

Published in final edited form as:

Circ Res. 2003 May 2; 92(8): 873–880. doi:10.1161/01.RES.0000069215.36389.8D.

Protein Kinase C ϵ Interacts With and Inhibits the Permeability Transition Pore in Cardiac Mitochondria

Christopher P. Baines, Chang-Xu Song, Yu-Ting Zheng, Guang-Wu Wang, Jun Zhang, Ou-Li Wang, Yiru Guo, Roberto Bolli, Ernest M. Cardwell, and Peipei Ping

Departments of Physiology and Biophysics and Medicine/Division of Cardiology (C.-X.S., Y.-T.Z., O.-L.W., Y.G., R.B.), University of Louisville, Louisville, Ky; Departments of Physiology and Medicine/Division of Cardiology (C.P.B., G.-W.W., J.Z., E.M.C., P.P.), University of California at Los Angeles, Los Angeles, Calif

Abstract

Although functional coupling between protein kinase C ϵ (PKC ϵ) and mitochondria has been implicated in the genesis of cardioprotection, the signal transduction mechanisms that enable this link and the identities of the mitochondrial proteins modulated by PKC ϵ remain unknown. Based on recent evidence that the mitochondrial permeability transition pore may be involved in ischemia/reperfusion injury, we hypothesized that protein-protein interactions between PKC ϵ and mitochondrial pore components may serve as a signaling mechanism to modulate pore function and thus engender cardioprotection. Coimmunoprecipitation and GST-based affinity pull-down from mouse cardiac mitochondria revealed interaction of PKC ϵ with components of the pore, namely voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT), and hexokinase II (HKII). VDAC1, ANT1, and HKII were present in the PKC ϵ complex at \approx 2%, \approx 0.2%, and \approx 1% of their total expression, respectively. Moreover, in vitro studies demonstrated that PKC ϵ can directly bind and phosphorylate VDAC1. Incubation of isolated cardiac mitochondria with recombinant PKC ϵ resulted in a significant inhibition of Ca²⁺-induced mitochondrial swelling, an index of pore opening. Furthermore, cardiac-specific expression of active PKC ϵ in mice, which is cardioprotective, greatly increased interaction of PKC ϵ with the pore components and inhibited Ca²⁺-induced pore opening. In contrast, cardiac expression of kinase-inactive PKC ϵ did not affect pore opening. Finally, administration of the pore opener atracyloside significantly attenuated the infarct-sparing effect of PKC ϵ transgenesis. Collectively, these data demonstrate that PKC ϵ forms physical interactions with components of the cardiac mitochondrial pore. This in turn inhibits the pathological function of the pore and contributes to PKC ϵ -induced cardioprotection.

Keywords

mitochondria; signal transduction; permeability transition pore; cardioprotection

The mitochondrion has received considerable attention as the target of multiple forms of protection, such as preconditioning (PC), against myocardial ischemia/reperfusion injury. Pharmacological studies have implicated a role for mitochondrial K_{ATP} channel opening in

© 2003 American Heart Association, Inc.

Correspondence to: Christopher P. Baines, PhD, Department of Physiology and Biophysics, UCLA School of Medicine, MRL Building, Suite 1609 at CVRL, 675 CE Young Dr, Los Angeles, CA 90095. CPBaines@gmx.net.

This manuscript was sent to Stephen F. Vatner, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

cardioprotection,¹⁻⁴ and PC has been shown to preserve mitochondrial function^{5,6} and to reduce mitochondrial cytochrome *c* release during ischemia/reperfusion.^{7,8} In addition to mitochondria, multiple studies have also demonstrated that activation of protein kinase C ϵ (PKC ϵ) is critical for the protective phenotype.⁹⁻¹³ Furthermore, cardioprotective stimuli can induce translocation of PKC ϵ to mitochondria.^{14,15} However, little is known regarding whether this signaling kinase and mitochondria are functionally linked and if so, the specific manner and the molecular mechanisms by which they exert cardioprotection. Recently, we have reported mitochondrial localization of PKC ϵ in the mouse heart, and that mitochondrial PKC ϵ expression and activity were enhanced in mice with cardiac-specific expression of active PKC ϵ .¹⁶ Accordingly, we postulate that mitochondrial proteins are targets for PKC ϵ , and that PKC ϵ -dependent modulation of these proteins plays a key role in protection against ischemic injury. However, the molecular identities of the mitochondrial proteins modulated by PKC ϵ have yet to be ascribed.

One potential target is the mitochondrial permeability transition pore. The mitochondrial pore is a multiprotein complex formed at the contact sites between the inner and outer mitochondrial membranes.¹⁷⁻¹⁹ Its core components are the voltage-dependent anion channel (VDAC) in the outer membrane and adenine nucleotide translocase (ANT) in the inner membrane. The pore complex also includes hexokinase, which binds to VDAC, and cyclophilin D, which binds to ANT.¹⁷⁻¹⁹ The current paradigm is that the VDAC-ANT-cyclophilin D complex is the functional pore, with hexokinase enabling modulation of the pore by glucose.^{17,18} While the physiological role of the pore is still not clear, pathological opening of the pore is known to cause mitochondrial permeability transition, and consequently plays a critical role in the progression of both apoptotic and necrotic cell death.¹⁷⁻¹⁹ Pore opening induces mitochondrial swelling, causing outer membrane rupture and the release of apoptogenic proteins such as cytochrome *c* and Smac/DIABLO. In addition, the inner membrane potential collapses, thus inhibiting ATP synthesis, which if prolonged will instead induce necrotic death.

The conditions that elicit pore opening are identical to those that exist in the ischemic heart, namely high Ca²⁺, low glucose and ATP, and high P_i.^{18,19} Consequently, a role for the mitochondrial pore in ischemia/reperfusion-induced cardiac cell death has been proposed.^{18,19} Several studies report that pore opening may contribute to ischemic injury and that inhibition of the pore may be involved in cardioprotection.^{3,8,20-24} Moreover, it has been shown that protein kinase A (PKA) can phosphorylate VDAC,^{25,26} and that protein kinase G (PKG) can inhibit the pore in brain mitochondria,²⁷ suggesting that kinases can regulate pore opening. Nevertheless, the molecular events by which cardiac protective stimuli or signaling molecules modulate the mitochondrial pore have never been defined.

Building on the concept of signal transduction being mediated by the formation of multiprotein complexes,^{28,29} we hypothesized that mitochondrial PKC ϵ can directly interact with, and inhibit opening of, the mitochondrial permeability transition pore in the heart, and that this functional coupling contributes to the cardioprotective actions of PKC ϵ . In the present study, we determined that PKC ϵ can interact with and phosphorylate key components of the mitochondrial pore in the mouse heart. Moreover, we found that transgenic activation of PKC ϵ enhances signaling complex formation between PKC ϵ and the pore, concomitant with inhibition of pore opening. Finally, prevention of pore inhibition with atractyloside significantly impairs the cardioprotective effects of PKC ϵ transgenesis against ischemia/reperfusion injury.

Materials and Methods

All procedures were performed in accordance with the University of Louisville IACUC guidelines, which conform with the NIH *Guide for the Care and Use of Laboratory Animals*.

Antibodies and Chemicals

Anti-PKC ϵ (1:1000) and GST (1:2000) were purchased from BD Pharmingen; anti-VDAC1 (1:2000) from Calbiochem; anti-ANT1 (1:500) and hexokinase II (1:1000) from Santa Cruz Biotechnology; and anti-cyclophilin D (1:1000) from Affinity Bioreagents. Glucose 6-phosphate dehydrogenase (G6PDH) and protease inhibitor cocktail were obtained from Roche Diagnostics, atractyloside from Calbiochem, and recombinant PKC ϵ from BioMol.

