Anesthesiology 2006; 104:503–10

Impact of In Vivo *Preconditioning by Isoflurane on Adenosine Triphosphate–sensitive Potassium Channels in the Rat Heart*

Lasting Modulation of Nucleotide Sensitivity during Early Memory Period

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Background: **The early memory of anesthetic-induced preconditioning (APC) is a period when myocardial protection continues even after removal of the anesthetic. Because adenosine triphosphate–sensitive potassium (K_{ATP}) channels are important mediators of APC, the authors investigated the hypothesis that the memory involves channel priming by isoflurane *via* a long-term modulation of the sensitivity to intracellular adenosine nucleotides.**

Methods: **Ventricular cardiomyocytes were obtained from the rat hearts after 30 min *in vivo* APC with 1.4% isoflurane and from control non-APC rat hearts. Whole cell and excised inside-out patch clamp techniques were used to study the sarcolemmal K_{ATP} channel. Membrane expression of K_{ATP} channel proteins, the pore-forming inward rectifier Kir6.2, and the regulatory sulfonylurea receptor SUR2A were assessed in APC and non-APC hearts by Western blotting.**

Results: **Activation of whole cell K_{ATP} current by isoflurane was enhanced after *in vivo* APC. At the single-channel level, this was paralleled by a 12-fold decrease in adenosine 5′-triphosphate sensitivity and a 3-fold decrease in adenosine 5′-diphosphate sensitivity, without changing the probability of channel opening or single-channel conductance. The membrane expression of Kir6.2 and SUR2A subunits was not altered by *in vivo* APC. A direct *in vitro* application of isoflurane to excised membrane patches increased the channel open probability and produced a 4-fold decrease in adenosine 5′-triphosphate sensitivity only of channels in non-APC myocytes.**

Conclusions: ***In vivo*** **APC by isoflurane decreases sensitivity of the sarcolemmal K_{ATP} channel to inhibition by adenosine 5′-triphosphate and decreases adenosine 5′-diphosphate sensitivity. These effects persist even after discontinuation of the anesthetic, suggesting a possible novel factor that may contribute to the mechanism of early memory of APC.**

ANESTHETIC preconditioning (APC) is a phenomenon whereby a brief exposure to volatile anesthetics protects the heart by delaying and reducing myocardial injury caused by subsequent prolonged ischemia.[1,2] Characteristic to APC is a memory phase, or an early window of protection, during which the cardioprotective effects persist even after discontinuation of the anesthetic.[1] The early memory phase has been shown to last at least 30–60 min. However, the exact time frame of anesthetic-induced early protection remains to be determined. A growing body of evidence indicates that the mechanism of APC involves activation of several intracellular signaling pathways and opening of adenosine triphosphate–sensitive potassium (K_{ATP}) channels in the sarcolemma ($sarcK_{ATP}$) and in the inner membrane of mitochondria ($mitoK_{ATP}$).[3] These channels seem to play essential and distinct roles in the mechanism of myocardial protection afforded by ischemic, hypoxic, and pharmacologic preconditioning.[4–12]

In a recent functional study, we demonstrated that isoflurane may protect isolated rat cardiomyocytes from the oxidative stress–induced cell death *via* a mechanism that involves activation of the $sarcK_{ATP}$ channels.[13] Further, we showed that APC memory could be reproduced under *in vitro* conditions in a model of isolated ventricular cardiomyocytes. Whole cell patch clamp studies demonstrated that a period of 10–30 min after isoflurane exposure is characterized by enhanced sensitivity of the $sarcK_{ATP}$ channel to its openers. This effect was mediated by the novel δ isoform of protein kinase C.[14] These studies suggested that early memory of APC may involve isoflurane priming of the $sarcK_{ATP}$ channel.

In the current study, we focused on the mechanism of isoflurane interaction with the $sarcK_{ATP}$ channel and investigated the hypothesis that isoflurane priming involves a long-lasting modulation of channel sensitivity to intracellular nucleotides, adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP), the important regulators of K_{ATP} channels.[15–17]

Materials and Methods

The experimental protocols of the current study were reviewed and approved by the Animal Care and Use Committee of the Medical College of Wisconsin, Milwaukee, Wisconsin. All conformed to the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health.[18]

In Vivo *APC*

To produce APC *in vivo*, rats were subjected to 30 min of inhaled isoflurane (1.4%, equivalent to 1 minimum alveolar concentration in rats). Isoflurane was delivered to the anesthesia chamber from the vaporizer (SurgiVet, Inc., Waukesha, WI) in a mixture of air and 30% O_2. The

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Received from the Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin. Submitted for publication July 13, 2005. Accepted for publication November 17, 2005. Supported in part by grant Nos. HL 034708 and PO1 GM066730 (to Dr. Bosnjak) from the National Institutes of Health, Bethesda, Maryland.

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end-tidal concentrations of isoflurane were monitored using a multigas analyzer Poet II (Criticare Systems, Inc., Waukesha, WI). After APC, rats were allowed to recover for 30 min before the hearts were harvested and the ventricular myocytes were isolated. Therefore, approximately 2.5 h passed between the *in vivo* APC and initiation of patch clamp experiments.

