Isoflurane activates rat mitochondrial ATP-sensitive K\textsuperscript{+} channels reconstituted in lipid bilayers

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Nakae, Yuri, Wai-Meng Kwok, Zeljko J. Bosnjak, and Ming Tao Jiang. Isoflurane activates rat mitochondrial ATP-sensitive K\textsuperscript{+} channels reconstituted in lipid bilayers. Am J Physiol Heart Circ Physiol 284: H1865–H1871, 2003. First published February 6, 2003; 10.1152/ajpheart.01031.2002.—Activation of mitochondrial ATP-sensitive K\textsuperscript{+} (mitoK\textsubscript{ATP}) channels is critical in myocardial protection induced by preconditioning with volatile anesthetics or brief periods of ischemia. In this study, we characterized rat mitoK\textsubscript{ATP} channels reconstituted in lipid bilayers and examined their direct regulation by isoflurane. Mitochondria and the inner membrane fraction were isolated from rat ventricles and fused into lipid bilayers. On the basis of their inhibition by 5-hydroxydecanoate (5-HD)/ATP or activation by diazoxide, mitoK\textsubscript{ATP} channels of several conductance states were observed in symmetrical (150 mM) potassium glutamate (26, 47, 66, 83, and 105 pS). Isoflurane (0.8 mM) increased the cumulative open probability from 0.09 ± 0.02 at baseline to 0.50 ± 0.09 (P < 0.05, n = 5), which was inhibited by 5-HD. Isoflurane caused a dose-dependent rightward shift in ATP inhibition of mitoK\textsubscript{ATP} channels, which increased the IC\textsubscript{50} for ATP from 335 ± 4 to 940 ± 34 \mu M at 0.8 mM (P < 0.05, n = 5–8). We conclude that direct activation of the mitoK\textsubscript{ATP} channel by isoflurane is likely to contribute to volatile anesthetic-induced myocardial preconditioning.

METHODS

This study was conducted according to National Institutes of Health standards (NIH Pub. No. 95-23, Revised 1996) and was approved by the Institutional Animal Use and Care Committee.

Isolation of mitochondria. Cardiac mitochondria were isolated according to the procedure of Solem and Wallace with modifications (26). Briefly, ventricles from fresh rat hearts were cut into small pieces in 6 volumes of ice-cold isolation buffer containing 30 mM MOPS (pH 7.2), 200 mM mannitol, 50 mM sucrose, 5 mM KH\textsubscript{2}PO\textsubscript{4}, 0.1% BSA, and 1 mM EGTA in the presence of protease inhibitor cocktails (Sigma). The tissues were then homogenized with a PT10 Polytron (Brinkman Instruments; Westbury, NY) for 15 s for three times at 30-s intervals. The homogenates were centrifuged at 3,000 \( g \) for 10 min. The supernatants were then centrifuged at 8,000 \( g \) for 20 min. The pellet was resuspended in the isolation medium without EGTA and centrifuged again at 8,000 \( g \). The final pellet enriched in mitochondria was suspended in the

MitoK\textsubscript{ATP} channels have been implicated as critical effectors/mediators of cardioprotection.

Previous studies (3, 11, 13, 14, 23, 25, 27) have shown that preconditioning by brief exposure to volatile anesthetic isoflurane (Iso) followed by washout protects the myocardium against subsequent ischemic injury, which mimics the ischemic preconditioning phenomenon. Experimental evidence suggests that the cardioprotective effect of Iso is mediated through adenosine receptors, protein kinase C (PKC), and K\textsubscript{ATP} channels in hearts from humans (25) or animals (11, 13), which is blocked by 5-HD (23). Our previous study has shown that Iso increases mitochondrial flavoprotein fluorescence in guinea pig ventricular myocytes (14). Iso also induces delayed myocardial protection in isolated rabbit hearts (27). However, the proposed role of mitoK\textsubscript{ATP} channels in APC in previous studies is all based on pharmacological or indirect evidence (i.e., flavoprotein fluorescence). In this study, we investigated the effect of Iso on native mitoK\textsubscript{ATP} channels reconstituted in lipid bilayers. Our results are the first to indicate that Iso directly activates mitoK\textsubscript{ATP} channels, which likely contributes to its myocardial protective effect in APC. Preliminary work has been previously presented elsewhere (12, 17).

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isolation medium without EGTA and BSA and stored on ice for preparation of mitochondrial inner membranes.

