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Reactive Oxygen Species Precede Protein Kinase C-δ Activation Independent of Adenosine Triphosphate–sensitive Mitochondrial Channel Opening in Sevoflurane-induced Cardioprotection

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Background: **In the current study, the authors investigated the distinct role and relative order of protein kinase C (PKC)-δ, adenosine triphosphate–sensitive mitochondrial K^+ (mito K^+_{ATP}) channels, and reactive oxygen species (ROS) in the signal transduction of sevoflurane-induced cardioprotection and specifically addressed their mechanistic link.**

Methods: **Isolated rat trabeculae were preconditioned with 3.8% sevoflurane and subsequently subjected to an ischemic protocol by superfusion of trabeculae with hypoxic, glucose-free buffer (40 min) followed by 60 min of reperfusion. In addition, the acute affect of sevoflurane on PKC-δ and PKC-ϵ translocation and nitrotyrosine formation was established with use of immunofluorescent analysis. The inhibitors chelerythrine (6 μM), rottlerin (1 μM), 5-hydroxydecanoic acid sodium (100 μM), and n-(2-mercaptopropionyl)-glycine (300 μM) were used to study the particular role of PKC, PKC-δ, mito K^+_{ATP}, and ROS in sevoflurane-related intracellular signaling.**

Results: **Preconditioning of trabeculae with sevoflurane preserved contractile function after ischemia. This contractile preservation was dependent on PKC-δ activation, mito K^+_{ATP} channel opening, and ROS production. In addition, on acute stimulation by sevoflurane, PKC-δ but not PKC-ϵ translocated to the sarcolemmal membrane. This translocation was inhibited by PKC inhibitors and ROS scavenging but not by inhibition of mito K^+_{ATP} channels. Furthermore, sevoflurane directly induced nitrosylation of sarcolemmal proteins, suggesting the formation of peroxynitrite.**

Conclusions: **In sevoflurane-induced cardioprotection, ROS release but not mito K^+_{ATP} channel opening precedes PKC-δ activation. Sevoflurane induces sarcolemmal nitrotyrosine formation, which might be involved in the recruitment of PKC-δ to the cell membrane.**

THE volatile anesthetic sevoflurane makes the heart more resistant to ischemia and reperfusion damage in experimental models[1–3] as well as in humans.[4,5] We and others have shown that protein kinase C (PKC), adenosine triphosphate–sensitive mitochondrial K^+ (mito K^+_{ATP}) channels, and reactive oxygen species (ROS) are necessary in the signal transduction of volatile anesthetic–induced cardioprotection.[3,6,7] Volatile anesthetics directly activate PKC[8] and induce intracellular ROS production,[7] either through modulation of mito K^+_{ATP} function[9] or through modulation of the electron transport chain.[10] ROS modulates PKC function directly *via* oxidative modification or indirectly *via* tyrosine phosphorylation.[11,12] In addition, mito K^+_{ATP} channels can be directly activated by ROS.[13] Consequently, a complex mechanistic link exists between these three signaling molecules, and so far, their relative order in anesthetic-induced cardioprotection has not been established.

Cardiomyocytes express a variety of PKC isoforms modifying the function of a wide range of (end-effector) target substrates by phosphorylation. The involved PKC isoforms that contribute to preconditioning differ among species and by the type of preconditioning stimulus.[14,15] In particular, the class of novel PKCs, *i.e.*, PKC-δ and PKC-ϵ, are involved in several preconditioning stimuli.[16–20] Recently, an essential role for PKC-δ activation was acknowledged in the signal transduction of isoflurane-induced cardioprotection in isolated rat hearts.[21] Furthermore, it has been shown that PKC-ϵ activation is necessary as a signaling molecule in sevoflurane-induced cardioprotection in guinea pig hearts.[22] In addition, different translocation patterns in response to volatile anesthetics have been shown for these PKC isoforms. In response to isoflurane, PKC-δ translocates to mitochondria, whereas PKC-ϵ translocates to the intercalated discs in rat myocardium.[21] However, in human atrial tissue, sevoflurane induced PKC-δ translocation toward the sarcolemma in addition to translocation of PKC-ϵ to nuclei, intercalated discs, and mitochondriae.[23]

In the current study, we studied the contribution of the PKC-δ and PKC-ϵ isoforms in isolated rat trabeculae in the signal transduction of sevoflurane-induced cardioprotection. Second, we investigated whether PKC isoform translocation and/or activation seems to be upstream or downstream of ROS formation and opening of mito K^+_{ATP} channels. Our study provides more insight in the sequence of intracellular signaling involved in sevoflurane-induced cardioprotection.

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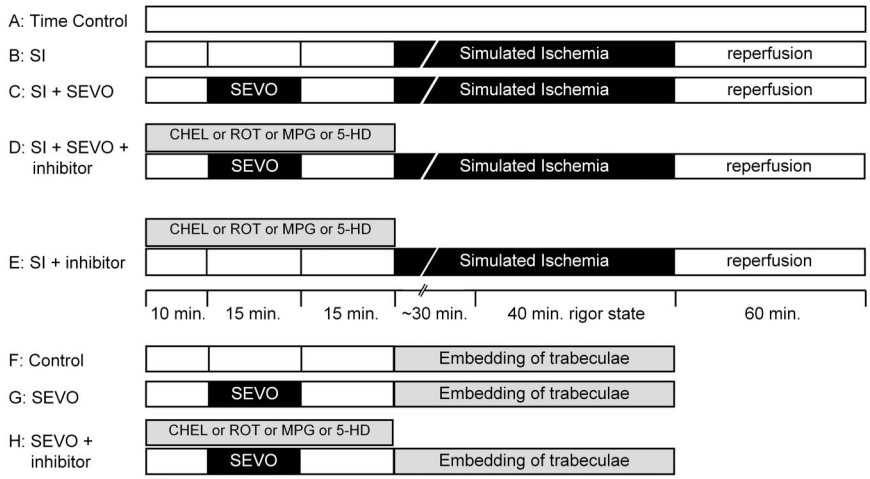

Fig. 1. Overview of the applied experimental protocols for 17 experimental groups of randomized isolated trabeculae. The protocols for functional contractile experiments are indicated as protocols *A–E* and protocols for immunofluorescent staining are depicted in *F–H*. 5-HD = 5-hydroxydecanoic acid sodium; CHEL = chelerythrine; MPG = n-(2-mercaptopropionyl)-glycine; ROT = rottlerin; SEVO = sevoflurane; SI = simulated ischemia.

