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Isoflurane-induced Facilitation of the Cardiac Sarcolemmal K_{ATP} Channel

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Background: **Volatile anesthetics have cardioprotective effects that mimic ischemic preconditioning, including the involvement of adenosine triphosphate–sensitive potassium (K_{ATP}) channels. However, evidence for a direct effect of volatile anesthetic on the K_{ATP} channel is limited. In this study, the effects of isoflurane on the cardiac sarcolemmal K_{ATP} channel were investigated.**

Methods: **Single ventricular myocytes were enzymatically isolated from guinea pig hearts. Whole cell and single-channel configurations, specifically the cell-attached and inside-out patch mode, of the patch clamp technique were used to monitor sarcolemmal K_{ATP} channel current.**

Results: **In the cell-attached patch configuration, 2,4-dinitrophenol (150 μM) opened the sarcolemmal K_{ATP} channel. Isoflurane (0.5 mM) further increased channel open probability and the number of active channels in the patch. In contrast, in the inside-out patch experiments, isoflurane had no significant effect on the K_{ATP} channel activated by low ATP (0.2–0.5 mM). In addition, isoflurane had no effect on the K_{ATP} channel when activated by adenosine diphosphate, adenosine + guanosine triphosphate, bimakalim, and 2,4-dinitrophenol under inside-out patch configurations. When K_{ATP} current was monitored in the whole cell mode, isoflurane alone was unable to elicit channel opening. However, during sustained protein kinase C activation by 12,13-dibutyrate, isoflurane activated the K_{ATP} current that was sensitive to glibenclamide. In contrast, isoflurane had no effect on the K_{ATP} channel activated by 12,13-dibutyrate in a cell-free environment.**

Conclusions: **Isoflurane facilitated the opening of the sarcolemmal K_{ATP} channel in the intact cell, but not in an excised, inside-out patch. The isoflurane effect was not due to a direct interaction with the K_{ATP} channel protein, but required an intracellular component, likely including the translocation of specific protein kinase C isoforms. This suggests that the sarcolemmal K_{ATP} channel may have a significant role in anesthetic-induced preconditioning.**

BRIEF periods of sublethal ischemia reduce the amount of myocyte necrosis produced by a subsequent sustained period of ischemia.[1] This phenomenon, termed ischemic preconditioning (IPC), has been shown to be cardioprotective in several mammalian models, including dogs,[2,3] rabbits,[4] rats,[5] pigs,[6] and humans.[7] Although the underlying mechanism is still unclear, much has been documented of the involvement of cardiac sarcolemmal adenosine triphosphate–sensitive potassium (K_{ATP}) channels in IPC. Several studies have shown that glibenclamide, a potent K_{ATP} channel blocker, abolishes the beneficial effects of IPC,[8] while K_{ATP} channel openers, such as bimakalim, pinacidil, and cromakalim, mimicked the cardioprotective effects of IPC.[9,10] Recent studies have also implicated the contribution of the mitochondrial K_{ATP} channel in IPC.[11] However, the relative contributions of the cardiac sarcolemmal and mitochondrial K_{ATP} channels in IPC are not clear. Recent evidence suggests that the mitochondrial K_{ATP} channel may play a more significant role in cardioprotection than the sarcolemmal channel.[12] Yet the contribution by the sarcolemmal K_{ATP} channel can not be irrefutably discounted. The various signaling pathways that underlie cardioprotection can potentially modulate the mitochondrial and sarcolemmal K_{ATP} channels. Both channel types may turn out to be important in cardioprotection by IPC.[13]

Volatile anesthetics, particularly isoflurane, were recently found to mimic IPC of the heart.[14,15] The cellular mechanisms underlying anesthetic-induced preconditioning are not known but may parallel those of IPC. Since the isoflurane effects were abolished by glibenclamide, the K_{ATP} channels have been implicated. However, direct evidence at the cellular and molecular levels of the involvement of these channels in anesthetic-induced cardioprotection is limited.[16,17]

Several endogenous factors also modulate the sarcolemmal K_{ATP} channel. Ischemic and hypoxic factors, such as an increase in adenosine diphosphate (ADP),[18] production of adenosine,[19] and changes in pH[20] regulate K_{ATP} channel openings. Thus, despite the emergence of the role of the mitochondrial K_{ATP} channel in cardioprotection, effects on the sarcolemmal K_{ATP} channel by these endogenous factors make the channel an attractive end effector of cardioprotection. In anesthetic-induced cardioprotection, a direct action of isoflurane on the cardiac sarcolemmal K_{ATP} channel has been reported.[16] Isoflurane decreased channel activity but also diminished the channel's sensitivity to ATP. Our previous study showed that isoflurane and halothane differentially modulated the sarcolemmal K_{ATP} channel.[21] Under whole cell conditions, isoflurane potentiated the opening of 2,4-dinitrophenol– and pinacidil-activated K_{ATP} channel current (I_{KATP}), while halothane inhibited the 2,4-dinitrophenol–activated I_{KATP} and had no effect on the pinacidil-activated I_{KATP}.

The goal of the present study was to directly investigate the potentiating effects of isoflurane on the cardiac

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sarcolemmal K_{ATP} channel at the single-channel level and to determine whether modulators of the channel affected the anesthetic action.

Materials and Methods

Preparation of Isolated Cardiac Ventricular Myocyte

After approval was obtained from the Institutional Animal Care and Use Committee, single ventricular cells were isolated from enzymatically treated adult guinea pig (200–300 g) hearts. The procedure is a modification of that of Mitra and Morad,[22] which has been previously described.[23] In brief, immediately after thoracotomy, the heart was rapidly mounted on a Langendorff apparatus and perfused retrogradely through the aorta with warm (37°C) oxygenated buffer containing the Joklik medium and 0.25 mg/ml collagenase (Gibco Life Technologies, Grand Island, NY) and 0.13 mg/ml protease (Sigma Chemical Co., St. Louis, MO) for 8–12 min. The isolated myocytes were washed and stored in a standard Tyrode solution. Only cells with clear borders and well-defined striations were selected and used for experiments within 12 h after isolation.

