KO mice might be a consequence of polymorphism in the genetic background or potential confounding effects of linked genes (40, 41). We were aware that few other approaches selectively block PKCe function under physiological conditions. For example, pharmacological agents such as chelerythrine chloride exert biological effects besides PKC inhibition (7, 8). Similarly, transgenic expression of the first variable (V1) region of PKCe in adult mouse hearts prevents PKCe translocation but progressively impairs contractility (1). Accordingly, we used protein transduction methods to introduce peptide modulators of PKCe translocation acutely into hearts.
A

PKC\(\alpha\) & Wild-type & PKC\(\alpha\) KO & soluble & particulate

PKC\(\delta\) & Wild-type & PKC\(\delta\) KO & soluble & particulate

PKC\(\epsilon\) & Wild-type & PKC\(\epsilon\) KO & soluble & particulate

B

WT & KO & WT/PMA

C

WT & KO & WT

D

PKC\(\delta\) - std, Wild-type, PKC\(\epsilon\) KO

FIG. 6. PKC\(\delta\) expression and activation in PKC\(\epsilon\) KO hearts. A, Western blot confirming absence of PKC\(\epsilon\) expression in PKC\(\epsilon\) KO hearts. PKC\(\delta\) protein increased in PKC\(\epsilon\) KO hearts. B, immunofluorescence staining showing nuclear localization (arrows) of PKC\(\delta\) in all of the myocytes prepared from WT hearts. PKC\(\delta\) localized to perinuclear sites (arrowheads) in all of the myocytes from PKC\(\epsilon\) KO hearts and in WT myocytes treated with phorbol 12-myristate 13-acetate (PMA). Confocal images representative of three independent preparations. C, disruption of the PKC\(\epsilon\) gene did not increase co-localization (right) of PKC\(\delta\) and mitochondria in myocytes dual-labeled with MitoTracker Red (left) and PKC\(\delta\)-selective antibodies (center). Confocal images representative of three independent culture preparations. D, Western blot confirming PKC\(\delta\) localization to mitochondria did not increase in PKC\(\epsilon\) KO hearts. Each lane represents the mitochondrial fraction from a single mouse heart.

from C57BL/6J mice. We observed effects on PKC\(\epsilon\) localization predicted by in vitro studies (5, 11, 32) without any impairment of base-line hemodynamic function. Inhibition of PKC\(\epsilon\) translocation prevented reduction of infarction size caused by preconditioning. Conversely, direct activation of PKC\(\epsilon\) translocation induced resistance to injury. Thus, complementary
approaches of gene targeting and pharmacological modulation of interactions between PKCε and its anchoring proteins independently established a requirement for this signaling pathway in acute cardioprotection.

In vivo myocardial ischemia causes release of norepinephrine from sympathetic effenter nerves and up-regulation of α₁-adrenergic receptors (42). Stimulation of α₁-adrenergic receptor activates numerous signaling pathways within ventricular myocytes including mitochondrial K_{ATP} channels (28), glucose transporters (42), and PKC isozymes (9). However, it is unclear which of the pathways protect acutely against injury and which of them contribute chronically to myocardial pathology. In this study, phenylephrine pretreatment caused activation and translocation of PKCε, but not PKCδ, to myocyte particulate fractions (data not shown). Beneficial effects of α₁-adrenergic receptor stimulation were blocked in PKCε KO hearts and in C57BL/6J hearts after perfusion with PKCε antagonist. This is the first demonstration of a requirement for PKCε signaling in cardioprotection mediated by α₁-adrenergic receptors. Our findings suggest that PKCε KO mice will be useful for future investigation of exercise-induced protection, a strategy that reduces ischemia-reperfusion injury in association with norepinephrine release (43).

Changes in expression of one PKC isozyme have been shown to alter expression of other PKC isozymes within the same tissue. For example, Ways et al. (44) observed that transfection of human MCF-7 breast cancer cells with PKCε enhanced the neoplastic phenotype, increased endogenous expression of PKCβ, and reduced expression of PKCδ and PKCγ. Targeted disruption of the PKCε gene did not alter expression of other PKC isozymes in mouse dorsal root ganglia or central nervous system tissues (16). However, Western analysis of PKCε null-mutant hearts in this study revealed up-regulation of PKCε protein expression. Using confocal microscopy techniques not employed previously, we found consistent localization of PKCε to perinuclear regions in PKCε KO myocytes, sites identical to those in wild-type myocytes after activation by phorbol ester and in neonatal rat cardiac myocytes after hypoxic preconditioning or norepinephrine stimulation (1). These findings supported chronic activation of PKCε in hearts from mice lacking PKCε. Definitive evaluation of compensatory changes in cardiac signaling triggered by PKCε gene ablation awaits microarray analysis of null-mutant hearts. Our data favor PKCε as one mediator of normal myocardial development, particularly in the context of compromised PKCε function. A similar role for PKCδ in maintenance of cytoskeletal integrity was proposed by Hahn et al. (14) based on a mouse model of myofibrillar cardiomyopathy.