Materials and Methods

Animals

The study was performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the VU University Medical Center (Amsterdam, The Netherlands). Male Wistar rats (weight, 250–400 g; Harlan Netherland, Horst, The Netherlands) were anesthetized with sodium pentobarbital (80 mg/kg intraperitoneally; Ceva Sante Animale BV, Maassluis, The Netherlands). After 1,000 U heparin (Leo Pharma, Breda, The Netherlands) was intravenously injected, the heart was quickly removed and subsequently retrogradely perfused through the aorta with Tyrode buffer (95% O_2 and 5% CO_2, pH 7.4) at room temperature. Standard solution contained 120 mM NaCl, 1.22 mM $MgSO_4.7H_2O$, 1.99 mM NaH_2PO_4, 27.0 mM $NaHCO_3$, 5.0 mM KCl, 1 mM $CaCl_2$, and 10 mM glucose. During dissection, 30 mM 2,3-butanedione monoxime and 15 mM KCl was added to the solution to protect the myocardium.[24] Subsequently, a suitable right ventricular trabecula (length, 2–5 mm; diameter, < 0.2 mm),[25] running from the free ventricle wall to the tricuspid valve, was carefully dissected.

Experimental Setup for Isolated Trabeculae

Muscles were mounted between a force transducer (AE801; SensoNor, Horten, Norway) and a micromanipulator in an airtight organ bath. After mounting, superfusion was started with normal Tyrode buffer at 27°C with a flow of 2 ml/min, and trabeculae were stimulated with two platinum field electrodes (duration, 5 ms; stimulation frequency, 0.5 Hz). The stimulation voltage was set at two times the stimulation threshold. Subsequently, the length of individual trabeculae was set at 95% of the maximal length as determined by a force–length relation, followed by a stabilization period of 40 min. The stimulation frequency and temperature were then decreased to 0.2 Hz and 24°C, respectively, followed by another 20 min of stabilization. After this stabilization period, the initial developed contractile force ($F_{dev,start}$) and maximal force ($F_{max,start}$), as determined by a post-extrasystolic potentiation protocol, were recorded. Post-extrasystolic potentiation determines the contractile reserve of trabeculae by maximally filling the sarcoplasmic reticulum with Ca^{2+}.[26] Trabeculae that did not stabilize, spontaneously contracted, or had a saturation ($= F_{dev}/F_{max}$) less than 25% or greater than 75% were excluded. The contractile forces measured by the force transducer were digitized and recorded with a sampling rate of 500 Hz.

Measurements of Contractile Force

Trabeculae were randomly assigned to distinct experimental groups as represented in figure 1. Trabeculae (except for time controls) were subjected to simulated ischemia (SI) by switching to superfusion with hypoxic glucose-free Tyrode (95% N_2 and 5% CO_2, partial pressure of oxygen [Po_2] $\leq$ 4 mmHg) and by increasing the stimulation frequency to 1 Hz. Ischemia decreased F_{dev} to zero followed by an increase in passive force after starting SI. When the passive force increased to 50% of $F_{max,start}$ (= start of rigor), trabeculae were subjected to SI for another 40 min and were subsequently reperfused for 60 min with normal oxygenated buffer solution. After reperfusion, recovery of F_{dev} ($F_{dev,rec}$) and F_{max} ($F_{max,rec}$) were determined and expressed as percentages of $F_{dev,start}$ and $F_{max,start}$, respectively. Except for the time and inhibitor control experiments, trabeculae were preconditioned for 15 min with normal Tyrode saturated with 3.8 vol% vaporized sevoflurane (Sevorane; Abbott B.V., Hoofddorp, The Netherlands) 30 min before the onset of SI. The volume percentage of sevoflurane in the gas phase above the Tyrode was continuously monitored by a calibrated infrared anesthetic monitor (Capnomac Ultima; Datex, Helsinki, Finland). Sevoflurane was washed out 15 min before the onset of SI.

In four additional groups, trabeculae were superfused with the PKC catalytic site inhibitor chelerythrine (6 μM; Sigma-Aldrich Chemie BV, Zwijndrecht, The Nether-

Table 1. General Characteristics of the Isolated Trabeculae

	CSA, mm^2	$F_{dev,start}$, mN/mm^2	$F_{dev,start}/F_{max,start}$, %	F_{pas}/F_{max}, %	Time to Rigor, min
Time	0.060 ± 0.02	51 ± 9.0	62 ± 5	3.1 ± 0.27	—
SI	0.038 ± 0.01	50 ± 11	47 ± 7	3.0 ± 0.32	26 ± 3
SI + sevoflurane	0.053 ± 0.02	59 ± 13	45 ± 6	3.8 ± 1.3	30 ± 3
SI + sevoflurane + chelerythrine	0.059 ± 0.01	26 ± 4.2	49 ± 8	2.9 ± 0.26	25 ± 2
SI + sevoflurane + rottlerin	0.077 ± 0.03	55 ± 22	44 ± 6	3.4 ± 0.52	20 ± 3
SI + sevoflurane + MPG	0.041 ± 0.008	45 ± 5.1	49 ± 8	3.4 ± 0.46	42 ± 5*
SI + sevoflurane + 5-HD	0.048 ± 0.01	73 ± 0.34	54 ± 3	3.6 ± 0.53	26 ± 1
SI + chelerythrine	0.061 ± 0.02	55 ± 22	48 ± 13	3.9 ± 0.45	25 ± 1
SI + rottlerin	0.073 ± 0.02	52 ± 8.9	56 ± 6	5.0 ± 1.1	20 ± 3
SI + MPG	0.054 ± 0.02	60 ± 14.0	64 ± 3	3.1 ± 0.21	36 ± 6
SI + 5-HD	0.0452 ± 0.02	52 ± 14	43 ± 10	5.9 ± 1.2	33 ± 7

* $P < 0.05$ compared with the experimental group simulated ischemia (SI) + sevoflurane + rottlerin and SI + rottlerin.

CSA = cross-sectional area; $F_{dev,start}$ = initial developed force; $F_{dev,start}/F_{max,start}$ = ratio between initial developed force and initial maximal force as determined by post-extrasystolic potentiation (PESP); F_{pas}/F_{max} = ratio between initial passive force and initial maximal force as determined by PESP; 5-HD = 5-hydroxy decanoic acid sodium; MPG = n-(2-mercaptopropionyl)-glycine.

lands), the specific inhibitor of PKC-δ rottlerin (1 μM; Sigma-Aldrich Chemie BV), the ROS scavenger n-(2-mercaptopropionyl)-glycine (MPG; 300 μM; Sigma-Aldrich Chemie BV), or the mito K^+_{ATP} channel inhibitor 5-hydroxydecanoic acid sodium (5-HD; 100 μM; Sigma-Aldrich Chemie BV) 10 min before preconditioning with sevoflurane until the onset of SI (fig. 1). The intrinsic effects of the distinct inhibitors on $F_{dev,rec}$ after SI were studied in individual inhibitor control experiments.

