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Isoflurane Activates Sarcolemmal Adenosine Triphosphate–sensitive Potassium Channels in Vascular Smooth Muscle Cells

A Role for Protein Kinase A

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Background: **Recent evidence indicates that vascular adenosine triphosphate–sensitive potassium (K_{ATP}) channels in vascular smooth muscle cells are critical in the regulation of vascular tonus under both physiologic and pathophysiologic conditions. Studies of the interaction of volatile anesthetics with vascular K_{ATP} channels have been limited. In the current study, the authors investigated the molecular mechanism of isoflurane's action on vascular K_{ATP} channels.**

Methods: **Electrophysiologic experiments were performed using cell-attached and inside-out patch clamp techniques to monitor native vascular K_{ATP} channels, and recombinant K_{ATP} channels comprised of inwardly rectifying potassium channel subunits (Kir6.1) and the sulfonylurea receptor (SUR2B). Isometric tension experiments were performed in rat thoracic aortic rings without endothelium.**

Results: **Application of isoflurane (0.5 mM) to the bath solution during cell-attached recordings induced a significant increase in K_{ATP} channel activity, which was greatly reduced by pretreatment with a selective inhibitor of protein kinase A (PKA), Rp-cAMPS (100 μM). In inside-out patches, isoflurane did not activate K_{ATP} channels. Isoflurane significantly activated wild-type recombinant SUR2B/Kir6.1 in cell-attached patches. Isoflurane-induced activation of wild-type channels was diminished in the PKA-insensitive mutant SUR2B-T633A/Kir6.1, SUR2B-S1465A/Kir6.1, and SUR2B/Kir6.1-S385A. In addition, the authors demonstrated that isoflurane-induced PKA activation was associated with isoflurane-induced decreases in isometric tension in the rat aorta.**

Conclusion: **These results indicate that isoflurane activates K_{ATP} channels *via* PKA activation. PKA-dependent vasodilation induced by isoflurane also was observed in isometric tension experiments. Analysis of expressed vascular-type K_{ATP} channels suggested that PKA-mediated phosphorylation of both Kir6.1 and SUR2B subunits plays a pivotal role in isoflurane-induced vascular K_{ATP} channel activation.**

ADENOSINE triphosphate–sensitive potassium (K_{ATP}) channels are present in both cardiomyocytes and vascular smooth muscle cells (VSMCs).[1] The cardiac K_{ATP} channel is composed of a sulfonylurea receptor (SUR) 2A and an inwardly rectifying K^+ channel subunit (Kir) 6.2, whereas the vascular K_{ATP} channel seems to be a complex of SUR2B and Kir6.1.[1,2] Different combinations of Kir6.x and SUR.x yield tissue-specific K_{ATP} channel subtypes with different features and distinct functional properties.[3] We previously reported that there were tissue-specific effects of either local or intravenous anesthetics on K_{ATP} channels, and that they had different effects on K_{ATP} channels in different tissures.[4–6]

Volatile anesthetics protect the myocardium against myocardial ischemia and reperfusion injury through a signal transduction pathway that includes protein kinase C (PKC) and mitochondrial and sarcolemmal K_{ATP} channels.[7,8] Volatile anesthetics also produce coronary vasodilatation by activating vascular K_{ATP} channels[9,10] and hyperpolarization in part through a protein kinase A (PKA)–induced K_{ATP} channel activation in vascular smooth muscle.[11] Recent evidence indicates that vascular K_{ATP} channels are critical in the regulation of vascular tonus, especially in the coronary circulation, and the K_{ATP} channel disruption may cause sudden death.[12,13] Therefore, isoflurane-induced vascular K_{ATP} channel opening may induce cardioprotection differently than anesthetic preconditioning. However, the precise molecular mechanism of isoflurane-induced vascular K_{ATP} channel opening has not been investigated.

The first objective of the current study was to determine, using patch clamp techniques, whether isoflurane activated K_{ATP} channels *via* PKA activation in native VSMCs. The second was to analyze the electrophysiologic effects of isoflurane on vascular K_{ATP} channel activity and to determine the underlying molecular mechanism, using transient transfection of recombinant K_{ATP} channels in combination with patch clamp techniques. The third was to determine whether enhancement of PKA activity by isoflurane played a pivotal role in isoflurane-induced vasodilation in rat aortic rings.

Materials and Methods

This study was approved by the Animal Investigation Committee of Tokushima University (Tokushima, Japan) and was conducted according to the animal use guidelines of the American Physiologic Society (Bethesda, Maryland).

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Cell Preparation

Male Wistar rats (weight, 250–300 g) were anesthetized with ether, and 1.0 U/g heparin was injected intraperitoneally 30 min before surgery. Aortas were dissected and longitudinally opened, and endothelium and adventitia were removed. The tissue was then minced into small pieces in normal Tyrode solution. The pieces were then explanted on glass coverslips in tissue culture dishes filled with medium 199 (Nissui Chemicals, Tokyo, Japan), supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY), 100 μg/ml streptomycin, and 100 μg/ml penicillin and stored in a carbon dioxide incubator (5% CO_2, 37°C). Single smooth muscle cells migrated out of the tissues and adhered to the coverslips within a few days. After being cultured for 6–10 days, they were used for electrophysiologic recordings.