Solutions

The isolated myocytes were initially placed in a standard Tyrode solution that contained the following ingredients: 132 mM NaCl, 4.8 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM dextrose, and 10 mM HEPES, with pH adjusted to 7.3 with NaOH. After establishing a gigaohm seal, the external solution was changed to one that was appropriate for the various patch configurations as mentioned below.

For experiments in the cell-attached mode, a sodium-free external K^+ solution was used containing 132 mM *N*-methyl-D-glucamine, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, and 5 mM KCl, with pH adjusted to 7.4 with HCl. The standard pipette solution for the cell-attached mode was identical to the external solution.

For experiments in the excised, inside-out patch configuration, the bath solution (corresponding to the intracellular side) contained 60 mM K-glutamate, 50 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 11 mM EGTA, and 0.2–3 mM K_2ATP, with pH adjusted to 7.4 with KOH. The final K^+ concentration was 140 mM. The pipette solution corresponding to the extracellular side was the same as the one described above for the cell-attached experiments.

For experiments in the whole cell mode, the external bath solution was similar to the one used in the cell-attached patch experiments. Nisoldipine (200 nM), supplied by Miles Pentex (West Haven, CT), was also added to block the L-type Ca channel current. The internal pipette solution was similar to the intracellular solution used in the inside-out patch experiments, with intracellular ATP kept at 0.5 mM.

Several modulators of the sarcolemmal K_{ATP} channel were used. 2,4-Dinitrophenol, ATP, ADP, adenosine, and guanosine triphosphate (GTP) were all obtained from Sigma Chemical Co. and dissolved in the appropriate buffer solutions for the patch clamp experiments. The potassium channel opener, bimakalim, was provided by Garrett Gross, Ph.D. (Professor, Department of Pharmacology and Toxicology, Medical College of Wisconsin). Bimakalim was prepared as a 10-mM stock solution in dimethyl sulfoxide and diluted to a concentration of 5 μM in the appropriate buffer before use.

The volatile anesthetic isoflurane (Ohmeda Caribe Inc., Liberty Corner, NJ) was mixed by adding known aliquots of concentrated anesthetics to graduated syringes with the appropriate bath solutions. Isoflurane superfusion was achieved using a syringe pump with a constant flow of 1 ml/min. Clinically relevant concentrations of isoflurane (0.5 and 1.0 mM, equivalent to 1.048 and 2.095 vol%, respectively) were used. To determine anesthetic concentrations, 1 ml of the superfusate was collected in a metal-capped 2-ml glass vial at the end of each experiment. The superfusate concentration of the anesthetic was then determined by gas chromatography (head-space analysis) utilizing flame ionization detection Perkin-Elmer Sigma 3B gas chromatograph.

Recording Procedure and Data Analysis

K_{ATP} channel activity was monitored using the whole cell, cell-attached, and excised, inside-out patch configurations of the patch clamp technique. Patch pipettes with resistances ranging from 2 to 10 MΩ were pulled from borosilicate glass (Garner Glass, Claremont, CA) using a programmable micropipette puller (Sachs-Flaming PC-84; Sutter Instruments, Novato, CA). Pipette tips were heat polished using a microforge (MF-83; Narishige, Tokyo, Japan). Current was measured using a patch clamp amplifier (EPC-7; List, Darmstadt, Germany) interfaced to a computer *via* an Axon Instrument 1200A Digidata board (Axon Instruments, Foster City, CA). Data acquisition and analysis were performed using the pClamp software package versions 6.0.3 and 8.0 (Axon Instruments). Additional data and statistical analyses were performed on Origin (OriginLab, Northampton, MA).

Currents recorded in the cell-attached and inside-out patch configurations were low pass-filtered at 500 Hz and sampled at 1 kHz. An opening was interpreted as a crossing of a 50% threshold level from the baseline to the first open channel amplitude. Channel activity was recorded in 2-min durations. For the cell-attached patch experiments, K_{ATP} channel activity was monitored at a pipette (command) potential (V_{pip}) of −40 mV. Since both the pipette and bath solutions contained 5 mM