Cell Isolation

The experiments in this study were conducted in ventricular cardiomyocytes isolated from the hearts of 66 control non-APC rats and 54 APC rats. Adult male Wistar rats (150–250 g) were heparinized and anesthetized with 100 mg/kg intraperitoneal thiobutabarbital (Inactin, Sigma, St. Louis, MO). Ventricular myocytes were obtained from the hearts by enzymatic dissociation with collagenase (type II; Invitrogen, Carlsberg, CA) and protease (type XIV; Sigma-Aldrich, St. Louis, MO) as described previously.[19] Isolated myocytes were kept in the Tyrode solution at room temperature (20°–22°C) and were used for patch clamp experiments within 6 h.

Solutions

Tyrode solution contained 132 mM NaCl, 5.0 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM HEPES, and 5 mM glucose, adjusted to pH 7.3 with NaOH. For whole cell patch clamp recordings, the intracellular/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, adjusted to pH 7.2 with KOH. The extracellular/bath solution contained 132 mM *N*-methyl-D-glucamine, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES, adjusted to pH 7.4 with HCl. The L-type Ca channels were blocked by 200 nM nisoldipine (Miles-Pentex, West Haven, CT). A 10-mM stock of pinacidil and a 1-mM stock of glibenclamide were prepared in dimethyl sulfoxide. After dilution in recording buffer, the final concentrations of dimethyl sulfoxide were 0.05% and 0.1% for pinacidil and glibenclamide, respectively. In control experiments, dimethyl sulfoxide at 0.1% did not activate sarcK_{ATP} channel current (I_{KATP}) when present in the bath solution during the time course experiments and did not alter whole cell I_{KATP} elicited by pinacidil (not shown).

For single-channel recordings in the inside-out patch configuration, the intracellular solution bathing the cytosolic side of membrane patches contained 140 mM KCl, 0.5 mM $MgCl_2$, 1.0 mM EGTA, 10 mM HEPES, and 100 μM K_2ATP, at pH 7.2 adjusted with KOH. When ATP sensitivity was investigated, the concentration–response curves were constructed at 0, 1, 10, 100, 500, 1,000, 2,000, and 3,000 μM intracellular ATP. The pipette solution facing the extracellular side of the membrane patches contained 140 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 10 mM HEPES, at pH 7.4 adjusted with KOH. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

To investigate the *in vitro* effects of isoflurane on K_{ATP} channels in the whole cell or single-channel experiments, liquid isoflurane (Baxter Healthcare Corp., Deerfield, IL) was dispersed in the bath solution by sonication. Anesthetic-containing buffer was stored in glass syringe reservoirs and was delivered to the recording chamber by a gravity-fed perfusion system. The average concentration of isoflurane in the recording chamber was 0.56 ± 0.1 mM, equivalent to 1.2 vol% at 22°C. Concentrations of isoflurane in the bath solution sampled from the recording chamber were determined by the headspace sampling chromatography method using Shimadzu GC 8A gas chromatograph (Shimadzu, Kyoto, Japan).

Whole Cell Experiments

Whole cell K_{ATP} currents were recorded in a voltage clamp configuration. Patch pipettes were pulled from borosilicate glass tubing (Garner Glass, Claremont, CA) using a horizontal PC-84 puller (Sutter, Novato, CA). Pipette tips were heat-polished (microforge MF-83; Narishige, Tokyo, Japan). The resistance of pipettes immersed in the bath solution was 1.5–2.5 MΩ. Recordings were made at room temperature using an EPC-7 patch clamp amplifier (List, Darmstadt-Eberstadt, Germany) and a Digidata 1322A interface (Axon Instruments, Foster City, CA) for sampling the data and providing command voltages to the headstage. The I_{KATP} was monitored over time by applying every 15 s a 200-ms voltage step to 0 mV from a holding potential of −40 mV. Voltage protocols were generated with pClamp9 software (Axon Instruments, Foster City, CA). Current traces were filtered at 3 kHz through a four-pole Bessel filter and digitized at 1 kHz. Amplitude of steady state macroscopic current was measured at the end of 200-ms voltage steps, and current was normalized to cell capacitance to obtain current density (pA/pF). To ensure equilibration between the pipette solution and the cytosol, a period of 30 min was allowed at the beginning of each whole cell experiment before application of isoflurane or pinacidil.

Single-channel Experiments

For single-channel recordings from excised membrane patches in the inside-out configuration,[20] the resistance of patch pipettes filled with extracellular solution was 7–12 MΩ. Channel activity was monitored continuously at room temperature using an EPC-7 amplifier, and 60 s recordings were made at each experimental step. The current signal was low-pass filtered at 500 Hz through an eight-pole Bessel filter and sampled at a rate of 1 kHz. The K_{ATP} channel was identified by unitary conductance, sensitivity to inhibition by intracellular ATP, and blockade by glibenclamide (1 μM). In single-channel analysis, half-amplitude threshold crossing was used for detecting the open state. The all-points amplitude histograms were constructed from 60-s recordings, and

channel open-state probability (Po) was determined from the ratios of the area under the peaks in the amplitude histograms fitted by a multi-Gaussian distribution. Because of a variable number of channels in patches, Po was reported as cumulative Po, *i.e.*, a fraction of the total time the channels were in the open state over the total time of recording. ATP concentration–response curves were constructed after normalizing Po to that measured in ATP-free buffer. In ADP experiments, Po measured in the individual groups was normalized to respective controls and was reported as relative Po. Data obtained with whole cell and single-channel protocols were analyzed using pClamp9 software and Origin7 software (OriginLab, Northampton, MA).

