Background: Volatile anesthetics can protect the myocardium against ischemic injury by opening the adenosine triphosphate (ATP)-sensitive potassium (K\textsubscript{ATP}) channels. However, direct evidence for anesthetic–channel interaction is still limited, and little is known about the role K\textsubscript{ATP} channel modulators play in this effect. Because pH is one of the regulators of K\textsubscript{ATP} channels, the authors tested the hypothesis that intracellular pH (pHi) modulates the direct interaction of isoflurane with the cardiac K\textsubscript{ATP} channel.

Methods: The effects of isoflurane on sarcolemmal K\textsubscript{ATP} channels were investigated at pH 7.4 and pH 6.8 in excised inside-out membrane patches from ventricular myocytes of guinea pig hearts.

Results: At pH 7.4, intracellular ATP (1–1,000 μM) inhibited K\textsubscript{ATP} channels and decreased channel open probability (Po) in a concentration-dependent manner with an IC\textsubscript{50} of 8 ± 1.5 μM, and isoflurane (0.5 mM) either had no effect or decreased channel activity. At pH 6.8, which itself facilitates channel opening, isoflurane enhanced channel activity by increasing Po and reduced channel sensitivity to ATP, with IC\textsubscript{50} shifting from 8 ± 1.2 to 45 ± 5.6 μM. When applied to the channels activated at pH 6.8, isoflurane (0.5 mM) increased Po and further reduced ATP sensitivity, shifting IC\textsubscript{50} to 110 ± 10.0 μM.

Conclusions: Changes in pHi appear to modulate isoflurane interaction with the cardiac K\textsubscript{ATP} channel. At pH 6.8, which itself facilitates channel opening, isoflurane enhances channel activity by increasing Po and reduces sensitivity to inhibition by ATP without changing the unitary amplitude of single channel current or the conductance. These results support the hypothesis of direct isoflurane–K\textsubscript{ATP} channel interaction that may play a role in cardioprotection by volatile anesthetics.

The adenosine triphosphate (ATP)-sensitive potassium (K\textsubscript{ATP}) channels are thought to play an important role in anesthetic preconditioning, a protection afforded by volatile anesthetics against ischemia and reperfusion injury. Although cellular mechanisms of this protection remain the focus of many investigations, direct evidence for interaction of volatile anesthetics with the K\textsubscript{ATP} channel is still limited. Such an interaction is clinically important because volatile anesthetics may mimic or enhance the protective mechanism of K\textsubscript{ATP} channel opening.

A characteristic property of K\textsubscript{ATP} channels is their sensitivity to inhibition by physiologic concentrations of intracellular ATP ([ATP]i) that is controlled by a number of cytosolic factors including nucleotide diphosphates (ADP), phospholipids such as phosphatidylinositol 4,5-biphosphate (PIP2), and intracellular protons. Intracellular pH (pHi) modulates the activity of native K\textsubscript{ATP} channels in pancreatic β cells, skeletal muscle, heart, and cloned K\textsubscript{ATP} channels in Xenopus oocyte expression system. The acidic species of K\textsubscript{ATP} channel modulators have been implicated in the mechanism of pH-dependent regulation of sensitivity to ATP. An interaction of intracellular protons with ATP in regulating channel activity has been also suggested recently. High sensitivity to activation by intracellular protons implies an important role for K\textsubscript{ATP} channels in regulation of cellular excitability during various metabolic stresses that often are accompanied by a decrease in pH. However, whether pH is a factor in volatile anesthetic–K\textsubscript{ATP} channel interaction has not been established.

We have recently reported that during whole cell or cell-attached patch clamp conditions isoflurane potentiates the cardiac K\textsubscript{ATP} channel current (I\textsubscript{KATP}) and increases open probability (Po) of channels previously activated by an uncoupler of oxidative phosphorylation, 2,4-dinitrophenol, and the K\textsubscript{ATP} channel opener, pinacolyl. Potentiation, however, did not occur in the inside-out patches where at pH 7.4 isoflurane either had no effect or decreased channel activity. Differential on-cell versus cell-free effects of isoflurane suggested that other intracellular factors might be involved in anesthetic potentiation.