PKC ϵ Transgenic Mice

The cardiac-targeted PKC ϵ transgenic mice have been extensively described.^{13,16,30} The α MHC promoter was used to drive cardiac expression of either an active (A159E; AE) or a dominant-negative (K436R and A159E; DN) PKC ϵ mutant in Institute for Cancer Research mice. Both lines are free of hypertrophy and display normal cardiac function.^{13,30} Mice were studied at 9 to 12 weeks.

Constructs and Recombinant Proteins

Mouse heart VDAC1 and ANT1, and rat heart cyclophilin D cDNAs were obtained by RT-PCR and subcloned into pCDNA3. Mouse hexokinase II cDNA was a generous gift from Sami Heikkinen (University of Kuopio, Finland) and was subcloned into pCDNA3. Recombinant [³⁵S]-labeled VDAC1, ANT1, hexokinase II (HKII), and cyclophilin D were generated by in vitro transcription and translation using the TNT rabbit reticulocyte lysate system (Promega). VDAC1 was subcloned into pAcGHLT and recombinant glutathione *S*-transferase (GST)-VDAC1 fusion protein generated using the baculovirus expression system (BD Pharmingen). The fusion protein (which contains a His6 tag) was then purified from the insect cell lysates on a Ni-NTA column. GST-PKC ϵ was generated using the same system.^{13,31}

Mitochondrial Isolation

Mouse cardiac mitochondria were isolated by differential centrifugation.¹⁶ After washing twice, mitochondria were then either directly used for swelling assays or were incubated in lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl [pH 7.4], 1% NP-40, 1 mmol/L EDTA, 1 mmol/L Na₃VO₄, and protease inhibitor). Contamination of the mitochondrial fraction by other subcellular compartments was minimal (data not shown), as determined by lactate dehydrogenase activity (cytosol), and Western blotting for nuclear mitotic apparatus protein (nuclei) and the dihydropyridine receptor (sarcolemma). Protein concentrations were determined using the Bradford method.

Western Blotting

Mitochondrial proteins (50 to 100 μ g) were resolved on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. After blocking with 5% milk, the membranes were immunoblotted using the ECL detection system (Amersham). Linearity was maintained by taking and quantifying multiple exposures of each membrane.

Coimmunoprecipitation

Coimmunoprecipitation was carried out as described.^{13,16} Briefly, 500 μ g mitochondrial protein was incubated with 1 μ g of either PKC ϵ , VDAC1, ANT1, or hexokinase II antibodies plus 20 μ L Protein A/G-Agarose (Santa Cruz) overnight at 4°C. Nonspecific IgG

was used as a negative control. After washing three times with lysis buffer, the complexes were subjected to SDS-PAGE and immunoblotting.

GST-Based Affinity Pull-Down Assays

GST-based affinity pull-down assays were performed as previously reported.^{13,31} Briefly, 1 μg of GST-PKC ϵ or GST-VDAC1 was immobilized on glutathione-Sepharose beads, mixed with either 50 μL of [³⁵S]-labeled in vitro translation mix or 500 μg cardiac mitochondrial protein, and incubated in binding buffer (0.2% Triton X-100, 150 mmol/L NaCl, 20 mmol/L Tris-HCl [pH 7.4], 1 mmol/L EDTA, 1 mmol/L EGTA, 0.2 mmol/L Na₃VO₄, and protease inhibitor). GST-null proteins were used as negative controls. The GST-protein complexes were then washed, resolved by SDS-PAGE, and analyzed via autoradiography or immunoblotting.

In Vitro Phosphorylation of VDAC

Recombinant PKC ϵ was incubated with GST-VDAC1 in phosphorylation cocktail (50 mmol/L Tris-HCl [pH 7.5], 0.1 mmol/L ATP, 2.3 mmol/L HEPES, 28.8 $\mu\text{g}/\text{mL}$ α -phosphatidyl-L-serine, 3 $\mu\text{g}/\text{mL}$ PMA, 7.5 mmol/L DTT, 3 mmol/L CaAc, 5.5 mmol/L MgCl₂, and 0.5 μCi [γ -³²P]-ATP) for 15 minutes at 37°C.^{13,31} Phosphorylation was then analyzed by SDS-PAGE and autoradiography.

Mitochondrial Hexokinase Activity

Hexokinase activity was determined by analyzing the glucose-6-phosphate-coupled conversion of NADP to NADPH.³² Ten microliters of mitochondrial extract was added to 990 μL of assay buffer (100 mmol/L Tris-HCl [pH 8.0], 0.5 mmol/L EDTA, 10 mmol/L ATP, 10 mmol/L MgCl₂, 2 mmol/L glucose, 0.1 mmol/L NADP, and 0.1 U/mL G6PDH). NADPH formation was then followed spectrophotometrically at 340 nm.

Mitochondrial Pore Opening

Opening of the mitochondrial pore was determined by Ca²⁺-induced swelling of isolated cardiac mitochondria.^{20–24} Opening of the pore causes mitochondrial swelling, which is measured as a reduction in A₅₂₀. Isolated cardiac mitochondria were resuspended in swelling buffer (120 mmol/L KCl, 10 mmol/L Tris-HCl [pH 7.4], 20 mmol/L MOPS, and 5 mmol/L KH₂PO₄) to a final concentration of 0.25 mg/mL. Absorbance was measured spectrophotometrically at 520 nm and pore opening induced by 200 $\mu\text{mol}/\text{L}$ CaCl₂. To confirm that the absorbance decreases were due to opening of the pore, mitochondria were incubated with either 30 nmol/L cyclosporin A or 10 $\mu\text{mol}/\text{L}$ MgCl₂, two pore inhibitors, 5 minutes before the addition of CaCl₂. In additional experiments, mitochondria were incubated with 2 μg recombinant PKC ϵ plus 1 $\mu\text{mol}/\text{L}$ PMA 15 minutes before the addition of CaCl₂.

Myocardial Infarction

The mouse model of myocardial infarction has been previously described in detail.^{13,33} Briefly, myocardial infarction was produced in pentobarbital-anesthetized mice by a 30-minute coronary occlusion followed by a 4-hour reperfusion. Physiological variables, including temperature, arterial pressure, heart rate, and blood gases, were maintained within normal limits. At the conclusion of the study, the occluded/reperfused coronary bed and the infarcted region were identified by postmortem perfusion of pthalo blue and tetrazolium chloride, respectively. Infarct size was calculated by videoplanimetry and expressed as a percentage of the ischemic zone. The pore activator atractyloside (25 mg/kg, IV) was administered 15 minutes before ischemia.

Statistical Analysis

Data are expressed as mean±SEM. Biochemical data were compared using unpaired *t* tests, and infarct size data were compared by one-way ANOVA followed by *t* test. *P*<0.05 was considered significant.

Results

PKC ϵ Interacts With the Mitochondrial Pore In Vivo

Examination of mouse cardiac mitochondria demonstrated robust expression of VDAC1, ANT1, hexokinase II, and cyclophilin D, alongside PKC ϵ (Figure 1, n=8). Moreover, immunoprecipitation of mitochondrial PKC ϵ followed by Western blotting revealed the interaction of PKC ϵ with VDAC1, ANT1, and hexokinase II, but not cyclophilin D (Figure 2, n=8). The amounts of VDAC1, ANT1, and HKII in the PKC ϵ complex were estimated to be \approx 2%, \approx 0.2%, and \approx 1% of their total mitochondrial expression, respectively.

