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Preconditioning by Isoflurane Induces Lasting Sensitization of the Cardiac Sarcolemmal Adenosine Triphosphate–sensitive Potassium Channel by a Protein Kinase C-δ–mediated Mechanism

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Background: **Cardioprotective effects of volatile anesthetics in anesthetic-induced preconditioning involve activation of the cardiac sarcolemmal adenosine triphosphate–sensitive potassium (sarcK$_{ATP}$) channels. This study addressed the memory phase of anesthetic preconditioning by investigating whether brief exposure to isoflurane produces lasting sensitization of the sarcK$_{ATP}$ channel and whether protein kinase C mediates this effect.**

Methods: **Whole cell sarcK$_{ATP}$ channel current (I$_{KATP}$) was monitored from single isolated rat ventricular cardiomyocytes. Pinacidil was used to open the channel, and the magnitude of activated I$_{KATP}$ was an indicator of channel's ability to open. Involvement of protein kinase C was investigated using chelerythrine and isoform-specific peptide inhibitors and activators of protein kinase C-δ and protein kinase C-ε.**

Results: **The mean density of I$_{KATP}$ elicited by pinacidil (5 μM) in anesthetic-free conditions was 3.8 ± 3.7 pA/pF (n = 11). After 10 min of exposure to isoflurane (0.56 mM) and 10 or 30 min of anesthetic washout, pinacidil-elicited I$_{KATP}$ was increased to 15.6 ± 11.3 pA/pF (n = 12; $P < 0.05$) and 11.8 ± 3.9 pA/pF (n = 6; $P < 0.05$), respectively. In the presence of chelerythrine (5 μM), isoflurane did not potentiate channel opening, and I$_{KATP}$ was 6.6 ± 4.6 pA/pF (n = 11). Application of protein kinase C-δ peptide inhibitor also abolished isoflurane-induced sensitization of sarcK$_{ATP}$ channel, and I$_{KATP}$ was 7.7 ± 5.4 pA/pF (n = 12). In contrast, protein kinase C-ε peptide inhibitor did not affect channel sensitization, and pinacidil-elicited current was 14.8 ± 9.6 pA/pF (n = 12). Interestingly, when both protein kinase C-δ and protein kinase C-ε activators were applied instead of isoflurane, they sensitized the channel to the same extent as isoflurane (18.9 ± 7.2 pA/pF, n = 11, and 18.6 ± 11.1 pA/pF, n = 10, respectively).**

Conclusion: **Isoflurane induces prolonged sensitization of the sarcK$_{ATP}$ channel to opening that persists even after anesthetic withdrawal. Our results indicate that protein kinase C-δ, rather than protein kinase C-ε, is a likely mediator of isoflurane effects, although both protein kinase C-δ and protein kinase C-ε can modulate the channel function.**

ANESTHETIC-INDUCED preconditioning (APC) is a phenomenon whereby a brief exposure to volatile anesthetics protects the heart against ischemia and reperfusion injury by delaying the onset of myocardial damage, reducing myocardial infarct size and improving recovery of contractile function.[1,2] This cardioprotection closely resembles the acute ischemic preconditioning in that there is an early memory phase during which the protective effect persists even after anesthetic withdrawal. Evidence is accumulating that memory of APC involves intracellular kinases, reactive oxygen species, and the adenosine triphosphate–sensitive potassium (K$_{ATP}$) channels,[3] but the exact mechanism is still unknown.

There are two distinct populations of K$_{ATP}$ channels in cardiac myocytes, the sarcolemmal (sarcK$_{ATP}$) channel that is located in the plasma membrane, and the mitochondrial channel located in the inner mitochondrial membrane. Both are thought to play an important role in cardioprotection, although their relative contribution to APC is still a subject of debate.[4]

SarcK$_{ATP}$ channels are abundant in the plasma membrane of cardiomyocytes.[5] Under normal metabolic conditions, these channels are closed. However, during metabolic stress, such as ischemia, they open. SarcK$_{ATP}$ channels are regulated by intracellular nucleotides and are inhibited by intracellular adenosine triphosphate (ATP) but activated by intracellular adenosine diphosphate. Therefore, by coupling cellular metabolic state to changes in membrane potential, these channels may regulate various cellular functions.[6-8] Contribution of sarcK$_{ATP}$ channels to the preconditioning phenomenon was demonstrated in a study by Suzuki *et al.* [9] There, ischemic preconditioning was unable to protect the hearts of Kir6.2 knockout mice that lack sarcK$_{ATP}$ channels. The importance of sarcK$_{ATP}$ channel activation in APC has also been demonstrated.[10] Studies indicate that preconditioning stimuli may modulate function of sarcK$_{ATP}$ channels during the memory phase; however, the number of these studies is limited.[11,12]

One of the central mediators in the preconditioning phenomenon is protein kinase C (PKC).[13] Activation of PKC represents an essential step in both ischemic preconditioning and APC. For example, it was shown that blockade of PKC abolishes cardioprotection by ischemic preconditioning,[14] whereas activation of PKC can induce the preconditioned state.[3] Furthermore, PKC is an important modulator of the sarcK$_{ATP}$ channel activity.[15,16] Channel phosphorylation by PKC increases sarcK$_{ATP}$ channel current (I$_{KATP}$) at physiologic concentrations of ATP[16] and, under certain conditions, may induce opening of the sarcK$_{ATP}$ channels.[17] Of seven PKC isoforms identified in cardiac myocytes,[18] only two

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novel isoforms, δ and ε, seem to play a role in APC.[4] When activated, these enzymes translocate from the cytosol to the membranes. Specificity of the translocation is ensured by isoform-specific receptors for PKC that are present at the translocation sites.[19] However, findings regarding translocation sites of PKC-δ and PKC-ε in APC are still controversial. A study in isolated rat hearts showed that after anesthetic exposure, PKC-ε translocates to sarcolemma, and PKC-δ translocates to mitochondria.[20] In contrast, the *in vivo* APC in rat hearts caused translocation of PKC-ε to mitochondria and PKC-δ to the sarcolemma.[21] Thus, there is no clear evidence to indicate which PKC isoform translocates to the sarcolemma and is involved in modulation of cardiac sarcK_{ATP} channel after APC.

