

Anesthetic-induced Preconditioning Delays Opening of Mitochondrial Permeability Transition Pore via *Protein Kinase C-ε–mediated Pathway*

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Background: **Cardioprotection by volatile anesthetic-induced preconditioning (APC) involves activation of protein kinase C (PKC). This study investigated the importance of APC-activated PKC in delaying mitochondrial permeability transition pore (mPTP) opening.**

Methods: **Rat ventricular myocytes were exposed to isoflurane in the presence or absence of nonselective PKC inhibitor chelerythrine or isoform-specific inhibitors of PKC-δ (rottlerin) and PKC-ε (myristoylated PKC-ε V1–2 peptide), and the mPTP opening time was measured by using confocal microscopy. Ca^{2+}-induced mPTP opening was measured in mitochondria isolated from rats exposed to isoflurane in the presence and absence of chelerythrine or in mitochondria directly treated with isoflurane after isolation. Translocation of PKC-ε was assessed in APC and control cardiomyocytes by Western blotting.**

Results: **In cardiomyocytes, APC prolonged time necessary to induce mPTP opening (261 ± 26 s APC *vs.* 216 ± 27 s control; $P < 0.05$), and chelerythrine abolished this delay to 213 ± 22 s. The effect of isoflurane was also abolished when PKC-ε inhibitor was applied (210 ± 22 s) but not in the presence of PKC-δ inhibitor (269 ± 31 s). Western blotting revealed translocation of PKC-ε toward mitochondria in APC cells. The Ca^{2+} concentration required for mPTP opening was significantly higher in mitochondria from APC rats (45 ± 8 $\mu M \cdot mg^{-1}$ control *vs.* 64 ± 8 $\mu M \cdot mg^{-1}$ APC), and APC effect was reversed with chelerythrine. In contrast, isoflurane did not protect directly treated mitochondria.**

Conclusion: **APC induces delay of mPTP opening through PKC-ε–mediated inhibition of mPTP opening, but not through PKC-δ. These results point to the connection between cytosolic and mitochondrial components of cardioprotection by isoflurane.**

ANESTHETIC preconditioning (APC) describes the protection of myocardium from ischemia and reperfusion injury triggered by exposure to volatile anesthetics before an ischemic event.[1,2] Cellular signaling of APC is complex, and in many aspects, comparable to that of ischemic preconditioning.[3] Studies have shown that protein kinase C (PKC), mitochondrial and sarcolemmal adenosine triphosphate (ATP)–sensitive K channels, and reactive oxygen species (ROS) play a pivotal role in the signal transduction cascade in APC.[1,4–6] Mitochondria are an integral part in the mechanism of cell death as well as cellular protection by preconditioning.[7] APC decreases mitochondrial Ca^{2+} overload and ROS production during reperfusion.[8,9] Isoflurane has recently been shown to induce mild mitochondrial uncoupling that was preserved after anesthetic washout.[10] Moreover, it has been suggested that APC decreases myocyte death through inhibition of mitochondrial permeability transition pore (mPTP) opening,[11] which is one of the main determinants of cell death and the end effector of ischemia and reperfusion injury.[7,12] Interestingly, PKC has been suggested to participate in ischemic preconditioning-induced suppression of mPTP opening.[12,13]

PKC is considered a major signaling component of APC,[4,14–17] and isoforms PKC-δ and PKC-ε are the most relevant for APC.[18,19] It has been shown that PKC-ε is the primary cardioprotective PKC isoform, whereas PKC-δ promotes injury.[20,21] Blockade of PKC abolishes cardioprotection by ischemic preconditioning, though activation of PKC can induce the preconditioned state.[12] When activated during preconditioning, PKC isoforms translocate from the cytosol to the membranes. Recent evidence suggests that PKC-ε is targeted to the mitochondria.[22] In fact, PKC-ε has been shown to be associated with many mitochondrial proteins, including components believed to constitute mPTP.[23] Such interaction of PKC-ε with mPTP causes inhibition of Ca^{2+}-induced mitochondrial swelling, an index of pore opening *in vitro*. However, the exact cellular distribution of PKC isoforms after APC remains controversial, and translocation and activation of PKC isoforms is dependent on the stimulus used, the experimental conditions, and the animal model.[14,24,25]

The role of PKC isoforms in isoflurane-induced delay of mPTP opening has not been investigated. Thus, the current study tested the hypothesis that PKC participates in the signaling pathways involved in isoflurane-induced delay of mPTP opening. We specifically sought to verify whether PKC-specific isoforms ε and δ mediate this effect. Our study provides information to explain intracellular signaling between mitochondria and the cytosol that is necessary for isoflurane-induced cardioprotection.

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Received from the Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin. Submitted for publication November 20, 2008. Accepted for publication March 9, 2009. Supported in part by Grants P01GM066730 and R01HL034708 from the National Institutes of Health, Bethesda, Maryland (to Dr. Bosnjak).

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Materials and Methods

Experimental procedures and protocols used in this study were in accordance with the Institutional Animal Care and Use Committee of the Medical College of Wisconsin (Milwaukee, Wisconsin). All conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 95–23, Revised 1996).[26]

Isolation of Cardiomyocytes

Ventricular cardiomyocytes were isolated from hearts of adult male Wistar rats (150–250 g) by enzymatic dissociation as previously described.[5,6] Cells were resuspended in HEPES-buffered Tyrode solution (in mM): 132 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, 5 D-glucose, 10 HEPES, pH 7.4. Cells were stored at room temperature. Myocytes were allowed to recover for 1 h and were used for experiments within 5 h. Only rod-shaped, quiescent myocytes with visible striations and no visible membrane damage were used for experiments.

Opening of mPTP in Cardiomyocytes

To induce mPTP pore opening, cardiomyocytes were loaded with the fluorescent probe tetramethylrhodamine ethylester (TMRE, 100 nM; Invitrogen, Carlsbad, CA) for 25 min at room temperature. TMRE, a lipophilic cation, accumulates preferentially into mitochondria. On laser-illumination, TMRE generates ROS within mitochondria, which leads to opening of mPTP.[12,27–30] In some experiments, after incubation with TMRE, adult rat myocytes were loaded with calcein-AM (1.0 μM; Invitrogen) and cobalt chloride (2.0 mM; Sigma-Aldrich, St. Louis, MO) for 15 min at room temperature. Calcein-AM is de-esterified and distributed in mitochondria and cytosol, where cytosolic calcein fluorescence is quenched by cobalt chloride so that only the mitochondrial dye is seen. Selected regions of the myocyte (50 μm^2) were subjected to laser-induced oxidative stress until mPTP opening had occurred, visualized as a collapse of mitochondrial membrane potential ($\Delta\Psi_m$),[31,32] and release of the fluorescent dye calcein (620 Da) from mitochondria.[33] Calcein release was used to verify the opening of mPTP independently from changes of $\Delta\Psi_m$.