PKC ϵ Interacts With and Phosphorylates VDAC In Vitro

In order to ascertain which of the pore proteins bound directly to PKC ϵ , [³⁵S]-labeled recombinant VDAC1, ANT1, hexokinase II, and cyclophilin D were produced by in vitro translation and incubated with either GST or GST-PKC ϵ (Figure 3A, n=3). GST-PKC ϵ was able to bind recombinant VDAC1 more efficiently than GST alone, binding 3.1% of available VDAC1 compared with 0.7%, respectively. In contrast, GST-PKC ϵ and GST bound equally to ANT1, binding 2.6% and 2.1%, respectively. Binding of GST-PKC ϵ to hexokinase II and cyclophilin D was negligible. The relatively high nonspecific binding of ANT1 to GST was somewhat of a concern. However, interaction of ANT with GST-VDAC1 was significantly more robust than GST alone (data not shown), indicating that specific ANT-protein interactions could still be observed with this system. Analysis of the murine VDAC1 sequence revealed 8 putative PKC phosphorylation sites, and recombinant PKC ϵ was able to phosphorylate GST-VDAC1 in a dose-dependent manner (Figure 3B, n=3).

PKC ϵ Inhibits Opening of the Mitochondrial Pore In Vitro

We tested whether binding of PKC ϵ exerts a functional effect on the mitochondrial pore. Mouse cardiac mitochondria were isolated and Ca²⁺-induced pore opening assessed by measuring mitochondrial swelling. Opening of the pore causes swelling of mitochondria, which is observed as a decrease in absorbance.^{20–24} In nontreated mitochondria, Ca²⁺ evoked a large decrease in A₅₂₀ (Figure 4, n=3). This effect was inhibited by cyclosporin A and Mg²⁺, two chemically disparate pore inhibitors, confirming that the decrease in absorbance was due to pore opening. In mitochondria treated with recombinant PKC ϵ plus PMA, but neither PKC ϵ nor PMA alone, the absorbance decrease was also significantly attenuated (Figure 4), demonstrating that PKC ϵ can inhibit the mitochondrial pore. Moreover, this effect of PKC ϵ was independent of changes in mitochondrial hexokinase activity (data not shown).

Interaction of PKC ϵ With the Mitochondrial Pore Is Increased in PKC ϵ Transgenic Mice

We next examined the interaction of PKC ϵ with the mitochondrial pore in the active (AE) and dominant-negative (DN) PKC ϵ mouse lines. The AE line exhibits cardioprotection, whereas the DN line does not.^{13,16} Moreover, while mitochondrial expression of PKC ϵ is increased in both lines, mitochondrial PKC ϵ activity is increased \approx 3- to 4-fold in the AE mice and decreased by about half in the DN mice.¹⁶ Western blot analysis revealed no significant changes in VDAC1, ANT1, and hexokinase II expression in either AE or DN mitochondria (Figure 5, n=6). Interestingly, cyclophilin D expression was increased in the AE mice and decreased in the DN mice (1.7±0.3-fold and 0.3±0.1-fold versus non-

transgenic, respectively). Immunoprecipitation of VDAC1, ANT1, or hexokinase II followed by blotting for PKC ϵ demonstrated increased interaction of PKC ϵ with these pore constituents in the AE mice (Figure 6, n=6). This enhanced complex formation between PKC ϵ and the pore was also evident in the DN mice (Figure 6, n=6), indicating that catalytic activity is not required for these interactions.

Opening of the Mitochondrial Pore Is Inhibited in PKC ϵ Transgenic Mice

We also examined whether cardiac pore function was modulated in the PKC ϵ transgenic mice. Analysis of mitochondria isolated from the different mouse lines demonstrated a reduction in Ca²⁺-induced swelling in AE mitochondria when compared with mitochondria isolated from nontransgenic mice (Figure 7, n=4). In contrast, swelling was unaffected in the DN mitochondria. Mitochondrial hexokinase activity was unchanged in both the AE and DN hearts (data not shown).

Atractyloside Attenuates Cardioprotection in PKC ϵ Transgenic Mice

Activation of PKC ϵ protects the heart against ischemia/reperfusion injury.⁹⁻¹³ We therefore examined whether inhibition of the mitochondrial pore by PKC ϵ contributes to the cardioprotective phenotype observed in the AE mice. In nontransgenic mice subjected to 30 minutes of regional ischemia/4 hours of reperfusion, myocardial infarction was 42.5±4.7% of the ischemic region (Figure 8, n=5 to 6). In contrast, the degree of infarction was significantly reduced to 21.7±3.6% in the AE mice. Atractyloside is an ANT ligand that places the translocase in a conformation that is more conducive to pore opening.¹⁸ Thus, atractyloside would counteract any inhibitory force exerted on the pore. Administration of atractyloside (25 mg/kg IV) 15 minutes before the ischemic period had no significant effect on infarction in nontransgenic animals (47.2±6.8%). However, atractyloside markedly attenuated the protection observed in the AE mice (37.4±2.2%).

Discussion

We have previously demonstrated the constitutive expression of PKC ϵ in cardiac mitochondria,¹⁶ and cardioprotective stimuli induce PKC ϵ translocation to the mitochondrial compartment.^{14,15} Together, these data indicate that PKC ϵ interacts with and modulates multiple mitochondrial proteins. Of these, the mitochondrial permeability transition pore complex was an attractive candidate as emerging evidence indicates that pore opening contributes to myocardial ischemic injury.^{3,8,20-24} Therefore, inhibition of the mitochondrial pore by PKC ϵ would represent a novel mechanism by which PKC ϵ mediates cardioprotection.

PKC ϵ Interacts With Mitochondrial Permeability Transition Pore

Coimmunoprecipitation from mouse cardiac mitochondria demonstrated interaction of PKC ϵ with VDAC1, ANT1, and hexokinase II *in vivo*. However, we found that only VDAC1 was a direct binding partner with PKC ϵ *in vitro*. Consequently, it appears that PKC ϵ is able to bind to the pore in mitochondria via interaction with VDAC, and that the ability of ANT and hexokinase to coprecipitate with PKC ϵ is presumably through interaction with VDAC.¹⁷⁻¹⁹ To our knowledge, this is the first time that direct interaction of a signaling kinase with components of the mitochondrial pore has been demonstrated. The amounts of VDAC1, ANT1, and hexokinase II in the PKC ϵ complex were estimated to be very small in relation to their total mitochondrial levels. However, these numbers only reflect the levels of each pore protein in the PKC ϵ complex under basal conditions, and our data would indicate that these amounts would be greatly enhanced under conditions where PKC ϵ is stimulated. Moreover, the numbers of each protein that is physically part of every pore would only be a fraction of the total pool. Consequently, we believe that the amounts of

pore-specific VDAC, ANT, and hexokinase II interacting with PKC ϵ are higher than the figures would indicate and are sufficient to be functionally relevant. In addition to interacting with the pore, we found that recombinant PKC ϵ could phosphorylate VDAC in a dose-dependent manner, consistent with previous studies showing that PKA can phosphorylate this protein.^{25,26}

