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Mechanisms of Isoflurane-mediated Hyperpolarization of Vascular Smooth Muscle in Chronically Hypertensive and Normotensive Conditions

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Background: **The purpose of this study was to compare the effects of isoflurane on membrane and intracellular mechanisms that regulate vascular smooth muscle (VSM) transmembrane potential (E_m; which is related to VSM tone) in the spontaneously hypertensive rat (SHR) model of essential hypertension and its normotensive Wistar-Kyoto (WKY) control.**

Methods: **Vascular smooth muscle E_m values were measured *in situ* in locally denervated, superfused, intact, small (200–300-μm OD) mesenteric arteries and veins in anesthetized 9–12-week-old SHR and WKY. Effects of 1.0 minimum alveolar concentration (0.60 mM) superfused isoflurane on VSM E_m were measured before and during superfusion with specific inhibitors of VSM calcium-activated (K_{Ca}) and adenosine triphosphate–regulated (K_{ATP}) potassium channels, and with endogenous mediators of vasodilatation (nitric oxide, cyclic guanosine monophosphate, protein kinase G, cyclic adenosine monophosphate, and protein kinase A).**

Results: **Isoflurane significantly hyperpolarized small arteries (5 ± 3.4 mV) and veins (6 ± 4.7 mV) (pooled SHR and WKY, mean ± SD). Inhibition of K_{Ca} and K_{ATP} channels, cyclic adenosine monophosphate, and protein kinase A, but not nitric oxide, cyclic guanosine monophosphate, and protein kinase G, abolished such hyperpolarization equally in SHR and WKY vessels.**

Conclusions: **Isoflurane-induced *in situ* VSM hyperpolarization in denervated, small mesenteric vessels involves a similar activation of K_{Ca} and K_{ATP} channels and cyclic adenosine monophosphate, but not nitric oxide or cyclic guanosine monophosphate, second messenger pathways in both SHR and WKY. A greater isoflurane-induced VSM hyperpolarization (observed previously in neurally intact SHR vessels) suggests enhanced inhibition of elevated sympathetic neural input as a major mechanism underlying such hyperpolarization (and coupled relaxation) in this neurogenic model of hypertension.**

VOLATILE anesthetic–mediated vasorelaxation[1] and its associated depressor effect are well recognized in normotensive subjects[2] together with related exaggerated alterations in hemodynamic control in chronic hypertension.[3] However, the mechanisms underlying these effects in normotensive and hypertensive subjects are poorly understood at the level of the vascular smooth muscle (VSM) cell.

We previously observed both a neurally dependent and independent component of isoflurane-induced *in situ* VSM hyperpolarization in mesenteric vessels of the spontaneously hypertensive rat (SHR) and its normotensive Wistar-Kyoto (WKY) control.[4] This model exhibits many of the pathophysiologic changes that occur in human essential hypertension[5] and has been used by other investigators to study the cardiovascular effects of anesthetics in hypertension.[6] Changes in resting membrane potential (E_m) over the physiologic range are closely coupled to corresponding alterations in VSM tone.[7–10]

A principal determinant of VSM E_m is the relatively large membrane permeability for potassium ions (K^+).[9,10] In past *in situ* studies using selective inhibitors of different VSM K^+ channel subtypes, we determined that isoflurane-induced hyperpolarization in denervated mesenteric vessels of normotensive Sprague-Dawley rats is mediated by enhanced or maintained opening of calcium-activated (K_{Ca}) and adenosine triphosphate–regulated (K_{ATP}) potassium channels. Voltage-dependent (K_V) or inwardly rectifying (K_{IR}) potassium channels were not involved.[11] The nitric oxide (NO), cyclic guanosine monophosphate (cGMP), and cyclic adenosine monophosphate (cAMP) second messenger pathways have been implicated in the regulation of K_{Ca} and K_{ATP} channel activity in VSM.[9,12]

The underlying hypothesis for the present study was that volatile anesthetics produce hypotension, at least in part, *via* a second messenger–mediated VSM hyperpolarization (and resultant vasodilatation) that is enhanced in hypertensive subjects. Support for this hypothesis is an enhanced K^+ current[13] and a reduced arterial vasodilator effect by endothelial factors such as NO in hypertension.[14] Anesthetics have also been shown to alter endothelium-derived NO control of VSM that involve effects on cGMP second messenger pathways.[15] However, such reported effects are not consistent.[16,17] Hence, it is not clear whether alterations in the cGMP vasodilator pathway contribute to the differential vascu-

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lar responses in hypertensive subjects (*vs.* normotensive subjects).

The first objective of the present study was to determine if isoflurane induces a K_{Ca}- and K_{ATP}-dependent VSM hyperpolarization in SHR and WKY rats. The second was to determine if the mechanism of isoflurane-induced VSM hyperpolarization (and therefore vasorelaxation) includes the NO, cGMP, or cAMP second messenger pathways that are involved in the regulation of potassium channel activity. The third objective was to determine if differences exist between SHR and WKY in the effects of isoflurane on such K^+ channel regulation of VSM E_m.