Immunohistochemical Analysis

Intracellular translocation and/or activation of distinct signaling molecules was visualized by immunofluorescent staining and subsequent three-dimensional digital imaging microscopy. Isolated trabeculae were subjected to similar protocols as compared with the force measurement protocols including the application of inhibitors. However, trabeculae were immediately embedded in gelatin, rapidly frozen in liquid nitrogen, and stored in −80°C until further use after the inhibitor washout period. For immunofluorescent staining, sections were paraformaldehyde fixated and incubated with a primary antibody (rabbit) raised against rat PKC-δ (662–673) or PKC-ϵ (728–737; both obtained from Research & Diagnostic Antibodies, Benicia, CA). Sections were then incubated with a fluorescein isothiocyanate–labeled swine anti-rabbit secondary antibody (F0205; DakoCytomation BV, Heverlee, The Netherlands). Simultaneously, sections were counterstained for the sarcolemma with 10% (vol/vol) wheat germ agglutinin (W-7024; Molecular Probes, Leiden, The Netherlands). Finally, nuclei were stained with 4',6 diamidino-2-phenylindole (DAPI)–containing mounting medium (H1200, Vectashield; Vector Laboratories, Burlingame, CA). Separate sections were incubated with a primary antibody (rabbit) raised against rat nitrotyrosine (A-21285; Molecular Probes) and then incubated with a secondary antibody as described above.

Sections were qualitatively analyzed with use of an inverted digital imaging microscopy workstation (Axiovert 200 Marianas; Carl Zeiss, Sliedrecht, The Netherlands) equipped with a motorized stage and multiple fluorescent channels. A cooled charge-coupled device camera (Cooke Sensicam; Cooke Co., Eugene, OR) was used to record images. Exposures, objective, montage, and pixel binning were automatically recorded and stored in memory. Dedicated imaging and analysis software (SlideBook, version 3.11) was obtained from Intelligent Imaging Innovations (Denver, CO) and included advanced deconvolution techniques.

Statistical Analysis

The sample size of each experimental group was 6, except for the inhibitor control groups (n = 5) and the groups for immunofluorescent analysis (n = 3). Data were evaluated for normal distribution, and a one-way analysis of variance was performed, followed by a Tukey *post hoc* analysis, to determine differences between the experimental groups. A *P* value less than 0.05 was considered to reflect a significant difference. All values are represented as mean ± SEM.

Results

General Characteristics

Table 1 shows the initial general trabeculae characteristics for each experimental group. Minor variation was detected in trabeculae cross-sectional areas among groups. $F_{dev,start}/F_{max,start}$ values were similar between experimental groups, implying that trabeculae had on average comparable contractile force reserves. Furthermore, passive force at 95% of the maximal length expressed as a percentage of $F_{max,start}$ was similar between groups before the onset of SI. SI resulted in an immediate decrease of F_{dev} to zero, followed by an increase in passive force, ultimately leading to a rigor state. On

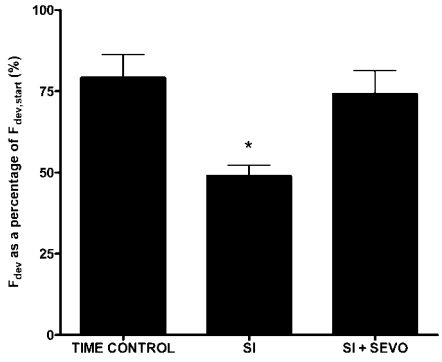

Fig. 2. The recovery of force after simulated ischemia (SI) for trabeculae subjected to sevoflurane (SEVO) preconditioning. Recovery of force ($F_{dev,rec}$) is expressed as percentage of initial force ($F_{dev,start}$) for different groups. SI decreased $F_{dev,rec}$ compared with time controls, which can be restored by preconditioning with sevoflurane. * $P < 0.05$ *versus* time control.

average, the time to onset of the rigor state was 28 min. MPG increased the time to rigor, but we found an insignificant correlation between time to rigor and $F_{dev,rec}$ or $F_{max,rec}$.

Sevoflurane-induced Cardioprotection

Figure 2 depicts the effects of sevoflurane on recovery of force after SI. In time controls, F_{dev} and F_{max} were decreased after approximately 170 min to 80 ± 9 and 81 ± 2% of $F_{dev,start}$ and $F_{max,start}$, respectively. SI reduced the recovery of force ($F_{dev,rec}$) to 49 ± 3% ($P < 0.01$ *vs.* time control). In addition, the recovery of F_{max} was decreased to 65 ± 4% ($P < 0.05$ *vs.* time control), whereas SI did not affect passive force. Sevoflurane itself induced negative inotropy and reduced F_{dev} to 50% of $F_{dev,start}$. However, this negative inotropy was completely reversed after the washout of sevoflurane, 15 min before SI. Preconditioning with sevoflurane improved the ischemic injury–related decrease in $F_{dev,rec}$ to 74 ± 7% ($P < 0.05$ *vs.* SI) but did not improve $F_{max,rec}$ (56 ± 4%; $P > 0.05$ *vs.* SI).

Sevoflurane-induced PKC-δ Translocation and Activation

Figures 3 and 4 represent functional and immunohistochemical data, respectively, on the role of PKC-δ in sevoflurane-induced cardioprotection. Figure 3 shows that chelerythrine (a) and rottlerin (b) both abolished sevoflurane-induced preservation of $F_{dev,rec}$ (36 ± 4 and 46 ± 4% for chelerythrine and rottlerin, respectively; both $P < 0.01$ *vs.* sevoflurane). Figure 4 shows the translocation pattern of PKC-δ in response to sevoflurane in both longitudinal (a and d) and cross-sectional (b, c, and e–h) sections. In control sections, a cytosolic localization of the PKC-δ isoform can be distinguished (a–c). In addition, the red sarcolemmal counterstain is visualized in panel c. In contrast, in trabeculae exposed to sevoflurane, PKC-δ is present as a striation-like translocation pattern in the longitudinal sections (d, *white arrows*). In cross-sectional sections, PKC-δ is visualized in a patchy distribution following distinct linear membrane structures of the cardiomyocytes (e, *white arrows*). The sarcolemmal counterstain colocalizes with PKC-δ (f), which is indicated by the yellow color. Interestingly, the sevoflurane-induced translocation of PKC-δ to the sarcolemma was blocked by both chelerythrine (g) and rottlerin (h).