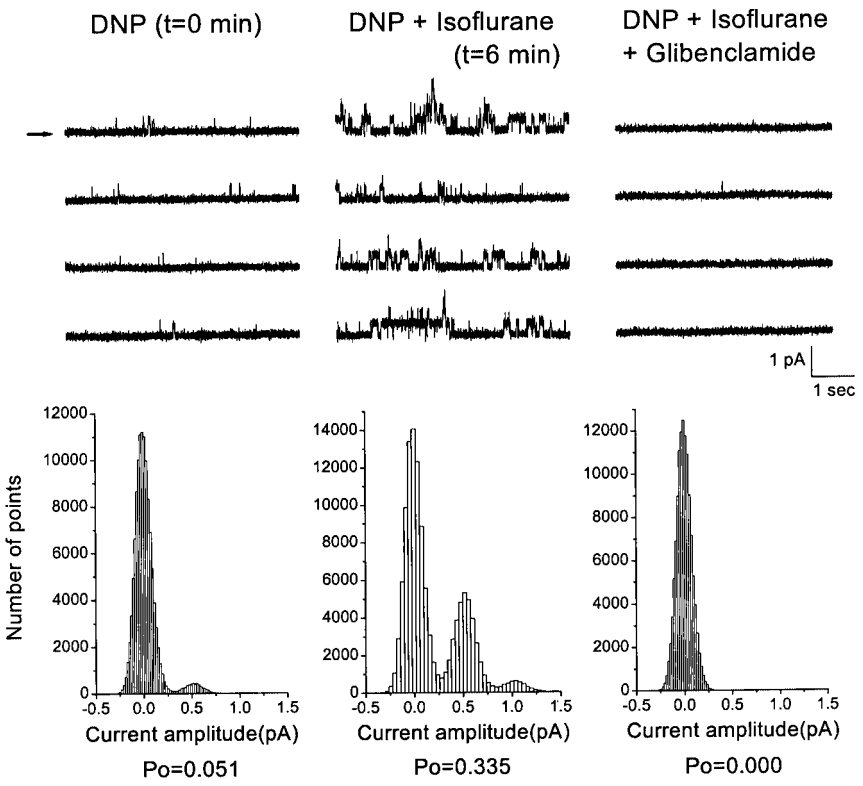

Fig. 1. Effect of isoflurane on the sarcolemmal K_{ATP} channel activated by 2,4-dinitrophenol (DNP). Channel activity was monitored in the cell-attached patch mode at a membrane potential of −46 mV. The pipette and bath solution contained 5 mM K. Single channel traces and corresponding amplitude histograms are shown in DNP, DNP + isoflurane, and DNP + isoflurane + glibenclamide. The times shown in the brackets denote the period at which channel activity was recorded. The arrow indicates zero current level.

potassium, the resting membrane potential ($V_{resting}$) was calculated to be −86 mV. Taking into account the polarity of the pipette potential relative to the membrane potential, the resultant membrane potential was −46 mV according to the standard relation for a cell-attached patch: $V_m = V_{resting} - V_{pip}$. For the excised, inside-out patch experiments, K_{ATP} channel activity was monitored at a membrane potential of 0 mV in external 5-mM K and internal 140-mM K concentrations. The K_{ATP} channels were identified by channel conductance and by their sensitivity to ATP and glibenclamide. All recordings were made at room temperature (20–25°C).

Because of multiple channels in a patch, open probability (Po) was calculated as a cumulative Po, which is defined as a fraction of the total length of time the channels were in an open state during the total recording duration. Po was determined from the ratios of the area under the peaks in the all-points amplitude histogram fitted with a Gaussian function. This provided us with a qualitative but comparative way of monitoring effects of isoflurane on K_{ATP} channel activity.

Whole cell K_{ATP} current was monitored during a 100-ms test pulse to 0 mV from a holding potential of −40 mV. Current was measured at 15-s intervals and amplitude at the end of the test pulse was plotted to monitor changes over time.

All data are presented as means ± SEM. Statistical significance of the data were evaluated using analysis of variance and paired Student *t* test. Differences were considered to be significant at $P < 0.05$.

Results

Effects of Isoflurane on K_{ATP} Channel Activated by 2,4-Dinitrophenol

The effects of isoflurane on the sarcolemmal K_{ATP} channel were first investigated in the presence of 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation. The cell-attached patch configuration was used. During control conditions, due to millimolar concentrations of ATP, the K_{ATP} channel was inactive. Figure 1 shows representative traces and corresponding all-points amplitude histograms of K_{ATP} channel activity recorded in the presence of 2,4-dinitrophenol, 2,4-dinitrophenol + isoflurane, and 2,4-dinitrophenol + isoflurane + glibenclamide. The effect of 150 μM 2,4-dinitrophenol was evident after approximately 5–10 min as K_{ATP} channels were activated. Recording of the K_{ATP} channel activity was then commenced (denoted by t = 0 min in fig. 1) and continued for 2–4 min before application of the anesthetic. The subsequent application of isoflurane (0.5 mM) in the continued presence of 2,4-dinitrophenol appeared to increase the cumulative Po of the K_{ATP} channel. Glibenclamide (500 nM), a potent inhibitor of the K_{ATP} channel, completely blocked the channel activity. In 8 patches, isoflurane significantly increased the cumulative channel Po from 0.036 ± 0.022 to 0.410 ± 0.086 ($P < 0.05$). The increase in Po of the K_{ATP} channel in the presence of both 2,4-dinitrophenol and isoflurane is also reflected in the opening of more channels present in the membrane patch, from 2

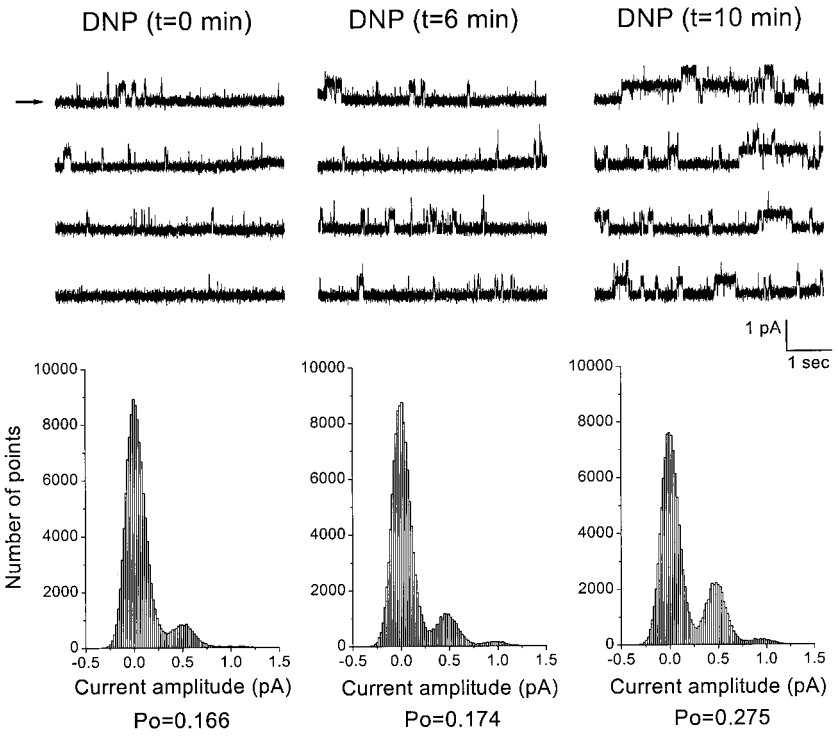

Fig. 2. Time course of the effect of 2,4-dinitrophenol (DNP) on the K_{ATP} channel. The time-dependent effect of DNP on K_{ATP} channel activity was monitored in a cell-attached patch as described in figure 1. The times shown in the brackets denote the period at which channel activity was recorded. The sample recording was obtained from one patch exposed to 150 μM DNP and monitored over the time period depicted.