**Preparation of mitochondrial inner membranes.** Submitochondrial fraction enriched in inner membranes was prepared as reported (2, 18). The mitochondria obtained from above were osmotically shocked by incubating in 10 mM phosphate buffer (pH 7.4) for 20 min and then in 20% sucrose for another 15 min. The membrane was sonicated with a sonicator (Dual Horn for model 550, Fisher Scientific; Hanover Park, IL) for 30 s for three times and centrifuged at 8,000 g for 10 min. The supernatants containing submitochondrial particles were fractionated using a continuous sucrose gradient (30–60%) and centrifuged at 80,000 g overnight in a SW28 rotor (Beckman). The inner mitochondrial membrane (which is enriched in the heavy fraction) was suspended with the isolation medium without EGTA and centrifuged at 184,000 g for 30 min. The final pellet-enriched inner mitochondrial membranes were suspended in the isolation medium without EGTA and BSA and stored at −80°C in small aliquots until use.

**Reconstitution of mitoK<sub>ATP</sub> channels into lipid bilayers.** Inner mitochondrial membranes were fused into lipid bilayers as reported previously for ryanodine receptors (12) with modifications. Briefly, 1-α-phosphatidylethanolamine (from the heart) and 1-α-phosphatidylserine (from the brain) in chloroform were mixed in a 1:1 (wt/wt) ratio, dried under nitrogen, and suspended in n-decane for a final concentration of 20 mg/ml. A delrin cup (with an aperture of 250 μm between the cis and trans chambers, 0.8 ml volume) was placed into a holder. Both chambers contained a symmetrical solution of 30 mM MOPS (pH 7.4), 150 mM potassium glutamate, 1 mM EGTA, 50 μM K<sub>ATP</sub>, and 0.2 mM MgCl<sub>2</sub>. Ag/AgCl electrodes were placed into each chamber via agar salt bridges and connected to the headstage of the bilayer clamp amplifier (BC-525C, Warner Instruments; Hamden, CT). The cis chamber was held at virtual ground, whereas the trans chamber was at command. The experiments were performed at room temperature (23–25°C). Lipid bilayers were formed by painting the aperture with the lipids, and the mitochondrial membranes were added into the cis chamber. After the appearance of cation current, indicating successful fusion, single channel currents at a holding potential of +40 mV (trans/cis, −40 mV by convention) were collected using an Axon Digidata 1332 AD/DA interface (Axon Instruments; Union City, CA) with the pCLAMP program (version 8.01, Axon Instruments) on a personal computer (Pentium II). The currents were filtered at 0.5 kHz with an eight-pole Bessel filter and digitized at 2.5 kHz. The channel activity accumulated over 2-min intervals was expressed in cumulative channel open probability (NP<sub>c</sub>·P<sub>c</sub>, where N is the apparent number of channels and P<sub>c</sub> is the mean open state probability). NP<sub>c</sub> was determined from amplitude histograms after multiple Gaussian curve fitting (Origin 6.0, Microcal Software; Northampton, MA).

**Identification of mitoK<sub>ATP</sub> channel reconstituted in lipid bilayers.** MitoK<sub>ATP</sub> channels were identified by their inhibition with 5-HD (a selective mitoK<sub>ATP</sub> channel inhibitor) and/ or ATP and activation with diazoxide (a mitoK<sub>ATP</sub> channel opener). All modulators were added to the cis chamber during continuous stirring for 1 min. Single-channel current amplitude at +40 mV (trans/cis) was used for determination of chord conductance. The voltage-current relationship was obtained at various holding potentials and used to calculate the slope conductance with linear regression with Origin 6.0.

**Effect of Iso on mitoK<sub>ATP</sub> channel reconstituted in lipid bilayers.** A stock solution of Iso (14.5 mM) was made by mixing excess Iso with the identical buffer used for channel reconstitution. The Iso concentrations in the stock solution and cis chamber were measured by gas chromatography (GC-8A, Shimazu; Columbia, MO).

After the appearance of K<sup>+</sup>-conducting current in bilayers, an aliquot of Iso from the stock solution was added to the cis chamber under stirring. The channel activities were monitored for up to 10 min, and the identity of the mitoK<sub>ATP</sub> channels was confirmed by their inhibition with 5-HD and/or ATP in the end. HMR1098, a sarcolemmal K<sub>ATP</sub> channel inhibitor, was used to distinguish the sarcolemmal K<sub>ATP</sub> channels from the mitoK<sub>ATP</sub> channel. To examine the effect of Iso on ATP-dependent inhibition of mitoK<sub>ATP</sub> channels, ATP from 50 μM to 2.5 mM was cumulatively added every 2 min in the presence and absence of Iso. An ATP inhibition curve was obtained from normalized NP<sub>o</sub> and the logarithm of the ATP concentration using Hill equation. The IC<sub>50</sub> and Hill coefficient (n<sub>H</sub>) were determined (Origin 6.0). NP<sub>o</sub> was normalized to the NP<sub>o</sub> at baseline (50 μM ATP).