Methods

Animal Preparation

All protocols in this study were reviewed and approved by the Animal Care and Use Committee at the Medical College of Wisconsin. A total of 218 SHR and WKY rats were studied. All animals were between 8 and 13 weeks of age and weighed between 250 and 350 g. Anesthesia was induced with 40 mg/kg intraperitoneal ketamine followed by 20 mg/kg intraperitoneal pentobarbital to facilitate surgical preparation. Subsequently, basal anesthesia was maintained throughout the course of each experimental protocol by intravenous pentobarbital administration at a constant infusion rate of 15–30 $mg \cdot kg^{-1} \cdot h^{-1}$ in physiologic salt solution (PSS) containing 2% bovine serum albumin. In each animal the femoral artery and vein were cannulated for mean arterial blood pressure (MAP) measurement and intravenous access, respectively. In addition, a tracheotomy was performed through which ventilation was controlled with a model 680 rodent respirator (Harvard Apparatus Co., South Natick, MA). Respiratory rate and tidal volumes were adjusted to maintain end-tidal carbon dioxide between 30 and 35 mmHg.

During all of the experiments, the animals breathed an inspired oxygen concentration of 30% (in an oxygen–nitrogen mixture) to reduce any possibility of hypoxia-induced effects on the VSM.[18] Minute ventilation was adjusted as described above and the end-tidal carbon dioxide was measured with a POET 2 infrared capnograph and end-tidal agent monitor (Criticare Systems, Inc. Waukesha, WI).

Blood Vessel Preparation

A midline laparotomy was performed through which a loop of terminal ileum and its attached mesentery were externalized and placed on a movable, temperature-regulated microscope stage that was mounted on a micro-G vibration-free table (Technical Mfg. Co., Woburn, MA). Small (200–300 μm) mesenteric arteries and paired veins were identified. After dissection of the perivascular fat (without disturbing luminal blood flow or adventitial innervation), the surrounding connective tissue of the vessels was attached to the silastic rubber floor of the chamber with 125-μm-diameter stainless steel pins. Smaller (50 μm) pins were used to line each of the vessels on both sides to minimize pulse movement artifact. While in the chamber, the vessels were continuously superfused with PSS composed of 119 mM NaCl, 4.7 mM KCl, 1.17 mM $MgSO_4$, 1.6 mM $CaCl_2$, 24.0 mM $NaHCO_3$, 1.18 mM NaH_2PO_4, 0.026 mM EDTA. The superfusate was continuously aerated with a gas mixture of nitrogen, oxygen, and carbon dioxide to maintain pH between 7.35 and 7.45, carbon dioxide partial pressure between 35 and 45 mmHg, and oxygen partial pressure between 75 and 100 mmHg. The temperature of the PSS was maintained between 36 and 37°C.

For each protocol, isoflurane was delivered locally (not systemically) to the *in situ* vessel preparation *via* the PSS superfusate. The oxygen–nitrogen– carbon dioxide mixture aerating the superfusate was used as a carrier gas to deliver the isoflurane to the PSS through an Ohio Medical Products vaporizer (Airco Inc., Madison, WI). Concentrations of isoflurane were measured in PSS and blood samples with a Shymadzu model GC-8A gas chromatograph (Shimadzu Co., Kyoto, Japan). The vaporizer settings were adjusted to produce PSS isoflurane concentrations that averaged 0.6 mM. This concentration corresponded to the mean concentration that was measured in blood when 1 MAC isoflurane was administered systemically by inhalation.[19]

Local sympathetic denervation of each paired mesenteric artery and vein preparation in the present study was accomplished by a 20-min superfusion of the vessel preparations with 300 μg/ml 6-hydroxydopamine. Local application of 6-hydroxydopamine produces changes consistent with perivascular denervation, including inhibition of contractile responses to field stimulation, blockade of H^3-norepinephrine uptake by sympathetic nerve endings, and histologic changes consistent with adrenergic nerve degeneration.[20,21] Before denervation by this technique, the preparation was pretreated with PSS containing 10^{-6} M phentolamine for 5 min to inhibit the vasoconstriction from catecholamines locally released by the 6-hydroxydopamine superfusion.[22]

Vascular Smooth Muscle Transmembrane Potential Measurements

In each preparation, single-cell VSM E_ms were measured *in situ* by advancing 3 M KCl–filled glass micropipette electrodes into the VSM layer of the mesenteric arteries and veins from the adventitial side. The tip diameter of the micropipettes was approximately 0.1 μm with an impedance range of approximately 40–60 MΩ. Micropipettes were pulled from borosilicate glass with a Model P-97 Brown/Flaming micropipette puller (Sutter Instrument Company, Novato, CA). Electrodes were advanced