Molecular Biology and Transfection

HEK293 cells were transiently cotransfected with plasmids encoding SUR2B, and Kir6.1, which together comprise the two subunits of smooth muscle K_{ATP} channels, using lipofectamine and Opti-MEM1. Mutagenesis was conducted using a Site-Directed Mutagenesis system (Invitrogen Corp., Carlsbad, CA). All DNA products were sequenced using a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 377 DNA sequencer (Applied Biosystems) to confirm the validity of their sequences. For electrophysiologic recordings, HEK293 cells were plated on glass coverslips, and Kir and SUR subunits were cotransfected, along with a complementary DNA encoding green fluorescent protein (pEGFP) as a reporter gene. After transfection, cells were cultured for 48–72 h before electrophysiologic recordings were taken.

Electrophysiologic Measurements

Cell-attached and inside-out patch configurations were applied to record the current through single channels using a patch clamp amplifier, as previously described.[14] In cell-attached configurations, the bathing solution was composed of the following: 140 mM KCl, 10 mM HEPES, 5.5 mM dextrose, and 1 mM EGTA. The pipette solution contained 140 mM KCl, 10 mM HEPES, and 5.5 mM dextrose. For inside-out configurations, the bathing solution (intracellular solution) contained 140 mM KCl, 10 mM HEPES, 5.5 mM dextrose, 1 mM $MgCl_2$, 1 mM EGTA, 0.5 mM MgADP, and 0.5 mM MgATP. The pipette solution (extracellular medium) was of the same composition as that used in cell-attached experiments. The pH of all solutions was adjusted to 7.3–7.4 with KOH. Recordings were made at 36° ± 0.5°C. Patch pipettes were pulled with an electrode puller (PP-830; Narishige, Tokyo, Japan). The resistance of pipettes filled with internal solution and immersed in the Tyrode solution was 5–7 MΩ. The sampling frequency of the single-channel data was 5 KHz with a low-pass filter (1 KHz).

Channel currents were recorded with a patch clamp amplifier (CEZ 2200; Nihon Kohden, Tokyo, Japan) and stored in a personal computer (Aptiva; International Business Machine Corporation, Armonk, NY) equipped with an analog-to-digital converter (DigiData 1200; Axon Instruments, Foster, CA). pClamp version 7 software (Axon Instruments) was used for data acquisition and analysis. The open probability (Po) was determined from current amplitude histograms and was calculated as follows:

$$Po = \frac{\sum_{j=1}^{N} t_j \cdot j}{T_d \cdot N},$$

where t_j is the time spent at current levels corresponding to j = 0, 1, 2, N channels in the open state; T_d is the duration of the recording; and N is the number of channels active in the patch. Recordings of 2–3 min were analyzed to determine Po. Channel activity was expressed as NPo.

Isometric Tension Experiments

Experiments were performed on 2.5-mm thoracic aortic rings obtained from male Wistar rats (250–300 g) anesthetized with ether. The rings were bathed in modified Krebs-Ringer's bicarbonate solution (control solution), consisting of the following: 118.3 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25.0 mM $NaHCO_3$, 0.026 mM calcium EDTA, and 11.1 mM glucose. For all rings, the endothelium was removed mechanically, and removal was confirmed by the absence of relaxation in response to acetylcholine (10^{-2} mM). Several rings cut from the same artery were studied in parallel, with each ring connected to an isometric force transducer (Micro easy magnus UC-2A; Kishimoto Medical Instruments Co., Ltd., Kyoto, Japan) and suspended in an organ chamber filled with 2 ml of the control solution (37°C, pH 7.4) infused with 95% O_2–5% CO_2. Arteries were gradually stretched to the optimal point of the length–tension curve, as determined by contraction with phenylephrine (3×10^{-4} mM). For most of the arteries studied, optimal tension was achieved with the equivalent to the applied mass of 1.0 g. Preparations were equilibrated for 90 min. During submaximal contractions in response to phenylephrine (3×10^{-4} mM), relaxation after administration of isoflurane was recorded. Vasorelaxation was expressed as a percentage of the maximal relaxation in response to papaverine (0.3 mM), which was added at the end of the experiments to produce maximal relaxation (100%) of the arteries.

Isoflurane Delivery

For patch clamp experiments, isoflurane was mixed by adding defined aliquots of concentrated isoflurane with the appropriate bathing solutions into graduated sy-

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ringes. Isoflurane superfusion was achieved using a syringe pump with a constant flow of 1 ml/min. A clinically relevant concentration of isoflurane, 0.5 mM was used, equivalent to 2.4 vol% or 1.7 minimum alveolar concentration (MAC). To determine isoflurane concentrations, 1.5 ml of the superfusate was collected in a metal-capped 3-ml glass vial at the end of each experiment. The concentration of isoflurane in the superfusate was then determined to be 0.49 ± 0.04 mM by gas chromatography (G-3500; Hitachi, Tokyo, Japan).