Western Blotting

Ventricular myocardium was snap-frozen in liquid nitrogen and stored at −80°C. Tissue (300 mg) was homogenized on ice in 2 ml buffer composed of 10 mM Tris HCl, 150 mM KCl, 20 μM $CaCl_2$, 250 mM sucrose, 2 mM DTT, and a cocktail of protease inhibitors (Roche, Nutley, NJ). Homogenate was centrifuged for 10 min at 7,700*g*, and the supernatant was centrifuged again for 1 h at 100,000*g* and +4°C to separate the ventricular membrane fractions. The pellets were resuspended in tris/ethylenediaminetetraacetic acid buffer containing 1% SDS at pH 7.4, and total protein concentration was determined with DC protein assay (BIO-RAD, Hercules, CA). Equal amounts of proteins (100 μg) from non-APC and APC hearts were separated on a 4–20% polyacrylamide gel and transferred to immunoblot membrane (BIO-RAD). Equal loading was confirmed by Ponceau staining. The membrane was blocked with 5% fat-free milk and incubated with a goat polyclonal anti-Kir6.2 G-16 antibody (sc-11228) or a rabbit polyclonal anti-SUR2 H-80 antibody (sc-25684) each at 1:100 dilution. The secondary antibodies were horseradish peroxidase–conjugated anti-goat immunoglobulin G and anti-rabbit immunoglobulin G for detection of Kir6.2 and SUR2, respectively. Both were used at 1:20,000 dilution. All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Chemiluminescence was enhanced by SuperSignal West Pico Luminol/Enhancer (Pierce, Rockford, IL).

Statistical Analysis

Data are reported as mean ± SD. Statistical analyses were performed using the Student two-tailed *t* test for comparison of two experimental groups. Multiple groups were compared using one-way analysis of variance with the Scheffé test for multiple pairwise comparisons. Differences were considered significant at $P < 0.05$. The statistic tests were provided in the Origin7 software.

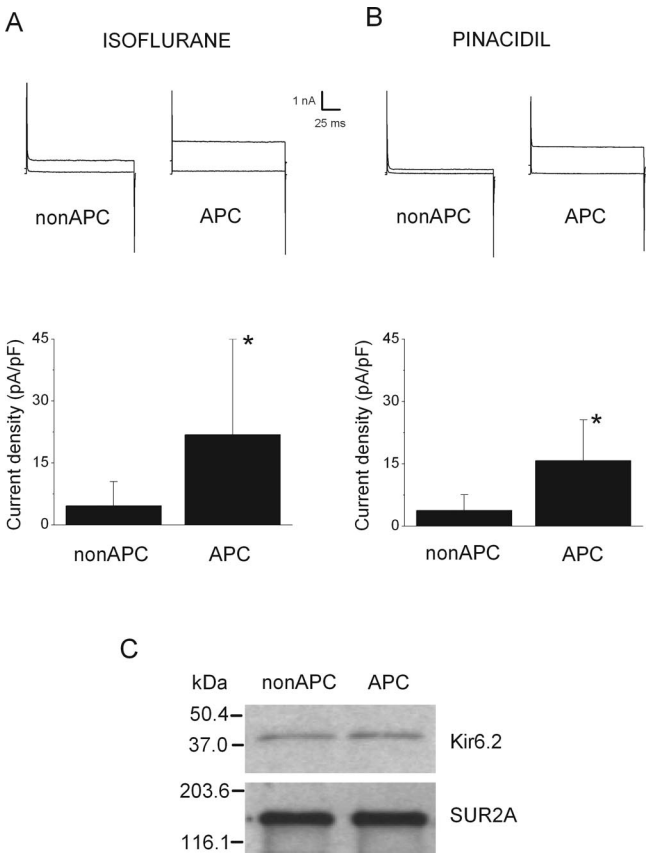

Fig. 1. Activation of the whole cell sarcolemmal adenosine triphosphate–sensitive potassium channel current (I_{KATP}) by isoflurane and pinacidil is enhanced after *in vivo* anesthetic preconditioning (APC). I_{KATP} was monitored during a 200-ms voltage step to 0 mV applied every 15 s from a holding potential of −40 mV. (*Upper panels*) Traces of I_{KATP} recorded at baseline and at steady state of activation by 0.5 mM isoflurane (*A*) and 5 μM pinacidil (*B*). The magnitude of I_{KATP} was greater in APC than in control, non-APC myocytes. (*Lower panels* in *A* and *B*) Summary data for density (mean ± SD) of I_{KATP} elicited by isoflurane and pinacidil (* $P < 0.05$, n = 10–12/group). (*C*) Representative Western blots show no difference in expression of channel subunits Kir6.2 and SUR2A in the membrane fraction from APC and non-APC rat hearts.