In the present study, we tested the hypothesis that pHi is one of the endogenous factors modulating isoflurane interaction with the cardiac K\textsubscript{ATP} channel. Because intracellular acidosis, an effect characteristic of early ischemia, may also occur peroperatively before or during administration of the volatile anesthetic agents, we investigated whether decreasing pHi alters interaction of isoflurane with the K\textsubscript{ATP} channel regarding modulation of channel Po and sensitivity to inhibition by [ATP]i.

Materials and Methods

Cell Isolation

After approval by the Institutional Animal Use and Care Committee of the Medical College of Wisconsin, single ventricular myocytes were isolated from guinea pig hearts by enzymatic dissociation with collagenase Type II (Gibco/Invitrogen, Life Technologies, Grand Island, NY) and protease Type XIV (Sigma, St Louis, MO) as...
reported previously. Ventricular myocytes were stored in modified Tyrode solution, and only calcium-tolerant, rod-shaped cells with distinct cross-striations were used for experiments within 8 h after isolation.

Solutions
The modified Tyrode solution contained NaCl, 132 mm; KCl, 4.8 mm; MgCl₂, 1.2 mm; CaCl₂, 1 mm; HEPES, 10 mm; and glucose, 5 mm; at pH adjusted to 7.4 with NaOH.

For single channel recordings in the inside-out patch clamp configuration, the bath solution facing the intracellular side of membrane patches contained KCl, 140 mm; MgCl₂, 0.5 mm; EGTA, 1 mm; HEPES, 10 mm; and variable 0–1 mm K₂ATP, at pH 7.4 or pH 6.8 adjusted with KOH or HCl. The pipette solution facing the extracellular side of membrane patches contained KCl, 140 mm; MgCl₂, 0.5 mm; CaCl₂, 0.5 mm; and HEPES, 10 mm at pH 7.4 adjusted with KOH. All chemicals were purchased from Sigma (St. Louis, MO).

Isoflurane (Baxter Healthcare, Deerfield, IL) was delivered to the recording chamber in the bath solution. Anesthetic solution was prepared by adding a measured aliquot of isoflurane to a known volume of bath solution and dispersing it by sonication. This solution was then transferred into a gas-tight glass syringe reservoir to be transferred into a gas-tight glass syringe reservoir to be dispersed by sonication. This solution was then aliquoted into the bath solution sampled directly from the recording chamber.

Single Channel Recordings and Analysis
Ventricular cells were placed in a RC-16 recording chamber (Warner, Hamden, CT) on the stage of an inverted IMT-2 microscope (Olympus, Tokyo, Japan). Single KATP channel activity was monitored in the inside-out configuration of the patch clamp technique at 20–23°C. Patch pipettes were pulled from borosilicate glass tubing (Garner Glass, Claremont, CA) with a horizontal PC-84 micropipette puller (Sutter Instruments, Novato, CA). The tips were heat-polished with an MF-83 microforge (Narishige, Tokyo, Japan). Pipettes had resistances of 7–12 MΩ when filled with the extracellular solution. After gigaseal formation, the inside-out patches were excised by rapidly pulling the pipette away from the cell. In this configuration, the intracellular side of the membrane patch was directly exposed to the intracellular bath solution. Channel activity was recorded using a List EPC-7 amplifier (ALC Scientific Instruments, Westbury, NY) interfaced to a personal computer through a Digiata 1200B (Axon Instruments, Foster City, CA). Data were acquired using pClamp8 software (Axon Instruments, Foster City, CA) and Origin6 software (OriginLab, Northampton, MA).