In isometric tension experiments, isoflurane was introduced into the gas mixture using an agent-specific vaporizer (I-MkII; Acoma, Tokyo, Japan). The concentration of the resulting gas mixture was monitored and adjusted using an anesthetic agent monitor (Capnomac Ultima; Datex, Helsinki, Finland). The concentration of isoflurane in Krebs-Ringer's bicarbonate solution was measured by gas chromatography and determined to be 0.16 ± 0.03 mM (0.8% or 0.6 MAC), 0.31 ± 0.04 mM (1.5% or 1.1 MAC), 0.47 ± 0.05 mM (2.3% or 1.6 MAC), and 0.60 ± 0.08 mM (2.9% or 2.1 MAC) at isoflurane concentrations of 1, 2, 3, and 4%, respectively, in the gas mixture.

Drugs

Pinacidil, glibenclamide, iberiotoxin, and chlorophenylthio-cAMP (CPT-cAMP) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Calphostin C and Rp-cAMPS were obtained from Calbiochem (San Diego, CA). The catalytic subunit of protein kinase A (c-PKA) was from Promega (Madison, WI). Pinacidil, glibenclamide, calphostin C, and iberiotoxin were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was less than 0.05%; dimethyl sulfoxide at a twofold higher concentration was shown not to affect both native and recombinant K_{ATP} channel currents and baseline vasoconstriction in ring segment of artery. Rp-cAMPS, CPT-cAMP, and c-PKA were dissolved into distilled water.

Statistical Analysis

Data are expressed as mean ± SD. Statistical analysis was performed using either the Student *t* test or repeated-measures analysis of variance, followed by the Scheffé test for multiple comparisons. Differences were considered to be statistically significant when *P* was less than 0.05.

Results

Effects of Isoflurane on K_{ATP} Channel Activity in Native VSMCs

To assess the effects of isoflurane on K_{ATP} channels in native VSMCs, we measured single channel currents in cell-attached and inside-out patch clamp configurations.

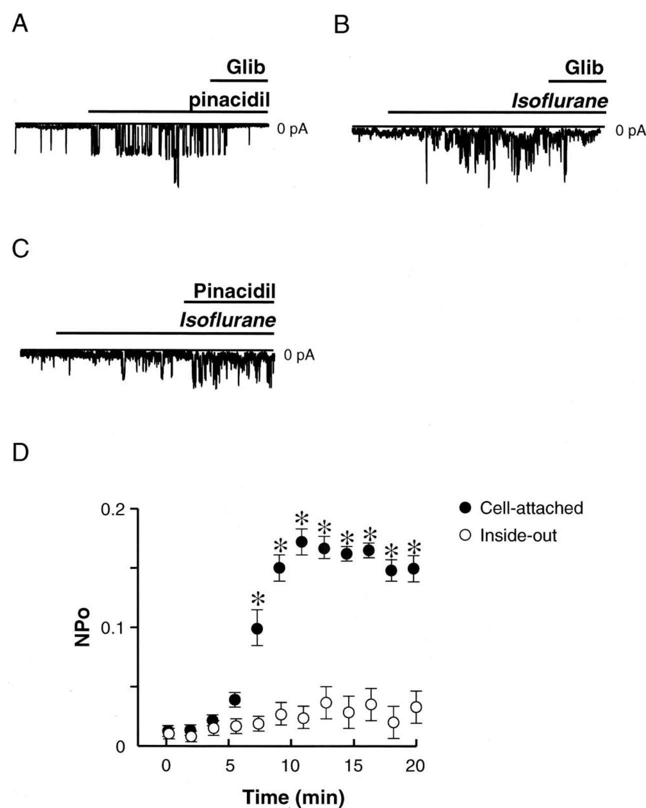

Fig. 1. Effects of isoflurane on the adenosine triphosphate–sensitive potassium (K_{ATP}) channel activities in native vascular smooth muscle cells. (*A*) Single-channel characteristics of K_{ATP} channels in the cell-attached configurations. Membrane potentials were clamped at −60 mV. Zero current levels are indicated by the *horizontal lines* marked "0 pA." Pinacidil (100 μM) and glibenclamide (Glib) (3 μM) were superfused to the bath solution as indicated by the *horizontal solid bars*. Effects of isoflurane on the K_{ATP} channel activities in the cell-attached (*B*) and inside-out (*C*) configuration. (*D*) The relation between NPo and time for the traces shown in cell-attached (●) and inside-out (○) configuration. Each *vertical bar* constitutes measurements from 12 patches (mean ± SD). * $P < 0.05$ *versus* baseline.

As shown in figure 1A, spontaneous single channel activity was observed infrequently in the cell-attached configuration. However, application of 100 μM pinacidil, a selective K_{ATP} channel opener, to the bath solution significantly activated K^+-selective channels. This channel activity was completely blocked by 3 μM glibenclamide, a specific K_{ATP} channel blocker.