Results

Isoflurane and Pinacidil Sensitivity of SarcK_{ATP} Channel

Figure 1 summarizes the results of whole cell experiments investigating the effects of isoflurane (fig. 1A) and the K_{ATP} channel opener, pinacidil (fig. 1B), on the rat I_{KATP} in the presence of 0.5 mM intracellular ATP. In control non-APC myocytes, 0.5 mM isoflurane had variable effects by eliciting I_{KATP} in some cells while producing no effect in others. The density of I_{KATP} activated by isoflurane was 4.6 ± 5.9 pA/pF (n = 12 cells), and 5 μM pinacidil-elicited current was 3.8 ± 3.7 pA/pF (n = 11). By contrast, in APC myocytes, the isoflurane- and pinacidil-elicited I_{KATP} was increased to 21.9 ± 23.0 pA/pF (n = 11) and 15.8 ± 9.8 pA/pF (n = 10), respectively (figs. 1A and B, lower panels). As illustrated in figure 1C, Western blotting revealed no change in

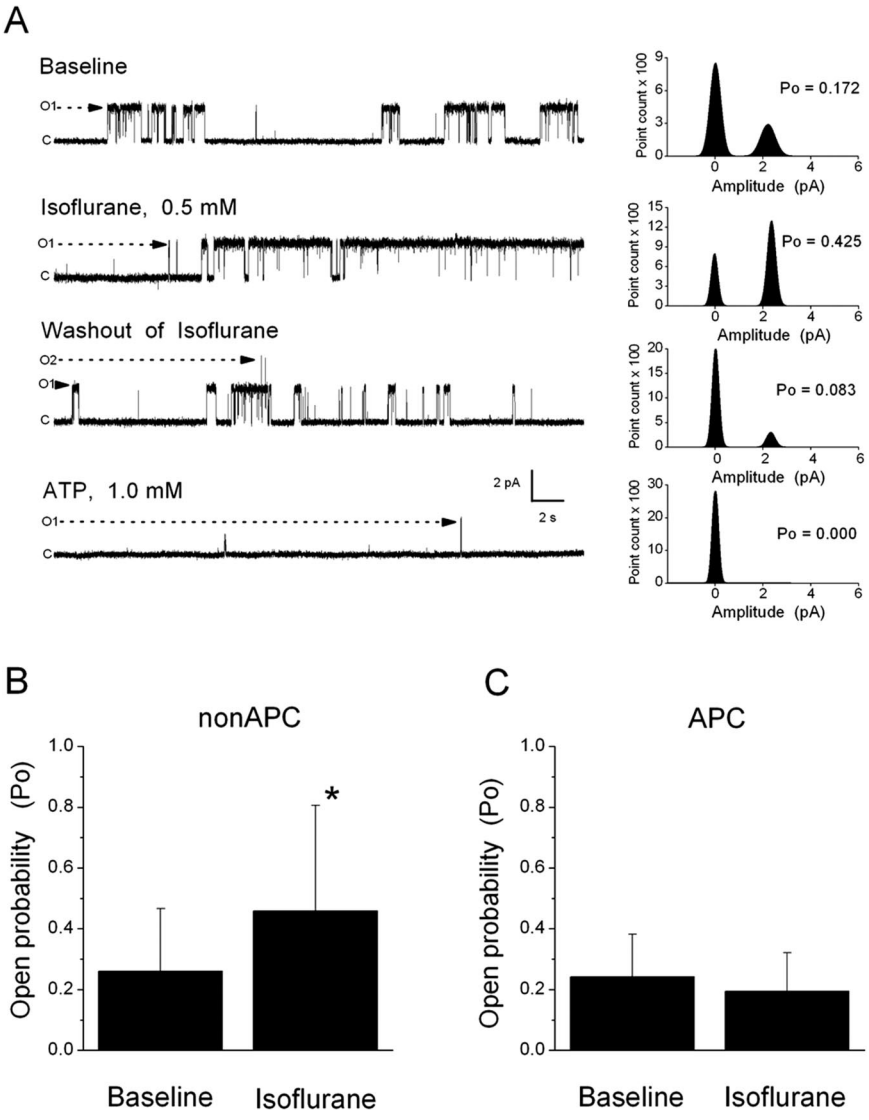

Fig. 2. Effects of isoflurane on open probability (Po) of single sarcolemmal adenosine triphosphate–sensitive potassium channel. Channel activity was monitored at +40 mV in symmetrical 145 mM K^+. (*A*, *left*) Single-channel records from an inside-out patch from a non–anesthetic preconditioning (APC) myocyte. *Upper deflection* indicates the levels of channel opening (O1 and O2) from the closed level (C). Corresponding amplitude histograms are shown in *A*, *right*. Isoflurane (0.5 mM) increased channel Po in a reversible manner. Blockade with 1 mM adenosine 5′-triphosphate (ATP) confirmed identity of adenosine triphosphate–sensitive potassium channel. (*B*) Summary data (mean ± SD) for isoflurane effect on non-APC channel Po (* $P < 0.05$, n = 18). (*C*) Baseline Po of APC channels was no different from Po of non-APC channels and was unaffected by secondary *in vitro* treatment with isoflurane (n = 12).

expression of channel proteins Kir6.2 and SUR2A in the membrane fractions from APC hearts compared with non-APC hearts (n = 4/group). These results indicate that *in vivo* APC, *i.e.*, a previous *in vivo* exposure of rats to isoflurane, enhances sensitivity of the rat sarcK_{ATP} channel to opening without changing membrane expression of the channel subunits, and this effect persists for at least several hours after discontinuation of the anesthetic.