**Identification of rat cardiac mitoK<sub>ATP</sub> channels reconstituted in lipid bilayers.** We first identified the mitoK<sub>ATP</sub> channels based on their sensitivity to 5-HD, ATP, and/or diazoxide. Figure 1A shows original single channel recordings of a cluster of mitoK<sub>ATP</sub> channels that were active at baseline (Fig. 1A1). The addition of ATP (0.5 mM; Fig. 1A2) completely inhibited their activities. Diazoxide at 50 μM (Fig. 1A3) reactivated the channels and increased the peak current, which was largely blocked with the subsequent addition of 5-HD (100 μM; Fig. 1A4). As shown in Fig. 1B, the initial mitoK<sub>ATP</sub> openings at baseline (Fig. 1B1) declined gradually to a complete closure (Fig. 1B2), likely due to rundown. The application of diazoxide (100 μM, Fig. 1B3) activated a channel with a current amplitude of 1.8 pA at +40 mV (trans/cis) after 5 min (chord conductance of 46 pS). 5-HD (100 μM, Fig. 1B4) inhibited its opening despite the continuous presence of diazoxide. These effects of diazoxide and 5-HD were similar to those reported in reconstituted bovine K<sub>ATP</sub> channels in bilayers (30). However, in contrast, we found that ATP was effective in inhibiting mitoK<sub>ATP</sub> channel activities when added to the cis chamber. The time course of diazoxide effect is shown in Fig. 1C. Marked activation of K<sub>ATP</sub> channels occurred 6 min after diazoxide application, which was blocked by 5-HD after 5 min of application. Cumulative data from sev-
Fig. 1. Effects of diazoxide and 5-hydroxydecanoate (5-HD) on mitochondrial ATP-sensitive K⁺ (mitoK<sub>ATP</sub>) channels reconstituted in lipid bilayers. A: original recordings of mitoK<sub>ATP</sub> channel activity in lipid bilayers. Upward deflection represents channel opening at a holding potential of +40 mV (trans); C, channels closed. A cluster of mitoK<sub>ATP</sub> channels in lipid bilayers were active at baseline (1), inhibited by 0.5 mM ATP (2), and then activated by 50 μM diazoxide (3), which was blocked by 5-HD (4). Data are representative of 3 observations. B: original recordings of mitoK<sub>ATP</sub> channel activity in a separate experiment. At baseline (1), channel openings were seen initially and then run down (2); the addition of 50 μM diazoxide to the cis chamber activated a channel with a chord conductance of 46 pS (inset), a segment of which is shown with an expanded scale (inset). This channel was subsequently inhibited by 100 μM 5-HD (4). Data are representative of 3 observations. C: time course of mitoK<sub>ATP</sub> channel modulation by diazoxide and 5-HD. D: cumulative data on diazoxide activation and 5-HD inhibition of the cumulative open probability (N<sub>Po</sub>) of mitoK<sub>ATP</sub> channels. Baseline, 50 μM ATP. Diazoxide (50–100 μM) increased N<sub>Po</sub>, and 5-HD (100–200 μM) blocked diazoxide-induced activation (n = 5 each). *P < 0.05 vs. baseline; †P < 0.05 vs. diazoxide.

Several experiments are summarized in Fig. 1D. Diazoxide (100 μM) increased N<sub>Po</sub> from 0.01 ± 0.01 at baseline (50 μM ATP) to 0.47 ± 0.12 (P < 0.05, n = 5). 5-HD (100 μM) almost completely inhibited diazoxide-induced activation (P < 0.05). These experiments confirmed the identity of mitoK<sub>ATP</sub> channels in lipid bilayers.

Several conductance states were observed in the reconstituted mitoK<sub>ATP</sub> channels. Figure 2 shows representative recordings of a mitoK<sub>ATP</sub> channel opening at various holding potentials (Fig. 2A) and its corresponding current-voltage relationship (Fig. 2B). This channel showed a slope conductance of 58 pS, which exhibited no significant rectification in the presence of low [Mg<sup>2+</sup>] (0.2 mM). Of 33 channels with clearly defined conductance states observed at +40 mV, 5 peaks of chord conductance were seen at 26 ± 2 pS (n = 9), 47 ± 2 pS (n = 10), 66 ± 2 pS (n = 5), 83 ± 2 pS (n = 4), and 105 ± 5 pS (n = 5).