Application of isoflurane (0.5 mM) to the bath solution during cell-attached recordings induced a significant increase in K_{ATP} channel activity (fig. 1B). The subsequent addition of 3 μM glibenclamide induced an immediate and complete reversal of the effects of isoflurane (fig. 1B). In contrast, in the inside-out patches, bath application of isoflurane did not activate K_{ATP} channels (fig. 1C). Figure 1D shows the relation between NPo and time for both cell-attached and inside-out configurations. In the cell-attached patches, there was a delay of approximately 5–10 min after bath application of isoflurane before steady state K_{ATP} channel activation occurred. One minute after application of isoflurane, the NPo value

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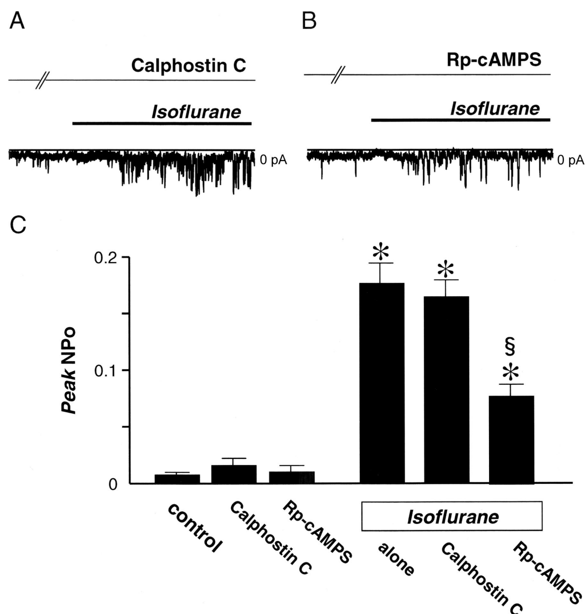

Fig. 2. Effect of protein kinase inhibitors on isoflurane-induced adenosine triphosphate–sensitive potassium channel activation in native vascular smooth muscle cells. (*A*) Effects of pretreatment (10 min) with calphostin C, a selective protein kinase C inhibitor. (*B*) Effects of pretreatment (10 min) with Rp-cAMPS, a selective protein kinase A inhibitor. Membrane potentials were clamped at −60 mV. Zero current levels are indicated by the *horizontal lines* marked "0 pA." Isoflurane was superfused to the bath solution as indicated by the *horizontal solid bars*. (*C*) Summary of changes in NPo. Each *vertical bar* constitutes measurements from 15–20 patches (mean ± SD). * $P < 0.05$ *versus* baseline. § $P < 0.05$ *versus* isoflurane alone.

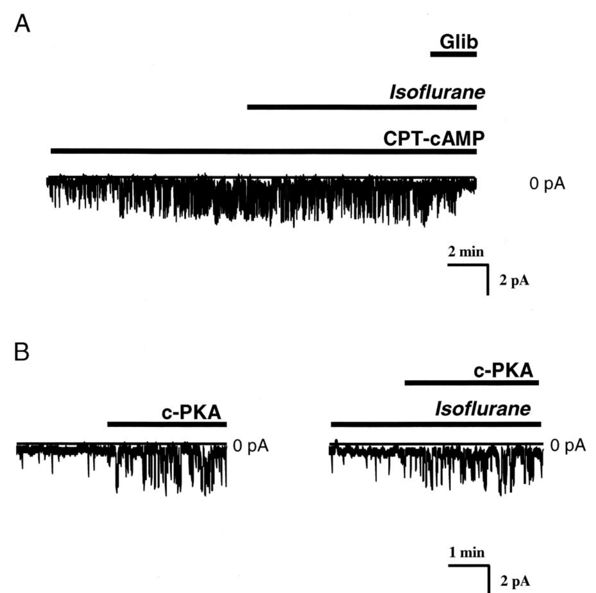

Fig. 3. Protein kinase A (PKA)–mediated adenosine triphosphate–sensitive potassium channel activation in native vascular smooth muscle cells. (*A*) Changes in the channel activity in response to a membrane permeable activator of PKA-dependent cAMP pathway, CPT-cAMP, added to the bath at 100 μM in cell-attached configuration. (*B*) Direct stimulatory effect of the catalytic subunit of PKA, c-PKA (100 U/ml), to the bath in inside-out patch configuration. Membrane potentials were clamped at −60 mV. Zero current levels are indicated by the *horizontal lines* marked "0 pA." Study drug was added to the bath solution as indicated by the *horizontal solid bars*.

was 0.012 ± 0.004, and this increased after 17–20 min of 0.5 mM isoflurane to 0.18 ± 0.03 (n = 15, $P < 0.05$; fig. 1D). Identical control experiments in the absence of isoflurane resulted in no K_{ATP} activation or inhibition at the same time periods in either cell-attached or inside-out configurations.