to 3.9 ± 0.5 (n = 8). However, the single-channel conductance was not affected. The chord conductances in 2,4-dinitrophenol alone and in the presence of 2,4-dinitrophenol + isoflurane were 12.1 ± 0.6 and 12.3 ± 0.3 pS, respectively, in agreement with that previously reported under physiologic conditions.[24]

Since 2,4-dinitrophenol gradually decreases the intracellular ATP concentration, the effect of isoflurane on the K_{ATP} channel may, in part, be due to a time-dependent 2,4-dinitrophenol effect. To test this hypothesis, the effects of 2,4-dinitrophenol alone on the K_{ATP} channel were monitored over time and compared with those in the presence of isoflurane. Figure 2 demonstrates K_{ATP} channel activity monitored during a 10-min period in the presence of 2,4-dinitrophenol alone. Between the t = 0- and 6-min periods, there was no marked change in channel Po. However, Po was increased at the t = 10-min mark. This is in contrast to the result of 2,4-dinitrophenol + isoflurane demonstrated in figure 1. At the t = 6-min mark, channel Po was higher with the addition of isoflurane compared with 2,4-dinitrophenol alone at the t = 0-min mark. The results summarized in figure 3 show the data for 2,4-dinitrophenol alone superimposed on the 2,4-dinitrophenol + isoflurane data. The time at which initial K_{ATP} channel activity was observed in the presence of 2,4-dinitrophenol is denoted by t = 0 min. In the 2,4-dinitrophenol–alone group, a time-dependent increase in channel Po was observed only during the t = 10-min period. However, in the 2,4-dinitrophenol + isoflurane group, the application of isoflurane at the t = 6-min mark significantly increased channel Po. There was a significant difference in channel Po between the 2,4-dinitrophenol–alone group and 2,4-dinitrophenol + isoflurane group at t = 6 min. At time t = 10 min, upon washout of isoflurane, channel activity remained high. Consequently, at the t = 10-min mark, the time-dependent effect of 2,4-dinitrophenol was evident. These results suggest a kinetic difference in K_{ATP} channel opening between the 2,4-dinitrophenol–alone and 2,4-dinitrophenol + isoflurane groups. In the presence of isoflurane, the opening of the K_{ATP} channel by 2,4-dinitrophenol was accelerated.

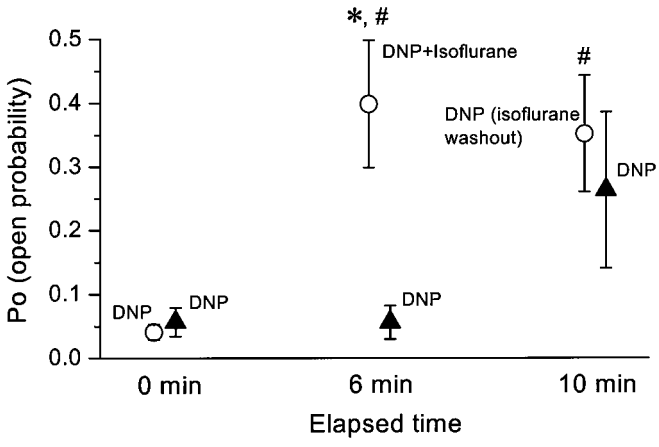

Fig. 3. The kinetic effect of isoflurane on 2,4-dinitrophenol (DNP)-activated K_{ATP} channel. Cumulative channel open probability is plotted against time for the DNP group (▲, n = 6) and the DNP + isoflurane group (○, n = 8). For the DNP + isoflurane group, isoflurane was added during the 6-min mark and washed out at the 10-min mark, as denoted on the plot. *P < 0.01 *versus* DNP group; #P < 0.01 *versus* 0 min of isoflurane + DNP group.

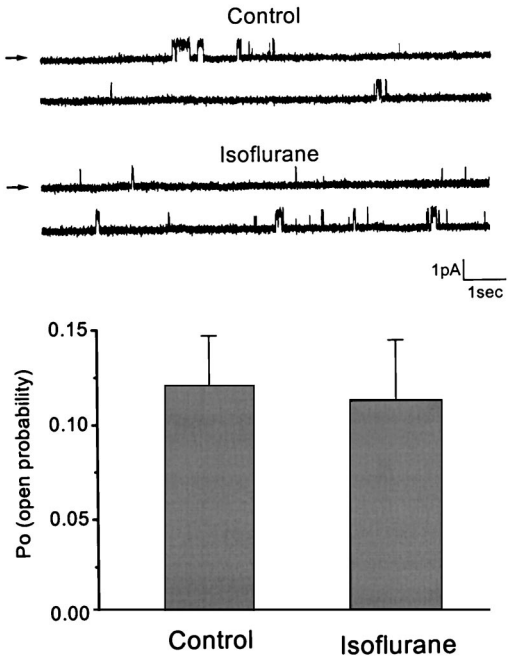

Fig. 4. Effect of isoflurane on the K_{ATP} channel activated by low adenosine triphosphate (ATP). (*Top*) Single-channel current traces monitored in the inside-out, excised patch mode at a membrane potential of 0 mV in external 5 mM K and internal 140 mM K. Control condition was obtained in 0.2 mM ATP. Isoflurane (0.5 mM) was added to the internal (bath) solution. The arrows indicate zero current level. (*Bottom*) Summarized data from 7 patches. Control ATP concentrations were 0.2–0.5 mM.