Confocal Microscopy

Cells were imaged in a polycarbonate recording chamber (Warner Instruments, Hamden, CT) using a confocal microscope (Eclipse TE2000-U; Nikon, Tokyo, Japan) with a 60×/1.4 oil-immersion objective (Nikon). For TMRE fluorescence, cells were illuminated by use of the 543-nm emission line of a HeNe laser. The emitted fluorescence was collected at 590 nm. Calcein was excited with the 488-nm line of an Argon laser, and fluorescence intensity was recorded at 520 nm. Images were recorded with EZ-C1 2.10 software (Nikon). The zoom function was used to select the region of interest (50 μm^2), and that region was scanned at 3.5-s intervals, typically between 90 and 120 image frames. The scanning speed was set to a pixel dwell time of 1.92 μs. The recorded image sequence (512 × 512 pixels) was used to yield changes in $\Delta\Psi_m$ signal throughout the recording. A set of neutral-density (ND4 and ND8) filters was adjusted to minimize dye bleaching. To ensure comparability between experiments, all settings of the confocal microscope (pinhole size, detector sensitivity, pixel dwell time, and laser power) were identical in all experiments.

Image Processing

Images were analyzed using MetaMorph 6.2 software (Universal Imaging, West Chester, PA) and the NIH ImageJ software 1.41 (National Institutes of Health, Bethesda, MD). Intensity of a cell-free area was subtracted as background. After background subtraction, image series were corrected for photobleaching by normalization to a monoexponential decay that was calculated from the average intensities for the whole recording. Time required to induce mPTP opening (t_{mPTP}) was determined from $\Delta\Psi_m$ recordings. The peak signal value over recorded region (50 μm^2) was normalized as 100%, and the lowest value as 0%. After normalization, the time required for a 50% decrease in signal was calculated and denoted as t_{mPTP}. In some experiments, the opening of mPTP was determined by constructing pseudolinescans or x, t plots vertically to wave of TMRE dissipation. With this analysis, each time frame is presented as a single pixel plotted on y axis (representing time), and the x axis represents the length of the selected region.

Experimental Protocol

Isolated cardiomyocytes were placed in a recording chamber on the stage of the confocal microscope, and cells were allowed to settle for 10 min. APC was induced by exposing myocytes to isoflurane (Baxter, Deerfield, IL) dispersed in Tyrode solution by sonication and delivered to cardiomyocytes using the airtight glass syringe and polyethylene tubing system. Isoflurane was administered for 20 min before 5 min of washout. Control cells did not receive isoflurane. To investigate the involvement of the PKC, APC was performed in the presence of the isoform-nonselective PKC inhibitor chelerythrine (1 μM; Sigma-Aldrich). To determine the PKC subclasses contribution, isoform-specific PKC-δ inhibitor rottlerin (0.2 μM; Sigma-Aldrich) and PKC-ε inhibitor myristoylated PKC-ε V1–2 peptide (εV1–2, 1 μM; Biomol Research, Plymouth, PA) were applied during APC. To confirm contribution of mPTP, myocytes were also treated with the inhibitors of mPTP cyclosporin A (0.5 μM; Calbiochem, La Jolla, CA) and bongkrekic acid (50 μM; A.G. Scientific, San Diego, CA). To verify that ROS triggered mPTP opening, ROS scavenger 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox,

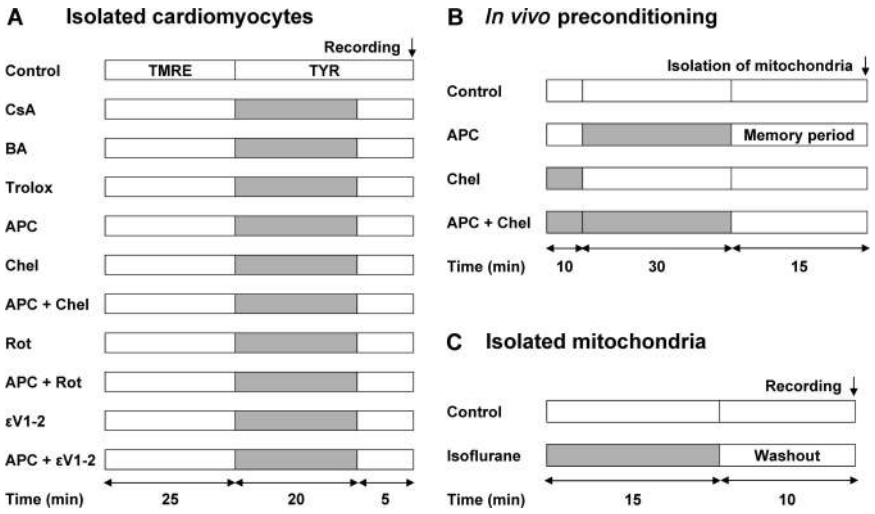

Fig. 1. Schematic illustration depicting the experimental protocols used in this study. Shaded regions indicate the application of drugs being tested. APC = isoflurane-induced preconditioning; BA = Bongkrekic acid; Chel = Chelerythrine; CsA = Cyclosporin A; Rot = Rottlerin; TMRE = Tetramethylrhodamine ethyl ester; TYR = Tyrode solution; εV1–2 = myristoylated PKC-ε V1–2 inhibitor.

2 mM; Calbiochem) was used. All experiments were conducted at room temperature. The protocols for all experimental groups are illustrated in figure 1. At the end of each experiment, samples of buffer containing isoflurane were collected and analyzed by gas chromatography (Gas chromatograph GC-8A; Shimadzu, Kyoto, Japan). The average concentration of isoflurane used in this study was 0.63 ± 0.3 mM.