Effects of Protein Kinase Inhibitor on Isoflurane-induced K_{ATP} Channel Activation in Native VSMCs

The effect of bath-applied isoflurane was observed in cell-attached patches, but no effect was seen in inside-out patches. This was attributed to the absence of PKA activity in the excised patches. Furthermore, there was a slow onset of channel activation after isoflurane treatment. These characteristics suggested that isoflurane mediated its effects indirectly, *via* an intracellular signaling pathway. It is well known that protein kinases play important roles in the physiologic regulation of K_{ATP} channels. Therefore, we examined whether isoflurane-induced activation of K_{ATP} channels was affected when kinase activity was suppressed. Pretreatment (10 min) of native VSMCs with calphostin C (500 nM), a selective PKC inhibitor, added to the bath solution did not affect isoflurane-induced K_{ATP} channel activation (n = 12; fig. 2A). We also examined the effect of Rp-cAMPS, a highly selective inhibitor of PKA (*K*i = 10 μM), which acts by binding to the PKA regulatory subunit. Pretreatment (10 min) with Rp-cAMPS (100 μM) greatly reduced isoflurane-induced K_{ATP} channel activation (n = 12; fig. 2B). Neither protein kinase inhibitor had any effect on baseline or pinacidil-induced K_{ATP} channel activity. Isoflurane-induced changes in NPo in the presence of protein kinase inhibitors are summarized in figure 2C.

PKA-mediated K_{ATP} Channel Activation in Native VSMCs

To demonstrate whether PKA involved in the activation of vascular K_{ATP} channel currents by isoflurane, we studied the effects of CPT-cAMP, a membrane permeable activator of PKA-dependent cAMP pathway, on these currents in cell-attached patches. As shown in figure 3A, the bath application of 100 μM CPT-cAMP gradually activated K_{ATP} channel currents, and over 10 min after bath application of CPT-cAMP, NPo value reached steady state level with an NPo value of 0.21 ± 0.07 (n = 7). Figure 3A also shows that isoflurane did not further activate steady state currents induced by CPT-cAMP.

In addition to cell-attached conditions, we also examined the direct effects of PKA in inside-out patches (fig. 3B). When 100 U/ml c-PKA, a catalytic subunit of PKA, was added to the bath, the K_{ATP} channel currents were markedly activated (n = 6). Further, application of c-PKA restored the isoflurane effect in inside-out patches (n = 5).

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Effects of Isoflurane on Mutated SUR2b/Kir6.1 Channels

Recent study demonstrated that PKA may directly activate SUR2B/Kir6.1 channels by multisite phosphorylation of these channels: two sites in SUR2B (threonine residue at position 633 and serine residue at position 1465) and one site in Kir6.1 (serine residue at position 385).[15] We mutated these three sites (threonine residue at SUR2B position 633 to alanine, SUR2B-T633A/Kir6.1; serine residue at position SUR2B 1465 to alanine, SUR2B-S1465/Kir 6.1; and serine residue at position Kir6.1 385 to alanine, SUR2B/Kir6.1-S385A) to examine the importance of PKA phosphorylation for isoflurane-induced vascular K_{ATP} channels.

Similar to native vascular K_{ATP} channel, application of isoflurane (0.5 mM) to bath solution during cell-attached recordings induced an increase in wild-type SUR2B/Kir6.1 channels (fig. 4A). However, figure 4A also shows that disrupting PKA phosphorylation sites significantly decreased isoflurane-induced wild-type SUR2B/Kir6.1 channel activation. CTP-cAMP (100 μM)– and isoflurane (0.5 mM)–induced increases in NPo value of wild-type SUR2B/Kir6.1, SUR2B-T633A/Kir6.1, SUR2B-S1465A/Kir6.1, and SUR2B/Kir6.1-S385A are summarized in figure 4B. All three point mutation of PKA phosphorylation sites abolished CTP-cAMP–induced wild-type SUR2B/Kir6.1 channel activation, and diminished isoflurane-induced channel activation.

Effects of Isoflurane on Vasodilation

We also studied the vasodilative effect of isoflurane in ring segments of the artery. In a rat aortic artery precontracted submaximally with phenylephrine (3×10^{-4} mM), the degree of vasorelaxation induced by 1–4% isoflurane was recorded with or without pretreatment (10 min) of Rp-cAMPS (100 μM). As shown in figure 5, isoflurane significantly decreased isometric forces in a concentration-dependent manner. However, pretreatment with Rp-cAMPS significantly inhibited isoflurane-induced vasodilation at each concentration of isoflurane (fig. 5). Rp-cAMPS alone had no effect on baseline vasoconstriction (data not shown).

Next, to elucidate the role of K^+ channels in the vasorelaxant mechanisms of isoflurane, we tested the effects of K_{ATP} channel blocker glibenclamide and of the calcium-activated K^+ (K_{Ca}) channel blocker iberiotoxin on isoflurane-induced vasorelaxation. The concentration–response curves for vasorelaxation induced by 1–4% isoflurane with glibenclamide (1 μM) or iberiotoxin (100 nM) are shown in figure 6. Both glibenclamide and iberiotoxin significantly attenuated isoflurane induced vasorelaxation at an almost similar potency.