Effect of Isoflurane on K_{ATP} Channel Activated by Low Adenosine Triphosphate

To better control the recording environment, experiments using the excised, inside-out patch configuration were carried out. Furthermore, due to the kinetic effect of isoflurane in the presence of 2,4-dinitrophenol on K_{ATP} channel Po, all subsequent excised patch experiments were conducted in a similar time course. The activation of the K_{ATP} channel by various agents and the effect of isoflurane in the continued presence of these agents were monitored within a 6-min period.

In the initial set of inside-out patch experiments, low internal ATP concentrations were used to elicit K_{ATP} channel activity. This circumvented the use of 2,4-dinitrophenol to activate the channel. Figure 4 shows the effect of isoflurane on the K_{ATP} channel activated by low ATP on the internal side of the membrane. Control conditions were obtained in 0.2–0.5 mM ATP. Isoflurane (0.5 mM) was added to the internal solution. In the presence of isoflurane, no change in channel open probability was observed. There were no significant differences in the cumulative open probability in the control condition and in the presence of isoflurane.

Effect of Isoflurane on K_{ATP} Channel Activated by Elevated Adenosine Diphosphate

Since the initial cell-attached patch experiments showed an acceleration of the opening of K_{ATP} channels by isoflurane in the presence of 2,4-dinitrophenol, the result from the excised patch suggested that this effect required intracellular agents. Therefore, the isoflurane effect on modulators of the K_{ATP} channel was tested. ADP is one of the endogenous modulators known to shift the channel's sensitivity to ATP.[18] Figure 5 shows the effect of isoflurane on the K_{ATP} channel activated by ADP. Control condition was obtained in 0.5 mM ATP on the internal side of the membrane. The addition of 0.1 mM ADP to the internal side increased the channel's cumulative Po. However, a subsequent addition of 0.5 mM isoflurane did not significantly affect Po. The cumulative Po in 0.1 mM ADP was 0.32 ± 0.09 and in isoflurane was 0.35 ± 0.10 .

Effect of Isoflurane on K_{ATP} Channel Activated by Adenosine

The next series of experiments tested whether isoflurane interacted with the membrane delimited coupling of the adenosine receptor and the sarcolemmal K_{ATP} channel. Adenosine modulates the K_{ATP} channel *via* G_i protein activation under existing GTP[19] as demonstrated in figures 6A and B. In the absence of adenosine, GTP applied from internal side of the membrane did not affect K_{ATP} activity. In the presence of adenosine

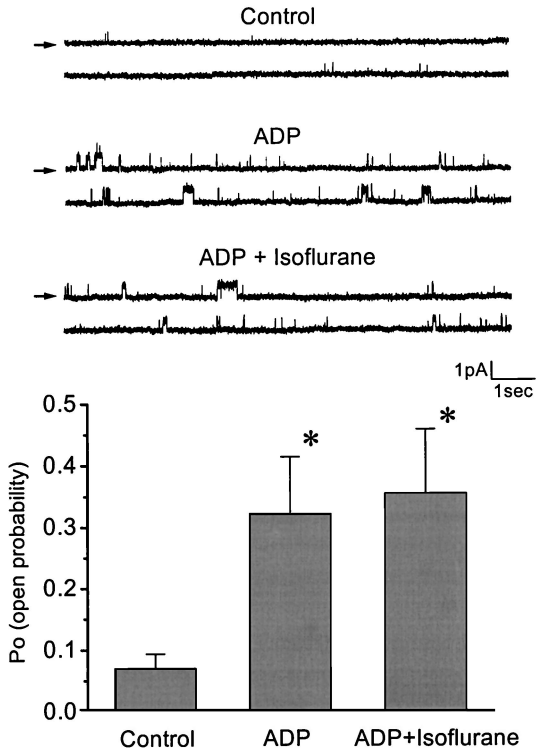

Fig. 5. Effect of isoflurane on the K_{ATP} channel activated by adenosine diphosphate (ADP). (*Top*) Single-channel current traces monitored in the inside-out patch mode in control, ADP, ADP + isoflurane. Control condition was obtained in 0.5 mM ATP. ADP (0.1 mM) and isoflurane (0.5 mM) were added to the internal (bath) solution. The arrows indicate zero current level. (*Bottom*) Summarized data from 10 patches. *$P < 0.05$ *versus* control group.

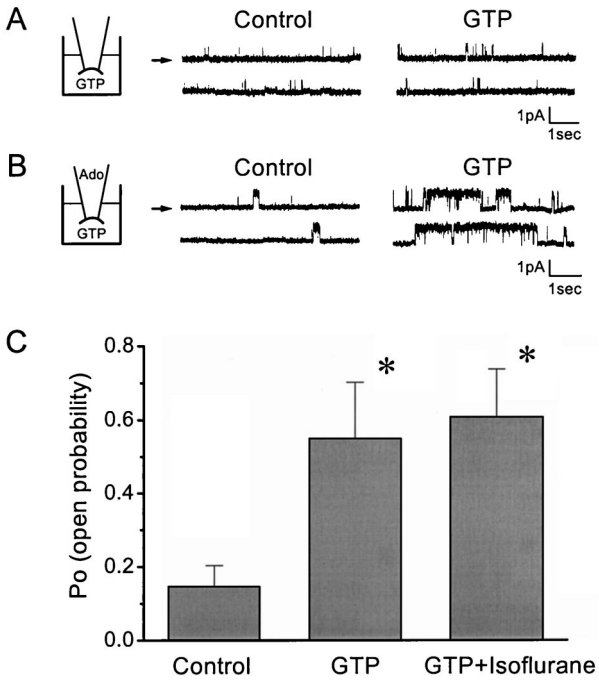

Fig. 6. Effect of adenosine and guanosine triphosphate (GTP) on the K_{ATP} channel. Single-channel current traces were monitored in the inside-out patch mode. The effect of GTP (200 μM) in the absence (*A*) and presence (*B*) of adenosine in the pipette solution is shown. The arrows indicate zero current level. (*C*) Summarized data for the isoflurane effect on K_{ATP} channel open probability in the presence of adenosine and GTP. *$P < 0.05$ *versus* control group, n = 5.