Recent studies in ventricular myocytes have demonstrated that isoflurane sensitizes the sarcK_{ATP} channel to the openers through multiple mechanisms that involve adenosine, phosphatidylinositol-4,5-bisphosphate, and reactive oxygen species–mediated pathways.[22,23] However, whether these channels are modulated during the memory phase of APC has not been addressed.

In the current study, we investigated the hypothesis that the memory phase of isoflurane-induced APC involves a prolonged enhancement of the sarcK_{ATP} channel ability to open. We tested whether isoflurane exposure modulates channel function more permanently and whether the channel sensitization persists even after anesthetic withdrawal. Furthermore, we tested the hypothesis that PKC contributes to this lasting channel sensitization and that PKC isoforms δ and ε mediate this effect.

Materials and Methods

The animal use and experimental protocols of this study were approved by the Animal Use and Care Committee of the Medical College of Wisconsin (Milwaukee, Wisconsin).

Cell Isolation

Ventricular myocytes were isolated from hearts of adult male Wistar rats (150–250 g) by enzymatic dissociation with 0.5 mg/ml collagenase type II (Invitrogen, Carlsberg, CA) and 0.25 mg/ml protease XIV (Sigma-Aldrich, St. Louis, MO) as described previously.[24] After isolation, the myocytes were stored in the Tyrode solution at 20°–22°C and used for patch clamp experiments within 5 h.

Solutions

The modified Tyrode solution had the following composition: 132 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM HEPES, 5 mM glucose, and 20 mM taurine, adjusted to pH 7.4 with NaOH. The pipette solution contained 60 mM K-glutamate, 50 mM KCl, 10 mM HEPES, 1 mM $CaCl_2$, 11 mM EGTA, and 0.5 mM K_2ATP, at pH 7.2 adjusted with KOH. The bath solution contained 132 mM *N*-methyl-D-glucamine, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM KCl, and 10 mM HEPES at pH 7.4 adjusted with HCl. Nisoldipine (Miles-Pentex, West Haven, CT) was added to the external solution at 200 nM to block the L-type Ca channels. A 10-mM stock of pinacidil and a 100-mM stock of levcromakalim were prepared in dimethyl sulfoxide (DMSO). The K_{ATP} channel blocker glibenclamide was also prepared in DMSO as a 1-mM stock. After dilution in the recording buffer, the final concentrations of DMSO were 0.05, 0.01, and 0.1% for pinacidil, levcromakalim, and glibenclamide, respectively. In control experiments, we tested whether 0.1% DMSO has an effect on rat I_{KATP}. DMSO alone did not activate I_{KATP} when present in the bath solution during 1-h-long time course experiments, and the whole cell I_{KATP} elicited by pinacidil was not affected by DMSO. For the latter experiments, the stock of pinacidil was made in 0.1N HCl, and DMSO was applied to the cells when pinacidil-activated current reached the steady state level. Chelerythrine, an isoform-nonselective PKC inhibitor, was dissolved in distilled water to make a 5-mM stock solution. Peptide inhibitors of PKC-ε (KIEI 1-1; KAI Pharmaceuticals, San Francisco, CA) and PKC-δ (deltaV1.1/Antennapeida carrier; a gift from Daria Mochly-Rosen, Ph.D., Professor and Chair, Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, California) were prepared as 10-μM stock solutions in distilled water. The PKC-δ peptide activator (KAD1-1), PKC-ε peptide activator (KAE1-1), and inactive carrier peptide (C1), all from KAI Pharmaceuticals, were prepared as 10-μM stock solutions in distilled water. All stock solutions were stored at −20°C and thawed immediately before experiments. The drugs were applied to myocytes in the bath solution, except chelerythrine, which was applied in the pipette solution. Isoflurane (Abbott Laboratories, North Chicago, IL) was dispersed in the bath solution by sonication and delivered to a recording chamber *via* perfusate from the airtight glass syringes. At the end of each experiment, samples of isoflurane-containing solution were collected from the recording chamber, and the concentration of isoflurane in the perfusate was analyzed by gas chromatography (Shimadzu, Kyoto, Japan). The average concentration of isoflurane used in this study was 0.56 ± 0.1 mM, equivalent to 1.2 vol% at 22°C.

Electrophysiologic Recordings

The I_{KATP} was recorded in the whole cell configuration of the patch clamp technique using an EPC-7 amplifier (List, Darmstadt-Eberstadt, Germany), and a Digidata 1322A interface (Axon Instruments, Foster City, CA). The pClamp9 software (Axon Instruments) was used for data acquisition and analysis. Pipettes were pulled from the borosilicate glass (Garner Glass, Claremont, CA) using a PC-84 puller (Sutter, Novato, CA) and were heat polished with an MF-83 microforge (Narishige, Tokyo, Japan). Resistance of the patch pipettes ranged from 1.5 to 2.5 MΩ. Experiments were performed in a recording chamber mounted on the stage of inverted Olympus IMT2 microscope (Tokyo, Japan). Only healthy-looking, rod-shaped, and quiescent myocytes with distinct cross-striations were chosen for patch clamp experiments. After a gigaohm seal was formed, the patch of membrane inside the pipette tip was ruptured, and the whole cell configuration was established. The series resistance was then electronically adjusted to obtain the fastest possible capacitance transient without causing ringing.