Signal transduction is mediated by the formation of dynamic complexes between signaling elements and their effectors,^{28,29} and we have demonstrated enhanced interactions of PKC ϵ with multiple proteins within the cardioprotected mouse heart.^{13,16,31,34} Consequently, if modulation of the mitochondrial pore by PKC ϵ is critical for cardioprotection, then we would expect enhanced PKC ϵ -pore interactions in protected hearts. To address this question, we used a genetic model of cardioprotection engendered by cardiac-specific expression of an active mutant of PKC ϵ ,^{13,30} in which mitochondrial PKC ϵ expression and activity are greatly enhanced.¹⁶ Coimmunoprecipitation revealed that mitochondrial PKC ϵ interactions with VDAC, ANT, and hexokinase II were greatly increased in the PKC ϵ transgenic mice when compared with controls. This enhanced complex formation between PKC ϵ and the pore proteins was also present in mouse hearts expressing DN PKC ϵ , consistent with our previous data showing that PKC ϵ interactions are activity-independent.^{16,31}

PKC ϵ Inhibits the Mitochondrial Permeability Transition Pore

A recent study demonstrated that PKG could inhibit the pore in isolated brain mitochondria,²⁷ suggesting that kinases can regulate pore opening. Therefore, by binding to and phosphorylating VDAC, PKC ϵ may exert a modulatory effect on the mitochondrial pore. Incubation of cardiac mitochondria with recombinant PKC ϵ resulted in a small but significant inhibition of Ca²⁺-induced mitochondrial swelling, an index of mitochondrial pore opening, thus demonstrating a direct inhibitory effect of PKC ϵ on the mitochondrial pore. The small magnitude of the inhibition is presumably a reflection of the artificial conditions under which PKC ϵ interacts with the mitochondria. For example, the phosphorylation reaction is reliant on what mitochondrial ATP is available after isolation, limitations of which would reduce the ability of PKC ϵ to phosphorylate the pore. Unfortunately, addition of exogenous ATP is precluded as ATP is a strong inhibitor of the pore.¹⁷ The amount of PKC ϵ may also be an issue, as the study in brain mitochondria used huge amounts of PKG for complete inhibition of the pore.²⁷

To circumvent these issues, pore function was also determined in the PKC ϵ transgenic mice, where phosphorylation will have occurred before mitochondrial isolation. In keeping with the *in vitro* data, we found that mitochondria isolated from hearts expressing active PKC ϵ exhibited a considerable suppression of Ca²⁺-induced pore opening. Together, these data demonstrate that both exogenous and endogenous PKC ϵ activation can inhibit the mitochondrial permeability transition pore in the mouse myocardium. Moreover, inhibition of the pore is only associated with the cardioprotective phenotype as pore function in mice expressing the DN PKC ϵ was no different from controls.

PKC ϵ -Mediated Pore Inhibition Contributes to Cardioprotection

Opening of the mitochondrial pore has been proposed as a mediator of cardiomyocyte death in response to ischemia/reperfusion.^{18,19} This is supported by experimental evidence of reperfusion-induced pore opening in isolated rat hearts.²¹ Moreover, pore inhibitors prevent ischemia/reperfusion-induced cell death in isolated cardiomyocytes or whole hearts.^{8,20,21,23,24}

The present study shows that the cardioprotected phenotype exhibited in the active PKC ϵ transgenic mice correlated with inhibition of the mitochondrial pore. We therefore wanted to

test whether these two circumstances were causally linked. The ANT protein exists in two states: the c-state and m-state in which the ATP/ADP binding site faces either the cytosol and the matrix, respectively.³⁵ The specific ANT ligand atractyloside places the translocase into the c-state, facilitating pore opening at high concentrations of the drug.³⁵ Therefore, we reasoned that the conformational changes induced by atractyloside would counteract the inhibition by PKC ϵ . Using a dose of atractyloside that did not itself affect myocardial infarction, protection against ischemia was significantly attenuated in the mice expressing active PKC ϵ . Thus, inhibition of the mitochondrial pore by PKC ϵ appears to mediate cardioprotection in these mice.

Mechanism of PKC ϵ -Mediated Inhibition of the Mitochondrial Pore

Although the specific mechanism by which PKC ϵ influences the VDAC protein and therefore the pore as a whole during cardioprotection is still unknown, several scenarios can be envisioned. One possibility is that PKC ϵ influences hexokinase activity, which can modulate pore opening.^{36,37} However, we saw no changes in hexokinase activity in mitochondria from PKC ϵ transgenic hearts or those treated with exogenous PKC ϵ . Another possibility is that phosphorylation of VDAC may inhibit the ion channel itself, thus reducing pore conductance. A third possibility is that PKC ϵ phosphorylation reduces the ability of VDAC to interact with ANT and/or hexokinase II, although preliminary studies suggest that this is not the case. A fourth possibility is that PKC ϵ -induced conformational changes may impair the Ca²⁺ sensitivity of the mitochondrial pore. Halestrap's group has proposed that binding of cyclophilin D to ANT facilitates opening of the pore by increasing Ca²⁺ sensitivity.³⁵ As we did not find cyclophilin D in the PKC ϵ -pore complex, PKC ϵ may induce changes within the pore that prevent binding of cyclophilin D and reduce Ca²⁺ sensitivity. A final possibility is that PKC ϵ recruits inhibitory proteins into the pore complex. For example, we have previously shown that activation of a PKC ϵ -ERK module in mitochondria of the AE transgenic mice leads to inactivation of the proapoptotic protein Bad.¹⁶ Inactivation of Bad would release the antiapoptotic protein Bcl-X_L, which can bind to and inhibit the mitochondrial pore.³⁸ The parallel phosphorylation of VDAC by PKC ϵ may act to facilitate the binding of Bcl-X_L to the pore. Future studies will address these issues.

Experimental Limitations

There are several experimental limitations that should be taken into account. The first is the use of the PKC ϵ transgenic model of protection. Classical PC is an acute phenomenon that relies on posttranslational modifications rather than gene expression. In contrast, the genetic model is associated with chronic changes in protein expression and activity and therefore cannot be directly compared with ischemic PC. Indeed, cyclophilin D was upregulated in the AE mice and down-regulated in the DN mice. While the obvious interpretation is that cyclophilin D expression is controlled by PKC ϵ activity, what effects these changes have on pore function are still not clear. Despite this, PC is known to induce mitochondrial PKC ϵ translocation^{14,15} and PC's protection is blocked by atractyloside,^{8,24} suggesting that PKC ϵ -induced inhibition of the pore may also contribute to PC. A second limitation is that while PKC ϵ can directly bind to and phosphorylate VDAC in vitro, this may not reflect what occurs in vivo. For instance, as PKC ϵ also forms a mitochondrial signaling module with ERK,¹⁶ it is feasible that under in vivo conditions ERK phosphorylates VDAC. The third limitation is that although we believe that the dose of atractyloside used is specific for ANT, we cannot completely rule out that its effect in the AE-PKC ϵ mice was due to the metabolic consequences of ANT inhibition rather than the effect on the pore. Further studies using non-ANT binding pore activators are warranted.

Summary

Very little is known about the pore and its regulation in the myocardium. Furthermore, how intracellular signaling networks couple to the mitochondrial pore has only just begun to be examined. Consequently, the present study provides new information as to how a signaling kinase can directly interact with and modulate function of the mitochondrial pore, and consequently contribute to a specific cardiac phenotype. Application of this paradigm to other signaling systems and cardiac phenotypes should enable us to gain novel insight into how mitochondrial dysfunction affects cardiovascular function and disease, and mechanisms by which this can be prevented.