Isoflurane Effect on Open Probability of SarcK_{ATP} Channel

To investigate whether whole cell effects of isoflurane could be explained by direct modulation of the channel, we performed excised inside-out patch clamp experiments in which channel activity was monitored at membrane potential of +40 mV in the continuous presence of 100 μM intracellular ATP. Figure 2A shows single-channel recordings from one inside-out patch and corresponding all-points histograms of unitary current amplitude. Po was determined from multiple Gaussian fits to the histograms. Isoflurane applied to the cytosolic surface of membrane patches enhanced channel opening. Figure 2B shows that in patches from non-APC myocytes, isoflurane increased Po to 0.459 ± 0.347 from the baseline of 0.261 ± 0.205 (n = 18). The effect was reversible upon anesthetic washout (not shown). This suggested that in normal non-APC rat myocytes, isoflurane may enhance I_{KATP}, partly by increasing channel Po. Interestingly, in the patches from APC myocytes (fig. 2C, baseline) Po was similar (0.242 ± 0.140, n = 12) to that measured in non-APC cells (fig. 2B, baseline) but was not altered during secondary *in vitro* exposure to isoflurane (0.195 ± 0.12, n = 12) as shown in figure 2C, isoflurane. These results suggest that enhanced activation of the whole cell I_{KATP} in APC myocytes cannot be attributed to increase in Po.

Isoflurane Effect on Single-channel Conductance

To test whether *in vivo* APC or *in vitro* application of isoflurane affects single-channel conductance, the uni-

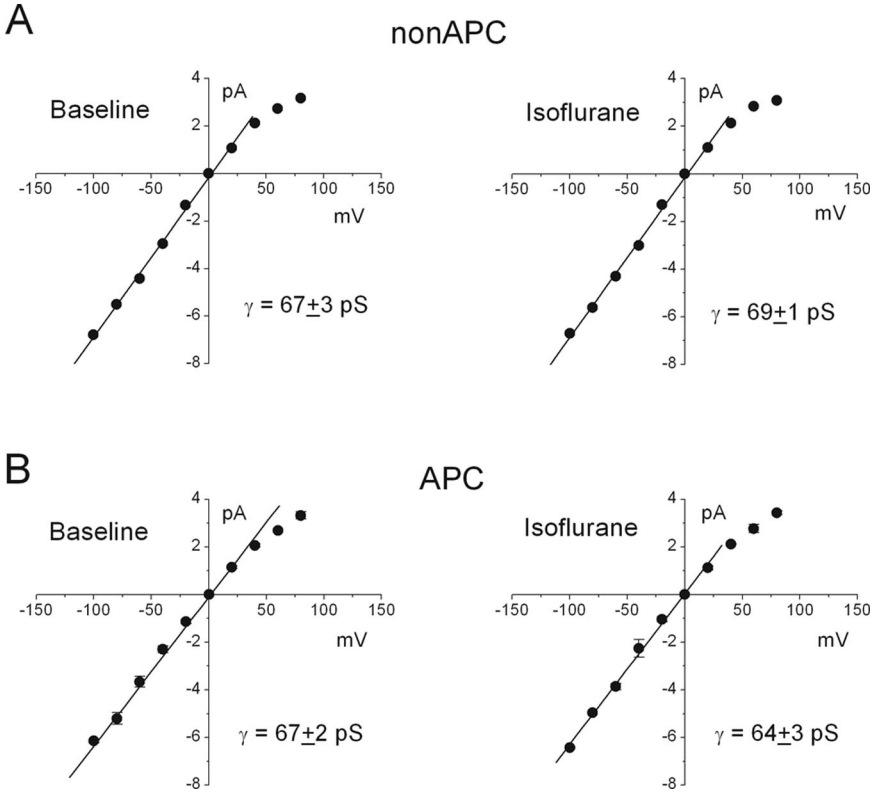

Fig. 3. Isoflurane does not affect single-channel conductance. Current–voltage relations were constructed from records of single sarcolemmal adenosine triphosphate–sensitive potassium channels at membrane potentials between −100 and +80 mV (n = 5/group). Slope conductance of the unitary current is shown in the graph fields. Application of isoflurane to patches from non–anesthetic preconditioning (APC) myocytes did not affect channel conductance (*A*). Conductance was not altered after *in vivo* APC with isoflurane (*B*, *left*) and during isoflurane treatment of patches from APC myocytes (*B*, *right*).

tary current was recorded from inside-out patches at membrane potentials between −100 and +80 mV in symmetrical 145 mM K^+. Current amplitudes, obtained from all-points amplitude histograms, were plotted against membrane potentials. A linear regression fit to current–voltage relation yielded the slope conductance. As shown in figure 3A, baseline conductance of channels in non-APC myocytes (67 ± 3 pS, n = 5) remained unchanged during patch exposure to 0.5 mM isoflurane (69 ± 1 pS, n = 5). Similar effect was found in APC myocytes (fig. 3B) where baseline single-channel conductance (67 ± 2 pS) was not significantly different from that measured during *in vitro* application of isoflurane (64 ± 3 pS, n = 5/group). These results indicated that isoflurane does not modulate the conductance of single K_{ATP} channels.