The membrane holding potential was set at −40 mV, and the whole cell I_{KATP} was monitored over time during a 200-ms depolarizing pulse to 0 mV applied every 15 s. Current amplitude was measured at the end of each voltage step and was normalized to cell capacitance to calculate current density, reported in pA/pF. This way, comparison of results obtained from different cells was possible. To assure equilibration of ATP between the pipette solution and the cytosol, at least 30 min was allowed at the beginning of each experiment, before application of pinacidil or levcromakalim. Current was measured at the point of steady state activation.

Statistical Analysis

Data were analyzed using Clampfit 9 software (Axon Instruments) and Origin 7 software (OriginLab, Northampton, MA). Results are expressed as mean ± SD. Statistical analysis was performed using paired and unpaired Student *t* tests. Differences were considered significant at $P < 0.05$.

Results

Effect of Isoflurane on the sarcK$_{ATP}$ Channel

Previous studies in isolated guinea pig cardiomyocytes have demonstrated that isoflurane potentiates whole cell I_{KATP} by sensitizing the channel to opening by pinacidil.[22] In the current study, the mechanism of isoflurane sensitization was further investigated in rat ventricular myocytes. I_{KATP} was measured in the presence of the cardiac K_{ATP} channel opener pinacidil (5 μM), and the magnitude of pinacidil-activated I_{KATP} was used as an indicator of channel readiness to open. Extracellularly applied pinacidil elicited an outward time-independent current that was sensitive to blockade by glibenclamide (1 μM), confirming identity of the I_{KATP}. Under control anesthetic-free conditions, pinacidil elicited I_{KATP} with density of 3.8 ± 3.7 pA/pF (n = 11; fig. 1A). However, when isoflurane was present before and during application of pinacidil (fig. 1B), opening of the channel was greatly enhanced, and I_{KATP} was increased to 17.2 ± 9.5 pA/pF (n = 6). This confirmed our previous findings in guinea pigs that showed sensitization and increased ability of the sarcK$_{ATP}$ channel to open in the presence of isoflurane.[22]

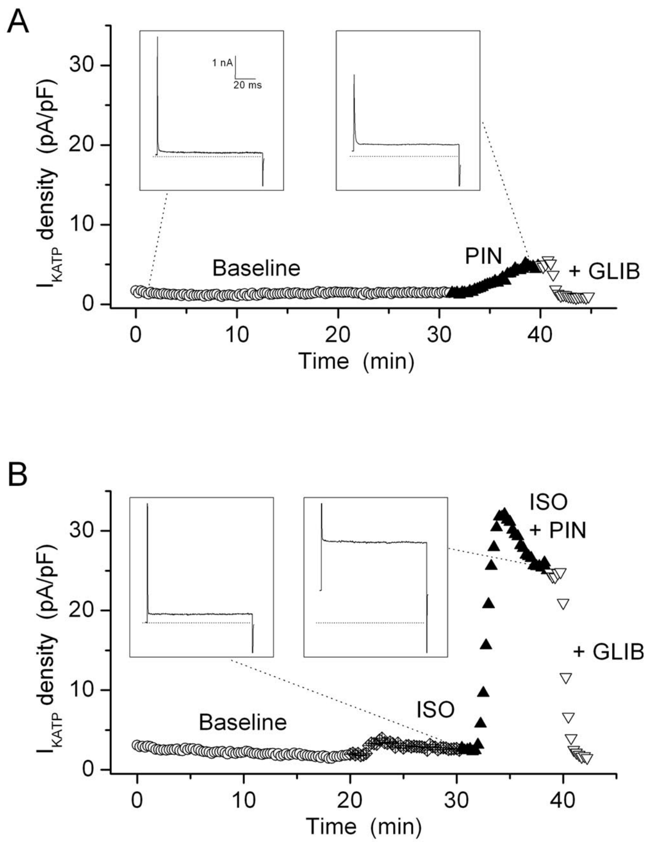

Fig. 1. Isoflurane (ISO) potentiates the whole cell sarcolemmal adenosine triphosphate–sensitive potassium channel current (I_{KATP}) activated by pinacidil (PIN) in rat ventricular myocytes. The current was elicited by a 200-ms pulse to 0 mV from a holding potential of −40 mV applied every 15 s. Current density (pA/pF) was plotted over time as shown in representative time course experiments. A period of 30 min (baseline) was allowed before application of pinacidil and/or isoflurane to assure equilibration between pipette solution containing 0.5 mM ATP and the cytosol. (*A*) Under control anesthetic-free conditions, 5 μM pinacidil elicited an outward current sensitive to inhibition by 1 μM glibenclamide (GLIB), indicating activation of the adenosine triphosphate–sensitive potassium channel. (*B*) In the continuous presence of 0.5 mM isoflurane, current activation by pinacidil was markedly enhanced. *Insets* show original traces of I_{KATP} recorded at the indicated points of experimental protocol.