(100 μM) on the external side of the membrane (in the pipette solution), GTP increased K_{ATP} channel activity. Figure 6C summarizes the effect of isoflurane on the K_{ATP} channel activated by adenosine and GTP. Control condition was obtained with 0.2–0.5 mM ATP on the internal side of the membrane. Adenosine (100 μM) was included in the pipette solution throughout the experiment. The addition of 0.2 mM GTP significantly increased Po from 0.15 ± 0.06 to 0.55 ± 0.15. However, addition of isoflurane (0.5 mM) did not result in any significant changes in Po.

Effect of Isoflurane on K_{ATP} Channel Activated by Bimakalim

Under excised patch conditions, isoflurane had no significant effects on the K_{ATP} channel activated by endogenous agents. This is in contrast to the enhancing effect isoflurane had on the K_{ATP} channel recorded under cell-attached patch conditions in the presence of 2,4-dinitrophenol. To test whether this difference was due to the method of channel activation, the effect of isoflurane on the K_{ATP} channel opened by bimakalim, a potassium channel opener,[25] was investigated using the excised, inside-out patch configuration. The results are summarized in figure 7A. Control condition was obtained with 3.0 mM ATP on the internal side of the membrane. During this condition, channel activity was mostly inhibited, with Po = 0.006 ± 0.003. Bimakalim (5.0 μM) significantly increased activity, with Po = 0.15 ± 0.04. Again, the addition of isoflurane did not change K_{ATP} channel activity.

Effect of 2,4-Dinitrophenol during Excised Patch Conditions

In the cell-attached patch configuration, activation of the K_{ATP} channel by 2,4-dinitrophenol occurs *via* uncoupling of oxidative phosphorylation and also by directly interacting with the K_{ATP} channel.[26] Consequently, additional experiments were conducted to test whether the isoflurane effect observed under cell-attached patch mode was dependent on a direct interaction of 2,4-dinitrophenol with the channel. Experiments were conducted in the inside-out patch configuration, and the effect of isoflurane on the 2,4-dinitrophenol–activated K_{ATP} channel was monitored. Figure 7B summarizes the results from these experiments. During control conditions obtained in 0.8–1.0 mM ATP on the internal side of the membrane, K_{ATP} channel activity was limited, with Po = 0.023 ± 0.007. When 150 μM 2,4-dinitrophenol

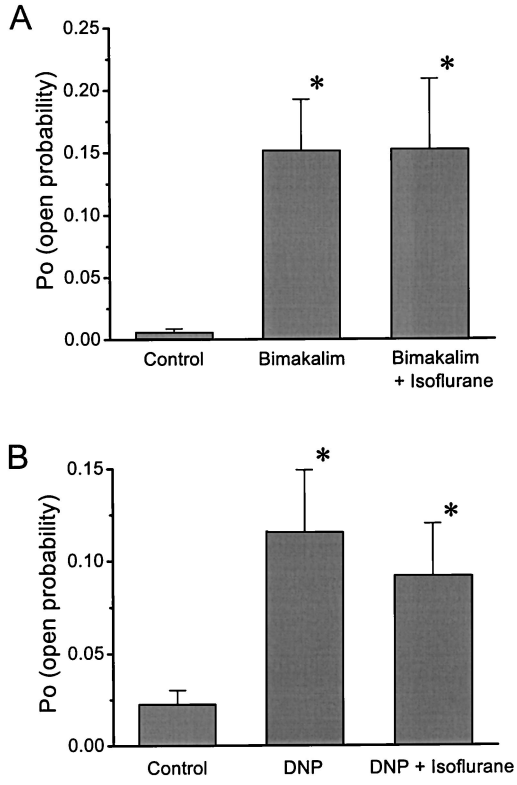

Fig. 7. Effect of isoflurane on the K_{ATP} channel activated bimakalim and 2,4-dinitrophenol (DNP). Channel activity was monitored in the inside-out patch mode. (*A*) Summary of the effect of isoflurane (0.5 mM) on the K_{ATP} channel activated by 5 μM bimakalim (n = 7). Control condition was obtained in 3.0 mM adenosine triphosphate (ATP). (*B*) Summary of the effect of isoflurane (0.5 mM) on the K_{ATP} channel activated by DNP (150–200 μM) (n = 8). Control condition was obtained with 0.8–1.0 mM ATP in the internal solution. DNP was added in the internal side of the membrane. *$P < 0.05$ *versus* control.

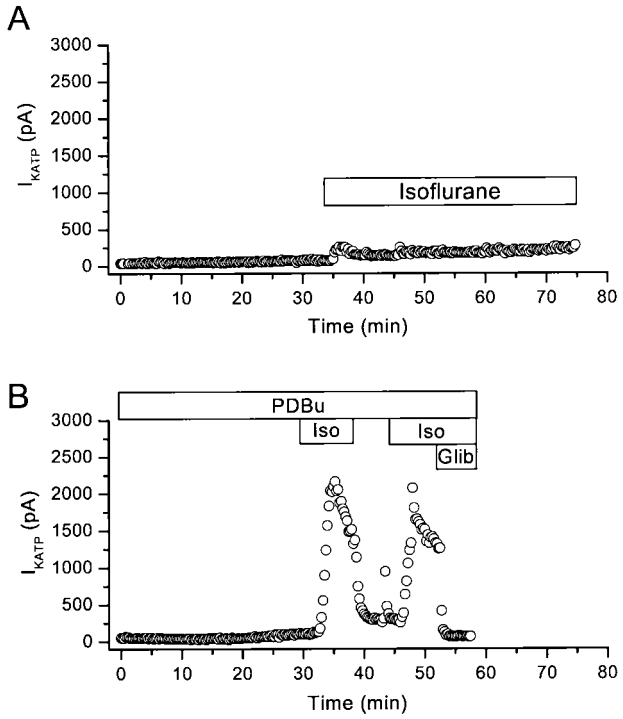

Fig. 8. Opening of the K_{ATP} channel by isoflurane during protein kinase C (PKC) activation. K_{ATP} current was monitored in the whole cell mode. Current amplitude was measured at the end of the 100-ms test pulse to 0 mV from a −40-mV holding potential. The intracellular adenosine triphosphate concentration was set at 0.5 mM. (*A*) Isoflurane (1.0 mM) alone was unable to elicit K_{ATP} current activation. (*B*) During continuous PKC activation by 12,13-dibutyrate (PDBu; 1 μM), isoflurane activated K_{ATP} current, which was subsequently blocked by glibenclamide (500 nM).

was applied to the internal side, Po increased significantly to 0.116 ± 0.034. Yet the addition of isoflurane had no further effect on K_{ATP} channel Po.