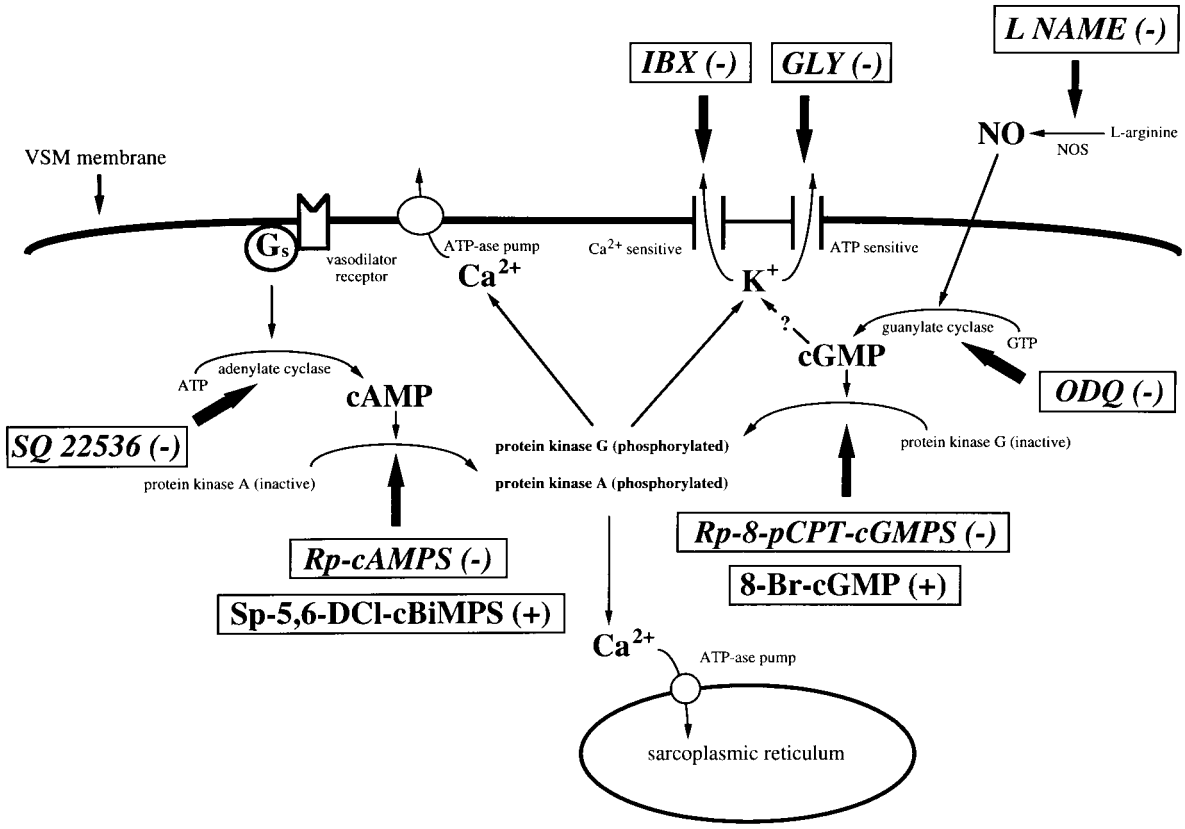

Fig. 1. Schematic illustrating non-neural, membrane, and intracellular mechanisms of vascular smooth muscle (VSM) control, which are potential targets for anesthetic action. Endogenously released nitric oxide (NO) is synthesized by NO synthase (eNOS) and diffuses across the VSM membrane to bind guanylate cyclase and mediate the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). cGMP phosphorylates protein kinase G, which stimulates membrane adenosine triphosphate-dependent (ATPase) pumps to transport calcium Ca^{2+} into the extracellular space and the sarcoplasmic reticulum. Membrane-bound Ca^{2+}-dependent and ATP-sensitive potassium (K^+) channels contribute to the regulation of VSM transmembrane potential (E_m) and thus to regulation of voltage-operated Ca^{2+} channels. In turn, such regulation significantly affects intracellular calcium ($[Ca^{2+}]_i$), particularly in VSM. In the current study, effects of isoflurane on these mechanisms were examined by concurrent administration of analogs and inhibitors that are selective for different levels of VSM control. L-NAME inhibits formation of NO by NO synthase (NOS). ODQ inhibits guanylate cyclase–induced synthesis of cGMP. Rp-8-pCPT-cGMPS inhibits cGMP-induced activation of protein kinase G. SQ22536 inhibits adenylate cyclase–induced synthesis of cyclic adenosine monophosphate (cAMP). Rp-cAMPS inhibits cAMP-induced activation of protein kinase A (PKA). K_{Ca} and K_{ATP} channels are blocked by iberiotoxin (IBX) and glybenclamide (Gly), respectively. 8-Br-cGMP and Sp-5,6-DCl-cBiMPS are membrane-permeable, hydrolysis-resistant cGMP and cAMP analogs, respectively.

manually using a hydraulic micro-micromanipulator (Trent Wells Inc., Coulterville, CA) and were connected to a Model 8100 biological amplifier (Dagan Corporation, Minneapolis, MN). Both arterial blood pressure and VSM E_ms were recorded with a Grass model RPS7C polygraph (Astro-Med/Grass Inc., West Warwick, RI) and a Superscope II (version 1.44) digital data acquisition system (GW Instruments Co., Somerville, MA).

Experimental Protocols

Figure 1 shows a schematic of the vasodilator second messenger pathways regulating VSM K^+ channel activity together with the sites of action of specific pharmacologic analogs and inhibitors used in the present study. Each analog and inhibitor concentration used was at least five times the reported ED_{50} or a concentration previously reported to be effective.[23–28] During each step in each protocol (see below), vessel preparations were superfused with PSS containing the specified equilibrated mixture of isoflurane, analog, inhibitor, or combination for a 20–30-min period before any E_m measurements.

Potassium Channel Inhibition. Two specific VSM membrane K^+ channel inhibitors were used to verify the participation of K^+ in the isoflurane-induced hyperpolarization of VSM in the SHR and WKY small mesenteric vessels. *In situ* VSM E_m and concurrent MAP were measured during sequential step intervals with PSS superfusate containing one of the following: (1) no agent (control); (2) 0.6 mM isoflurane; (3) either a K_{Ca} channel inhibitor (10^{-7} M iberiotoxin) or a K_{ATP} channel inhibitor (10^{-6} M glybenclamide)[9] after washout of isoflurane; or (4) isoflurane plus one of the channel inhibitors.