Exposure to Isoflurane Induces Prolonged Sensitization of the sarcK$_{ATP}$ Channel

The above experiments tested immediate effects of isoflurane on the sarcK$_{ATP}$ channel in rat myocytes. However, to date, no studies have addressed a possibility of persisting effects of volatile anesthetics on the sarcK$_{ATP}$ channel. Such lasting effects could explain the cardioprotection memory of APC. Therefore, we hypothesized that isoflurane induces a prolonged sensitization of the sarcK$_{ATP}$ channel, which persists even after anesthetic withdrawal. To test this hypothesis, the following exper-

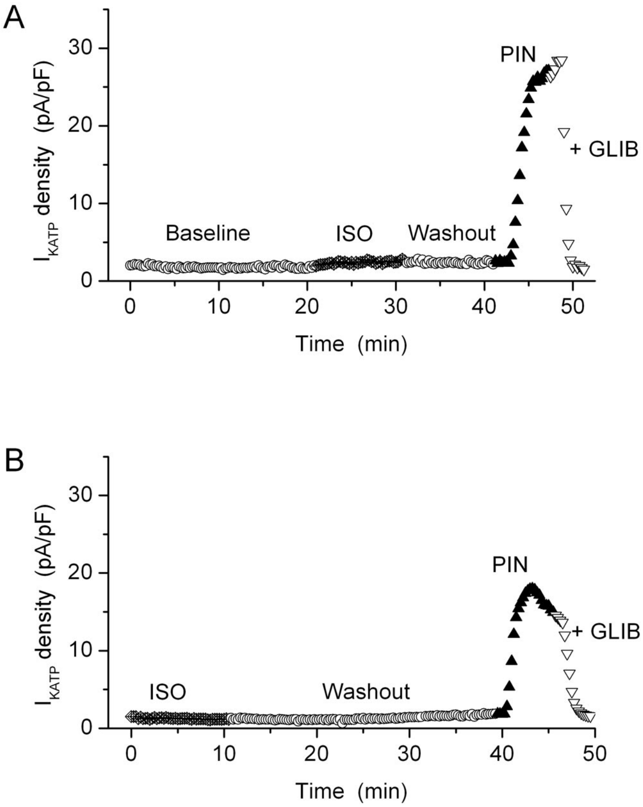

Fig. 2. Isoflurane (ISO) pretreatment results in prolonged potentiation of sarcolemmal adenosine triphosphate–sensitive potassium channel current (I_{KATP}). (*A*) Representative time course experiment in which a voltage clamped myocyte was subjected to 10 min of pretreatment with 0.5 mM isoflurane and 10 min of anesthetic washout before application of 5 μM pinacidil (PIN). After isoflurane pretreatment and its washout, the pinacidil-elicited I_{KATP} was greater compared with that in anesthetic-free control (fig. 1A). This suggested that the channel remains sensitized to opening even after withdrawal of the anesthetic. (*B*) Time course of I_{KATP} with 30 min of washout after 10 min of isoflurane exposure. Magnitude of pinacidil-activated I_{KATP} was still greater than in anesthetic-free control. GLIB = glibenclamide.

iments were performed. Voltage clamped myocytes were exposed to isoflurane for 10 min. Anesthetic was then washed out before application of pinacidil. Two different washout periods were tested: 10 and 30 min. A 10-min washout was sufficient to remove all anesthetic, which was confirmed by repeated gas chromatography measurements. After cell exposure to isoflurane and 10 min washout, the density of pinacidil-elicited I_{KATP} continued to be significantly higher (15.6 ± 11.3 pA/pF, n = 12; fig. 2A) compared with the anesthetic-free control. Even after 30 min of anesthetic washout, the sarcK_{ATP} channel sensitization was still present, although somewhat attenuated (11.8 ± 3.9 pA/pF, n = 6; fig. 2B). These results showed that channel sensitization by isoflurane persists even after anesthetic withdrawal (fig. 3). To confirm this finding, we used another cardiac-specific K_{ATP} channel opener, levcromakalim (10 μM). In control experiments, the magnitude of levcromakalim-activated I_{KATP} was 18.3 ± 5.8 pA/pF (n = 6; not shown). After cell pretreatment with isoflurane and 10 min washout of anesthetic, the density of levcromakalim-elicited I_{KATP} was significantly increased to 24.5 ± 3.5 pA/pF (n = 6; not shown), indicating that sensitization is not exclusive to pinacidil but can be extended to other K_{ATP} channel openers. These results showed that after isoflurane exposure and its washout, the sarcK_{ATP} channel remains sensitized to opening, suggesting a possible memory to previous anesthetic exposure.

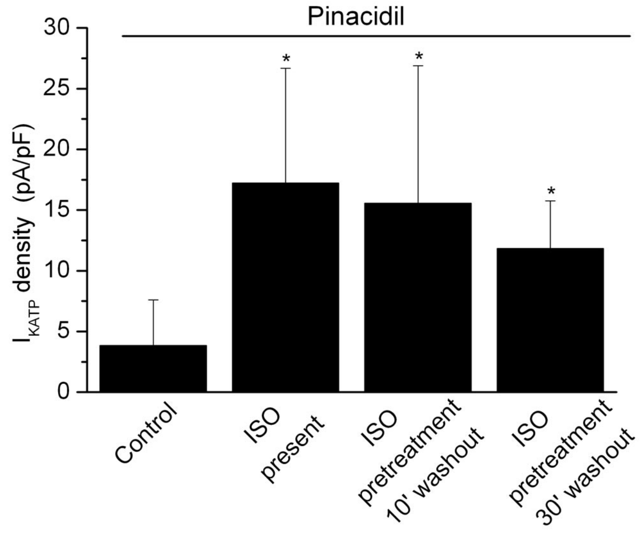

Fig. 3. Summary graph shows the mean values (± SD) for density of pinacidil-elicited sarcolemmal adenosine triphosphate–sensitive potassium channel current (I_{KATP}) measured in anesthetic-free controls (n = 11), in the continued presence of isoflurane (ISO; n = 6), after isoflurane pretreatment and 10 min of washout (n = 12), and after isoflurane pretreatment and 30 min of washout (n = 6). * Significant at $P < 0.05$ *versus* control.