At symmetric 140 mm K⁺ concentration, unitary outward current through single KATP channels was monitored at the transmembrane patch potential of +40 mV. The 60-s recordings were made at each experimental step. The KATP channels were identified by the single channel conductance, sensitivity to inhibition by [ATP]ᵢ, and blockade by glibenclamide (1 μM). A 50% threshold criterion was used for detecting the open state. Amplitude of single channel current was determined from the all-points amplitude histograms constructed from data segments of 60-s duration. Channel Po was determined from the ratios of the area under the peaks in the all-points amplitude histograms fitted with a Gaussian function. The number (N) of channels in each patch was estimated during brief exposure to ATP-free internal solution (0 ATP) at the end of the experiments. Po was calculated using the equation Po = [1 − (Pc)ⁿH] where Pc is the channel closed state probability. For measurements of ATP sensitivity, Po of each patch was normalized to Po determined at 0 ATP to control for variations in Po among patches. The sensitivity to ATP (1, 10, 50, 100, and 1,000 μM) was determined at pH 7.4 and pH 6.8 in the absence or presence of isoflurane. The experimental protocols were completed within 10–12 min after patch excision. To minimize channel rundown, we used the Ca²⁺-free and low Mg²⁺ intracellular solution and exposed each patch to 0 ATP only at the end of experimental protocols because even a brief exposure to ATP-free solution immediately after patch excision could accelerate rundown. Therefore, the number of channels in patches could have been underestimated in our study. Channel rundown occurred more frequently at pH 7.4. Decreasing pH to 6.8 tended to stabilize the channels and slow rundown. Recordings from patches exhibiting a significant rundown were excluded from analysis. For measurement of ATP sensitivity, the relationship between [ATP]ᵢ and Po was fitted by Hill equation:

Normalized Po = Po/Po_max = 1/(1 + ([ATP]ᵢ/IC₅₀)ⁿH)

where Po is channel open probability at any test [ATP]ᵢ; Po_max is open probability at 0 [ATP]; IC₅₀ is ATP concentration for half-maximal effect; and nH is Hill coefficient.
concentration-dependent manner. Figure 1 shows summary data for [ATP]-normalized Po relationship at pH 7.4 in the control and during application of 0.5 mM isoflurane. Each data point is a mean from four patches. During control conditions, increasing [ATP]i caused a concentration-dependent decrease in Po. Fitting mean data to the Hill equation yielded an IC 50 for ATP inhibition of 8 ± 1.5 μM and a Hill coefficient (nH) of 0.6 ± 0.05. When applied to the internal side of patches at pH 7.4, isoflurane decreased Po at [ATP]i less than 50 μM but had no marked effect on Po at [ATP]i greater than 50 μM. Figure 2 shows the sample traces of single KATP channel activity recorded at pH 7.4 in the control and during application of 0.5 mM isoflurane at 50 μM [ATP]i. In a patch containing five channels, isoflurane decreased channel activity in a reversible manner. As shown in figure 3, at 100 μM [ATP]i channel activity was much lower and little affected by isoflurane. The unitary amplitudes of 2.2 ± 0.1 pA (control) and 2.1 ± 0.1 pA (isoflurane) and the conductance were not altered by the anesthetic at pH 7.4.

**I sof lurane Effects on ATP Sensitivity at pH 6.8**

To test whether anesthetic–KATP channel interaction is modulated by pH, the effects of isoflurane were examined by decreasing pH from 7.4 to 6.8. The following protocol was carried out at each tested [ATP]i: control at pH 7.4, control at pH 6.8, isoflurane at pH 6.8, washout of isoflurane at pH 6.8, and 0 ATP at pH 6.8. This protocol also included a control baseline at pH 7.4 because decreasing pH itself is known to enhance opening of the cardiac KATP channels.12–14,16 Only one concentration of ATP was tested per patch. Figure 4 shows recordings at 100 μM [ATP]i from a patch containing three active channels. Infrequent at pH 7.4, channel opening increased markedly when decreasing pH to 6.8. Application of 0.5 mM isoflurane at pH 6.8 enhanced channel activity and further increased Po. Isoflurane effects were reversible during washout. Figure 5 shows summary data for [ATP]i–Po relationship obtained during the above condition where each patch was sequentially exposed to the internal solution at pH 7.4 and 6.8 and to 0.5 mM isoflurane at pH 6.8. Each data point is a mean from six patches. At pH 7.4, fitting the [ATP]i–Po relationship to the Hill equation yielded an IC 50 of 8 ± 1.2 μM and nH of 0.6 ± 0.04. Decreasing pH from 7.4 to 6.8 caused a rightward shift of the curve with IC 50 of 45 ± 5.6 μM and nH of 0.8 ± 0.1. The IC 50 value was approximately fivefold greater than that at pH 7.4, and both values were different from each other at P < 0.05. When applied at pH 6.8, isoflurane further decreased ATP sensitivity, and the curve shifted further to the right, yielding an IC 50 of 110 ± 10.0 μM and nH of 1.05 ± 0.12. The IC 50 value was more than twofold greater than that at pH 6.8 alone, and the values were significantly different at P < 0.05. Neither decreasing