Pretreatment of Rats and Isolation of Mitochondria

For measurement of mPTP opening in isolated mitochondria, rats were preconditioned *in vivo* with isoflurane in the absence or presence of chelerythrine. In anesthetized animals (Inactin, 100–150 mg/kg) tracheotomy was performed, and trachea was cannulated. Animals then underwent mechanical ventilation with a rodent ventilator (Harvard Apparatus 683, South Natick, MA), using air-oxygen mixture. In the APC group, isoflurane was administered for 30 min and discontinued 15 min before isolation of mitochondria. In the APC-chelerythrine group, chelerythrine was administered as intravenous bolus (5 mg/kg). Ten minutes after chelerythrine injection, rats received isoflurane. In chelerythrine group, rats received only chelerythrine. Control rats did not receive isoflurane or chelerythrine (fig. 1B). Isoflurane was administered *via* a vaporizer (Ohio Medical Products 100F, Madison, WI). Heart was excised after treatment, and mitochondria were isolated as described in the next paragraph. Isoflurane concentration was measured at the tip of the tracheotomy tube using an infrared gas analyzer that was calibrated with known standards. The concentration of isoflurane used for *in vivo* preconditioning was 1.4%.[34]

Cardiac mitochondria were isolated by homogenization and differential centrifugation as described previously.[10] The heart was quickly excised after thoracotomy, and the left ventricle was immersed in cold isolation buffer (in mM): 50 sucrose, 200 mannitol, 5 KH_2PO_4, 1 EGTA, 5 3-(N-morpholino)propanesulfonic acid, and 0.1% bovine serum albumin; pH 7.3 adjusted with KOH. The tissue was homogenized with Polytron homogenizer (IKA-Werke, Staufen, Germany), and mitochondria were then isolated by differential centrifugation. The final mitochondrial pellet was resuspended in cold isolation buffer without EGTA. Total protein concentration was assessed with detergent compatible protein kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Mitochondria were kept on ice and used within 4 h after isolation.

For isoflurane treatment of isolated mitochondria, the mitochondrial suspension was separated in two aliquots, which were subsequently diluted to 1 mg/ml mitochondria and incubated either with isoflurane (0.5 mM), which was dissolved in dimethyl sulfoxide, or with dimethyl sulfoxide alone for 15 min at room temperature. To remove isoflurane, both suspensions were then diluted in isolation buffer and centrifuged at 8000*g* for 10 min (fig. 1C).

Opening of mPTP in Isolated Mitochondria

Opening of mPTP in isolated cardiac mitochondria was assessed by measuring $\Delta\Psi_m$ using rhodamine 123 (50 nM; Invitrogen) in the presence of pyruvate (5 mM) and malate (5 mM). Excessive and/or prolonged mitochondrial Ca^{2+} accumulation is associated with opening of the mPTP, which accelerates ion exchanges across the inner mitochondrial membrane and eventually leads to a sudden loss of $\Delta\Psi_m$, indicating a massive depolarization due to mPTP opening. Isolated mitochondria (0.5 mg/ml) were exposed to 5 μM pulses of Ca^{2+} until $\Delta\Psi_m$ suddenly decreased. Specificity for mPTP was confirmed with cyclosporin A (1 μM). The concentration of Ca^{2+} ($\mu M\ mg^{-1}$ of mitochondrial protein) necessary to trigger mPTP pore opening was measured.

Western Blotting

Mitochondrial and cytosolic fractions from isoflurane-treated and untreated cardiomyocytes were prepared by

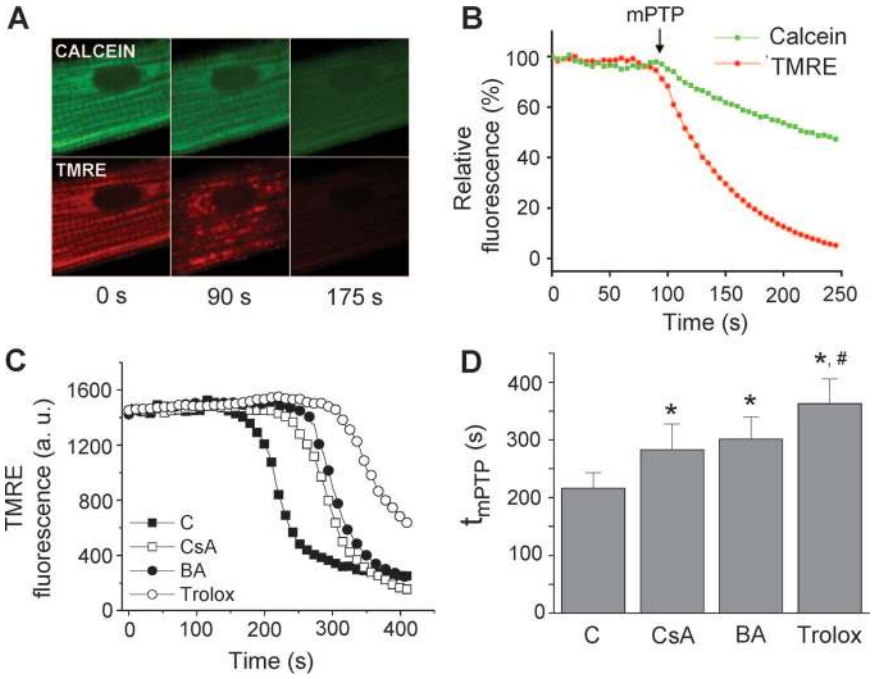

Fig. 2. Measurement of mitochondrial permeability transition pore (mPTP) opening in myocyte loaded with calcein and tetramethylrhodamine ethyl ester (TMRE). Myocytes were dual-loaded with TMRE (100 nM) and calcein-AM (1 μM) in the presence of cobalt chloride (2 mM). (*A*) Representative images at time 0 s denotes properties of mitochondria before laser illumination. Both TMRE and calcein are located inside the mitochondria, suggesting closed mPTP. Laser illumination induces redistribution of mitochondrial calcein to cytosol together with loss of TMRE signal at 90 s. At 175 s, the whole region of the myocyte (50 μm^2) has undergone global mitochondrial depolarization, and the mitochondrial localization of the calcein signal was lost. (*B*) Time course of calcein and TMRE fluorescence. The mean calcein signal decreases progressively with time of illumination concomitant with the loss of TMRE signal, indicating the opening of mPTP (*arrow*). (*C*) Original recordings showing the effect of reactive oxygen species (ROS) scavenger Trolox (2 mM) and blockers of mPTP cyclosporin A (0.5 μM) and bongkrekic acid (50 μM). (*D*) Summary graph compares average time necessary to decrease initial TMRE fluorescence to 50%. BA = bongkrekic acid; C = control; CsA = cyclosporin A; TMRE = tetramethylrhodamine ethyl ester; * $P < 0.05$ *versus* Control; # $P < 0.05$ *versus* CsA or BA. Values are means ± SD, n = 5.

differential centrifugation in 0.3 M mannitol, 0.1% bovine serum albumin, 2 mM EDTA, 10 mM HEPES, pH 7.4, as described previously.[35] Protein samples (50 μg) were separated on a 7.5% sodium dodecyl sulfate gel, and transfered to immunoblot membrane (Bio-Rad). Western blotting was performed using a 1:200 dilution of a rabbit polyclonal anti- PKC-ε antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were stripped and reprobed with an antibody against subunit I of cytochrome c oxidase (Santa Cruz Biotechnology) as a marker for mitochondria. The blots were scanned and analyzed with NIH ImageJ 1.41.