Nitric Oxide Synthase Inhibition. The effect of inhibition of NO synthesis on isoflurane-induced VSM hyperpolarization in the small mesenteric arteries and veins in both animal types was assessed by superfusion with the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME). *In situ* VSM E_m and concurrent MAP were measured sequentially during superfusion with PSS containing (1) no agent (control), (2) 0.6 mM isoflurane, (3) NO synthase inhibitor, or (4) isoflurane plus NO synthase inhibitor. Both a lower (50 μM)[29] and higher (1 mM)[30] concentration of L-NAME was used in separate animal–vessel preparations.

Cyclic Guanosine Monophosphate Pathway Inhibition. The effect of an inhibition of two specific steps in the cGMP pathway on isoflurane-induced VSM hyperpolarization in the small mesenteric arteries and veins was investigated in two separate studies. In the first, guanylate cyclase-induced synthesis of cGMP was inhibited with 15 μM H-[1,2,4] oxadiazolo [4,3,-a] quinoxalin-1one (ODQ).[12] In the second study, cGMP-mediated activation of protein kinase G (PKG) was inhibited with 2.5 μM (Rp)-8-(*para*–chlorophenylthio)guanosine-3',5'-cyclic monophosphorothioate (Rp-8-pCPT-cGMPS), an inhibitor of PKG phosphorylation (fig. 1).[12] *In situ* VSM E_m and MAP were measured sequentially during vessel superfusion with PSS containing (1) no agent (control), (2) 0.6 mM isoflurane, (3) one of the two specific cGMP pathway inhibitors (after washout of isoflurane), or (4) both isoflurane and one of the two inhibitors.

In addition to these studies, two control studies were conducted to verify the functional existence of PKG in these vessels and the efficacy of its inhibitors, respectively. In the first control study, the VSM E_m response to the membrane-permeable cGMP analog 8-bromoguanosine-3',5'-cyclic monophosphate (8-Br-cGMP) was measured. The concentration used in the PSS superfusate was 100 μM.[23] *In situ* VSM E_m and MAP were measured sequentially during vessel superfusion with PSS containing (1) no agent (control), (2) the cGMP analog, or (3) no agent (after washout of the analog). In the second control study, the efficacy of the inhibitor of PKG activation (Rp-8-pCPT-cGMPS) was verified. *In situ* VSM E_m and MAP were measured sequentially during superfusion with PSS containing (1) no agent (control), (2) the inhibitor of PKG activation, or (3) both the cGMP analog and the inhibitor of PKG activation.

Cyclic Adenosine Monophosphate Pathway Inhibition. Similar to the cGMP studies, the effect of an inhibition of two specific steps in the cAMP pathway on isoflurane-induced VSM hyperpolarization was investigated in two separate studies. In the first, adenyl cyclase-induced synthesis of cAMP was inhibited with 410 μM SQ22536.[24] In the second study, cAMP-mediated activation of protein kinase A (PKA) was inhibited with 24.5 μM Rp-adenosine-3',5'-cyclic monophosphorothioate (Rp-cAMPS), an inhibitor of PKA phosphorylation (fig. 1).[25] *In situ* VSM E_m and MAP were measured sequentially during vessel superfusion with PSS containing (1) no agent (control), (2) 0.6 mM isoflurane, (3) one of the two inhibitors (after isoflurane washout), or (4) both isoflurane and one of the two inhibitors.

In addition to these studies, two control studies were conducted to verify the functional existence of PKA in the VSM of these vessels and the efficacy of its inhibitors, respectively. In the first, E_m response to a membrane-permeable activator of PKA 0.15 μM Sp-5,6-dichloro-1-b-D-ribofuranosylbenzimidazole-3',5'-monophosphoro-thioate (Sp-5,6-DCl-cBiMPS)[26] was measured. This agent activates PKA *via* phosphorylation. *In situ* VSM E_m and MAP were measured sequentially during vessel superfusion with PSS containing (1) no agent (control), (2) the PKA activator, or (3) no agent (after washout of the activator). In the second control study, the efficacy of the inhibitor of PKA activation was verified. *In situ* VSM E_m and MAP were measured sequentially during vessel superfusion with PSS containing (1) no agent (control), (2) the inhibitor of PKA activation, or (3) both the PKA activator and its inhibitor.

Source of Chemical and Pharmacologic Agents

Sodium chloride, potassium chloride, magnesium sulfate, calcium chloride, sodium bicarbonate, sodium phosphate, ethylenediaminetetraacetic acid, D-glucose, 6-hydroxydopamine, phentolamine, and L-NAME were purchased from Sigma Chemical Co. (St. Louis, MO). ODQ, SQ22536, Rp-cAMPS, and Sp-5,6-DCl-cBiMPS were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). 8-Br-cGMP was purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA). Iberiotoxin and glybenclamide were purchased from Research Biochemicals International (Natick, MA). Sodium pentobarbital and isoflurane were purchased from Abbott Laboratories (North Chicago, IL). Ketamine hydrochloride was purchased from Phoenix Pharmaceutical, Inc. (St. Joseph, MO).

Statistical Analysis

The VSM E_m value reported for each step in each of the experimental protocols is the mean ± SD obtained from 8–12 vessel–animal preparations for both artery and vein. For each step in each experimental protocol, an individual VSM E_m value for a vessel–animal preparation is the numerical average of at least five stable (6–10 s) individual impalements. The mean ± SD of 8–12 of these individual values was then compared using a repeated-measures multiple analysis of variance. The "between" factor was animal type (with WKY and SHR as different levels), and the levels of the "within" factor were each of the three or four conditions defining that particular protocol. The MAP recorded simultaneously with VSM E_m was analyzed in identical fashion.