Acknowledgments

This study was supported by AHA Ohio Valley Affiliate Postdoctoral Fellowship 0120412B (C.P.B.), NIH HL-63901 and 65431 (P.P.), HL-43151, 55757, and 68088 (R.B.), University of Louisville Research Foundation, Jewish Hospital Research Foundation, Commonwealth of Kentucky Research Challenge Trust Fund, and by the Laubisch Endowment. We are extremely grateful to Sami Heikkinen, University of Kuopio, Finland, for his very generous gift of the mouse hexokinase II cDNA.

References

1. O'Rourke B. Myocardial K_{ATP} channels in preconditioning. *Circ Res.* 2000; 87:845–855. [PubMed: 11073879]
2. Baines CP, Liu GS, Birincioglu M, Critz SD, Cohen MV, Downey JM. Ischemic preconditioning depends on interaction between mitochondrial K_{ATP} channels and actin cytoskeleton. *Am J Physiol.* 1999; 276:H1361–H1368. [PubMed: 10199863]
3. Murata M, Akao M, O'Rourke B, Marban E. Mitochondrial ATP-sensitive potassium channels attenuate matrix Ca^{2+} overload during simulated ischemia and reperfusion: possible mechanism of cardioprotection. *Circ Res.* 2001; 89:891–898. [PubMed: 11701616]
4. Garlid KD, Paucek P, Yarov-Yarovoy V, Murray HN, Darbenzio RB, D'Alonzo AJ, Lodge NJ, Smith MA, Grover GJ. Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K^+ channels: possible mechanism of cardioprotection. *Circ Res.* 1997; 81:1072–1082. [PubMed: 9400389]
5. Fryer RM, Eells JT, Hsu AK, Henry MM, Gross GJ. Ischemic preconditioning in rats: role of mitochondrial K_{ATP} channel in preservation of mitochondrial function. *Am J Physiol.* 2000; 278:H305–H312.
6. Laclau MN, Boudina S, Thambo JB, Tariosse L, Gouverneur G, Bonoron-Adele S, Saks VA, Garlid KD, Dos Santos P. Cardioprotection by ischemic preconditioning preserves mitochondrial function and functional coupling between adenine nucleotide translocase and creatine kinase. *J Mol Cell Cardiol.* 2001; 33:947–956. [PubMed: 11343417]
7. Liu H, Zhang HY, Zhu X, Shao Z, Yao Z. Preconditioning blocks cardiocyte apoptosis: role of K_{ATP} channels and PKC ϵ . *Am J Physiol.* 2002; 282:H1380–H1386.
8. Xu M, Wang Y, Hirai K, Ayub A, Ashraf M. Calcium preconditioning inhibits mitochondrial permeability transition and apoptosis. *Am J Physiol.* 2001; 280:H899–H908.
9. Ping P, Takano H, Zhang J, Tang XL, Qiu Y, Li RC, Banerjee S, Dawn B, Balafonova Z, Bolli R. Isoform-selective activation of protein kinase C by nitric oxide in the heart of conscious rabbits: a signaling mechanism for both nitric oxide-induced and ischemia-induced preconditioning. *Circ Res.* 1999; 84:587–604. [PubMed: 10082480]
10. Ping P, Zhang J, Qiu Y, Tang XL, Manchikalapudi S, Cao X, Bolli R. Ischemic preconditioning induces selective translocation of protein kinase C isoforms ϵ and η in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. *Circ Res.* 1997; 81:404–414. [PubMed: 9285643]
11. Dorn GW 2nd, Souroujon MC, Liron T, Chen CH, Gray MO, Zhou HZ, Csukai M, Wu G, Lorenz JN, Mochly-Rosen D. Sustained in vivo cardiac protection by a rationally designed peptide that

- causes ϵ protein kinase C translocation. *Proc Natl Acad Sci U S A*. 1999; 96:12798–12803. [PubMed: 10536002]
12. Liu GS, Cohen MV, Mochly-Rosen D, Downey JM. Protein kinase C ϵ is responsible for the protection of preconditioning in rabbit cardiomyocytes. *J Mol Cell Cardiol*. 1999; 31:1937–1348. [PubMed: 10525430]
 13. Ping P, Song C, Zhang J, Guo Y, Cao X, Li RC, Wu W, Vondriska TM, Pass JM, Tang XL, Pierce WM, Bolli R. Formation of protein kinase C ϵ -Lck signaling modules confers cardioprotection. *J Clin Invest*. 2002; 109:499–507. [PubMed: 11854322]
 14. Zhang J, Bolli R, Lalli J, Tang X-L, Li RCX, Zheng Y, Pass J, Ping P. Ischemic preconditioning and phorbol ester redistribute protein kinase C ϵ to the nucleus, sarcolemmal membranes, and mitochondria in rabbit myocardium. *Circulation*. 1999; 100(suppl I):I-325. Abstract.
 15. Fryer RM, Wang Y, Hsu AK, Gross GJ. Essential activation of PKC δ in opioid-initiated cardioprotection. *Am J Physiol*. 2001; 280:H1346–H1353.
 16. Baines CP, Zhang J, Wang GW, Zheng YT, Xiu JX, Cardwell EM, Bolli R, Ping P. Mitochondrial PKC ϵ , and MAPK form signaling modules in the murine heart: enhanced mitochondrial PKC ϵ -MAPK interactions and differential MAPK activation in PKC ϵ -induced cardioprotection. *Circ Res*. 2002; 90:390–397. [PubMed: 11884367]
 17. Zamzami N, Kroemer G. The mitochondrion in apoptosis: how Pandora's box opens. *Nat Rev Mol Cell Biol*. 2001; 2:67–71. [PubMed: 11413468]
 18. Crompton M, Barksby E, Johnson N, Capano M. Mitochondrial inter-membrane junctional complexes and their involvement in cell death. *Biochimie*. 2002; 84:143–152. [PubMed: 12022945]
 19. Suleiman MS, Halestrap AP, Griffiths EJ. Mitochondria. a target for myocardial protection. *Pharmacol Ther*. 2001; 89:29–46. [PubMed: 11316512]
 20. Clarke SJ, McStay GP, Halestrap AP. Sangliferhin A acts as a potent inhibitor of the mitochondrial permeability transition and reperfusion injury of the heart by binding to cyclophilin-D at a different site from cyclosporin A. *J Biol Chem*. 2002; 277:34793–34799. [PubMed: 12095984]
 21. Di Lisa F, Menabo R, Canton M, Barile M, Bernardi P. Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic NAD⁺ and is a causative event in the death of myocytes in postischemic reperfusion of the heart. *J Biol Chem*. 2001; 276:2571–2575. [PubMed: 11073947]
 22. Korge P, Honda HM, Weiss JN. Protection of cardiac mitochondria by diazoxide and protein kinase C: implications for ischemic preconditioning. *Proc Natl Acad Sci U S A*. 2002; 99:3312–3317. [PubMed: 11867760]
 23. Tsuchida A, Sakamoto J, Miki T, Genda S, Matsumoto T, Ohnuma Y, Miura T. Inhibition of mitochondrial permeability transition pore protects cardiomyocytes from death during ischemia/reperfusion: a possible mechanism of cardioprotection by mitochondrial K_{ATP} activation. *Circulation*. 2001; 104(suppl II):II-16. Abstract.
 24. Hausenloy DJ, Maddock HL, Baxter GF, Yellon DM. Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? *Cardiovasc Res*. 2002; 55:534–543. [PubMed: 12160950]
 25. Bera AK, Ghosh S. Dual mode of gating of voltage-dependent anion channel as revealed by phosphorylation. *J Struct Biol*. 2001; 135:67–72. [PubMed: 11562167]
 26. Bera AK, Ghosh S, Das S. Mitochondrial VDAC can be phosphorylated by cyclic AMP-dependent protein kinase. *Biochem Biophys Res Commun*. 1995; 209:213–217. [PubMed: 7537039]
 27. Takuma K, Phuagphong P, Lee E, Mori K, Baba A, Matsuda T. Anti-apoptotic effect of cGMP in cultured astrocytes: inhibition by cGMP-dependent protein kinase of mitochondrial permeable transition pore. *J Biol Chem*. 2001; 276:48093–48099. [PubMed: 11677240]
 28. Schillace RV, Scott JD. Organization of kinases, phosphatases, and receptor signaling complexes. *J Clin Invest*. 1999; 103:761–765. [PubMed: 10079095]
 29. Hunter T. Signaling—2000 and beyond. *Cell*. 2000; 100:113–127. [PubMed: 10647936]
 30. Pass JM, Zheng Y, Wead WB, Zhang J, Li RC, Bolli R, Ping P. PKC ϵ activation induces dichotomous cardiac phenotypes and modulates PKC ϵ -RACK interactions and RACK expression. *Am J Physiol*. 2001; 280:H946–955.