Effect of Protein Kinase C on K_{ATP} Current Activation by Isoflurane

Results obtained from the excised patch experiments strongly suggest an intracellular component in the regulation of the K_{ATP} channel by isoflurane. Since activation of protein kinase C (PKC) is seen as a critical step in IPC,[27–29] the role of PKC activation on the isoflurane effect was investigated during whole cell conditions. For PKC activation, 12,13-dibutyrate (1 μM) was applied extracellularly throughout the course of the experiment. A time of 30 min was allowed to elapse before application of isoflurane to allow for diffusional exchange of the intracellular and pipette ATP, where the concentration was set at 0.5 mM. The experiment is depicted in figure 8. Isoflurane alone had no effect on membrane current and did not activate I_{KATP}, as was previously reported.[21] In contrast, isoflurane was able to elicit an outward current during sustained PKC activation by 12,13-dibutyrate. This outward current was blocked by glibenclamide, identifying it as the K_{ATP} current. Prior to the application of isoflurane, 12,13-dibutyrate alone did not activate I_{KATP}. The average isoflurane induced K_{ATP} current density in the presence of 12,13-dibutyrate was 14.1 ± 4.2 pA/pF (n = 6).

Effect of Protein Kinase C Activation during Excised Patch Conditions

The whole cell experiments with 12,13-dibutyrate showed that PKC activation facilitated the opening of the sarcolemmal K_{ATP} channel by isoflurane. To confirm whether PKC activation is sufficient to modulate the effect of isoflurane on the K_{ATP} channel, inside-out, excised patch experiments were carried out. Based on the whole cell experiment described above, isoflurane would be expected to increase K_{ATP} channel activity in a cell-free environment during activation of PKC. In an excised membrane patch, stimulation of endogenous PKC activity results in increased K_{ATP} channel activity.[30] As summarized in figure 9, application of 0.5 μM 12,13-dibutyrate to the intracellular side of the membrane patch resulted in an increase in Po from the control condition obtained in 500 μM ATP. Surprisingly, the subsequent application of isoflurane (0.5 mM) did not significantly increase channel Po. This result was obtained from 6 patches.

Discussion

The present study provides direct evidence at the single-channel level that isoflurane facilitates opening of the sarcolemmal K_{ATP} channel. Under cell-attached patch conditions, isoflurane, in combination with 2,4-dinitrophenol, increased the cumulative Po of the K_{ATP} channel. This increase appeared to be the result of an

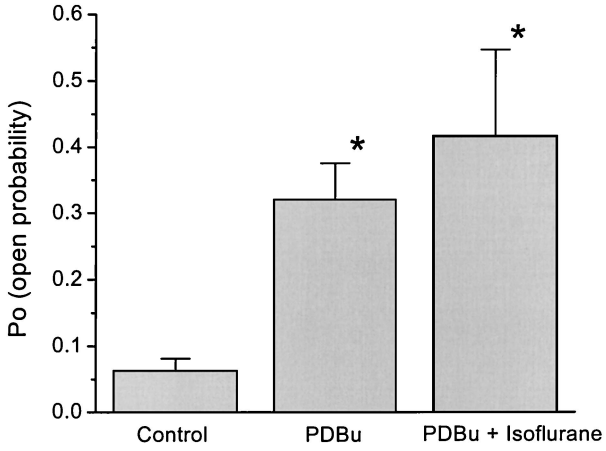

Fig. 9. Effect of protein kinase C (PKC) activation and isoflurane on the K_{ATP} channel in excised patches. A summary of the effect of isoflurane on the K_{ATP} channel activated by 12,13-dibutyrate (PDBu; 0.5 μM) is shown. The control condition was obtained in 500 μM adenosine triphosphate. Open probability was determined from channel recordings obtained from inside-out patches (n = 6). *$P < 0.05$ *versus* control.

acceleration of channel opening in the presence of 2,4-dinitrophenol. The result confirms our previous observation that isoflurane potentiated the K_{ATP} current at the whole cell level.[21] 2,4-Dinitrophenol, an uncoupler of oxidative phosphorylation, inhibits ATP synthesis at the mitochondrial membrane and mimics cellular hypoxia. A drawback in using 2,4-dinitrophenol is that the depletion of ATP occurs gradually. Despite the time-dependent effect of 2,4-dinitrophenol, the time-control experiments showed that the enhancing effect of isoflurane hastened the opening of the K_{ATP} channel. Thus, these results show that isoflurane can enhance K_{ATP} channel opening during conditions where intracellular ATP concentration is decreased.