The first two groups of mean E_m measurements were

the denervated control and the superfused isoflurane in all protocols involving isoflurane administration. Thus, the effect of superfused isoflurane on VSM E_m and MAP was determined by comparing pooled denervated control data to pooled superfused isoflurane data using repeated-measures analyses of variance. WKY *versus* SHR served as the between factor and control *versus* isoflurane served as the repeated factor.

Mean blood concentrations of isoflurane were determined by pooling all measurements from individual animal–vessel preparations during each of the three experimental conditions (*i.e.*, before, during, and after superfusion of blood vessels with isoflurane, respectively). The mean values in these three groups were compared using a one-way analysis of variance.

All *t* tests in the current study were calculated using the Stat-View program for Macintosh computers (SAS Institute, Cary, NC). All analyses of variance were calculated using the Super ANOVA program for Macintosh computers (Abacus Concepts, Berkeley, CA). The significance of differences between individual means of specific groups was evaluated using the *post hoc* least significance difference test. $P \leq 0.05$ was used to define the significance of all differences.

Results

Effect of Isoflurane on Mean Arterial Blood Pressure and Vascular Smooth Muscle Transmembrane Potential

The mean ± SD isoflurane concentration in the PSS superfusate was 0.6 ± 0.1 mM for the isoflurane step and 0.6 ± 0.2 mM for the isoflurane + inhibitor step (n = 66 WKY and 69 SHR vessel preparations). Combining all protocols, the mean ± SD baseline MAP during pentobarbital anesthesia for SHR (158 ± 26 mmHg) was significantly greater than for WKY (114 ± 16 mmHg). Neither SHR nor WKY MAP was significantly altered by local superfusion with isoflurane. The maximum variation (from the initial baseline MAP) within any single protocol was 15% (observed for SHR in the study of the effect of PKA activation on VSM E_m). Such MAP variations included both increases and decreases and were not correlated with any of the observed changes in VSM E_m.

The *in situ* venous VSM was significantly more polarized than the arterial VSM in each animal type. In addition, the isoflurane-induced hyperpolarization response was significant and similar between SHR and WKY for each vessel type. For the SHR artery, the pooled mean VSM E_m ± SD before isoflurane superfusion was −37 ± 4.3 mV *versus* −41 ± 4.2 mV during isoflurane superfusion. For the WKY artery, corresponding values were −37 ± 4.2 mV *versus* −43 ± 3.4 mV, respectively. For the SHR vein, the pooled mean VSM E_m ± SD before isoflurane superfusion was −42 ± 5.7 mV *versus* −49 ± 5.8 mV during isoflurane superfusion. For the WKY vein, corresponding values were −40 ± 4.3 mV *versus* −46 ± 4.8 mV, respectively.

Effect of Potassium Channel Inhibition on Isoflurane-induced Vascular Smooth Muscle Hyperpolarization

Table 1 illustrates the effect of K^+ channel inhibitors on arterial and venous mean VSM E_m in SHR and WKY and the responses to isoflurane. Superfusion of the vessel preparations with either the K_{Ca} or K_{ATP} channel inhibitor (iberiotoxin or glybenclamide, respectively) significantly depolarized the VSM in each of the two

Table 1. Effect of Potassium Channel Inhibition on Isoflurane-induced Hyperpolarization of Vascular Smooth Muscle (VSM)

	Control	ISO	IBX	IBX + ISO
Artery				
WKY	41 ± 4.3	44 ± 4.4*	34 ± 4.8†	33 ± 4.5†‡
SHR	41 ± 2.5	43 ± 3.4*	34 ± 1.6†	34 ± 2.5†‡
Vein				
WKY	42 ± 3.6	45 ± 5.3	35 ± 2.9*†	36 ± 4.1†‡
SHR	43 ± 5.6	47 ± 4.7*	38 ± 7.3†	38 ± 6.2†‡
	Control	ISO	GLY	GLY + ISO
Artery				
WKY	40 ± 3.0	43 ± 3.5*	35 ± 3.1†	36 ± 2.1†
SHR	40 ± 2.0	43 ± 1.9*	35 ± 0.6†	36 ± 2.1†
Vein				
WKY	42 ± 4.1	44 ± 4.7*	36 ± 4.0†	36 ± 3.8†
SHR	44 ± 4.3	47 ± 5.8*	40 ± 5.5†	41 ± 6.6†

Values are mean mV ± SD. For sequential measurements of *in situ* VSM transmembrane potential, physiologic salt solution superfusate contained the following: (1) no agent (denervated control), (2) 0.6 mM isoflurane (ISO), (3) K^+_{Ca} channel inhibitor, 10^{-7} M iberiotoxin (IBX), or K^+_{ATP} channel inhibitor, 10^{-6} M glybenclamide (GLY) (after washout of isoflurane), (4) inhibitor + isoflurane.

* Different from control, † different from both control and isoflurane; $P \leq 0.05$; n = 8.

WKY = Wistar Kyoto; SHR = spontaneously hypertensive rat.