31. Song C, Vondriska TM, Wang GW, Klein JB, Cao X, Zhang J, Kang YJ, D'Souza S, Ping P. Molecular conformation dictates signaling module formation: example of PKC ϵ and Src tyrosine kinase. *Am J Physiol.* 2002; 282:H1166–H1171.
32. Riddle SR, Ahmad A, Ahmad S, Deeb SS, Malkki M, Schneider BK, Allen CB, White CW. Hypoxia induces hexokinase II gene expression in human lung cell line A549. *Am J Physiol.* 2000; 278:L407–L416.
33. Guo Y, Wu WJ, Qiu Y, Tang XL, Yang Z, Bolli R. Demonstration of an early and a late phase of ischemic preconditioning in mice. *Am J Physiol.* 1998; 275:H1375–H1387. [PubMed: 9746488]
34. Ping P, Zhang J, Pierce WM, Bolli R. Functional proteomic analysis of protein kinase C ϵ signaling complexes in the normal heart and during cardioprotection. *Circ Res.* 2001; 88:59–62. [PubMed: 11139474]
35. Halestrap AP, McStay GP, Clarke SJ. The permeability transition pore complex: another view. *Biochimie.* 2002; 84:153–166. [PubMed: 12022946]
36. Pastorino JG, Shulga N, Hoek JB. Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome *c* release and apoptosis. *J Biol Chem.* 2002; 277:7610–7618. [PubMed: 11751859]
37. Gottlob K, Majewski N, Kennedy S, Kandel E, Robey RB, Hay N. Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes Dev.* 2001; 15:1406–1418. [PubMed: 11390360]
38. Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC. *Nature.* 1999; 399:483–487. [PubMed: 10365962]

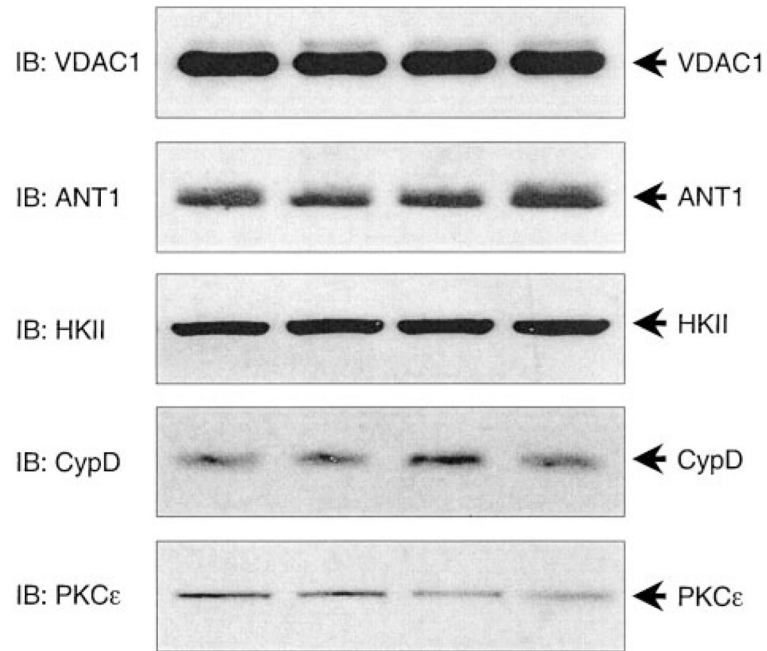


Figure 1. Expression of individual mitochondrial pore proteins in mouse cardiac mitochondria. Mitochondrial lysates from control mouse hearts were subjected to SDS-PAGE followed by Western immunoblotting with (from top to bottom) anti-VDAC1, ANT1, HKII, CypD, and PKCε. Each lane represents an individual heart (n=8). IB indicates immunoblotting.

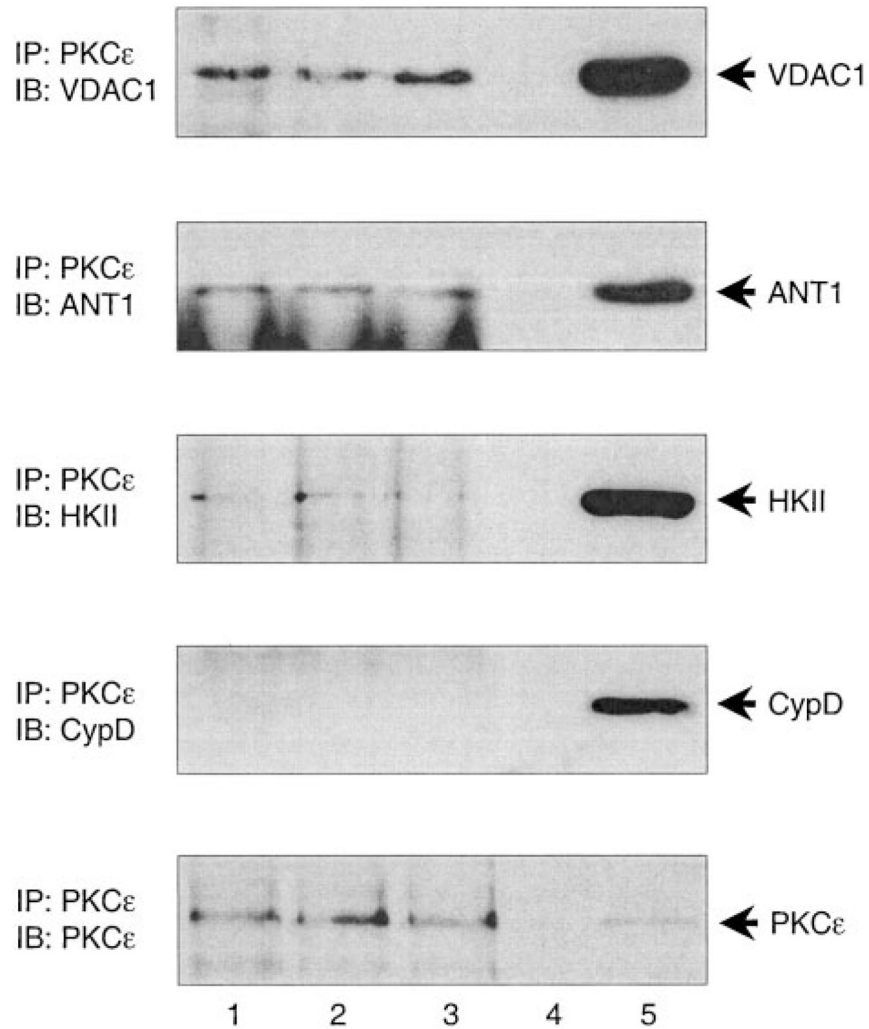


Figure 2.