The mitoK<sub>ATP</sub> channels constituted ~30% of the total K⁺-conducting cation channels we observed in the bilayers. Sarcolemmal K<sub>ATP</sub> channels based on their blockade by a specific blocker, HMR1098 (30 μM), were excluded, and the incidence was rare (2 in ~100 successful cation channel incorporations). Fewer than 6% of cation channels were stimulated by ATP and inhibited by ruthenium red or ryanodine, an alkaloid blocking the ryanodine receptors from the sarcoplasmic reticulum (SR). Thus contamination from SR membranes is minimal, and some of those ryanodine receptors may also come from the mitochondria, as shown recently (2).

Effects of isoflurane on reconstituted mitoK<sub>ATP</sub> channels. We then investigated the direct effect of Iso on mitoK<sub>ATP</sub> channels. Aliquots of Iso stock solution (14.5 mM) were added to the cis chamber and stirred for 1 min to obtain a final concentration of 0.4 and 0.8 mM. Its concentration remained stable for at least 10 min (data not shown). Figure 3A shows the effect of Iso on K<sub>ATP</sub> channel activity. At baseline, the channels were active. However, 5 min after the addition of 0.8 mM Iso, the peak channel current was increased, likely due to increased P<sub>o</sub> as well as the number of channels being open. The effect of Iso was completely blocked by 5-HD, confirming their identity as mitoK<sub>ATP</sub> channels. We also tested a sarcolemmal K<sub>ATP</sub> blocker, HMR1098, in
some experiments. As shown in Fig. 3B, a single K\textsubscript{ATP} channel was active with a current of $\approx 2$ pA at baseline. The addition of Iso at 0.8 mM enhanced the peak current amplitude to $\approx 10$ pA, indicating more channels being open, which were silent at baseline. That addition of HMR1098 (25 \textmu{}M) had no effect on the channel openings, but 5-HD at 100 \textmu{}M was effective in blocking the channel openings, confirming their identity as mitoK\textsubscript{ATP} channels. Figure 3C shows the time course of the effect of Iso on mitoK\textsubscript{ATP} channels. Its maximum stimulatory effect was seen at 6 min after administration, which was blocked by 5-HD (100 \textmu{}M). Cumulative data from several experiments are summarized in Fig. 3D. Iso (0.8 mM) increased $N_{Po}$ from $0.09 \pm 0.02$ at baseline to $0.50 \pm 0.09$ ($n = 5$, $P < 0.05$). 5-HD (100 \textmu{}M) inhibited the effects of Iso to $0.08 \pm 0.02$ ($n = 5$, $P < 0.05$ compared with Iso).

We further investigated whether Iso may affect ATP-dependent inhibition of mitoK\textsubscript{ATP} channels. Figure 4A shows representative recordings of the effect of ATP on the mitoK\textsubscript{ATP} channel activity induced by Iso. Iso (0.4 mM) increased the channel activity from baseline (50 \textmu{}M ATP). ATP at 0.5 and 1.0 mM inhibited the Iso-induced channel activity dose dependently. Normalized $N_{Po}$ from several experiments are summarized in Fig. 4B. Under control conditions (50 \textmu{}M ATP), ATP inhibited the mitoK\textsubscript{ATP} activity in a dose-dependent manner. The IC\textsubscript{50} was 335 ± 4 \textmu{}M, and $n_{H}$ was 5.4 ±
0.2 (n = 8). Iso at 0.4 and 0.8 mM caused a rightward shift in the ATP inhibition curve, with an increased IC$_{50}$ of 485 ± 17 μM (n = 5) and 940 ± 34 μM (n = 5), respectively. $n_H$ decreased to 2.1 ± 0.1 and 3.2 ± 0.4 at 0.4 and 0.8 mM Iso, respectively (n = 5 each, P < 0.05 vs. control).