Table 2. Effect of Nitric Oxide Synthase Inhibition (L-NAME) on Isoflurane-induced Hyperpolarization of Vascular Smooth Muscle (VSM)

	Control	ISO	50 μM L-NAME	50 μM L-NAME + ISO
Artery				
WKY	40 ± 1.7	43 ± 1.6*	38 ± 2.0†	42 ± 1.7*†
SHR	41 ± 2.2	44 ± 2.9*	39 ± 1.8†	43 ± 1.6*‡
Vein				
WKY	43 ± 3.6	45 ± 3.5*	40 ± 4.0†	44 ± 3.7‡
SHR	45 ± 3.7	47 ± 3.2*	42 ± 3.8†	46 ± 2.0*‡
	Control	ISO	1 mM L-NAME	1 mM L-NAME + ISO
Artery				
WKY	35 ± 4.0	44 ± 4.3*	37 ± 3.7§	42 ± 4.2*‡
SHR	34 ± 1.8	39 ± 2.6*	35 ± 3.2§	41 ± 3.1*‡
Vein				
WKY	37 ± 2.6	46 ± 5.0*	38 ± 3.0§	45 ± 4.3*‡
SHR	43 ± 3.3	50 ± 3.2*	39 ± 5.0†	50 ± 3.7*‡

Values are mean mV ± SD. For sequential measurements of *in situ* VSM transmembrane potential, physiologic salt solution superfusate contained the following: (1) no agent (denervated control), (2) 0.6 mM isoflurane (ISO), (3) either 50 μM or 1 mM L-NAME (after washout of isoflurane), (4) L-NAME + isoflurane.

* Different from control, † different from both control and isoflurane, § different from isoflurane, ‡ different from L-NAME; $P \leq 0.05$; n = 8.

WKY = Wistar Kyoto; SHR = spontaneously hypertensive rat.

vessel types in both SHR and WKY. In addition, when superfused concurrently with isoflurane, both inhibitors abolished the isoflurane-induced VSM hyperpolarization in each of the two vessel types in both SHR and WKY.

Effect of Nitric Oxide Synthase Inhibition on Isoflurane-induced Hyperpolarization of Vascular Smooth Muscle Transmembrane Potential

Table 2 illustrates the effect of two concentrations of the NO synthase inhibitor (L-NAME) on arterial and venous VSM E_m in SHR and WKY and the VSM E_m response to isoflurane. When superfused alone (*i.e.*, after washout of isoflurane), the 50-μM concentration caused a small but significant depolarization in each vessel type relative to its respective control VSM E_m. However, at the 1-mM concentration, such depolarization was not consistent, occurring only in the SHR vein. Of particular note is that addition of either concentration of the inhibitor to the superfusate containing isoflurane did not significantly attenuate the isoflurane-induced hyperpolarization in either the SHR or WKY vessels.

Effect of Cyclic Guanosine Monophosphate Pathway Inhibition on Isoflurane-induced Hyperpolarization of Vascular Smooth Muscle Transmembrane Potential

The data in table 3 support the functional existence of the cGMP pathway in the VSM of small mesenteric vessels in both SHR and WKY. The cGMP analog (8-Br-cGMP) significantly hyperpolarized VSM in each vessel type in both SHR and WKY. In addition, the efficacy of the inhibitor of PKG activation (2.5 μM Rp-8-pCPT-cGMPS) was verified by its ability to block the hyperpolarization produced by the cGMP analog. Superfusion with the inhibitor of PKG activation (Rp-8-pCPT-cGMPS) or with the inhibitor of guanylate cyclase–induced synthesis of cGMP (ODQ; after washout of isoflurane) produced a depolarizing response in each vessel type when compared with its respective VSM E_m control value. These responses were variable in that the depolarization produced by these two inhibitors was not significant for all vessel types. However, in both vessel types of both WKY and SHR, the isoflurane-induced hyperpolarization was not significantly attenuated either by the inhibitor of PKG activation or cGMP synthesis.

Effect of Cyclic Adenosine Monophosphate Pathway Inhibition on Isoflurane-induced Hyperpolarization of Vascular Smooth Muscle Transmembrane Potential

The data in table 4 support the functional existence of the cAMP pathway in the VSM of small mesenteric vessels in both SHR and WKY. The membrane-permeable activator of PKA (Sp-5,6-DCl-cBiMPS) significantly hyperpolarized VSM in each vessel type in both SHR and WKY. In addition, the efficacy of the inhibitor of PKA activation (24.5 μM Rp-cAMPS) was verified by its ability to inhibit the hyperpolarization induced by the PKA activator. It is particularly important to note that the isoflurane-induced VSM hyperpolarization was abolished in the presence of either the inhibitor of cAMP synthesis (SQ22536) or the inhibitor of PKA activation (Rp-cAMPS).

Discussion

Potassium channel activity in the VSM membrane is the primary regulator of VSM E_m over the normal *in situ*

Table 3. Effect of cGMP Pathway Analogs and Inhibitors on Isoflurane-induced Hyperpolarization of Vascular Smooth Muscle (VSM)