PKC ϵ interacts with mitochondrial pore proteins in vivo. Mitochondrial lysates from control mouse hearts were immunoprecipitated with anti-PKC ϵ antibody. The complexes were subjected to SDS-PAGE followed by Western immunoblotting with (from top to bottom) anti-VDAC1, ANT1, HKII, CypD, and PKC ϵ antibodies. Lanes 1 through 3 contain PKC ϵ immunoprecipitates from 3 individual hearts; lane 4 is blank; and lane 5 contains cardiac mitochondrial lysate as positive control (n=8). IP indicates immunoprecipitation; IB, immunoblotting.

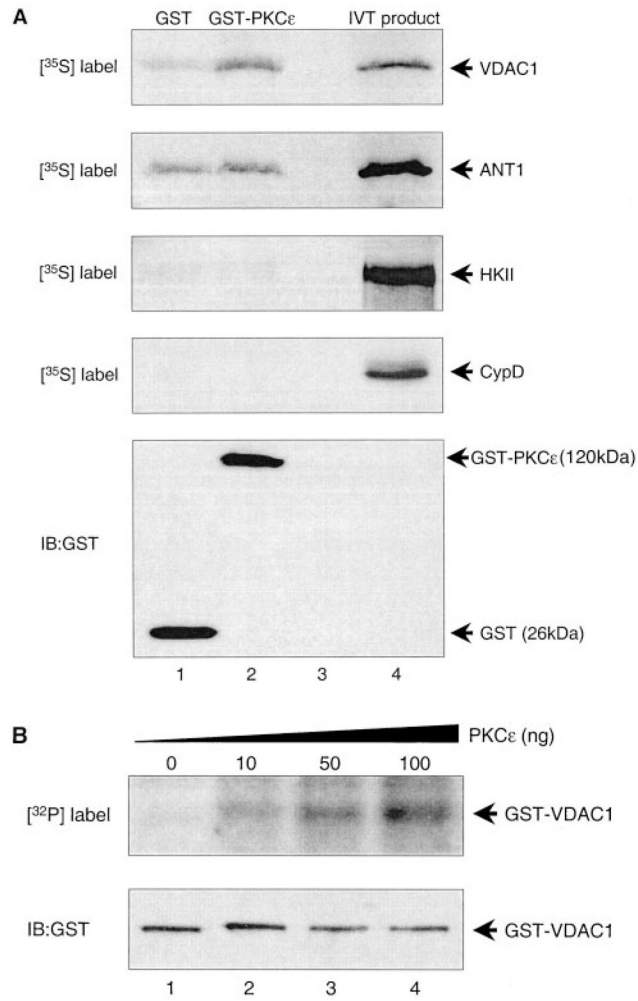


Figure 3.

PKC ϵ interacts with and phosphorylates VDAC in vitro. A, Equal amounts of recombinant GST or GST-PKC ϵ fusion proteins conjugated to glutathione-Sepharose beads were incubated with mixes containing [³⁵S]-labeled VDAC1, ANT1, HKII, or CypD produced by in vitro translation (IVT). The complexes were pulled down and subjected to SDS-PAGE and either autoradiography (top 4 panels) or immunoblotting with anti-GST antibody (bottom panel). Lane 1 contains GST alone plus the respective in vitro translation product; lane 2 contains GST-PKC ϵ plus the respective in vitro translation product; lane 3 is blank; and lane 4 contains the respective in vitro translation product as positive control (n=3). B, Recombinant GST-VDAC1 fusion protein was incubated with γ [³²P]-ATP plus increasing amounts of recombinant PKC ϵ and subjected to SDS-PAGE and either autoradiography (top panel) or immunoblotting with anti-GST antibody (bottom panel). Lanes 1 through 4 contain radio-labeled GST-VDAC1 after incubation with 0, 10, 50, and 100 ng of recombinant PKC ϵ , respectively (n=3). IB indicates immunoblotting.

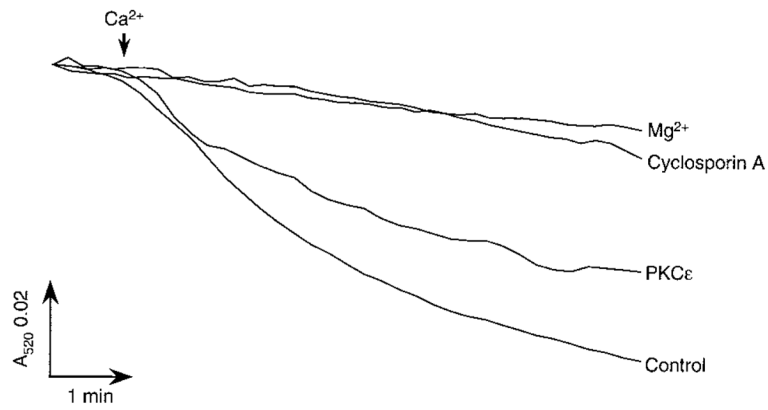


Figure 4. Recombinant PKC ϵ inhibits swelling in mouse cardiac mitochondria. Mitochondria were isolated from nontransgenic mouse hearts and swelling was induced by 200 $\mu\text{mol/L}$ CaCl₂ and measured spectrophotometrically as a decrease in A₅₂₀. In some experiments, mitochondria were incubated with either the pore inhibitors cyclosporin A (30 nmol/L) or MgCl₂ (10 $\mu\text{mol/L}$) 5 minutes before calcium was added, or with recombinant PKC ϵ (2 μg) plus PMA (1 $\mu\text{mol/L}$) 15 minutes before calcium was added. Figure shows representative traces for control, cyclosporin A-, Mg²⁺-, and PKC ϵ -treated mitochondria (n=3).

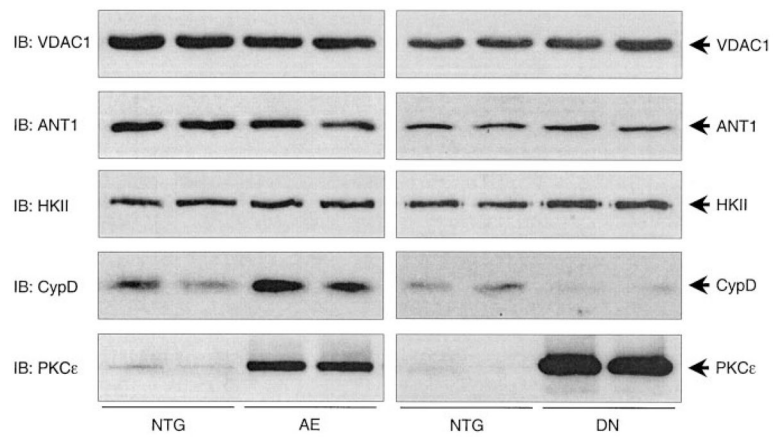


Figure 5. Expression of PKC ϵ and pore proteins in mitochondria from mice with cardiac-specific expression of active and inactive PKC ϵ . Mitochondrial lysates from NTG, AE, and DN mouse hearts were subjected to SDS-PAGE followed by Western immunoblotting with (from top to bottom) anti-VDAC1, ANT1, HKII, CypD, and PKC ϵ antibodies (n=6). IB indicates immunoblotting.