Lack of Sarcolemmal PKC-ϵ Translocation in Sevoflurane-induced Cardioprotection

Figure 5 shows the distribution of PKC-ϵ in control (a and b) and sevoflurane-exposed trabeculae (c and d). In all panels, green represents specific PKC-ϵ staining, and

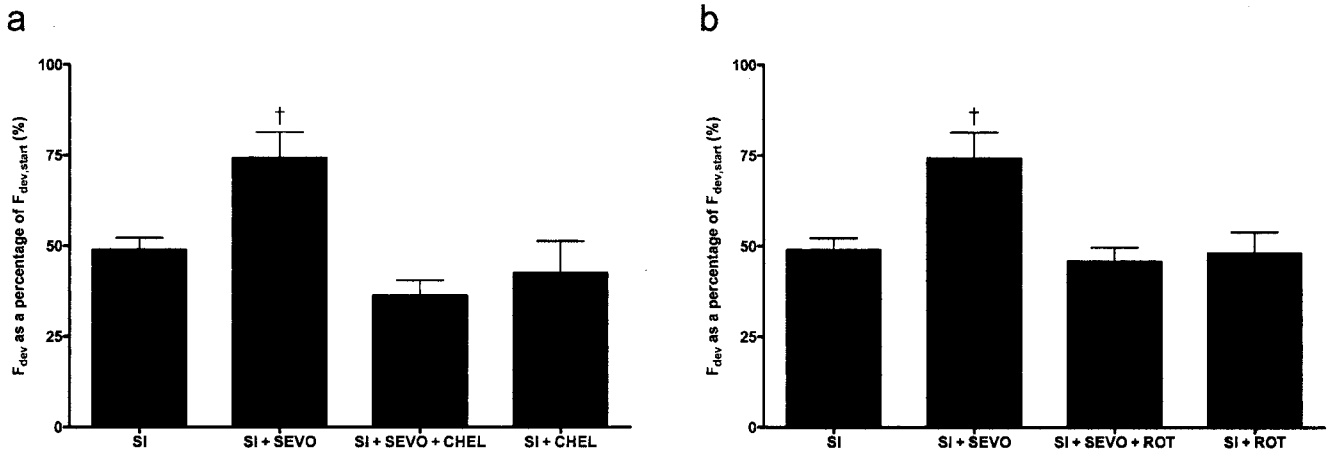

Fig. 3. The role of protein kinase C-δ in sevoflurane (SEVO)-induced cardioprotection in functional experiments. Recovery of force ($F_{dev,rec}$) is expressed as percentage of initial force ($F_{dev,start}$) for different groups. (*a*) Chelerythrine (CHEL) abolished the protective effect of sevoflurane on contractile function as is represented but showed no intrinsic protective effect. (*b*) Similar results were observed for the protein kinase C-δ–specific inhibitor rottlerin (ROT). † $P < 0.05$ *versus* simulated ischemia (SI), SI + SEVO + inhibitor, and SI + inhibitor.

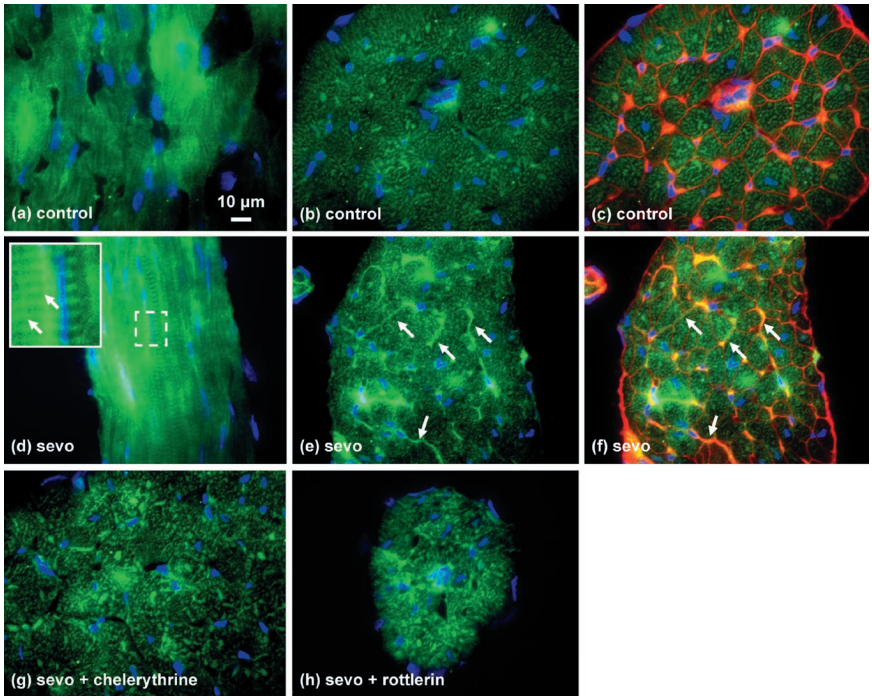

Fig. 4. Three-dimensional digital imaging microscopy analysis of protein kinase C (PKC)-δ translocation patterns in response to sevoflurane (sevo). In all panels, *green* represents specific PKC-δ staining, and *blue* represents the nuclear 4',6 diamidino-2-phenylindole staining. In *panels c* and *f*, *red* represents the wheat germ agglutinin staining of the sarcolemmal glycocalyx. Control trabecula show a cytosolic location of PKC-δ in longitudinal sections (*a*) as well as in cross-sectional sections (*b* and *c*). In trabecula exposed to sevoflurane, PKC-δ is localized in striation-like patterns and linear structures in longitudinal sections (*d*, *inset*, *white arrows*). In cross-sectional sections, PKC-δ is translocated to linear, patchy structures (*e*, *white arrows*). The *red* sarcolemmal counterstain is changed into *yellow*, indicating sevoflurane-induced colocalization of PKC-δ in the cardiomyocyte sarcolemmal membrane (*f*). This specific translocation of PKC-δ in response to sevoflurane was abolished by chelerythrine (*g*) and rottlerin (*h*).

blue indicates the nuclear DAPI staining. In panels b and d, the sarcolemma has been counterstained by wheat germ agglutinin (red color). In control trabeculae, PKC-ϵ is localized in the perinuclear area of both cardiomyocytes and interstitial cells, as is shown by the green color surrounding the nuclei of cardiomyocytes. Sevoflurane exposure results in a reduction of perinuclear PKC-ϵ in cardiomyocytes but not in interstitial cells.