	Control		8-Br-cGMP	Washout
Protocol 1				
Artery				
WKY	39 ± 3.4		43 ± 3.2*	37 ± 2.8
SHR	40 ± 2.9		45 ± 4.1*	39 ± 2.8
Vein				
WKY	40 ± 3.7		45 ± 4.4*	40 ± 3.4
SHR	43 ± 3.0		49 ± 4.4*	43 ± 3.5
	Control		Rp-8-pCPT-cGMPS	Rp-8-pCPT-cGMPS + 8Br-cGMP
Protocol 2				
Artery				
WKY	38 ± 3.0		32 ± 2.1†	33 ± 4.0†
SHR	37 ± 4.0		33 ± 2.6†	33 ± 2.4†
Vein				
WKY	41 ± 2.7		34 ± 5.4†	34 ± 6.0†
SHR	41 ± 3.3		37 ± 4.2†	36 ± 3.1†
	Control	ISO	Rp-8-pCPT-cGMPS	Rp-8-pCPT-cGMPS + ISO
Protocol 3				
Artery				
WKY	36 ± 4.1	42 ± 3.9†	35 ± 3.4‡	39 ± 5.1
SHR	34 ± 3.4	39 ± 3.8†	33 ± 2.7‡	39 ± 4.9†
Vein				
WKY	40 ± 6.6	46 ± 6.0†	35 ± 5.9†‡	43 ± 6.3†
SHR	39 ± 3.6	49 ± 6.3†	37 ± 6.4‡	49 ± 4.8†
	Control	ISO	ODQ	ODQ + ISO
Protocol 4				
Artery				
WKY	38 ± 4.8	43 ± 3.5†	34 ± 2.9†‡	43 ± 3.4†
SHR	36 ± 6.0	43 ± 5.8†	33 ± 4.1‡	44 ± 4.4†
Vein				
WKY	40 ± 4.1	46 ± 5.3†	34 ± 2.7†‡	46 ± 5.6†
SHR	42 ± 9.8	48 ± 7.4†	34 ± 5.7†‡	48 ± 5.9†

Values are mean mV ± SD. For sequential measurements of *in situ* VSM transmembrane potential, physiologic salt solution superfusate contained the following: protocol 1: (1) no agent (denervated control), (2) 100 μM 8-Br-cGMP (cGMP analog), (3) no agent (washout); protocol 2: (1) no agent (denervated control), (2) 2.5 μM Rp-8-pCPT-cGMPS (protein kinase G inhibitor), (3) 2.5 μM Rp-8-pCPT-cGMPS plus 100 μM 8-Br-cGMP; protocol 3: (1) no agent (denervated control), (2) 0.6 mM isoflurane (ISO), (3) 2.5 μM Rp-8-pCPT-cGMPS (after washout of isoflurane), (4) 2.5 μM Rp-8-pCPT-cGMPS plus 0.6 mM isoflurane; protocol 4: (1) no agent (denervated control), (2) 0.6 mM isoflurane, (3) 15 μM ODQ (inhibitor of cGMP synthesis; after washout of isoflurane), (4) 15 μM ODQ plus isoflurane.

* Different from control and washout, † different from control, ‡ different from isoflurane and Rp-8-pCPT-cGMPS + isoflurane or isoflurane and ODQ + isoflurane; $P \leq 0.05$; n = 8–15 for ODQ, and n = 8 for all other protocols.

WKY = Wistar Kyoto; SHR = spontaneously hypertensive rat.

physiologic range.[7,8,10,31] In the present study we observed an inhibition of isoflurane-induced *in situ* VSM hyperpolarization in small mesenteric blood vessels of SHR and WKY after direct block of VSM K_{Ca} or K_{ATP} channels (with iberiotoxin or glybenclamide, respectively). Attention was focused on K_{Ca} and K_{ATP} channels only, because in a previous study with normotensive Sprague-Dawley rats,[19] we observed that only these channels (and not K_V or K_{IR} channels) were activated by volatile anesthetics to produce *in situ* VSM hyperpolarization. These observations suggested that neurally independent mechanisms of VSM hyperpolarization and inhibition of VSM tone by volatile anesthetics (in both hypertensive and normotensive subjects) result from enhanced K_{Ca} and K_{ATP} channel activation. However, the possible role of NO, cGMP, and cAMP second messenger vasodilator pathways in mediating such anesthetic-induced effects was not clear.

A major finding of the present study is the observed abolition of isoflurane-induced VSM hyperpolarization by inhibition of cAMP synthesis or cAMP-mediated activation of PKA (with SQ22536 or Rp-cAMPS, respectively). Other investigators have shown that phosphorylated PKA activates K_{Ca} and K_{ATP} channels.[10,32] Abolition of isoflurane-induced VSM hyperpolarization by inhibition of components in the cAMP pathway suggests that volatile anesthetics can enhance activity of components in the cAMP second messenger system in addition to a

Table 4. Effect of cAMP Pathway Analogs and Inhibitors on Isoflurane-induced Hyperpolarization of Vascular Smooth Muscle (VSM)

	Control		Sp-5,6-DCl-cBimps	Washout
Protocol 1				
Artery				
WKY	36 ± 3.1		48 ± 5.5*	38 ± 2.8
SHR	36 ± 1.8		42 ± 3.0*	36 ± 4.7
Vein				
WKY	37 ± 3.8		47 ± 5.0*	38 ± 5.2
SHR	39 ± 4.1		48 ± 6.0*	38 ± 5.0
	Control		Rp cAMPS	Rp cAMPs + Sp-5,6-DCl-cBimps
Protocol 2				
Artery				
WKY	37 ± 5.9		36 ± 4.9	37 ± 4.7
SHR	35 ± 2.8		34 ± 2.6	33 ± 3.0
Vein				
WKY	39 ± 5.2		39 ± 7.1	39 ± 8.0
SHR	43 ± 6.2		41 ± 7.9	41 ± 9.2
	Control	ISO	Rp cAMPS	Rp cAMPS + ISO
Protocol 3				
Artery				
WKY	35 ± 1.9	42 ± 3.1†	35 ± 4.0	36 ± 2.0
SHR	35 ± 2.0	40 ± 4.3†	35 ± 3.2	35 ± 3.4
Vein				
WKY	39 ± 3.4	48 ± 5.0†	39 ± 4.9	39 ± 4.0
SHR	41 ± 2.8	51 ± 5.9†	41 ± 4.3	42 ± 6.3
	Control	ISO	SQ22536	SQ22536 + ISO
Protocol 4				
Artery				
WKY	34 ± 2.4	41 ± 2.6†	35 ± 3.7	35 ± 4.6
SHR	34 ± 2.8	39 ± 4.0†	33 ± 4.0	35 ± 4.8
Vein				
WKY	37 ± 3.5	44 ± 3.4†	36 ± 3.1	36 ± 3.4
SHR	43 ± 4.3	53 ± 5.4†	42 ± 4.6	41 ± 6.1