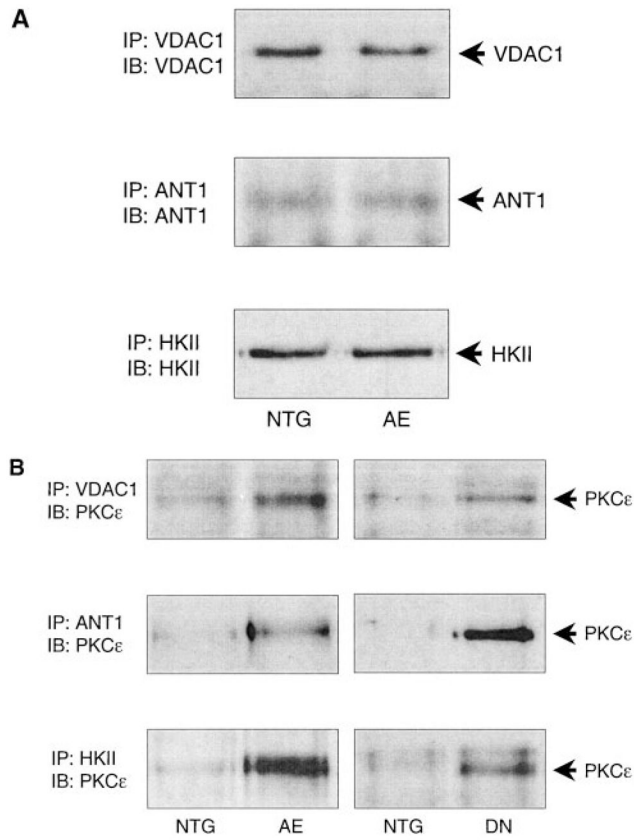


Figure 6. PKC ϵ interaction with pore proteins is enhanced in mitochondria from mice with cardiac-specific expression of active and inactive PKC ϵ . A, Mitochondrial lysates from NTG and AE mouse hearts were immunoprecipitated with anti-VDAC1 (top), ANT1 (middle), or HKII (bottom) antibodies. The complexes were subjected to SDS-PAGE followed by Western immunoblotting with anti-VDAC1 (top), ANT1 (middle), and HKII (bottom) antibodies (n=6). B, Mitochondrial lysates from NTG, AE, and DN mouse hearts were immunoprecipitated with anti-VDAC1 (top), ANT1 (middle), or HKII (bottom) antibodies. The complexes were subjected to SDS-PAGE followed by Western immunoblotting with anti-PKC ϵ antibody (n=6). IP indicates immunoprecipitation; IB, immunoblotting.

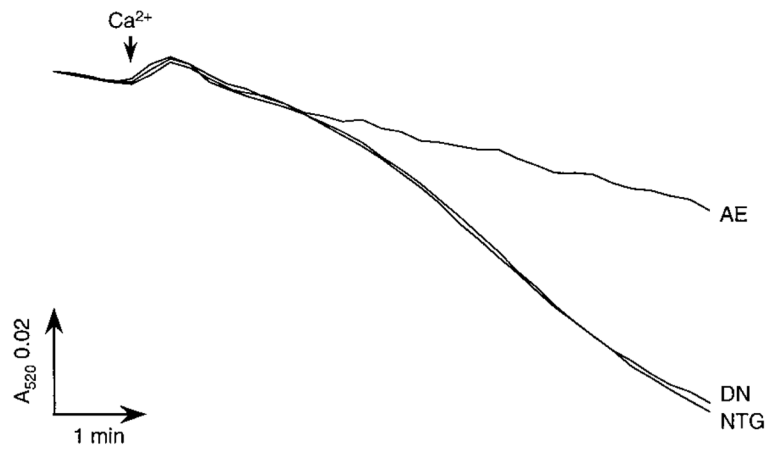


Figure 7. Mitochondrial swelling is inhibited in mice with cardiac-specific expression of active but not inactive PKC ϵ . Mitochondria were isolated from NTG, AE, and DN hearts, and swelling was induced by 200 μ mol/L $CaCl_2$ and measured spectrophotometrically as a decrease in A_{520} . Figure shows representative traces for NTG, AE, and DN mitochondria (n=4).

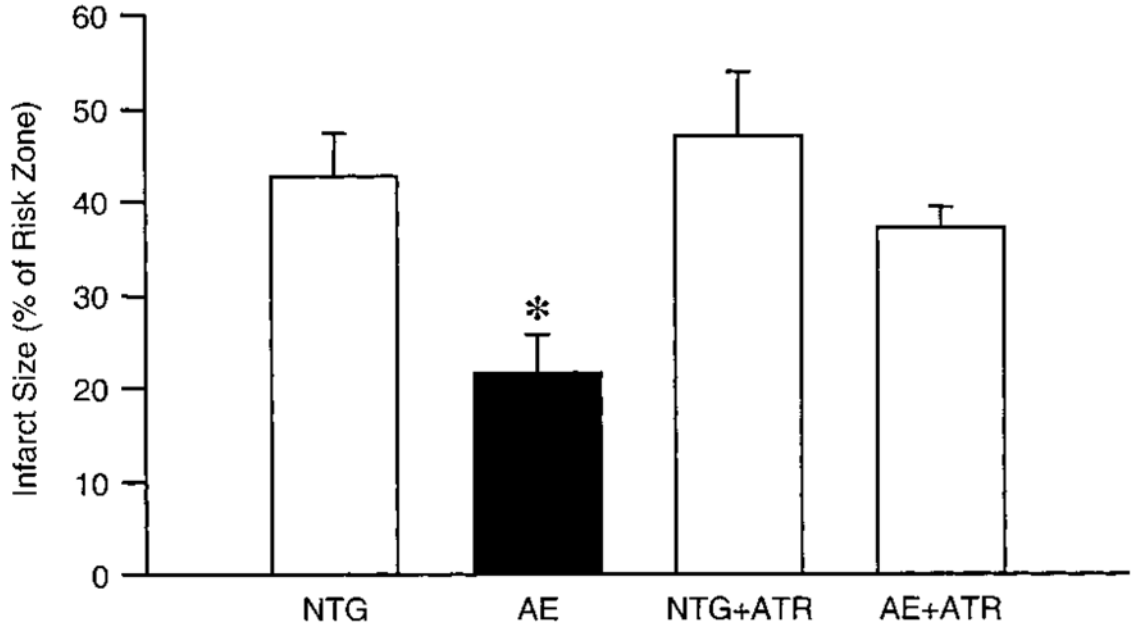


Figure 8. Atractyloside inhibits cardioprotection in mice with cardiac-specific expression of active PKC ϵ . Anesthetized NTG and AE mice were subjected to 30 minutes of coronary occlusion followed by 4 hours reperfusion, and the resultant infarct size was determined by postmortem tetrazolium staining. Atractyloside (ATR) was administered as a bolus injection (25 mg/kg, IV) 15 minutes before the coronary occlusion. Data are represented as mean \pm SEM. * P <0.05 compared with NTG (n=5 to 6).