The results from the excised, inside-out patch studies suggest that the effect of isoflurane on the K_{ATP} channel requires an intracellular component. Isoflurane had no effect on the K_{ATP} channel that was previously activated by low intracellular ATP. This suggested that isoflurane does not interact directly with the channel protein but requires an intracellular component missing in a cell-free environment. Endogenous agents that are produced during ischemic conditions, ADP[18] and adenosine,[19] are also modulators of the K_{ATP} channel. Isoflurane, however, had no additional effect on the K_{ATP} channel activated by ADP or adenosine in an excised patch environment. Moreover, in an excised patch configuration, a 2,4-dinitrophenol–activated K_{ATP} channel was also not affected by isoflurane. This indicated that the isoflurane effect observed during cell-attached conditions were not due to the use of 2,4-dinitrophenol as an opener of the channel. K_{ATP} channels opened by a potassium channel opener, bimakalim, also was not affected by isoflurane in a cell-free environment. This further confirms the involvement of intracellular agents since in our previous study, isoflurane potentiated pinacidil-activated I_{KATP} recorded during a whole cell condition.[21]

The results obtained in this study appear to be in disagreement with those obtained by Han *et al.*[16] In their study, in an excised patch configuration, isoflurane increased the mean closed time of the K_{ATP} channel. However, isoflurane also shifted the K_{ATP} channel's sensitivity to ATP, making it less sensitive. The latter effect would result in an enhanced current flow at a particular intracellular ATP concentration. Due to multiple channels in a patch, no kinetic analysis of the channel activity was attempted in our study. Thus, in an excised patch condition, isoflurane may have subtle kinetic effects not evident by measuring Po alone. However, our study showed that isoflurane had no net effect on the K_{ATP} channel in a cell-free environment. In addition, species differences may account for the discrepancy in the mechanism of anesthetic action on the K_{ATP} channel, where rabbit ventricular myocytes were used in the study by Han *et al.*[16] Nevertheless, results from both studies support the hypothesis of K_{ATP} channel activation during anesthetic-induced cardioprotection. In rabbit ventricular myocytes, the mechanism may involve decreased sensitivity to ATP, and in guinea pig ventricular myocytes, it may involve an increase in channel Po *via* an intracellular signaling cascade.

A strong candidate that may be the missing link between isoflurane and the K_{ATP} channel is PKC. Recently, PKC has been implicated as a crucial component in IPC.[27–29] PKC also modulates the sarcolemmal K_{ATP} channel by shifting its sensitivity to intracellular ATP.[30] The whole cell experiments in this study demonstrated that PKC activation can "prime" the sarcolemmal K_{ATP} channel to opening by isoflurane. This may provide the appropriate environment for isoflurane to facilitate the opening of the K_{ATP} channel. However, surprisingly, in a cell-free environment, activation of PKC alone was not sufficient to enhance the opening of the K_{ATP} channel by isoflurane. Under this condition, the effect of PKC on the K_{ATP} channel is due to membrane-bound PKC. Hence, it is likely that the isoflurane effect on the sarcolemmal K_{ATP} channel involves the translocation of specific PKC isoforms. Studies have shown that, in IPC, translocation of the PKC-ϵ isoform has a pivotal role in cardioprotection.[31,32]

Another possible mechanism underlying the effect of isoflurane on the K_{ATP} channel is that isoflurane itself may decrease intracellular ATP concentration. The cell-attached patch experiments showed that isoflurane can facilitate opening of the K_{ATP} channel after applying 2,4-dinitrophenol to decrease intracellular ATP. Isoflurane alone, in the absence of 2,4-dinitrophenol, was unable to elicit channel activity. This may suggest that isoflurane enhances K_{ATP} channel opening by facilitating the decrease in intracellular ATP by 2,4-dinitrophenol. This is supported by the study that isoflurane may affect mitochondrial K_{ATP} channel,[33] and opening of this channel may decrease ATP synthesis.[34] However, other studies have reported that volatile anesthetics do not affect ATP concentrations in the heart or myocyte. For instance, halothane did not change ATP concentrations during normal conditions.[35] Another study showed that isoflurane and halothane did not change ATP concentrations during control, ischemia, and reperfusion compared to a nonanesthetic condition.[36] Other volatile anesthetics, enflurane and sevoflurane, also did not affect ATP concentrations after the reperfusion period.[37]

The effect of isoflurane from the 2,4-dinitrophenol and PKC experiments may be linked, although it is rather speculative at this point. It is possible that 2,4-dinitrophenol, *via* its effect on the mitochondria that results in the uncoupling of oxidative phosphorylation, can subsequently trigger an intracellular signaling cascade leading to translocation of a PKC isoform. This, in turn, can facilitate the opening of the K_{ATP} channel. The excised patch experiments with 2,4-dinitrophenol would support this hypothesis since there was no effect of isoflu-

rane on 2,4-dinitrophenol–activated channels in the cell-free environment. However, there are currently no data available showing translocation of a PKC isoform by 2,4-dinitrophenol.

The results from our experiments support those from the whole animal studies demonstrating that the cardioprotective effect of isoflurane may involve the sarcolemmal K_{ATP} channel.[14,38] Our results directly show at the single-channel level that isoflurane modulates K_{ATP} channel activity previously exposed to 2,4-dinitrophenol. Hence, the activation of the sarcolemmal K_{ATP} channel may be one of the underlying pathways involved in anesthetic preconditioning. Although the extent of the role of the sarcolemmal *versus* the mitochondrial K_{ATP} channel has not been clearly established in IPC, emerging evidence points toward the mitochondrial K_{ATP} channel playing a larger role.[13] However, the contribution of the sarcolemmal K_{ATP} channel cannot be ruled out. In a preliminary study on transgenic mice hearts with a targeted deletion of Kir6.2, the pore-forming subunit of the sarcolemmal K_{ATP} channel, the protective effects of IPC were abolished.[39] It is conceivable that one may be a trigger of cardioprotection and the other an effector. The various signaling pathways involved in cardioprotection may differentially modulate the sarcolemmal and mitochondrial K_{ATP} channels. Therefore, the result that isoflurane can modulate the sarcolemmal K_{ATP} channel, particularly during PKC activation, suggests a significant role for this channel in cardioprotection.

In conclusion, our studies show that isoflurane facilitated the opening of the sarcolemmal K_{ATP} channel. This volatile anesthetic effect is not due to a direct interaction with the channel protein, but involves an intracellular component, likely including the translocation of PKC isoforms.

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