**DISCUSSION**

This is the first study investigating the direct effect of volatile anesthetics on mitoK$_{ATP}$ channels reconstituted in lipid bilayers. Our results provide strong evidence that Iso directly activates rat cardiac mitoK$_{ATP}$ channels. This finding supports previous assertions that the mitoK$_{ATP}$ channels play a critical role in volatile anesthetic-mediated cardiac preconditioning (3, 11, 13, 14, 23, 25). As Iso activates the mitoK$_{ATP}$ channel despite the presence of millimolar ATP, its direct effect likely plays an important role in myocardial protection induced by APC. This effect is independent of its potential modulation of other signaling cascades such as adenosine receptors (25). Furthermore, our observation of five peaks for mitoK$_{ATP}$ conductance states suggests that native K$_{ATP}$ channels may exist in more than one molecular complex of sulfonylurea receptors (SUR) and Kir subunits. This possibility is supported by the recent observation that the cardiac mitoK$_{ATP}$ channel may be composed of Kir6.1 and Kir6.2 subunits as well as a novel small-molecular-weight SUR subunit (15).

**Regulation of mitoK$_{ATP}$ channels by ATP and Iso.** Although the ATP regulatory site of the mitoK$_{ATP}$ channel has been shown to be unipolar and cytosolic (29), others proposed that it faces the matrix side (10). As the ATP-inhibiting site is cytosolic and resides in the pore-forming Kir subunit of surface K$_{ATP}$ channel complex (28), we speculate that the ATP-inhibiting site on the Kir subunit of the mitoK$_{ATP}$ channels faces the cytosol. In the present study, ATP (and other modulators) was added to the cis chamber, which likely exerted its inhibitory effect via the cytosolic binding sites on the Kir subunits of mitoK$_{ATP}$ channels. Zhang et al. (30) observed that ATP-induced inhibition of mitoK$_{ATP}$ channels occurs only in the trans chamber and that K$^+$ flux favors flows from trans to cis (inward rectification). Thus it is possible that the cytosolic side of some mitoK$_{ATP}$ channels may be oriented toward the trans chamber after reconstitution in lipid bilayers or that another ATP-binding/inhibiting site is present on the matrix side as originally proposed (10). The orientation of the mitoK$_{ATP}$ channels reconstituted in proteoliposomes was shown to be dependent on the presence of Mg$^{2+}$ (29), which might also have affected their orientation during reconstitution into lipid bilayers.

The results of our current study showed that Iso directly activates K$_{ATP}$ channels. This is probably due to more channels being open as well as increased $P_o$ of each channel. The effect of Iso is likely due to its disruption of the allosteric interaction between the channel subunits, probably the pore-forming subunit Kir 6.x, and ATP, which reduced the positive cooperativity between ATP-binding sites (i.e., decreased $n_H$). Our observations also showed that Iso was capable of opening K$_{ATP}$ channels that were otherwise silent or inactive in the lipid bilayers. These channels were likely in the rundown mode or dephosphorylated. In addition, no other cytosolic factors are required for their activation by Iso. Therefore, the direct activation of mitoK$_{ATP}$ channels by Iso in bilayers is likely independent of their phosphorylation status or presence of other signaling components. The mechanisms by which opening of mitoK$_{ATP}$ channels protect the heart from ischemic injury are still controversial. Opening of the
mitoK<sub>ATP</sub> channels has been shown to trigger generation of oxygen and nitrogen free radicals, which in turn activate other cellular targets and induce myocardial protection (19, 21). Oxygen free radicals/superoxide may also exert a positive feedback effect directly on mitoK<sub>ATP</sub> channel activation (12, 30).

Limitations of the study. In this study, K<sub>ATP</sub> channels were identified based on their sensitivity to 5-HD/ATP inhibition and/or diazoxide activation. Recently, it has been shown that diazoxide and 5-HD may have mitoK<sub>ATP</sub> channel-independent targets. Diazoxide decreased succinate oxidation dose dependently, and 5-HD may be metabolized into its active form (8). Volatile anesthetics including Iso have also been shown to inhibit NADH/complex I of the electron transfer chain in mitochondria (1, 9, 24). In the present study, however, although enzymes involved in electron chain transfer may be part of the inner membrane vesicle fused into the bilayers, we did not add substrates such as NADH and succinate in our system. Thus we consider it unlikely that diazoxide or Iso induced significant production of free radicals that caused activation of the mitoK<sub>ATP</sub> channels reconstituted in lipid bilayers.

In summary, the native rat mitoK<sub>ATP</sub> channels of several conductance states were identified by their inhibition with ATP and 5-HD and activation with diazoxide after reconstitution into lipid bilayers. Iso directly stimulates the openings of mitoK<sub>ATP</sub> channels by reducing their ATP sensitivity. This mechanism likely contributes to volatile anesthetic-induced myocardial preconditioning.

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