Activation of PKC Is Necessary for Isoflurane-induced Sensitization of the sarcK_{ATP} Channel

To elucidate the mechanism of the isoflurane-induced memory, we investigated whether the PKC-mediated signaling is responsible for the prolonged channel sensitization. In these experiments, we first used chelerythrine (5 μM), an isoform-nonselective inhibitor of PKC.[25] When the sensitization experiments were conducted in the continued presence of chelerythrine, the density of pinacidil-activated I_{KATP} was decreased to 6.6 ± 4.6 pA/pF (n = 11; fig. 4A) and therefore was significantly lower than the current recorded without PKC inhibition (fig. 2). Chelerythrine alone, applied under control anesthetic-free conditions, did not affect pinacidil-activated I_{KATP}. Current density in these experiments was 4.0 ± 5.8 pA/pF (n = 6; fig. 4B). These results suggested that PKC is involved in prolonged channel sensitization by isoflurane.

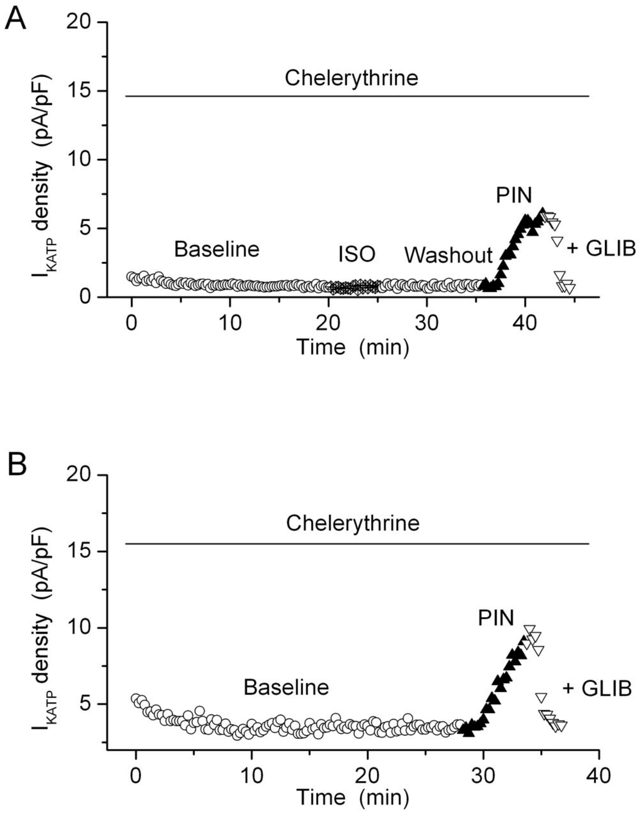

Fig. 4. Inhibitor of protein kinase C chelerythrine abolishes isoflurane (ISO)–induced potentiation of sarcolemmal adenosine triphosphate–sensitive potassium channel current (I_{KATP}). Shown are representative time course experiments conducted in the continued presence of 5 μM chelerythrine, which was applied to the myocytes in the pipette solution. (*A*) In the presence of chelerythrine, I_{KATP} elicited by 5 μM pinacidil (PIN) after pretreatment with isoflurane and its washout was markedly reduced, indicating that chelerythrine abolishes isoflurane-induced potentiation of I_{KATP}. (*B*) Chelerythrine had no effect on pinacidil-activated current under control, anesthetic-free conditions, confirming that this protein kinase C inhibitor alone does not affect channel opening by pinacidil. GLIB = glibenclamide.

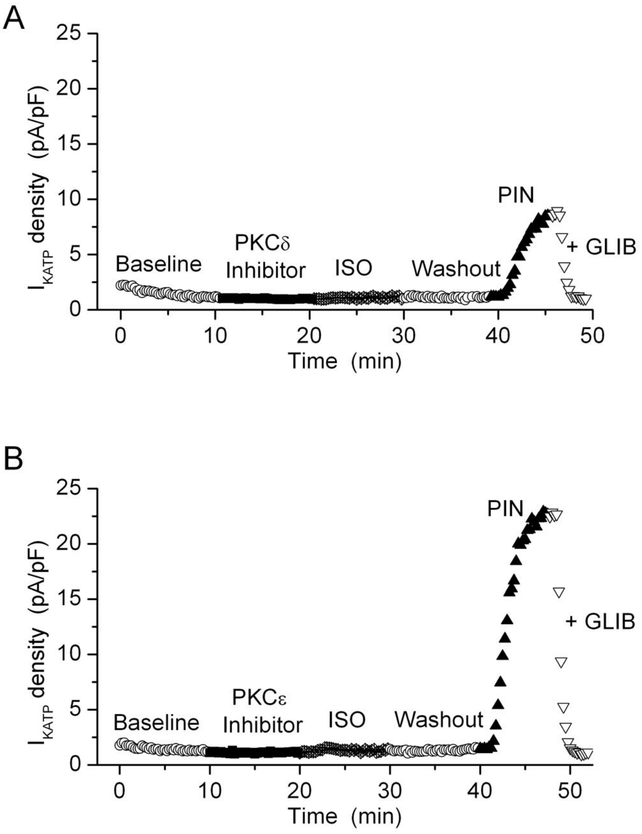

Fig. 5. Inhibition of protein kinase C (PKC)-δ but not PKC-ε abolishes isoflurane (ISO)–induced potentiation of sarcolemmal adenosine triphosphate–sensitive potassium channel current (I_{KATP}). Shown are representative time course experiments in which specific membrane-permeable peptide inhibitors of PKC-δ and PKC-ε were applied to myocytes in the external solution before isoflurane. (*A*) After treatment with inhibitor of PKC-δ (100 nM), isoflurane did not produce sensitization. (*B*) In contrast, treatment with inhibitor of PKC-ε (200 nM) did not alter the potentiation of I_{KATP} by isoflurane. GLIB = glibenclamide; PIN = pinacidil.