Discussion

In the current study, we demonstrated in native rat VSMCs that isoflurane significantly increased K_{ATP} channel currents in cell attached patches, but not excised inside-out patches. The isoflurane-induced increase in K_{ATP} channel current was partially inhibited by a selective PKA inhibitor, Rp-cAMPS, but not a selective PKC inhibitor, calphostin C. These results suggest that isoflurane activates vascular K_{ATP} channels through a signal transduction pathway that involves activation of PKA, but not PKC. Recombinant K_{ATP} channel studies in transiently transfected HEK293 cells suggested that PKA-mediated both Kir6.1 and SUR2B phosphorylation in response to isoflurane plays a pivotal role in activation of vascular K_{ATP} channels. Isometric tension experiments indicated that isoflurane-induced PKA activation is also involved in the mechanism of isoflurane-induced vasodilation of rat aortic ring preparations.

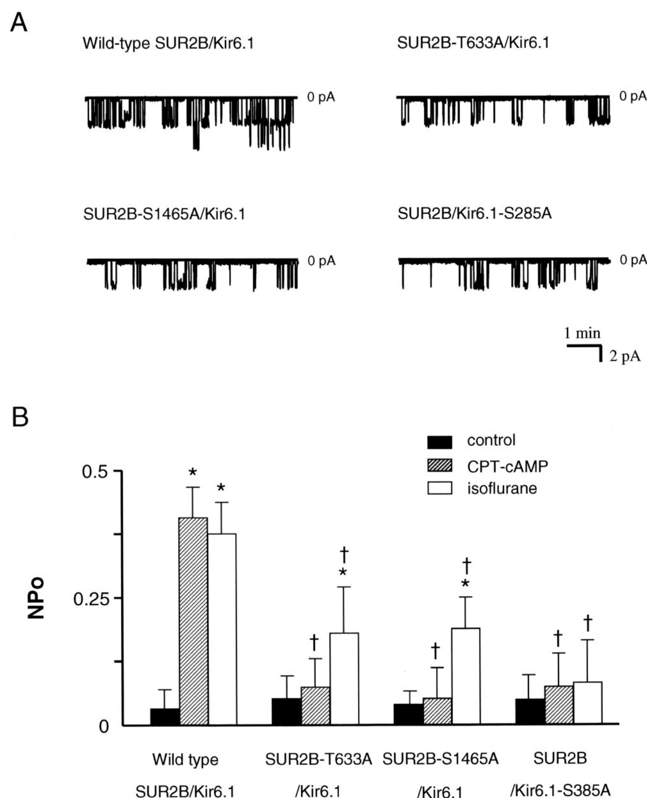

Fig. 4. Effects of isoflurane on the currents of recombinant adenosine triphosphate–sensitive potassium channels expressed in HEK293 cells in the cell-attached configuration. (*A*) Shown are representative examples of wild-type sulfonylurea receptor (SUR) 2B/inwardly rectifying potassium channel (Kir) 6.1 and mutated multisite phosphorylation of SUR2B/Kir6.1 channels (threonine residue at SUR2B position 633 to alanine, SUR2B-T633A/Kir6.1; serine residue at position SUR2B 1465 to alanine, SUR2B-S1465/Kir 6.1; and serine residue at position Kir6.1 385 to alanine, SUR2B/Kir6.1-S385A) currents obtained 10 min after the application of isoflurane. Membrane potentials were clamped at −60 mV. Zero current levels are indicated by the *horizontal lines* marked "0 pA." (*B*) Summary of CPT-cAMP and isoflurane effects on NPo of wild-type SUR2B/Kir6.1, SUR2B-T633A/Kir6.1, SUR2B-S1465/Kir 6.1, and SUR2B/Kir6.1-S385A channels. Each *vertical bar* constitutes measurements from 14 patches (mean ± SD). * $P < 0.05$ *versus* control (no application of drug). † $P < 0.05$ *versus* wild-type SUR2B/Kir6.1 channels.

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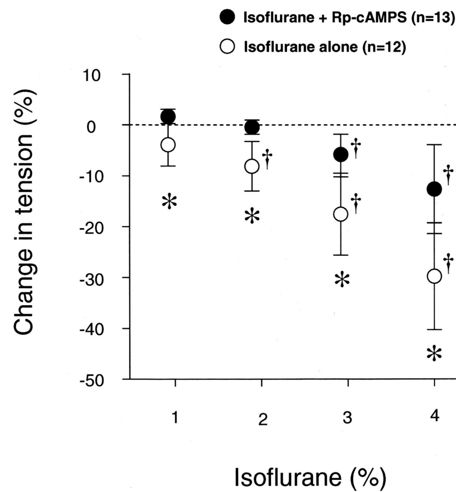

Fig. 5. Concentration-response curves to isoflurane (1–4%) in the presence or absence of pretreatment of Rp-cAMPS (100 μM), a selective protein kinase A inhibitor, obtained in rat thoracic aortas without endothelium. Data are shown as mean ± SD and expressed as percent of maximal relaxation induced by papaverine (3 × 10^{-4} M). † $P < 0.05$ *versus* 1% isoflurane value. * $P < 0.05$ between control rings and rings pretreated with Rp-cAMPS. n refers to the number of rats from which the aortas were obtained.