Statistical Analysis

Data were analyzed using Origin 7 software (OriginLab, Northampton, MA). Data are reported as means ± SD, and n refers to the number of experiments. In all experimental groups, cardiomyocytes or mitochondria were isolated from at least five different rats. Comparisons between groups were performed by one-way ANOVA and use of Tukey test for *post hoc* analysis. Differences with $P < 0.05$ were considered significant.

Results

Detection of mPTP Opening

Opening of mPTP can be detected in intact cells by measuring permeability of the inner mitochondrial membrane to the fluorescent dye calcein.[33] We tested whether photoexcitation induced dissipation of $\Delta\Psi_m$ coincides with calcein leakage from the mitochondria. Figure 2A shows a typical recording of TMRE fluorescence obtained from a 50 × 50-μm region, as assessed by confocal microscopy. Fluorescence at 590 nm (TMRE) and between 515 and 525 nm (calcein) was recorded simultaneously from the same region. Photoexcitation of the selected region induced calcein to move from mitochondria to the cytosol concurrent with the loss of $\Delta\Psi_m$. In the cytosol, calcein fluorescence was quenched by cobalt chloride, resulting in a more diffuse staining that indicated an opening of mPTP. Figure 2B illustrates TMRE and calcein fluorescence from the same regions plotted as a function of time. The role for the mPTP was also confirmed using mPTP blockers cyclosporin A and bongkrekic acid. As shown in figure 2C, dissipation of $\Delta\Psi_m$ was significantly delayed in the presence of 0.5 μM cyclosporin A and 50 μM bongkrekic acid, respectively. To investigate whether ROS formation was involved in the loss of $\Delta\Psi_m$, we examined the effect of the free radical scavenger Trolox.[28] Treatment with 2 mM Trolox significantly slowed $\Delta\Psi_m$ loss during laser-scan compared with control cell (fig. 2C). The data are summarized in figure 2D.

PKC Confers Isoflurane-induced Suppression of mPTP Opening

The effect of isoflurane on t_{mPTP} was first examined in the presence of isoform-nonspecific PKC inhibitor, chelerythrine (1 μM). Figure 3A shows representative recordings where APC by isoflurane produced a significant delay in t_{mPTP}. Chelerythrine prevented the isoflurane-

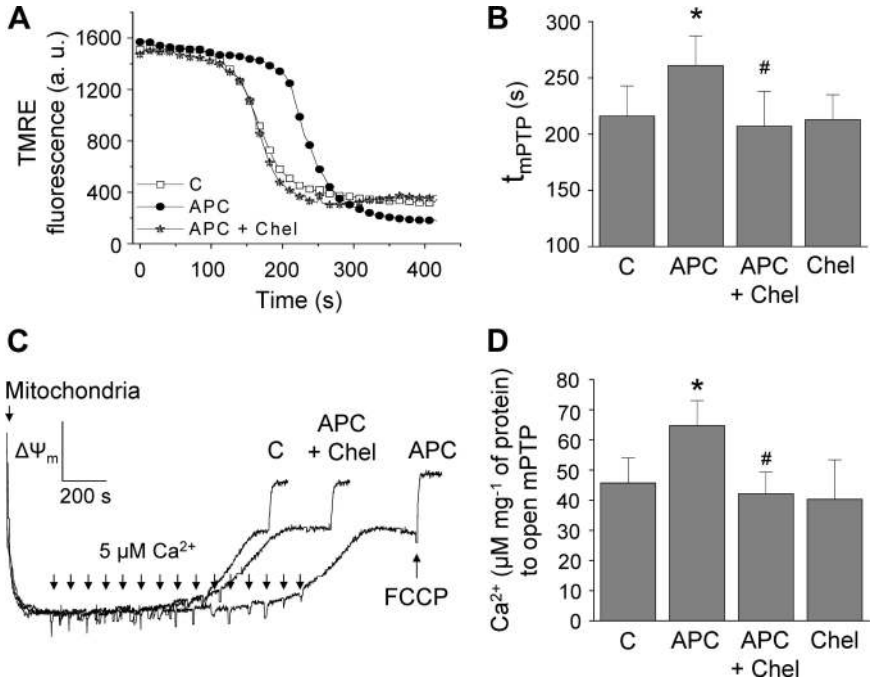

Fig. 3. Protein kinase C (PKC) inhibitor chelerythrine abolishes isoflurane-induced delay of mitochondrial permeability transition pore (mPTP) opening. (*A*) Representative recording of tetramethylrhodamine ethyl ester (TMRE) fluorescence from a 50-μm^2 region of myocyte. The cells were treated either with isoflurane (0.5 mM) or with isoflurane in the presence of chelerythrine (1 μM). (*B*) Summary of changes in mPTP opening (t_{mPTP}) in cardiomyocytes. (*C*) Original recordings showing the effect of APC on Ca^{2+} overload required for mPTP opening in isolated mitochondria. The Ca^{2+} concentration necessary to trigger massive depolarization of mitochondria due to mPTP opening was increased after APC. This effect was abolished when isoflurane was administered after rats received chelerythrine. (*D*) Summary graph shows Ca^{2+} concentration necessary to open mPTP in isolated mitochondria. APC = isoflurane-induced preconditioning; a. u. = arbitrary units; C = control; Chel = chelerythrine; FCCP = carbonylcyanide-p-trifluoromethoxyphenylhydrazone (mitochondrial uncoupler); $\Delta\Psi_m$ = mitochondrial membrane potential. * $P < 0.05$ *versus* control; # $P < 0.05$ *versus* APC. Values are means ± SD, n = 8 (myocytes), n = 5 (mitochondria).

induced effect. Chelerythrine at applied concentration had no significant effect on t_{mPTP} in control cells, as summarized in figure 3B.