Inhibition of PKC-δ but Not PKC-ε Abolishes Sensitization of the sarcK$_{ATP}$ Channel by Isoflurane

To determine which PKC isoform mediates isoflurane-induced channel sensitization, we used specific peptide inhibitors of PKC-ε and PKC-δ. These isoform-specific inhibitors of PKC translocation were applied in the extracellular solution before addition of isoflurane. As shown in figure 5A, after pretreatment with peptide inhibitor of PKC-δ (100 nM), isoflurane sensitization was abolished, and the pinacidil-activated I_{KATP} decreased significantly (7.7 ± 5.4 pA/pF, n = 12). By contrast, PKC-ε peptide inhibitor (200 nM) had no effect on isoflurane sensitization, and pinacidil-elicited current was 14.8 ± 9.6 pA/pF (n = 12; fig. 5B). These results suggest that PKC-δ mediates isoflurane-induced channel sensitization. As a positive control, we used specific peptide activators of PKC-δ and PKC-ε (200 nM) in place of isoflurane. Interestingly, both PKC-δ and PKC-ε activators were able to induce sarcK$_{ATP}$ channel sensitization. With PKC-δ activator, the density of I_{KATP} was 18.9 ± 7.2 pA/pF (n = 12; fig. 6A). With PKC-ε activator, the density of I_{KATP} was 18.6 ± 11.1 pA/pF (n = 10; fig. 6B). In addition, when PKC-δ and PKC-ε activators were administered before isoflurane exposure and washout, there was no additive effect, and pinacidil-elicited I_{KATP} was 15.6 ± 11.0 pA/pF (n = 7) and 13.5 ± 3.1 (n = 4), respectively (data not shown). As a negative control, we used an inactive peptide carrier C1 (200 nM), which did not affect isoflurane-induced channel sensitization, and under these conditions, pinacidil-elicited I_{KATP} was 15.2 ± 9.4 pA/pF (n = 5; fig. 7). This suggested that saturation of the effect is reached by the PKC-δ or PKC-ε activator and isoflurane alone. These results indicate that although both PKC-δ and PKC-ε activation are able to sensitize the sarcK$_{ATP}$ channel, activation of PKC-δ seems to be the event mediating isoflurane-induced sensitization of the sarcK$_{ATP}$ channel.

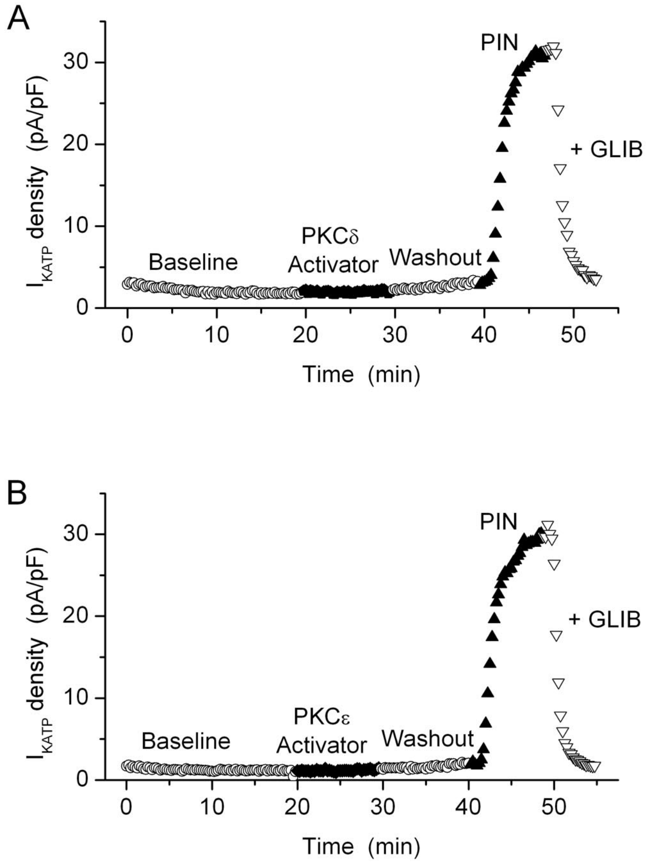

Fig. 6. Protein kinase C (PKC)-δ and PKC-ε activation sensitizes the sarcolemmal adenosine triphosphate–sensitive potassium channel and mimics isoflurane effects. Peptide activators of specific PKC isoforms were applied in the external solution before myocyte exposure to pinacidil (PIN). Shown are representative time courses. (*A*) After treatment with activator of PKC-δ, pinacidil-elicited sarcolemmal adenosine triphosphate–sensitive potassium channel current (I_{KATP}) was enhanced to the same extent as after isoflurane exposure. (*B*) Exposure to specific activator of PKC-ε also sensitized sarcolemmal adenosine triphosphate–sensitive potassium channel to pinacidil. GLIB = glibenclamide.