**Statistical Analysis**

Data are presented as mean ± SEM. Comparisons were made using paired or unpaired Student t test. Multiple group means were compared by analysis of variance with a Student–Newman–Keuls test. Differences with a two-tailed P < 0.05 were accepted as significant.

**Results**

The effects of isoflurane on the outward current through KATP channels were investigated at pH 7.4 and pH 6.8 in the inside-out patches from guinea pig ventricular myocytes at a symmetric 140 mM K+ concentration and the patch potential of +40 mV.

**ATP Sensitivity of Single KATP Channels and Isoflurane Effects at pH 7.4**

Membrane patches were excised into the intracellular solution containing 0.2 mM ATP. Multiple channel openings that appeared on patch excision decreased within 30–40 s, and thereafter only the activity of spontaneously operative channels was recorded. To assess ATP dependence of isoflurane effects, we first evaluated ATP sensitivity of the channel during control conditions at pH 7.4. The following protocol was carried out at each tested [ATP]i: control at pH 7.4, isoflurane at pH 7.4, washout of isoflurane at pH 7.4, and 0 ATP at pH 7.4. Only one concentration of ATP was tested per patch. ATPi inhibited channel activity and decreased Po in a
pHi to 6.8 nor application of isoflurane at pHi 6.8 altered the amplitude of unitary current, which was 2.2 ± 0.1 pA at pHi 7.4, 2.3 ± 0.1 pA at pHi 6.8, and 2.3 ± 0.1 at pHi 6.8 with isoflurane. Single channel conductance remained in a range of 55–57 pS.

Discussion

Results from this study provide direct evidence for isoflurane inhibition of ATP sensitivity of cardiac K_ATP channels at reduced pHi. In the inside-out patches at pHi 6.8, isoflurane potentiates single K_ATP channels by increasing Po and reduces channel sensitivity to ATP without affecting amplitude of unitary outward current and conductance.

Isoflurane alone did not activate whole cell I_KATP in human atrial and guinea pig ventricular myocytes, however, isoflurane potentiated the whole cell I_KATP preactivated by pinacidil or 2,4-dinitrophenol. In guinea pig ventricular myocytes, isoflurane increased Po of K_ATP channels in the cell-attached but not in the inside-out membrane patches. By contrast, isoflurane decreased the activity of single K_ATP channels in the inside-out patches from rabbit ventricular myocytes. The pHi in all of these studies was kept at 7.4.

In the present study, in the inside-out patches and at pHi 7.4, isoflurane inhibited single K_ATP channel activity and decreased Po at [ATP]i less than 50 μM in a concentration-independent manner but had no effect on channel activity at [ATP]i greater than 50 μM. This finding confirms the results of Fujimoto et al., who reported lack of isoflurane effects on KATP channel activity in the inside-out patches at 300 μM [ATP]. We also found that isoflurane does not decrease ATP sensitivity of the channel at pHi 7.4. This observation differs from that of Han et al., who reported a decrease in ATP sensitivity after exposure of inside-out patches to isoflurane at pHi 7.4. Reasons for this discrepancy are not clear, but taking aside species (rabbit vs. guinea pig) differences, they could be related to differences in the experimental design. First, Han et al. examined the effects of isoflurane at the membrane potential of −70 mV and thus investigated the inward current through K_ATP channels, whereas our studies focused on the unitary outward current at the membrane potential of 0 mV (Fujimoto et al.) and +40 mV (present study). This raises the question whether anesthetics may differentially modulate the inward versus outward conductance of the K_ATP channel. Second, our measurements were taken during the exposure to isoflurane, whereas Han et al. measured...
ATP sensitivity before and after application of isoflurane, as shown in their figure 1 (middle and lower) and figure 3A and B. Third, from their figures 1, 2A, and 3A it appears that ATP was absent during the patch exposure to isoflurane. Regardless of these differences, both studies have demonstrated that a volatile anesthetic, isoflurane, may directly interact with the sarcolemmal K<sub>ATP</sub> channel.