The impact of *in vivo* preconditioning on Ca^{2+}-induced mPTP opening in isolated mitochondria was also assessed. Figure 3C shows recordings where mitochondria were challenged with incremental Ca^{2+} concentrations up to the point of dissipation of $\Delta\Psi_m$, indicating mPTP opening. In mitochondria from control animals, the Ca^{2+} concentration required to open the mPTP was 45 ± 8 $\mu M \cdot mg^{-1}$ protein. After APC, the Ca^{2+} concentration required to open the mPTP was significantly increased to 64 ± 8 $\mu M \cdot mg^{-1}$ protein ($P < 0.05$). This increase was attenuated when isoflurane was administered in the presence of PKC inhibitor chelerythrine (5 mg/kg), thus confirming the results obtained using isolated myocytes (fig. 3D).

To assess the effect of isoflurane in the absence of cytosolic components, isolated mitochondria were directly exposed to isoflurane before inducing mPTP with Ca^{2+}. The time course in figure 4A demonstrates that isoflurane was unable to delay mPTP opening. The Ca^{2+} concentration required for mPTP opening was not significantly higher in isoflurane exposed compared to untreated mitochondria (45 ± 8 $\mu M \cdot mg^{-1}$ control *vs.* 47 ± 12 $\mu M \cdot mg^{-1}$ isoflurane). Figure 4B summarizes the effects of isoflurane on mPTP in isolated mitochondria.

Effects of PKC-δ and PKC-ε Inhibition

To distinguish which PKC subclass is required for the APC-induced t_{mPTP} delay, PKC-specific isoform inhibitors were applied. Cells were subjected to APC with isoflurane in the presence of isoform-specific blockers, rottlerin (0.2 μM), or εV1–2 (1 μM). As illustrated in figure 5A, the delay in t_{mPTP} was still observed after APC in the presence of rottlerin, suggesting that PKC-δ is not involved for APC-induced delay of t_{mPTP}. However, delay of mPTP opening was completely blocked by 1 μM εV1–2. Both rottlerin and εV1–2, at the concentrations used, had no significant effect on t_{mPTP} in control cells, as shown in figure 5B. As illustrated by Western blot in figure 6A, PKC-ε concentration was increased signifi-

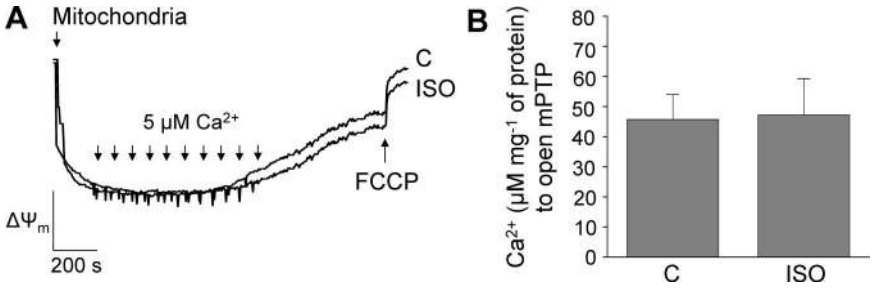

Fig. 4. Isoflurane applied directly to mitochondria is not protective against mitochondrial permeability transition pore (mPTP) opening. (*A*) Representative time courses of mPTP opening in mitochondria exposed to isoflurane directly (ISO) and control (C) mitochondria. The concentration of Ca^{2+} necessary to open mPTP was comparable in both groups. (*B*) Summary of the effect of ISO on the mPTP opening elicited by Ca^{2+} overload. FCCP = carbonylcyanide-p-trifluoromethoxyphenylhydrazone (mitochondrial uncoupler); ISO = isoflurane; $\Delta\Psi_m$ = mitochondrial membrane potential. Values are means ± SD; n = 7.

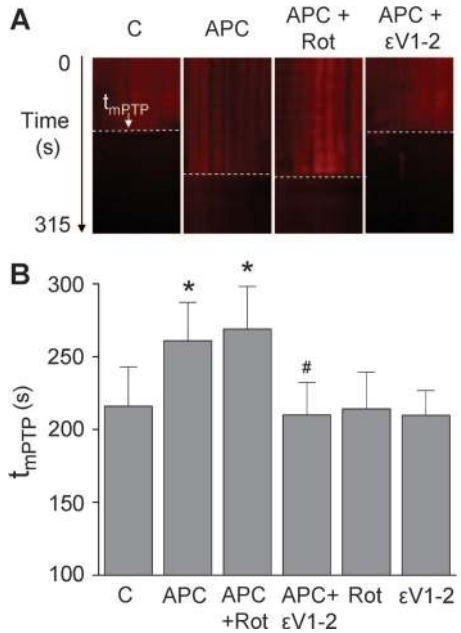

Fig. 5. Protein kinase C (PKC)-ε, but not PKC-δ, abolishes isoflurane-induced delay in mitochondrial permeability transition pore (mPTP) opening. (*A*) Representative pseudoline-scan images of tetramethylrhodamine ethyl ester (TMRE) fluorescence along mitochondrial rows; time progresses from *top* (total time = 315 s). The *dark regions* between vertical columns represent junctions between mitochondria. The sudden dissipation of TMRE fluorescence indicates $\Delta\Psi_m$ loss and mPTP opening (t_{mPTP}, *dotted line*). Isoflurane-induced suppression of mPTP opening was still observed when rottlerin was applied during the preconditioning period (APC + Rot), as evidenced by a persistent extension in the time required to induce mPTP opening compared with control myocytes. In contrast, specific PKC-ε inhibitor εV1–2 (APC + εV1–2) applied during the preconditioning period abolished isoflurane-induced t_{mPTP} delay. (*B*) Mean values for t_{mPTP}. Rottlerin (0.2 μM) and εV1–2 (1 μM) alone did not affect t_{mPTP} in nonpreconditioned myocytes. APC = isoflurane-induced preconditioning; C = control; Rot = rottlerin; εV1–2 = myristoylated PKC-εV1–2 inhibitor. * *P* < 0.05 *versus* control; # *P* < 0.05 *versus* APC. Values are means ± SD, n = 7.