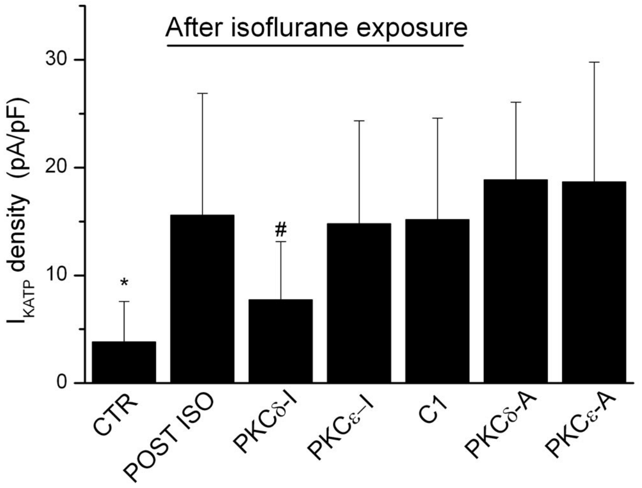

Fig. 7. Protein kinase C (PKC)-δ is involved in prolonged sensitization of the sarcolemmal adenosine triphosphate–sensitive potassium channel by isoflurane. Summary graph shows the mean values (± SD) of pinacidil-activated sarcolemmal adenosine triphosphate–sensitive potassium channel current (I_{KATP}) measured under various experimental conditions: in the absence of isoflurane (CTR, n = 11), after isoflurane pretreatment and its washout (POST ISO, n = 12), after pretreatment with PKC-δ inhibitor (PKCδ-I, n = 12), after pretreatment with PKC-ε inhibitor (PKCε-I, n = 12), in the presence of inactive peptide carrier (C1, n = 5), in the presence of specific PKC-δ activator (PKCδ-A, n = 11), and in the presence of specific PKC-ε activator (PKCε-A, n = 10). Peptide inhibitor of PKC-δ abolished isoflurane-induced sensitization, whereas inhibitor of PKC-ε and inactive peptide carrier C1 had no effect on channel sensitization. The peptide activators of PKC-δ and PKC-ε produced channel sensitization similar in magnitude to that induced by isoflurane. * $P < 0.05$ CTR *versus* POST ISO, PKCε-I, C1, PKCδ-A, and PKCε-A. # $P < 0.05$ PKCδ-I *versus* POST ISO.

Discussion

In the current study, we used an *in vitro* model of single isolated cardiac myocyte to investigate the mechanism of isoflurane-induced APC, in particular, the memory phase of early APC. We focused on the sarcK_{ATP} channel and tested the hypothesis that isoflurane produces lasting modulation of this channel. Our results show that in rat ventricular myocytes, isoflurane sensitizes the sarcK_{ATP} channel to opening as evidenced by the potentiation of I_{KATP}, which persists even after anesthetic withdrawal. This suggests that after exposure to anesthetic and its removal, the channel remains primed to opening, indicating a memory of the channel and/or signaling elements upstream. Furthermore, we demonstrated that the mechanism of isoflurane-induced memory involves specific activation of PKC-δ.

A distinct characteristic of preconditioning phenomenon is the memory phase when cardioprotection continues despite removal of the preconditioning stimulus. An early memory phase of APC, which starts immediately after anesthetic exposure and may continue up to 3 h, was the major interest of this study. The mechanism of early memory involves posttranslational modifications of the cellular elements rather then changes in the protein expression[26] and seems similar in all modes of cardiac preconditioning including APC. It involves activation of G protein–coupled receptors, protein kinases, reactive oxygen species, and the K_{ATP} channels. However, thus far, no studies have shown whether and how the putative end-effectors of preconditioning such as K_{ATP} channels are modulated during the memory phase of APC. In the current study, we showed that after APC, sarcK_{ATP} channel "remembers" previous exposure to anesthetic and remains sensitized to opening. Therefore, during potentially lethal ischemia–reperfusion injury, the primed channel could open more readily and/or to a greater extent and thereby exert its protective effects more efficiently. A similar effect was demonstrated in rabbit cardiomyocytes where agents that are able to precondition the myocardium, adenosine and phorbol 12-myristate 13-acetate, primed the sarcK_{ATP} channel to opening, thus decreasing the latency of channel opening, shortening action potential duration during metabolic inhibition, and tending to delay the onset of myocyte hypercontracture.[12] In addition, it has been demonstrated that in beating guinea pig cardiomyocytes preconditioned by a brief episode of ischemia, opening of the sarcK$_{ATP}$ channels during subsequent prolonged ischemia is greatly enhanced, and myocytes tolerate hypoxia better.[11]

The exact mechanism by which preconditioning primes the sarcK$_{ATP}$ channel to opening is not known but may involve multiple factors. In guinea pig cardiomyocytes, ischemic preconditioning increased the trafficking of sarcK$_{ATP}$ channels thereby up-regulating the number of channels in the plasma membrane.[11] Other studies indicated importance of the channel protein phosphorylation by PKC as a major mechanism of channel priming.[24,27] Results from our study support the latter hypothesis. PKC has been reported to potentiate sarcK$_{ATP}$ channel opening.[16,17] In the intact rabbit and human cardiomyocytes at low intracellular ATP, activation of PKC by phorbol 12,13-didecanoate elicited whole cell I$_{KATP}$.[17] Also, application of constitutively active PKC to excised inside-out membrane patches increased the sarcK$_{ATP}$ channel activity by reducing its sensitivity to ATP inhibition.[16] This effect was abolished in the presence of active protein phosphatase 2A, suggesting that direct phosphorylation of the channel by PKC is responsible for the potentiation of channel opening.[16] A phosphorylation site responsible for the PKC-induced modulation of sarcK$_{ATP}$ channel was found to be threonine residue T180 on the Kir6.2 subunit, the channel pore.[28]