Our results suggest that pH<sub>i</sub> may be one of the factors that modulate isoflurane–channel interaction. The K<sub>ATP</sub> channels are sensitive to changes in pH<sub>i</sub>, and a decrease in pH<sub>i</sub> is a potent stimulus for their activation by the mechanism that involves a decrease in sensitivity to inhibition by ATP. The optimal effect occurs at pH<sub>i</sub> 6.8 to 6.5, and further decrease or increase in pH<sub>i</sub> leads to channel inhibition. For instance, intracellular acidification to pH<sub>i</sub> less than 6.5 inhibits cardiac K<sub>ATP</sub> channels by inducing multiple subconductance levels. As anticipated, in our study decreasing pH<sub>i</sub> from 7.4 to 6.8 increased channel opening. This effect was the result of increased Po and reduced ATP sensitivity, as reflected by the rightward shift in the [ATP]<sub>i</sub>–Po curve with IC<sub>50</sub> increasing from 8 μM to 45 μM. Decreasing pH<sub>i</sub> to 6.8 did not affect the amplitude of unitary outward current, as also reported by others. The IC<sub>50</sub> values for ATP inhibition obtained by us at near physiologic and mild aci-dotic pH<sub>i</sub> are in the range of previously reported concentrations. However, these values are not identical, and our Hill coefficients are lower. This is not surprising because the experimental conditions vary among studies, and it is well known that many factors may alter ATP sensitivity of K<sub>ATP</sub> channels. These include the variations in experimental protocols and ionic conditions, the presence and concentration of monovalent and divalent ions (Ca<sup>2+</sup>, Mg<sup>2+</sup>) and glucose, differences in the range of pH<sub>i</sub> under study, outward or inward channel conductance under study, and species differences. In addition, pH<sub>i</sub> sensitivity of K<sub>ATP</sub> channels may be modified by ATP<sup>21</sup> and Mg<sup>2+</sup> ions.

Our study demonstrated that mild intracellular acidosis modulates direct interaction of isoflurane with the K<sub>ATP</sub> channel. At pH<sub>i</sub> 6.8, isoflurane potentiates channel activity by decreasing ATP sensitivity and shifting IC<sub>50</sub> for ATP inhibition from 45 μM to 110 μM. The mechanism by which intracellular acidosis modulates isoflurane–K<sub>ATP</sub> channel interaction is unknown, and we can only be speculative on this point. It has been established that pH sensing is an inherent property of Kir6.2 subunits of the K<sub>ATP</sub> channel. Three separate domains in the Kir6.2 protein—the N terminus, C terminus, and M2 domain—are involved in pH regulation, and the proton-sensing amino acid residues responsible for modulation of chan-
Intracellular protons appear to increase the activity of K_ATP channel by specifically binding to histidine (His-175) on the C-terminus of the Kir6.2 subunit, and this site is independent of the ATP-binding site, lysine (K185). Although independent, these sites appear to interact with each other, and allosteric modulation of the cloned K_ATP channels by ATP and H^+ has been recently demonstrated. Whether the allosteric modulation of channel activity by intracellular protons and ATP may play a role in isoflurane potentiation is not known. Because during ionic conditions of our study at pHi 6.8 isoflurane increased channel activity by reducing ATP sensitivity but did not affect unitary current amplitude or conductance, the channel pore is probably not targeted by the anesthetic. However, there is still a possibility of anesthetic interaction with the C terminus of the Kir6.2 subunit harboring the proton and ATP-binding sites. Furthermore, we cannot exclude a possibility of anesthetic interaction with the SUR2A subunit, modulating channel gating.

Previous findings from our laboratory and results of this study suggest that isoflurane may enhance opening of the K_ATP channel previously activated or modified by the action of intracellular channel regulators, and that pHi may be one of them.