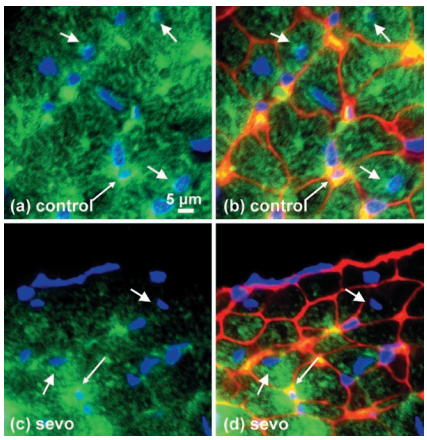

Fig. 5. Protein kinase C (PKC)-ϵ distribution in control (*a* and *b*) and sevoflurane (sevo)-exposed trabeculae (*c* and *d*). In all panels, *green* represents specific PKC-ϵ staining, and *blue* indicates the nuclear 4',6 diamidino-2-phenylindole staining. In *panels b* and *d*, *red* represents the wheat germ agglutinin staining of the sarcolemmal glycocalyx. In control trabeculae, PKC-ϵ is localized in the perinuclear area, as is demonstrated by the *green* surrounding the nuclei of both cardiomyocytes (*bold arrowheads*) and interstitial cells (*thin arrows*). Note that exposure to sevoflurane results in a reduction of perinuclear PKC-ϵ in cardiomyocytes but not in interstitial cells.

Role of ROS and Mito K^+_{ATP} Channels in Sevoflurane-induced Cardioprotection

The role of mito K^+_{ATP} channels and ROS production in sevoflurane-induced cardioprotection in recovery of force and PKC-δ translocation is shown in figures 6 and 7, respectively. Inhibition of mito K^+_{ATP} channels by 5-HD (fig. 6a) and ROS scavenging by MPG (fig. 6b) both abolished sevoflurane-induced cardioprotection. Neither inhibitor established modulation of F_{dev} or intrinsic cardioprotective properties. Although 5-HD inhibits sevoflurane-induced cardioprotection, figure 7 shows that 5-HD does not abolish sevoflurane-induced PKC-δ translocation to the sarcolemma (a and b). In contrast, ROS scavenging by MPG inhibits sevoflurane-induced translocation of PKC-δ (c and d). Figure 8 represents the sevoflurane-induced formation of nitrotyrosine, as indicated by green fluorescence. In time controls, nitrotyrosine was undetectable (a and b), whereas sevoflurane clearly induced sarcolemmal nitrosylation (c and d). The involvement of ROS in the peroxynitrite modification of sarcolemmal tyrosine residues was shown by the absence of nitrotyrosine when trabeculae were exposed to sevoflurane in the presence of MPG (e and f).

Discussion

In this study, we demonstrated that sevoflurane-induced cardioprotection in functional cardiac tissue depends on activation of the PKC-δ isoform. Furthermore, exposure of trabeculae to sevoflurane induces translocation of PKC-δ but not of PKC-ϵ from the cytosol to the cardiomyocyte sarcolemma. PKC-δ translocation acts *via*

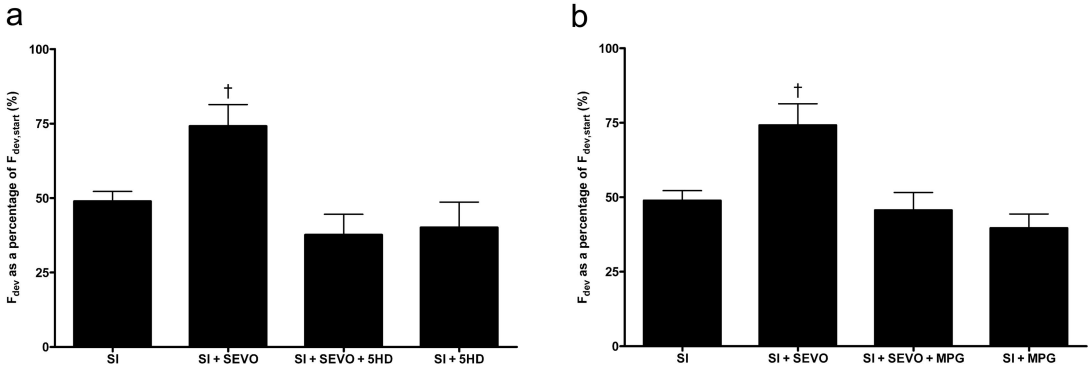

Fig. 6. The role of adenosine triphosphate–sensitive mitochondrial K^+ channels and reactive oxygen species in sevoflurane (SEVO)-induced cardioprotection in functional experiments. Recovery of force ($F_{dev,rec}$) is expressed as percentage of initial force ($F_{dev,start}$) for different groups. Selective inhibition of adenosine triphosphate–sensitive mitochondrial K^+ channels by 5-hydroxydecanoic acid sodium (5-HD) (*a*) and scavenging of reactive oxygen species by n-(2-mercaptopropionyl)-glycine (MPG) (*b*) abolished the cardioprotective effect of sevoflurane. 5-HD and MPG revealed no intrinsic cardioprotective effects. † $P < 0.05$ *versus* simulated ischemia (SI), SI + SEVO + inhibitor, and SI + inhibitor.

the production of ROS but not the opening of mito K^+_{ATP} channels. Finally, sevoflurane induces formation of sarcolemmal nitrotyrosine, which represents the reaction of peroxynitrite with tyrosine residues. To our knowledge, this is the first study showing the involvement of PKC-δ and its relative order compared with ROS and opening of mito K^+_{ATP} channels in sevoflurane-induced cardioprotection.

Several studies showed specific translocation patterns of various PKC isoforms in response to ischemic as well as pharmacologic preconditioning. Inagaki *et al.*[27] studied PKC translocation patterns of the cardioprotective agent JTV519 and demonstrated that this compound induced a translocation of PKC-δ but not of PKC-ϵ to the sarcolemma. Recently, Julier *et al.*[23] demonstrated that sevoflurane itself results in translocation of PKC-δ to the sarcolemma in human atrial tissue, in addition to translocation of PKC-ϵ to the intercalated discs and mitochondria. However, these sevoflurane-treated patients re-

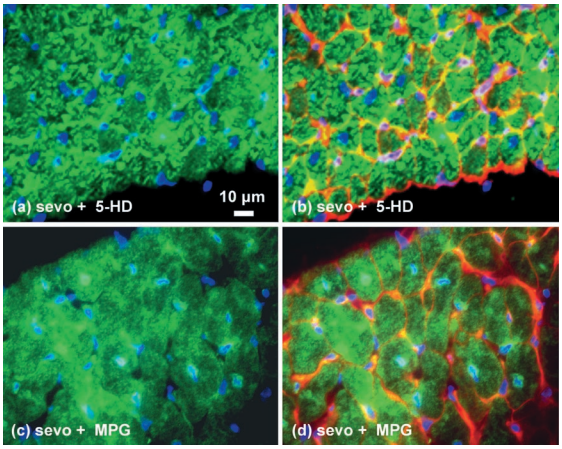

Fig. 7. Effect of inhibition of adenosine triphosphate–sensitive mitochondrial K^+ and reactive oxygen species scavenging on protein kinase C (PKC)-δ translocation in response to sevoflurane (sevo) in cross-sectional sections of trabeculae. In all panels, *green* indicates specific PKC-δ staining, and *blue* represents the nuclear 4',6 diamidino-2-phenylindole staining. *Red* represents the wheat germ agglutinin staining of the sarcolemmal glycocalyx. Although 5-hydroxydecanoic acid sodium (5-HD) inhibits sevoflurane-induced cardioprotection (fig. 6), *a* and *b* show that 5-HD does not abolish sevoflurane-induced PKC-δ translocation to the sarcolemma. In contrast, reactive oxygen species scavenging by n-(2-mercaptopropionyl)-glycine (MPG) inhibits sevoflurane-induced translocation of PKC-δ (*c* and *d*).