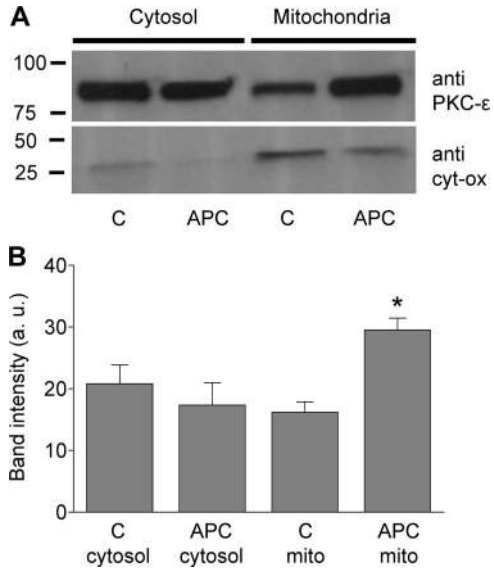

Fig. 6. Isoflurane preconditioning induces translocation of protein kinase C (PKC)-ε toward mitochondria. (*A*) Representative Western blot shows a higher abundance of PKC-ε in the mitochondrial (mito) fraction from APC compared to non-APC cardiomyocytes. (*B*) Graph shows summarized results. APC = isoflurane-induced preconditioning; a. u. = arbitrary units; C = control; cyt-ox = cytochrome c oxidase (mitochondrial marker); PKC-ε = protein kinase C-ε. * *P* < 0.05 *versus* control. Values are means ± SD, obtained from three independent experiments.

cantly after isoflurane exposure in the mitochondrial fraction of cardiomyocytes, whereas it was slightly decreased in the cytosol (n = 3/group, data summary in fig. 6B). These results indicate that APC induces translocation of PKC-ε toward mitochondria and that PKC-ε is involved in the mechanism of isoflurane-induced delay of mPTP opening.

Discussion

The current study investigated the role of PKC in isoflurane-induced delay in mPTP opening. Our results indicate that isoflurane treatment triggers PKC activation, which subsequently leads to inhibition of mPTP opening. More specifically, inhibition of PKC-ε translocation with εV1–2 abrogated the isoflurane-induced delay in mPTP opening, indicating a PKC-ε–dependent signal transduction pathway is involved. Accordingly, isoflurane caused translocation of PKC-ε toward mitochondria as evidenced by Western blotting. The fact that APC applied after chelerythrine *in vivo* was not effective in delaying mPTP opening in isolated mitochondria underscored the importance of PKC signaling towards mitochondria as a target. In addition, isoflurane exposure of isolated mitochondria was not protective against mPTP opening, further suggesting that cytosolic proteins such as PKC play an important role in signal transduction to mitochondria in other to delay mPTP opening. The protective effect of isoflurane observed in the current study appears to be in part independent of direct, depolarizing effects of the anesthetic on mitochondria, as the cardioprotective benefits of isoflurane were demonstrated in cells with the same initial $\Delta\Psi_m$.[10]

Mitochondria have been known as central mediators of cell survival in ischemia and reperfusion injury. Moreover, mPTP opening has been recognized as an important mediator of cell death.[7] Almost 30 yr ago, Hunter and Harworth described increased permeability of the inner mitochondrial membrane caused with high Ca^{2+}.[36] Later, the role of mPTP opening in myocardial ischemia and reperfusion injury was also reported.[37] The molecular composition of mPTP is still under debate. Many models consider that mPTP is a multi-protein complex that spans inner and outer mitochondrial membranes under certain conditions. Proposed components forming the pore include the voltage-dependent anion channel, adenine nucleotide transporter, and cyclophilin-D.[38] However, knockout experiments questioned the

involvement of voltage-dependent anion channel and adenine nucleotide transporter in mPTP formation. It is now thought that they may have a regulatory function on mPTP, whereas a critical role is still attributed to cyclophilin-D.[39] Pore opening results in the collapse of the $\Delta\Psi_m$, respiratory uncoupling, release of cytochrome c, and apoptosis-inducing factors.[7] Cardioprotection against ischemia and reperfusion injury by ischemic and pharmacologic preconditioning has been previously shown to involve prevention of mPTP opening.[12] Mitochondrial potassium channel opener diazoxide and PKC activation by phorbol-myristate acetate both had a protective effect against mPTP opening induced by high Ca^{2+} loads in isolated mitochondria.[13] Modulation of mitochondrial function has also been considered a key component of cardioprotection by volatile agents.[10] Moreover, Piriou *et al.* showed that APC by desflurane inhibits Ca^{2+}-induced mPTP opening in isolated mitochondria.[11]

The mechanisms by which anesthetics modulate mPTP opening are not completely elucidated. Previous studies demonstrated that the ischemic preconditioning-induced cardioprotection is PKC-dependent[4,40] and PKC isoform-specific, with δ and ε isoforms being the most important. Overexpression and association of PKC-ε with different mitochondrial proteins, including components of mPTP, leads to subsequent inhibition of mPTP.[41] It has also been reported that PKC delays the opening of mPTP through inhibition of glycogen synthase kinase-3β.[12] APC also causes activation of PKC[42] and, more specifically, translocation of PKC-ε and PKC-δ,[14,19,24] although controversy exists regarding the exact role of each isoform. APC with sevoflurane stimulates translocation of PKC-δ to mitochondria and PKC-ε to sarcolemma.[14] PKC-δ is translocated to mitochondria after preconditioning with opioids and adenosine.[43,44] In contrast, isoflurane caused PKC-ε translocation towards mitochondria and PKC-δ towards sarcolemmal membrane,[14,24] and increased PKC-ε phosphorylation.[19] In isolated hearts, pharmacologic inhibition of PKC-ε abolished sevoflurane-induced cardioprotection.[18] The importance of PKC-ε translocation toward mitochondria was also shown in human myocardial tissue samples of patients exposed to sevoflurane preconditioning undergoing on-pump coronary artery bypass graft surgery.[45] This is in agreement with our observation that PKC-ε is responsible for isoflurane-induced effect on mPTP. Interestingly, PKC-ε has been suggested to be constitutively present within cardiac mitochondria; as such, it directly confers protection without requiring translocation.[46] In our study, the observations that isolated mitochondria were protected from Ca^{2+}-induced mPTP opening after *in vivo* exposure to isoflurane and that protection was reversed by chelerythrine strongly support the role of cytosolic signaling pathways targeting mitochondria, in agreement with our PKC-ε translocation data. In fact, our results that mitochondria directly exposed to isoflurane did not exhibit delay in mPTP opening suggest that mitochondrial PKC-ε is not involved in the isoflurane effect on mPTP. Other groups have also found that translocation of PKC-ε is required for myocardial protection against ischemia and reperfusion injury.[23,40,47]