Effect of Isoflurane on ATP Sensitivity

Intracellular ATP regulates activity of the K_{ATP} channel by causing its closure,[15,16] and sensitivity to inhibition by ATP is modulated by many cellular factors. To investigate whether isoflurane affects ATP sensitivity, channel Po was measured at various concentrations of ATP in patches from APC and non-APC myocytes in the absence and the presence of 0.5 mM isoflurane. The relationship between normalized Po and ATP concentrations was fitted to a Hill equation yielding ATP concentration for half-maximal inhibition (IC_{50}) of the channel and the Hill coefficient (n_H). As shown in figure 4A, in patches from non-APC myocytes, IC_{50} for ATP inhibition was 38 ± 9 μM, and n_H was 0.6 (n = 10/data point). The *in vivo* APC had a dramatic effect on ATP sensitivity by shifting IC_{50} to 475 ± 62 μM and increasing n_H to 1.4 (n = 8–12/data point). However, a secondary *in vitro* application of isoflurane to patches from APC myocytes had no additional effect on channel ATP sensitivity, and IC_{50} was 469 ± 87 μM with n_H of 1.3 (n = 6–8/data point, not shown). In contrast to these findings, the channels in non-APC myocytes were sensitive to isoflurane. As shown in figure 4B, *in vitro* exposure to isoflurane shifted IC_{50} from 38 ± 9 to 137 ± 23 μM with n_H of 1.2 (n = 10/data point). These results show that the *in vivo* APC by isoflurane and acute *in vitro* treatment with isoflurane both markedly decrease ATP sensitivity of the rat cardiac sarcK_{ATP} channel. The magnitude of this effect, however, is different depending on the mode of isoflurane application.

Effect of Isoflurane on ADP Sensitivity

Intracellular ADP is an important modulator of the K_{ATP} channels that increases or inhibits its activity. Magnesium ADP enhances channel opening. We examined whether isoflurane modulates channel sensitivity to 20 and 200 μM intracellular ADP, the concentrations that are found in cardiac myocytes under normal and ischemic conditions, respectively.[21] Experiments were conducted in the continuous presence of 100 μM ATP. In each experimental group, Po was normalized to the respective control, and relative Po values were used for comparisons among groups. In the inside-out patches

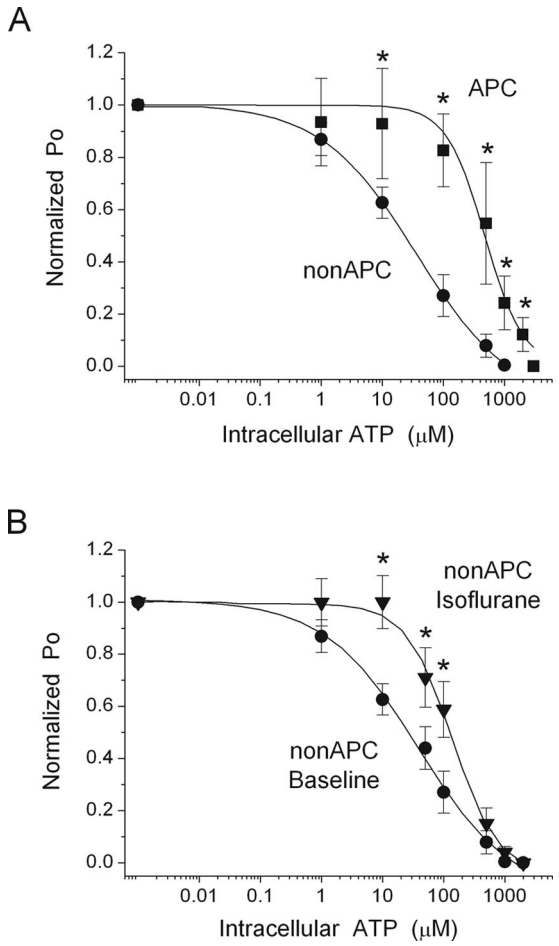

Fig. 4. Adenosine 5′-triphosphate (ATP) sensitivity of the sarcolemmal adenosine triphosphate–sensitive potassium (sarcK_{ATP}) channel is reduced after *in vivo* anesthetic preconditioning (APC) and during *in vitro* application of isoflurane to excised patches. (*A*) ATP concentration and open probability (Po) relations for channels in patches from non-APC and APC myocytes. ATP concentration for half-maximal channel closure (IC_{50}) was 38 μM (n = 10/data point) in non-APC myocytes and 475 μM (n = 8–12/data point) in APC myocytes. (*B*) In patches from non-APC myocytes, 0.5 mM isoflurane shifted IC_{50} from 38 to 137 μM (n = 10/data point, * $P < 0.05$).