Given the importance of PKCε activation in inducible cardioprotection, we were surprised to find that wild-type and PKCε KO hearts could not be distinguished by baseline susceptibility to ischemia-reperfusion injury. At least two models of cardiac PKCδ function in the absence of PKCε protein expression may explain these results. In the first model, PKCε signaling serves as one mediator of cardiotoxicity and isozyme-selective inhibition would improve contractile recovery and reduce myocardial necrosis during reperfusion. In the second model, PKCε signaling contributes to cardioprotection and isozyme-selective inhibition would further impair contractile recovery and increase infarction size. Although Western analysis and immunofluorescence microscopy data indicated chronic PKCε activation in PKCε KO myocytes, full function of up-regulated isozyme may require translocation to cellular sites and substrates not available under control conditions. For example, Zhao et al. (6) demonstrated that transfection of neonatal rat cardiac myocytes with wild-type PKCδ did not alter cell viability during simulated ischemia (6). These alternatives will be tested using adenosine A₁ receptor agonists, δ₁-opioid receptor agonists, and direct modulators of PKCδ translocation in experiments beyond the scope of the present investigation.

In summary, we developed complementary approaches for inhibition of PKCε function in intact hearts. We demonstrated for the first time that PKCε expression was not required for normal in vivo morphology and contractility under physiologic conditions. We established that PKCε activation was necessary for acute cardioprotection induced by ischemic preconditioning and by α₁-adrenergic receptor stimulation. Finally, we showed that increased PKCε expression in hearts lacking PKCε might have sustained normal cardiac growth but did not alter baseline resistance to oxidative stress. This study advances the understanding of the molecular mechanisms responsible for myocardial integrity as potential targets for prevention of ischemic heart disease.

REFERENCES
30. Xi, L., Hess, M. L., and Kukreja, R. C. (1998) *Mol. Cell. Biochem.* **186**, 69–77
31. Gustafsson, A. B., Sayen, M. E., Williams, S. D., Crow, M. T., and Gottlieb, R. A. (2002) *Circulation* **106**, 735–739
32. Souroujon, M. C., and Mochley-Rosen, D. (1998) *Nat. Biotechnol.* **16**, 919–924
33. Schwarze, S. R., Ho, A., Vucero-Akbari, A., and Dowdy, S. F. (1999) *Science* **285**, 1569–1572
34. Kraft, A. S., and Anderson, W. R. (1983) *Nature* **301**, 621–623
35. Ping, P., Zhang, J., Cao, X., Li, R. C. X., Kong, D., Tang, X. L., Qiu, Y., Manchikalapudi, S., Auchampach, J. A., Black, R. G., and Bolli, R. (1999) *Am. J. Physiol.* **276**, H1468–H1481
36. Sourir, A. T., Pennington, D. J., Rault, N. J. H., Latchman, D. S., Owen, M. J., and Marber, M. S. (2002) *Cardiovasc. Res.* **55**, 672–680
37. Caruso, M., Maitan, M. A., Bifulco, G., Miele, C., Vigliotta, G., Oriente, F., Formisano, P., and Beguinot, F. (2001) *J. Biol. Chem.* **276**, 45088–45097
38. Soeller, C., and Cannell, M. B. (1999) *Circ. Res.* **84**, 266–275
39. Castrillo, A., Pennington, D. J., Otto, F., Parker, P. J., Owen, M. J., and Bosca, L. (2001) *J. Exp. Med.* **194**, 1231–1242
40. Banbury Conference on Genetic Background in Mice. (1997) *Neuron* **19**, 755–759
41. Gerlai, R. (1996) *Trends Neurosci.* **19**, 177–181
42. Salvi, S. (2001) *Chest* **119**, 1242–1249
43. Tsuchimochi, H., Matsukawa, K., Komine, H., and Murata, J. (2002) *Am. J. Physiol.* **283**, H1896–H1906
44. Ways, D. K., Kukoly, C. A., DeVente, J., Hooker, J. L., Bryant, W. O., Posekany, K. J., Fletcher, D. J., Cook, P. P., and Parker, P. J. (1995) *J. Clin. Invest.* **95**, 1906–1915
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