Although isoflurane-induced vasodilation of different vascular tissues has been reported,[16,17] the exact cellular mechanisms underlying the vasorelaxant effect of isoflurane remains unclear. It was reported that isoflurane increased canine coronary flow, as measured by a Doppler flow probe, which was counteracted by glibenclamide, indicating that K_{ATP} channels were involved in isoflurane-induced vasodilatation.[9] Further, Kokita *et al.*[18] reported that isoflurane-induced hyperpolarization of rat small mesenteric vessels was coupled to activation of several potassium channels, including vascular K_{ATP} channels. However, direct evidence of isoflurane-induced activation of K_{ATP} channel currents in VSMCs has not been demonstrated. In the current study, we demonstrated for the first time that 0.5 mM isoflurane activated native sarcolemmal K_{ATP} channels in rat aortic VSMCs. Isoflurane-induced K_{ATP} channel activation was observed in the cell-attached, but not the excised inside-out, patch clamp configuration, suggesting that regulation of vascular K_{ATP} channels by isoflurane occurs by way of an intracellular signaling pathway. This is in contrast to the results of recent studies of rat cardiac myocytes, in which isoflurane activated rat cardiac K_{ATP} channels in excised patch clamp examinations, indicating that volatile isoflurane directly interacted with sarcolemmal K_{ATP} channels.[19] A recent study demonstrated a direct and pHi-dependent interaction of isoflurane with the nucleotide binding domain 1 of SUR2A, the regulatory subunit of the cardiac sarcolemmal K_{ATP} channel.[20] Other studies in guinea pig myocytes have shown that isoflurane enhanced sarcolemmal K_{ATP} channel currents in a whole cell patch clamp configuration by facilitating channel opening after initial activation, in the presence of the mitochondrial uncoupler 2,4-dinitrophenol, or the K_{ATP} channel opener pinacidil.[21,22] It was also shown that activation of PKC was required to facilitate opening of sarcolemmal K_{ATP} channels in cardiac myocytes.[22,23] Our results, however, indicate that isoflurane-induced activation of vascular K_{ATP} channels was independent of PKC activation. This suggests that different mechanisms involving the tissue specificity of K_{ATP} channels underlie isoflurane-induced activation of cardiac and vascular sarcolemmal K_{ATP} channels.

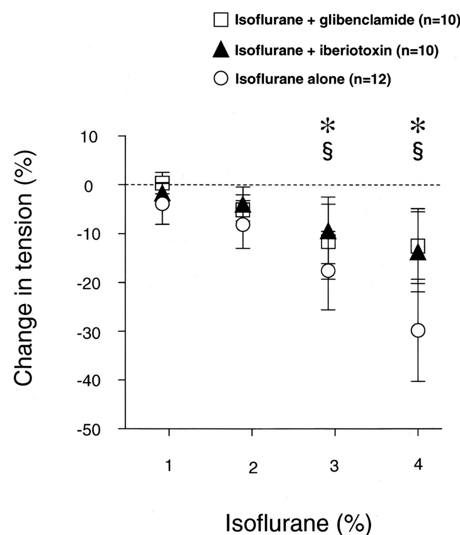

Fig. 6. Concentration–response curves to isoflurane (1–4%) in the presence or absence of pretreatment of glibenclamide (1 μM), a selective adenosine triphosphate–sensitive potassium channel blocker, or iberiotoxin (100 nM), a selective calcium-activated potassium channel blocker, obtained in rat thoracic aortas without endothelium. Data are shown as mean ± SD and expressed as percent of maximal relaxation induced by papaverine (3 × 10^{-4} M). * $P < 0.05$ between control rings and rings pretreated with glibenclamide. § $P < 0.05$ between control rings and rings pretreated with iberiotoxin. n refers to the number of rats from which the aortas were obtained.

There are multiple mechanisms of regulation of vascular K_{ATP} channels.[24–26] The effects of nucleotide triphosphates and diphosphates on channel function have long been recognized, and link channel activity to metabolism. In addition to metabolic regulation, phosphorylation by PKA and PKC directly modulates vascular K_{ATP} channel activity.[24–27] It was demonstrated that glibenclamide-sensitive currents induced by adenosine[28] and calcitonin gene–related peptide[29] were largely reduced by two PKA inhibitors, Rp-cAMPS and H89. Indeed, several vasodilators, including adenosine, calcitonin gene–related peptide, and β-adrenoreceptor agonists, have been suggested to activate vascular K_{ATP} channels through the cAMP–PKA signal transduction pathway.[24–26] Similar to these vasodilators, in our study, isoflurane-induced vascular K_{ATP} channel activation was diminished by a specific PKA inhibitor, Rp-cAMPS, suggesting that isoflurane-induced PKA activation is likely to affect vascular K_{ATP} channel protein phosphorylation and hence channel activity. This is in agreement with