Our study has a few limitations. We used a cellular model of oxidative stress to study the mechanism of APC-induced delay of mPTP opening.[12,28,31,37,48] This model simulates ROS production during the reperfusion of ischemic myocardium and may not include other contributors to mPTP opening in cardiomyocytes during reperfusion. On the other hand, our experiments on isolated mitochondria strongly support the protective effect of isoflurane on Ca^{2+}-induced mPTP formation. Rottlerin is known to inhibit PKC-δ more potently than other PKC isoforms,[14–16,24,49] but other unspecific inhibitory effects may also exist. We did not investigate the effect of isoflurane preconditioning or inhibition of PKC on the outcome of ischemia and reoxygenation injury. However, other studies have confirmed involvement of PKC in anesthetic preconditioning-induced protection against ischemia and reoxygenation injury.[18,19]

In conclusion, our study shows for the very first time that isoflurane activates PKC-ε–dependent signaling pathway targeted towards mitochondria, leading to a delay in mPTP opening under conditions of oxidative stress.

We thank Samantha J. Mueller, B.S., Research Technologist, for technical assistance in isolation of ventricular myocytes and David A. Schwabe, B.S., Research Technologist, for assistance in rat surgical procedures. We also thank Mary B. Ziebell, Research Technologist, for isoflurane measurements and Terri L. Misorski, A.A.S., Program Coordinator, for editorial assistance (all from Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin).

References

1. Stadnicka A, Marinovic J, Ljubkovic M, Bienengraeber MW, Bosnjak ZJ: Volatile anesthetic-induced cardiac preconditioning. J Anesth 2007; 21:212–9

2. Kersten JR, Schmeling TJ, Pagel PS, Gross GJ, Warltier DC: Isoflurane mimics ischemic preconditioning *via* activation of K(ATP) channels: Reduction of myocardial infarct size with an acute memory phase. ANESTHESIOLOGY 1997; 87:361–70

3. Murry CE, Jennings RB, Reimer KA: Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74:1124–36

4. Zaugg M, Lucchinetti E, Uecker M, Pasch T, Schaub MC: Anaesthetics and cardiac preconditioning. Part I, Signalling and cytoprotective mechanisms. Br J Anaesth 2003; 91:551–65

5. Marinovic J, Bosnjak ZJ, Stadnicka A: Preconditioning by isoflurane induces lasting sensitization of the cardiac sarcolemmal adenosine triphosphate-sensitive potassium channel by a protein kinase C-delta-mediated mechanism. ANESTHESIOLOGY 2005; 103:540–7

6. Aizawa K, Turner LA, Weihrauch D, Bosnjak ZJ, Kwok WM: Protein kinase C-epsilon primes the cardiac sarcolemmal adenosine triphosphate-sensitive potassium channel to modulation by isoflurane. ANESTHESIOLOGY 2004; 101:381–9

7. Honda HM, Ping P: Mitochondrial permeability transition in cardiac cell injury and death. Cardiovasc Drugs Ther 2006; 20:425–32

8. Riess ML, Camara AK, Novalija E, Chen Q, Rhodes SS, Stowe DF: Anesthetic preconditioning attenuates mitochondrial Ca2+ overload during ischemia in Guinea pig intact hearts: Reversal by 5-hydroxydecanoic acid. Anesth Analg 2002; 95:1540–6

9. Kevin LG, Novalija E, Riess ML, Camara AK, Rhodes SS, Stowe DF: Sevoflurane exposure generates superoxide but leads to decreased superoxide during ischemia and reperfusion in isolated hearts. Anesth Analg 2003; 96:949–55

10. Ljubkovic M, Mio Y, Marinovic J, Stadnicka A, Warltier DC, Bosnjak ZJ, Bienengraeber M: Isoflurane preconditioning uncouples mitochondria and protects against hypoxia-reoxygenation. Am J Physiol Cell Physiol 2007; 292: C1583–90

11. Piriou V, Chiari P, Gateau-Roesch O, Argaud L, Muntean D, Salles D, Loufouat J, Gueugniaud PY, Lehot JJ, Ovize M: Desflurane-induced preconditioning alters calcium-induced mitochondrial permeability transition. Anesthesiology 2004; 100:581–8

12. Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, Olson EN, Sollott SJ: Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. J Clin Invest 2004; 113:1535–49

13. 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; 99:3312–7

14. Uecker M, Da Silva R, Grampp T, Pasch T, Schaub MC, Zaugg M: Translocation of protein kinase C isoforms to subcellular targets in ischemic and anesthetic preconditioning. Anesthesiology 2003; 99:138–47

15. Bouwman RA, Musters RJ, van Beek-Harmsen BJ, de Lange JJ, Lamberts RR, Loer SA, Boer C: Sevoflurane-induced cardioprotection depends on PKC-alpha activation *via* production of reactive oxygen species. Br J Anaesth 2007; 99: 639–45

16. Bouwman RA, Musters RJ, van Beek-Harmsen BJ, de Lange JJ, Boer C: Reactive oxygen species precede protein kinase C-delta activation independent of adenosine triphosphate-sensitive mitochondrial channel opening in sevoflurane-induced cardioprotection. Anesthesiology 2004; 100:506–14

17. Toller WG, Montgomery MW, Pagel PS, Hettrick DA, Warltier DC, Kersten JR: Isoflurane-enhanced recovery of canine stunned myocardium: Role for protein kinase C? Anesthesiology 1999; 91:713–22

18. Novalija E, Kevin LG, Camara AK, Bosnjak ZJ, Kampine JP, Stowe DF: Reactive oxygen species precede the epsilon isoform of protein kinase C in the anesthetic preconditioning signaling cascade. Anesthesiology 2003; 99:421–8

19. Obal D, Weber NC, Zacharowski K, Toma O, Dettwiler S, Wolter JI, Kratz M, Mullenheim J, Preckel B, Schlack W: Role of protein kinase C-epsilon (PKCepsilon) in isoflurane-induced cardioprotection. Br J Anaesth 2005; 94:166–73

20. Chen L, Hahn H, Wu G, Chen C-H, Liron T, Schechtman D, Cavallaro G, Banci L, Guo Y, Bolli R, Dorn GW II, Mochly-Rosen D: Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and varepsilon PKC. Proc Natl Acad of Sci U S A 2001; 98:11114–9