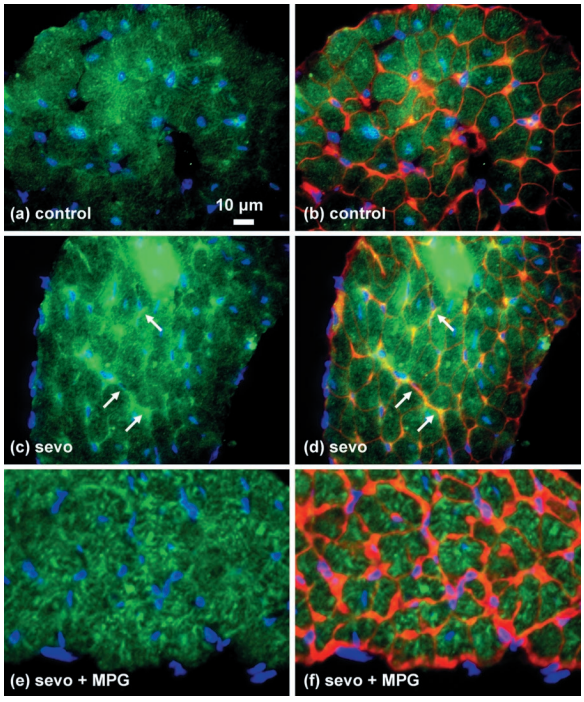

Fig. 8. Three-dimensional digital imaging microscopy analysis of nitrotyrosine in response to sevoflurane (sevo). In all panels, *green* indicates specific nitrotyrosine staining, and *blue* represents the nuclear 4',6 diamidino-2-phenylindole staining. *Red* represents the wheat germ agglutinin staining of the sarcolemmal glycocalyx. In time controls, nitrotyrosine was undetectable (*a* and *b*), whereas sevoflurane clearly induced sarcolemmal nitrosylation (*c* and *d*; see also *white arrows*). The involvement of reactive oxygen species in the formation of nitrotyrosine was demonstrated by the absence of nitrotyrosine when trabeculae were exposed to sevoflurane in the presence of n-(2-mercaptopropionyl)-glycine (MPG) (*e* and *f*).

ceived significantly more phenylephrine compared with control subjects to maintain blood pressure. Because α-adrenergic stimulation induces PKC translocation[17] and cardioprotection,[28] the sevoflurane-induced effects were biased by the phenylephrine-induced changes. We demonstrate with immunofluorescent analysis that sevoflurane itself induces translocation of PKC-δ to the sarcolemma. In addition, our functional experiments show that PKC-δ activation may be important for sevoflurane-induced cardioprotection. This is in agreement with observations showing that PKC-δ is primarily involved in pharmacologic preconditioning with opioids,[14] adenosine[18] and phenylephrine.[17] However, we do not rule out that other PKC isoforms, such as PKC-ϵ, are involved in sevoflurane-induced preconditioning as well.

Interestingly, a functional role for PKC-ϵ was recently established during sevoflurane-induced cardioprotection in guinea pig hearts in addition to studies showing that ischemic preconditioning is mediated by PKC-ϵ.[22,29–31] Some studies even indicate that PKC-δ inhibition is cardioprotective, as was demonstrated by the increased resistance against ischemia–reperfusion damage in mice hearts overexpressing a δ V1 inhibitor peptide.[32] Nevertheless, the latter study also established the necessity of an active form of the PKC-δ because overexpression of the δ V1 inhibitor peptide at intermediate or very high doses resulted in cytoskeletal deformations, a lethal phenotype and less protection to ischemia–reperfusion, respectively. Liu *et al.*[19] demonstrated that ischemic preconditioning resulted in a translocation of both PKC-δ and PKC-ϵ, whereas on pharmacologic preconditioning with opioids, only PKC-δ translocated. This is in accordance with our observations that sevoflurane itself does not cause sarcolemmal translocation of PKC-ϵ in trabeculae. In addition, Fryer *et al.*[14,15] showed in isolated rat hearts that opioid-induced cardioprotection but not ischemic preconditioning was abolished by pharmacologic inhibition of PKC-δ. However, in contrast, Uecker *et al.*[21] showed PKC-δ translocation to the mitochondria and PKC-ϵ to the sarcolemma in response to isoflurane. These observations indicate that distinct signal transduction pathways are involved not only in ischemic preconditioning and pharmacologic preconditioning, but also between different pharmacologic preconditioning stimuli.

Recently, a profound role for ROS in the signal transduction of volatile anesthetic–induced cardioprotection has been established.[3,7,33] Our results indicate that translocation of PKC-δ occurs downstream of ROS because PKC-δ translocation could be inhibited by MPG. This is in agreement with observations of Zhang *et al.*,[34] who showed that opioid-induced protection was mediated by ROS, which could not be blocked by rottlerin. In addition, Wang *et al.*[35] showed that diazoxide-induced protection in isolated rat hearts was abolished by chelerythrine. In contrast, other studies showed that ROS-mediated cardioprotection could not be blocked by PKC inhibition.[36] This suggests that ROS may also be involved in a signaling pathway independent from PKC. That sevoflurane indeed results in the production of ROS in our model was demonstrated by the positive nitrotyrosine staining of the cardiomyocyte sarcolemma on exposure to sevoflurane. Nitrotyrosine is induced when nitric oxide and superoxide form peroxynitrite, which can modify tyrosine residues to form nitrotyrosine. Others also demonstrated the involvement of ROS and nitrogen species as triggers of anesthetic preconditioning in isolated guinea pig hearts.[37]

It has been hypothesized that ROS production arises from the mitochondria as volatile anesthetics inhibit complex I of the electron transport chain. Whether opening of the mito K^+_{ATP} channels is essential for ROS production has not been completely elucidated. However, it was recently demonstrated that 5-HD does not inhibit sevoflurane-induced ROS production.[23] In contrast, during isoflurane-induced preconditioning, opening of the mito K^+_{ATP} channels was essential for ROS production triggering cardioprotection.[33] We demonstrate in functional trabeculae that opening of the mito K^+_{ATP} channels is essential for inducing the cardioprotective response but that opening of mito K^+_{ATP} channels is not essential for PKC-δ activation. With respect to the published literature, this supports that mito K^+_{ATP} channel opening is involved in sevoflurane-induced cardioprotection but is downstream of PKC-δ translocation. These data suggest that ROS production precedes PKC-δ activation and that PKC-δ activation precedes the opening of mito K^+_{ATP} channels in the signaling pathway of sevoflurane-induced cardioprotection.