Despite ample evidence demonstrating a direct interaction of PKC with sarcK$_{ATP}$ channel, thus far, there are no studies to show which isoform of PKC is involved in preconditioning-induced modulation of the sarcK$_{ATP}$ channel. The heart expresses several different isoforms of PKC: conventional (α and β), novel (δ, ε, and η), and atypical (λ and ζ).[29] Of these isoforms, only PKC-δ and -ε are indicated to be crucial for the cardioprotection afforded by APC. Volatile anesthetics were shown to activate δ and ε isoforms by inducing specific translocation of these isoforms to various cellular locations.[20,21,24,30–33] This translocation enables a close proximity of the PKC and its substrates and ensures the specificity of their interaction.[34] Specific translocation sites of δ and ε isoforms include sarcolemma and mitochondria, where two putative end-effectors of preconditioning, the sarcolemmal and mitochondrial K$_{ATP}$ channels, are located. PKC-δ was shown to translocate to the mitochondria and PKC-ε was shown to translocate to the sarcolemma in isolated rat hearts preconditioned by isoflurane *in vitro*.[20] However, in the hearts from rats preconditioned by isoflurane *in vivo*, PKC-δ translocated to the sarcolemma and PKC-ε translocated to the mitochondria.[21] Also, in the model of isolated rat trabeculae, sevoflurane induced translocation of PKC-δ to the sarcolemma, whereas PKC-ε distribution did not change.[30] Similar results were demonstrated in human atrial tissue samples after APC by sevoflurane, where PKC-δ translocated to the sarcolemma and PKC-ε translocated to the mitochondria, nuclei, and intercalated discs.[32] Findings from these and other studies[24] are contradictory, and this is most likely because of differences in the species used, preconditioning agents, and the type of experimental preparation. These conflicting results reflect the controversy and difficulty in drawing clear-cut conclusions regarding the specific role of each PKC isoform in the preconditioning phenomenon (including ischemic preconditioning and other forms of pharmacologic preconditioning).

In our study, inhibition of PKC-δ abolished isoflurane-induced sensitization of the sarcK$_{ATP}$ channel, but inhibition of PKC-ε had no effect. However, when activators of PKC-δ and PKC-ε were used instead of isoflurane, they sensitized the channel to the same extent as isoflurane. When PKC-δ and PKC-ε activators were used together with isoflurane, no additive effect was observed. Based on these findings, we conclude that activation of PKC-δ most likely mediates isoflurane effects. Activation of PKC-ε can also sensitize the sarcK$_{ATP}$ channel, but based on experiments using the peptide inhibitor of PKC-ε, it seems that this isoform does not mediate isoflurane effects on the sarcK$_{ATP}$ channel. That PKC-δ or PKC-ε activator and isoflurane do not have an additive effect might be explained by the saturation of the effect reached by the PKC-δ or PKC-ε activator and isoflurane alone. Takeishi *et al.* [35] demonstrated that specific isoforms of PKC respond differently to different stimuli (hypoxia, ischemia, oxidative stress, angiotensin II). Therefore, one possible explanation is that isoflurane specifically activates and translocates PKC-δ but not PKC-ε to the membrane. Another explanation is that activation of PKC-ε by the peptide activator may indirectly affect the sarcK$_{ATP}$ channel *via* other pathways, such as mitochondrial pathway, by initiating cross-talk between mitochondria and sarcK$_{ATP}$ channels. Evidence supporting this possibility was given by Aizawa *et al.*,[24] who demonstrated that priming action of PKC-ε activator on the sarcK$_{ATP}$ channel is blocked by the coadministration of 5-hydroxydecanoate, a mitochondrial K$_{ATP}$ channel blocker.

Opening of the cardiac sarcK$_{ATP}$ channels during ischemia–reperfusion reduces action potential duration and decreases calcium overload, thus being beneficial for cell survival. However, excessive sarcK$_{ATP}$ channel activation might predispose the heart to lethal arrhythmias.[36] Our study demonstrates that isoflurane exposure sensitizes the sarcK$_{ATP}$ channel to opening *via* a PKC-mediated mechanism. In this sensitized state, the sarcK$_{ATP}$ channel would open more readily during ischemia–reperfusion and exert its protective effects more efficiently. Furthermore, PKC may have additional beneficial effects. It has been demonstrated recently in adult rat myocytes and African green monkey kidney cells (COS-7 cells) expressing recombinant K_{ATP} channels that prolonged activation of PKC may prevent excessive and thus detrimental opening of the K_{ATP} channels by downregulating the number of channels in the plasma membrane.[37] This negative feedback involves channel internalization. Therefore, PKC may have multiple, complex effects on the K_{ATP} channel. By potentiating channel activation through phosphorylation and by regulating channel trafficking, PKC may tightly regulate activity of the sarcK_{ATP} channel during periods of ischemia and reperfusion.[37]

In conclusion, we report a novel finding that APC by isoflurane induces a prolonged potentiation of the sarcK_{ATP} channel activity that lasts even after withdrawal of isoflurane. Our results indicate that the δ isoform of PKC, rather than the ε isoform, is the mediator of isoflurane effects.

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