21. Churchill EN, Mochly-Rosen D: The roles of PKCdelta and epsilon isoenzymes in the regulation of myocardial ischaemia/reperfusion injury. Biochem Soc Trans 2007; 35:1040–2

22. Budas GR, Mochly-Rosen D: Mitochondrial protein kinase Cepsilon (PKCepsilon): emerging role in cardiac protection from ischaemic damage. Biochem Soc Trans 2007; 35:1052–4

23. Baines CP, Zhang J, Wang G-W, Zheng Y-T, Xiu JX, Cardwell EM, Bolli R, Ping P: Mitochondrial PKCε and MAPK form signaling Modules in the murine heart: Enhanced mitochondrial PKCε-MAPK interactions and differential MAPK activation in PKCε-induced cardioprotection. Circ Res 2002; 90:390–7

24. Ludwig LM, Weihrauch D, Kersten JR, Pagel PS, Warltier DC: Protein kinase C translocation and Src protein tyrosine kinase activation mediate isoflurane-induced preconditioning *in vivo*: Potential downstream targets of mitochondrial adenosine triphosphate-sensitive potassium channels and reactive oxygen species. Anesthesiology 2004; 100:532–9

25. Zaugg M, Lucchinetti E, Spahn DR, Pasch T, Schaub MC: Volatile anesthetics mimic cardiac preconditioning by priming the activation of mitochondrial K(ATP) channels *via* multiple signaling pathways. Anesthesiology 2002; 97:4–14

26. Guide for the Care and Use of Laboratory Animals/Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. 7th ed. Washington, DC, National Academy Press, 1996

27. Huser J, Blatter LA: Fluctuations in mitochondrial membrane potential caused by repetitive gating of the permeability transition pore. Biochem J 1999; 343:311–7

28. Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ: Reactive oxygen species (ROS)-induced ROS release: A new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. J Exp Med 2000; 192:1001–14

29. Huser J, Rechenmacher CE, Blatter LA: Imaging the permeability pore transition in single mitochondria. Biophys J 1998; 74:2129–37

30. Foote CS: Mechanisms of photosensitized oxidation: There are several different types of photosensitized oxidation which may be important in biological systems. Science 1968; 162:963–70

31. Duchen MR, Leyssens A, Crompton M: Transient mitochondrial depolarizations reflect focal sarcoplasmic reticular calcium release in single rat cardiomyocytes. J Cell Biol 1998; 142:975–88

32. Brady NR, Elmore SP, van Beek JJ, Krab K, Courtoy PJ, Hue L, Westerhoff HV: Coordinated behavior of mitochondria in both space and time: A reactive oxygen species-activated wave of mitochondrial depolarization. Biophys J 2004; 87:2022–34

33. Petronilli V, Miotto G, Canton M, Brini M, Colonna R, Bernardi P, Di Lisa F: Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. Biophys J 1999; 76:725–34

34. Mazze RI, Rice SA, Baden JM: Halothane, isoflurane, and enflurane MAC in pregnant and nonpregnant female and male mice and rats. Anesthesiology 1985; 62:339–41

35. Ljubkovic M, Marinovic J, Fuchs A, Bosnjak ZJ, Bienengraeber M: Targeted expression of Kir6.2 in mitochondria confers protection against hypoxic stress. J Physiol 2006; 577:17–29

36. Hunter DR, Haworth RA: The Ca2+-induced membrane transition in mitochondria. I. The protective mechanisms. Arch Biochem Biophys 1979; 195: 453–9

37. Crompton M, Costi A, Hayat L: Evidence for the presence of a reversible Ca2+-dependent pore activated by oxidative stress in heart mitochondria. Biochem J 1987; 245:915–8

38. Crompton M: The mitochondrial permeability transition pore and its role in cell death. Biochem J 1999; 341:233–49

39. Juhaszova M, Wang S, Zorov DB, Bradley Nuss H, Gleichmann M, Mattson MP, Sollott SJ: The identity and regulation of the mitochondrial permeability transition pore: Where the known meets the unknown. Ann N Y Acad Sci 2008; 1123:197–212

40. Inagaki K, Churchill E, Mochly-Rosen D: Epsilon protein kinase C as a potential therapeutic target for the ischemic heart. Cardiovasc Res 2006; 70: 222–30

41. Baines CP, Song CX, Zheng YT, Wang GW, Zhang J, Wang OL, Guo Y, Bolli R, Cardwell EM, Ping P: Protein kinase C-epsilon interacts with and inhibits the permeability transition pore in cardiac mitochondria. Circ Res 2003; 92:873–80

42. Cope DK, Impastato WK, Cohen MV, Downey JM: Volatile anesthetics protect the ischemic rabbit myocardium from infarction. Anesthesiology 1997; 86:699–709

43. Fryer RM, Wang Y, Hsu AK, Gross GJ: Essential activation of PKC-delta in opioid-initiated cardioprotection. Am J Physiol Heart Circ Physiol 2001; 280: H1346–53

44. Kudo M, Wang Y, Xu M, Ayub A, Ashraf M: Adenosine A(1) receptor mediates late preconditioning *via* activation of PKC-delta signaling pathway. Am J Physiol Heart Circ Physiol 2002; 283:H296–301

45. Julier K, da Silva R, Garcia C, Bestmann L, Frascarolo P, Zollinger A, Chassot PG, Schmid ER, Turina MI, von Segesser LK, Pasch T, Spahn DR, Zaugg M: Preconditioning by sevoflurane decreases biochemical markers for myocardial and renal dysfunction in coronary artery bypass graft surgery: A double-blinded, placebo-controlled, multicenter study. Anesthesiology 2003; 98:1315–27

46. Jaburek M, Costa AD, Burton JR, Costa CL, Garlid KD: Mitochondrial PKC epsilon and mitochondrial ATP-sensitive K+ channel copurify and coreconstitute to form a functioning signaling module in proteoliposomes. Circ Res 2006; 99:878–83

47. Ping P, Zhang J, Qiu Y, Tang XL, Manchikalapudi S, Cao X, Bolli R: Ischemic preconditioning induces selective translocation of protein kinase C isoforms epsilon and eta in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. Circ Res 1997; 81:404–14

48. Hausenloy DJ, Yellon DM, Mani-Babu S, Duchen MR: Preconditioning protects by inhibiting the mitochondrial permeability transition. Am J Physiol Heart Circ Physiol 2004; 287:H841–9

49. Davies SP, Reddy H, Caivano M, Cohen P: Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 2000; 351:95–105