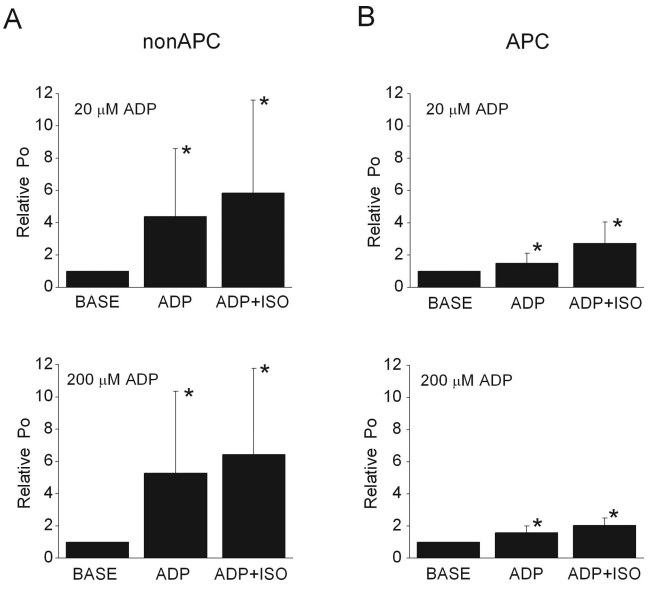

Fig. 5. Adenosine 5′-diphosphate (ADP) sensitivity is reduced after *in vivo* anesthetic preconditioning (APC) with isoflurane. Shown are mean values for relative open probability (Po) ± SD. (*A*, *upper* and *lower*) ADP alone increased Po of non-APC channels, and application of isoflurane together with ADP (ADP + ISO) further increased Po. (*B*, *upper* and *lower*) ADP sensitivity of channels from APC myocytes was markedly decreased compared with non-APC channels (* $P < 0.05$).

from non-APC myocytes, ADP activated the K_{ATP} channels (fig. 5A). There was a similar fivefold increase in Po in the presence of 20 μM (n = 8) and 200 μM (n = 6) ADP. However, application of isoflurane to ADP-activated channels produced little increase in Po, and a previous patch exposure to isoflurane did not modify ADP sensitivity of channels in patches from non-APC myocytes (n = 8, not shown). Characteristically, in patches from APC myocytes, channel responsiveness to both 20 μM (n = 9) and 200 μM (n = 6) ADP was significantly reduced (fig. 5B), without affecting isoflurane sensitivity.

Discussion

The results of the current study provide evidence for differential modulation of the rat sarcK_{ATP} channel by isoflurane under *in vivo* and *in vitro* conditions. A novel finding is that *in vivo* APC induces a long-lasting functional modification of channel gating by ATP, resulting in a dramatic 12-fold decrease in ATP sensitivity. Consequently, this will change the threshold for channel opening and enhance sensitivity of the channel to modulators and openers. Therefore, in the anesthetic preconditioned hearts, the K_{ATP} channels will be more likely to open during an ischemic or hypoxic episode. The reduced ATP sensitivity, an effect that lasted for at least 3–4 h after *in vivo* exposure to isoflurane, could in part be responsible for increased activation of the whole cell I_{KATP} by isoflurane or pinacidil in the APC myocytes. Acute *in vitro* application of isoflurane to membrane patches from non-APC myocytes produced a marked but less pronounced (4-fold) decrease in ATP sensitivity. The effect was observed within few minutes of exposure to the anesthetic, suggesting a possible direct action of isoflurane on the channel. This finding is consistent with a previous report by Han *et al.*,[22] where isoflurane decreased ATP sensitivity of the sarcK_{ATP} channel in the inside-out patches from rabbit myocytes. A similar effect was observed in guinea pigs, but only at mildly acidic intracellular pH.[23] Our study extends those observations by showing that the ATP sensitivity of sarcK_{ATP} channels in rat cardiomyocytes is markedly reduced during *in vitro* application of isoflurane even at normal intracellular pH, which might explain why in contrast to other species, isoflurane alone was able to elicit whole cell I_{KATP} in rat myocytes. Taken together, these findings

suggest species dependence and intracellular pH dependence of isoflurane effects on the sarcK_{ATP} channel.

Consistent with the report by Han *et al.*,[22] the single-channel conductance was not altered by isoflurane in our study. However, in contrast to findings in other species,[22,24] application of isoflurane to the inside-out membrane patches from rat non-APC myocytes increased the probability of channel opening. Characteristically, Po was increased during exposure to isoflurane but declined upon anesthetic washout. Because single-channel recordings were made at intracellular pH 7.2, which is in the range of normal physiologic intracellular pH in rat cardiomyocytes,[25] the effect of isoflurane on channel activity in rats seems different from those in guinea pigs, where isoflurane increased Po only at more acidic, ischemic-like pH of 6.8,[23] and in rabbits where isoflurane clearly had an inhibitory effect on Po at intracellular pH of 7.4.[22] The *in vivo* APC in rats produced no lasting change in channel activity, and Po values of channels in APC and non-APC myocytes were similar. The observation that Po of non-APC channels is enhanced only during exposure to isoflurane may also explain our findings in APC myocytes. It is conceivable that Po has been increased during *in vivo* exposure to isoflurane. However, after APC, when isoflurane is no longer present, channel activity returned to the control level. Interestingly, Po of the APC channels was not altered by the secondary *in vitro* exposure to isoflurane. One speculation is that *in vivo* APC could trigger another cellular mechanism, protecting against excessive channel opening that could be detrimental for the heart by compromising K^+ homeostasis. In addition to these findings, Western blotting revealed no differences in expression of K_{ATP} channel subunits in the membrane fractions from control non-APC rat hearts and APC hearts harvested 2 h after *in vivo* APC. Together, these results suggest that isoflurane-evoked enhanced activation of the whole cell I_{KATP} in APC myocytes cannot be explained by changes in membrane expression of channel subunits, or changes in Po and single-channel conductance. Rather, it could result from a pronounced and long-lasting decrease in channel sensitivity to inhibition by ATP (current study), together with activation of the intracellular signaling pathways mediated by phospholipids and protein kinase C.[14,26]