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previous data, showing the isoflurane-induced hyperpolarization of vascular smooth muscle cells occurred *via* activation of cAMP synthesis or PKA activation.[11]

In addition to its effect on vascular K_{ATP} channels, isoflurane activates calcium-activated potassium (K_{Ca}) channels to produce hyperpolarization of vascular smooth muscle cells *via* the cAMP–PKA cell signaling pathway.[11] In the current study, we studied the effects of a K_{ATP} channel blocker, glibenclamide, and a K_{Ca} channel blocker, iberiotoxin, on isoflurane-induced vasodilation. Both glibenclamide and iberiotoxin significantly attenuated isoflurane-induced vasodilation. Therefore, it is possible that the PKA-dependent isoflurane-induced vasodilation observed in the isometric tension experiments on rat aortic ring preparations in the current study resulted from activation of at least two types of potassium channels, K_{ATP} and K_{Ca} channels.

It was also reported that volatile anesthetics depressed L-type Ca^{2+} channel activity and subsequent Ca^{2+} entry by enhancing apparent channel inactivation.[30–32] Isoflurane depressed protein tyrosine phosphorylation–modulated contraction of vascular smooth muscle, especially that mediated by tyrosine-phosphorylated PCLγ-1 and mitogen-activated protein kinase signaling pathways.[33] These findings that isoflurane inhibited tyrosine phosphorylation–mediated vascular smooth muscle or that isoflurane-reduced L-type Ca^{2+} channel activity may be independent with PKA pathway. This may be responsible for the action that PKA inhibitor partially abolished isoflurane-induced K_{ATP} channel activity.

It was shown that S385 of the pore-forming Kir6.1 subunit and T633 or S1465 of SUR2B subunit represent putative PKA phosphorylation sites of SUR2B/Kir6.1 channels.[15] Quinn *et al.*[15] reported that these three sites in vascular K_{ATP} channels must be phosphorylated by PKA before activation of vascular K_{ATP} channels occurs. In the current study, similar to the report by Quinn *et al.*,[15] mutation of single PKA phosphorylation sites in Kir6.1 (alanine substituting for S385) and in SUR2B (alanine substituting for T633 or S1465) significantly diminished stimulatory effects of isoflurane on wild-type SUR2B/Kir6.1 channels during cell-attached recordings. These results suggest that multisite phosphorylation for PKA in both Kir6.1 and SUR2B subunits are required before isoflurane-induced K_{ATP} channel activation in VSMCs.

Recent studies on mice lacking different K_{ATP} channel subunits have begun to clarify the roles of vascular K_{ATP} channels in cardiovascular pathophysiology.[12,13] Impaired vascular smooth muscle function was a feature of Kir6.1- and SUR2-deficient mice, manifest as episodic coronary artery vasospasm and a high rate of sudden death.[12,13] A number of physiologic studies also reported that vascular K_{ATP} channels are involved in the maintenance of resting blood flow in a number of vascular beds, notably the coronary circulation, as well as in vasodilation in response to metabolic demand. These results indicate that isoflurane-induced vascular K_{ATP} channel activation might have advantageous properties, especially under ischemic conditions such as angina. Accordingly, volatile anesthetic–induced K_{ATP} channel opening in VSMCs may help to produce cardioprotection against myocardial ischemia and reperfusion injury in volatile anesthetic–induced preconditioning. However, further studies are necessary to clarify the influence of isoflurane on vascular K_{ATP} channels in clinical settings.

The limitation of our study is that just one concentration (0.5 mM) of isoflurane was used in patch clamp experiments. It is premature to make conclusion about its potency based on just one concentration of anesthetics. However, 0.5 mM isoflurane at 37°C corresponds with 2.4 vol%, which is a clinically relevant concentration. Another limitation is that there are no data showing isoflurane increases cAMP or PKA activity in VSMCs in the current study. CPT-cAMP could mimic isoflurane effect on K_{ATP} channels in cell-attached patches. Exposure to isoflurane did not enhance the K_{ATP} channel opening activated by CPT-cAMP. Furthermore, we showed that c-PKA could activate K_{ATP} channels with or without pretreatment of isoflurane in inside-out patches. These results strongly suggest that isoflurane-induced native vascular K_{ATP} channel activation is dependent on PKA.

In conclusion, we showed that 0.5 mM isoflurane activates K_{ATP} channels in native rat VSMCs *via* PKA activation. PKA-dependent vasodilation induced by isoflurane was also observed in isometric tension experiments using rat aortic ring preparations. Mutagenesis experiments using expressed SUR2B/Kir6.1 channels (vascular-type K_{ATP} channels) indicated that PKA-mediated phosphorylation of both Kir6.1 and SUR2B subunits plays a pivotal role in isoflurane-induced SUR2B/Kir6.1 channel activation. These results demonstrate a potential molecular mechanism of volatile anesthetic–induced vasodilation, through activation of vascular K_{ATP} channels.

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