The functional significance of the mitochondrial versus sarcolemmal K_ATP channels for cardioprotection remains controversial. Recent evidence suggests a role for mitochondrial K_ATP channels in the initiation of cardioprotection, but sarcolemmal K_ATP channels have also been indicated in the protection afforded by ischemic

Fig. 4. Effect of decreasing intracellular pH (pHi) from 7.4 to 6.8 on K_ATP channel activity in the absence and presence of isoflurane. (Upper) Sixty-second recordings of channel activity at 100 μM intracellular ATP ([ATP]) from an inside-out patch that was exposed sequentially to intracellular solution at pH 7.4, at pH 6.8, isoflurane at pH 6.8, washout of isoflurane at pH 6.8, and 0 ATP at pH 6.8. Decreasing pHi to 6.8 increased channel activity, and during these conditions, isoflurane further enhanced channel activity. Open probability (Po) values determined at each step of the experimental protocol are shown below traces. Dashed lines denote the closed state (C). Upward deflection indicates channel opening. (Lower) All-points amplitude histograms from recordings above. The amplitude of unitary outward current was not changed when decreasing pHi or during application of isoflurane at pHi 6.8.

Fig. 5. Summary data for intracellular ATP ([ATP])-normalized open probability (Po) relationship obtained at intracellular pH (pHi) 7.4, pH 6.8, and during isoflurane application at pH 6.8. Each data point is a mean ± SEM from six patches. Solid lines are Hill fits to the normalized Po data. Decreasing pH from 7.4 to 6.8 shifted the curve to the right, suggesting a decrease in ATP sensitivity. At pH 6.8, isoflurane caused further rightward shift in [ATP]-Po relationship, suggesting further decrease in ATP sensitivity. IC_{50} and Hill coefficient values are reported in Results.
preconditioning. Activation of the \( K_{ATP} \) channels is thought to be crucial for anesthetic preconditioning. However, the precise mechanism by which the enhancement of \( K_{ATP} \) channel activity by volatile anesthetics protects the myocardium is not yet established. Cardiac \( K_{ATP} \) channels are closed at physiologic [ATP]. During pathophysiologic conditions, such as ischemia, \( K_{ATP} \) channels activate during the first few minutes of the ischemic insult, long before any significant decrease in [ATP]. This suggests that other intracellular factors must be involved, and modulation of ATP sensitivity by factors targeting specifically the SUR subunit or the Kir6.x subunit has been demonstrated for ADP, PIP2, and pH. A transient decrease in pH that occurs during ischemia and accompanies other metabolic stresses may promote opening of sarcolemmal \( K_{ATP} \) channels by decreasing sensitivity to ATP, thus allowing isoflurane interaction with the channel, leading to further enhancement of channel activity. Recent studies implicated an important role of the adenylate kinase and creatine kinase-mediated phosphotransfer in communicating mitochondria-generated signals to the sarcolemmal \( K_{ATP} \) channels. Whether volatile anesthetics alter the signal transfer to the cell subsarcolemmal compartment and \( K_{ATP} \) channels is an open question.

Our results support a possible role of volatile anesthetics in ischemia because of early ischemic acidosis. However, as demonstrated in the animal models and in humans, volatile anesthetics precondition the myocardium independently of ischemia. In clinical settings, the pH dependence of \( K_{ATP} \) channel potentiation by isoflurane would likely play a protective role peripherally when various metabolic stresses may produce a transient decrease in pH. In conclusion, this study provides evidence for pH-dependent modulation of direct isoflurane interaction with the cardiac sarcolemmal \( K_{ATP} \) channel in guinea pig ventricular myocytes. Although at near physiologic pH isoflurane has no effect nor inhibits \( K_{ATP} \) channel, at reduced pH of 6.8, isoflurane increases channel Po and decreases sensitivity to inhibition by ATP as reflected by the rightward shift of the [ATP]–Po relationship.

References

37. Cason BA, Gamperl AK, Slocum RE, Hickey RF: Anesthetic-induced preconditioning: previous administration of isoflurane decreases myocardial infarct size in rabbits. ANESTHESIOLOGY 1997; 87:1182–90