Modulation of the K_{ATP} channel by ATP and ADP is complex and multifactorial.[16,17,27] Intracellular magnesium ADP is known to stimulate activity of the K_{ATP} channel. In our study, Po of non-APC channels was increased on application of both low nonischemic and high ischemic-like concentrations of ADP, but the magnitude of channel activation did not correlate well with the concentrations of ADP. One potential explanation is that ADP signaling requires much lower concentrations of the nucleotide, and our experiments could have been conducted at saturating levels of ADP. Further, the cardiac K_{ATP} channels seem much less sensitive to intracellular ADP compared with other channel isoforms, *e.g.*, the pancreatic β-cell K_{ATP} channels.[17] Nevertheless, the important finding is that *in vivo* APC with isoflurane renders the cardiac K_{ATP} channels significantly less sensitive to ADP. Because binding of magnesium ADP to the nucleotide binding domain 2 (NBD2) of the SUR subunit seems essential for activation of the K_{ATP} channel,[17] our experiments showing that *in vivo* APC with isoflurane causes a threefold decrease in ADP sensitivity would suggest attenuation rather than augmentation of channel opening. However, despite reduced ADP sensitivity, the whole cell experiments clearly showed increased activation of I_{KATP} after *in vivo* APC. One possible explanation is that the consequences of decreased sensitivity to ADP were overridden by a much greater effect of *in vivo* APC on channel ATP sensitivity that promoted channel opening.

The cardiac K_{ATP} channel is an octameric complex of two protein subunits, the inwardly rectifying K channel (Kir6.2) that forms the channel pore and the sulfonylurea receptor (SUR2A), a regulatory subunit. It is the SUR subunit that confers channel sensitivity to inhibitory sulfonylureas, to K_{ATP} channel openers, and to stimulation by Mg nucleotides. SUR subunit also modulates channel sensitivity to ATP.[16,17,27] The intracellular adenine nucleotides interact with both subunits of the channel. Binding of ATP to Kir6.2 causes channel closure, but complex interactions of magnesium ATP and magnesium ADP with two nucleotide binding domains of the SUR subunit modulate channel activity in response to changes in cellular metabolism.[17,28] Our study showing a selective long-lasting modulation by isoflurane of channel sensitivity to intracellular ATP and ADP suggests a possibility of direct anesthetic interaction with the channel subunits.

Anesthetic preconditioning mimics ischemic preconditioning in that both exhibit memory, an early and a late window of protection. The classic early memory of ischemic preconditioning lasts 1–2 h in anesthetized animals,[29,30] but it could be extended from 2 to 4 h in awake, conscious animals.[31] The early memory of APC is reported to last 30–60 min after discontinuation of the anesthetic *in vivo* [2] and 10–30 min in a model of isolated myocytes *in vitro*.[14] However, the exact time frame or limit of early memory of APC is not yet established. The mechanism of early memory of APC is thought to involve rapid posttranslational modification of existing intracellular mediators and signaling proteins.[9,12] Under the assumption that the mechanism of memory also involves a long-term modification of intrinsic properties of the channel protein, in particular, the sensitivity to inhibition by intracellular ATP and activation by ADP, the results from our patch clamp studies suggest that early memory may last 2–4 h after *in vivo* APC. Our recent experiments have shown that changes to the channel

properties are no longer detectable at approximately 6 h after APC when the ATP sensitivity returns to the control level (A. Stadnicka, Ph.D., unpublished observation, October 2005). A limitation of our study is that it is focused exclusively on the sarcK_{ATP} channel, whereas APC is known to involve numerous interactive factors and pathways. In addition, the design of our study does not allow us to make a definite link between our findings and cardioprotection. More studies are required to demonstrate this connection.

In conclusion, the results of the current study suggest a possible novel factor that might contribute to the mechanism of early memory of APC. We propose that this phase involves priming of the sarcK_{ATP} channel *via* isoflurane-induced long-lasting decrease in channel sensitivity to intracellular adenine nucleotides, in particular ATP, without changes in single-channel conductance, probability of channel opening, or membrane expression of the channel subunits. This modulator mechanism seems to be initiated upon exposure to isoflurane[14] and persists for several hours after discontinuation of the anesthetic.

The authors thank Mary Ziebell (Research Technologist, Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin) for isoflurane measurements.

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