Possible target substrates of PKC-δ during sevoflurane-induced cardioprotection remain speculative. Targets might include proteins involved in Ca^{2+} homeostasis, as we previously demonstrated by showing that preconditioning with norepinephrine attenuated Ca^{2+} overload and preserved Ca^{2+} reuptake activity of the sarcoplasmic reticulum.[28,38] In addition, PKC has been reported to modify the function of Ca^{2+}-handling proteins, such as L-type Ca^{2+} channels,[39] the ryanodine receptor,[40] sarcoplasmic Ca^{2+} adenosine triphosphatase,[41] and the Na^+–Ca^{2+} exchanger.[42] Furthermore, anesthetic preconditioning might also preserve mitochondrial function.[43] Future experiments in our laboratory will be directed toward unraveling the potential end-effector proteins of PKC-δ.

In our laboratory, the *in vitro* model of isolated rat trabecula has been extensively used in previous studies regarding ischemia and reperfusion injury and preconditioning in functional cardiac tissue.[3,28,38] Our experiments are performed under relatively hypothermic conditions (24°C) to ensure stability of trabeculae over time. This could influence signaling processes, such as translocation of PKC isoforms. However, it was reported

recently that during cold cardioplegia, translocation of PKC isoforms still occurred and that the induced cardioprotection is mediated by protein kinase C.[44–46] The negative inotropic effect of sevoflurane, when applied before SI, was completely restored after the washout period. There was no difference in F_{dev} after washout between groups. In studies regarding ischemic and anesthetic preconditioning, chelerythrine, rottlerin, MPG, and 5-HD are commonly used inhibitors for studying PKC, PKC-δ, ROS, and mito K^+_{ATP} channels, respectively. We used concentrations comparable with data found in the literature. Although rottlerin is reported to be a specific inhibitor of the PKC-δ isoform, one should be cautious of unspecific inhibitory effects.[47,48] However, it has been demonstrated by Keenan *et al.*[49] that PKC-δ is more potently inhibited by rottlerin than classic PKC isoforms. In addition, we show by immunofluorescent microscopy that rottlerin selectively inhibits sevoflurane-induced translocation of PKC-δ.

In summary, PKC-δ, mito K^+_{ATP} channels, and ROS are equally involved in sevoflurane-induced cardioprotection in isolated rat trabeculae. ROS production precedes PKC-δ translocation on stimulation with sevoflurane, while the opening of mito K^+_{ATP} channels occurs downstream of this translocation. Furthermore, sevoflurane exposure induces formation of peroxynitrite, which modifies tyrosine residues in the sarcolemma. We propose that sevoflurane directly induces ROS and peroxynitrite formation, which might result in recruitment of PKC-δ to the cardiomyocyte sarcolemma. Furthermore, ROS and peroxynitrite might also affect tyrosine residues in the PKC-δ isoform *via* tyrosine kinase, leading to activation of downstream signaling molecules, including mito K^+_{ATP} channels. The downstream localization of mito K^+_{ATP} channels in the signaling pathway of sevoflurane-induced cardioprotection suggests a role for mitochondria as crucial organelles for cardiac preservation.

References

1. Varadarajan SG, An J, Novalija E, Stowe DF: Sevoflurane before or after ischemia improves contractile and metabolic function while reducing myoplasmic Ca^{2+} loading in intact hearts. Anesthesiology 2002; 96:125–33
2. Coetzee JF, le Roux PJ, Genade S, Lochner A: Reduction of postischemic contractile dysfunction of the isolated rat heart by sevoflurane: Comparison with halothane. Anesth Analg 2000; 90:1089–97
3. de Ruijter W, Musters RJP, Boer C, Stienen GJM, Simonides WS, de Lange JJ: The cardioprotective effect of sevoflurane depends on protein kinase C activation, opening of mitochondrial K^+_{ATP} channels and the production of reactive oxygen species. Anesth Analg 2003; 97:1370–6
4. El Azab SR, Scheffer GJ, Rosseel PM, de Lange JJ: Induction and maintenance of anaesthesia with sevoflurane in comparison to high dose opioid during coronary artery bypass surgery. Eur J Anaesthesiol 2000; 17:336–8
5. De Hert SG, ten Broecke PW, Mertens E, van Sommeren EW, de Blier IG, Stockman BA, Rodrigus IE: Sevoflurane but not propofol preserves myocardial function in coronary surgery patients. Anesthesiology 2002; 97:42–9
6. Toller WG, Kersten JR, Pagel PS, Hettrick DA, Warltier DC: Sevoflurane reduces myocardial infarct size and decreases the time threshold for ischemic preconditioning in dogs. Anesthesiology 1999; 91:1437–46
7. 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
8. Rebecchi MJ, Pentyala SN: Anaesthetic actions on other targets: Protein kinase C and guanine nucleotide-binding proteins. Br J Anaesth 2002; 89:62–78
9. Sato T, O'Rourke B, Marban E: Modulation of mitochondrial ATP-dependent K^+ channels by protein kinase C. Circ Res 1998; 83:110–4
10. Hanley PJ, Ray J, Brandt U, Daut J: Halothane, isoflurane and sevoflurane inhibit NADH:ubiquinone oxidoreductase (complex I) of cardiac mitochondria. J Physiol 2002; 544:687–93
11. Gopalakrishna R, Jaken S: Protein kinase C signaling and oxidative stress. Free Radic Biol Med 2000; 28:1349–61
12. Konishi H, Yamauchi E, Taniguchi H, Yamamoto T, Matsuzaki H, Takemura Y, Ohmae K, Kikkawa U, Nishizuka Y: Phosphorylation sites of protein kinase C delta in H2O2-treated cells and its activation by tyrosine kinase in vitro. Proc Natl Acad Sci U S A 2001; 98:6587–92
13. Zhang DX, Chen YF, Campbell WB, Zou AP, Gross GJ, Li PL: Characteristics and superoxide-induced activation of reconstituted myocardial mitochondrial ATP-sensitive potassium channels. Circ Res 2001; 89:1177–83
14. 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
15. Fryer RM, Hsu AK, Wang Y, Henry M, Eells J, Gross GJ: PKC-delta inhibition does not block preconditioning-induced preservation in mitochondrial ATP synthesis and infarct size reduction in rats. Basic Res Cardiol 2002; 97:47–54
16. Miyawaki H, Ashraf M: Ca2+ as a mediator of ischemic preconditioning. Circ Res 1997; 80:790–9
17. Mitchell MB, Meng X, Ao L, Brown JM, Harken AH, Banerjee A: Preconditioning of isolated rat heart is mediated by protein kinase C. Circ Res 1995; 76:73–81
18. Kudo M, Wang Y, Xu M, Ayub A, Ashraf M: Adenosine A1 receptor mediates late preconditioning via activation of PKC-delta signaling pathway. Am J Physiol Heart Circ Physiol 2002; 283:H296–301
19. Liu H, Zhang HY, McPherson BC, Baman T, Roth S, Shao Z, Zhu X, Yao Z: Role of opioid delta1 receptors, mitochondrial K(ATP) channels, and protein kinase C during cardiocyte apoptosis. J Mol Cell Cardiol 2001; 33:2007–14
20. Inagaki K, Hahn HS, Dorn GW, Mochly-Rosen D: Additive protection of the ischemic heart ex vivo by combined treatment with delta-protein kinase C inhibitor and epsilon-protein kinase C activator. Circulation 2003; 108:869–75
21. 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
22. 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
23. Julier K, da Silva R, Garcia C, Bestman L, Frascarolo P, Zollinger A, Chassot P, 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
24. Mulieri LA, Hasenfuss G, Ittleman F, Blanchard EM, Alpert NR: Protection of human left ventricular myocardium from cutting injury with 2,3-butanedione monoxime. Circ Res 1989; 65:1441–9
25. Schouten VJ, ter Keurs HE: The force-frequency relationship in rat myocardium: The influence of muscle dimensions. Pflugers Arch 1986; 407:14–7
26. Schouten VJ, Allaart CP, Westerhof N: Effect of perfusion pressure on force of contraction in thin papillary muscles and trabeculae from rat heart. J Physiol 1992; 451:585–604
27. Inagaki K, Kihara Y, Hayashida W, Izumi T, Iwanaga Y, Yoneda T, Takeuchi Y, Suyama K, Muso E, Sasayama S: Anti-ischemic effect of a novel cardioprotective agent, JTV519, is mediated through specific activation of delta-isoform of protein kinase C in rat ventricular myocardium. Circulation 2000; 101:797–804
28. Musters RJ, van der Meulen ET, van der Laarse WJ, van Hardeveld C: Norepinephrine pretreatment attenuates Ca^{2+} overloading in rat trabeculae during subsequent metabolic inhibition: Improved contractile recovery via an alpha 1-adrenergic, PKC-dependent signaling mechanism. J Mol Cell Cardiol 1997; 29:1341–54
29. Gray MO, Karliner JS, Mochly-Rosen D: A selective epsilon-protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. J Biol Chem 1997; 272:30945–51
30. 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
31. 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
32. Hahn HS, Yussman MG, Toyokawa T, Marreez Y, Barrett TJ, Hilty KC, Osinska H, Robbins J, Dorn GW: Ischemic protection and myofibrillar cardiomyopathy: Dose-dependent effects of in vivo delta PKC inhibition. Circ Res 2002; 91:741–8
33. Tanaka K, Weihrauch D, Ludwig LM, Kersten JR, Pagel PS, Warltier DC:

Mitochondrial adenosine triphosphate–regulated potassium channel opening acts as a trigger for isoflurane-induced preconditioning by generating reactive oxygen species. ANESTHESIOLOGY 2003; 98:935–43

34. Zhang HY, McPherson BC, Liu H, Baman T, McPherson SS, Rock P, Yao Z: Role of nitric-oxide synthase, free radicals, and protein kinase C delta in opioid-induced cardioprotection. J Pharmacol Exp Ther 2002; 301:1012–9

35. Wang Y, Takashi E, Xu M, Ayub A, Ashraf M: Downregulation of protein kinase C inhibits activation of mitochondrial K(ATP) channels by diazoxide. Circulation 2001; 104:85–90

36. Das DK, Maulik N, Sato M, Ray PS: Reactive oxygen species function as second messenger during ischemic preconditioning of heart. Mol Cell Biochem 1999; 196:59–67

37. Novalija E, Varadarajan SG, Camara AK, An J, Chen Q, Riess ML, Hogg N, Stowe DF: Anesthetic preconditioning: Triggering role of reactive oxygen and nitrogen species in isolated hearts. Am J Physiol Heart Circ Physiol 2002; 283: H44–52

38. Musters RJ, van der Meulen ET, Zuidwijk M, Muller A, Simonides WS, Banerjee A, van Hardeveld C: PKC-dependent preconditioning with norepinephrine protects sarcoplasmic reticulum function in rat trabeculae following metabolic inhibition. J Mol Cell Cardiol 1999; 31:1083–94

39. Kamp TJ, Hell JW: Regulation of cardiac L-type calcium channels by protein kinase A and protein kinase C. Circ Res 2000; 87:1095–102

40. Takasago T, Imagawa T, Furukawa K, Ogurusu T, Shigekawa M: Regulation of the cardiac ryanodine receptor by protein kinase-dependent phosphorylation. J Biochem 1991; 109:163–70

41. Qu Y, Torchia J, Sen AK: Protein kinase C mediated activation and phosphorylation of Ca(2+)-pump in cardiac sarcolemma. Can J Physiol Pharmacol 1992; 70:1230–5

42. Stengl M, Mubagwa K, Carmeliet E, Flameng W: Phenylephrine-induced stimulation of Na+/Ca2+ exchange in rat ventricular myocytes. Cardiovasc Res 1998; 38:703–10

43. 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

44. Friehs I, Cao-Danh H, Takahashi S, Buenaventura P, Glynn P, McGowan FX, del Nido PJ: Adenosine prevents protein kinase C activation during hypothermic ischemia. Circulation 1997; 96(suppl):II-221–5

45. Nawas SI, Schwertz DW, Beck JM, Fogelson BG, Law WR: Mechanisms of myocardial protection by adenosine-supplemented cardioplegia: Differential response of calcium-independent protein kinase C isozymes. J Surg Res 2000; 89:163–8

46. Hedayati N, Schomisch SJ, Carino JL, Timothy SJ, Lesnefsky EJ, Cmolik BL: Cardioprotection by St Thomas' solution is mediated by protein kinase C and tyrosine kinase. J Surg Res 2003; 113:121–7

47. Gschwendt M, Muller HJ, Kielbassa K, Zang R, Kittstein W, Rincke G, Marks F: Rottlerin, a novel protein kinase inhibitor. Biochem Biophys Res Commun 1994; 199:93–8

48. 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

49. Keenan C, Goode N, Pears C: Isoform specificity of activators and inhibitors of protein kinase C gamma and delta. FEBS Lett 1997; 415:101–8