Values are mean mV ± SD. For sequential measurements of *in situ* VSM transmembrane potential, physiologic salt solution superfusate contained the following: protocol 1: (1) no agent (denervated control), (2) 0.15 μM Sp-5,6-DCl-cBimps (protein kinase A [PKA] activator), (3) no agent (washout); protocol 2: (1) no agent (denervated control), (2) 24.5 μM Rp cAMPS (PKA inhibitor), (3) 24.5 μM Rp cAMPS plus 0.15 μM Sp-5,6-DCl-cBimps; protocol 3: (1) no agent (denervated control), (2) 0.6 mM isoflurane (ISO), (3) 24.5 μM Rp cAMPS (after washout of isoflurane), (4) 24.5 μM Rp cAMPS plus 0.6 mM isoflurane; protocol 4: (1) no agent (denervated control), (2) 0.6 mM isoflurane, (3) 410 μM SQ22536 (inhibitor of cAMP synthesis; after washout of isoflurane), (4) 410 μM SQ22536 plus 0.6 mM isoflurane.

* Different from control and washout, † different from all other conditions in the Rp cAMPS or the SQ22536 protocol; $P \leq 0.05$; n = 8.

WKY = Wistar Kyoto; SHR = spontaneously hypertensive rat.

possible direct action on potassium channel proteins. This is in contrast to reported isoflurane-induced inhibition of cAMP-mediated vasodilatation of *in vitro* coronary arteries.[33] However, a cAMP-mediated VSM hyperpolarization is in agreement with a number of other studies that have demonstrated an augmentation of cAMP concentration and activity in VSM by volatile anesthetics.[34] The results of the present study suggest that the mechanisms underlying the observed isoflurane-induced VSM hyperpolarization (and presumed coupled relaxation) in the *in situ* small mesenteric resistance- and capacitance-regulating blood vessels of SHR and WKY include activation of a cAMP-mediated opening of VSM K_{Ca} and K_{ATP} channels.

The small but significant *in situ* VSM depolarization response to superfusion with 50 μM L-NAME (NO synthase inhibitor) observed after washout of isoflurane (table 2) provides some evidence that NO participates in the regulation of VSM E_m and tone in the SHR and WKY small mesenteric blood vessels. However, it is not clear why 1.0 mM L-NAME failed to produce a similar (or greater) depolarization response (except for the SHR vein). The data in table 3 provide relatively strong evidence for the existence of the cGMP-PKG pathway in the regulation of VSM E_m in these vessels (*e.g.*, hyperpolarizing response to 8-Br-cGMP [cGMP analog] and depolarizing response to Rp-8-pCPT-cGMPS [PKG inhibitor]). Perhaps the best recognized effect of cGMP is the phosphorylation (and activation) of cGMP-dependent PKG,[35] ultimately leading to an increased extrusion of intracel-

lular calcium ($[Ca^{2+}]_i$) by activated cell and sarcoplasmic reticulum membrane-bound Ca^{2+} pumps.[36] It is not clear if such reduction of $[Ca^{2+}]_i$ alone can alter the resting VSM E_m. However, some evidence suggests that either activated PKG or cGMP (or a cGMP derivative) hyperpolarizes VSM by activating VSM membrane potassium channels.[36] Volatile anesthetics have been reported to enhance NO-mediated relaxation of *in vitro* canine coronary arterial VSM[37] and increase the concentration of cGMP in canine cerebral arterial VSM.[15] However, other *in vitro* studies concluded that isoflurane does not enhance, but rather inhibits, endothelium-derived NO release[38,39] as well as cGMP-mediated VSM relaxation.[40] In the present study, no differences were observed in VSM E_m response to isoflurane before and during inhibition of NO synthesis or block of selective steps in the cGMP-PKG pathway in respective vessels of either animal type. This suggests that the isoflurane-induced VSM hyperpolarization does not involve components in the cGMP pathway.

We recognize that our conclusions in the present study are based on VSM E_m measurements rather than measurements of VSM contractility or changes in $[Ca^{2+}]_i$. However, VSM tone is closely coupled to both E_m and $[Ca^{2+}]_i$ and critically dependent on influx of extracellular calcium through voltage-sensitive calcium channels.[9] Such influx (and $[Ca^{2+}]_i$) are inversely proportional to potassium channel conductance and hence magnitude of VSM E_m. Thus, isoflurane-induced increases in the magnitude of VSM E_m should be inversely coupled to decreases in $[Ca^{2+}]_i$ and VSM tone.[41]

A second major observation in the present study is the lack of a significant difference between SHR and WKY in the effects of isoflurane on K^+ channel–mediated control of VSM E_m in respective small mesenteric blood vessels. The results in the present study complement those of our previous studies, in which we demonstrated a greater anesthetic-induced hyperpolarization in neurally intact (*vs.* denervated) vessel preparations in SHR and WKY.[19] Both studies strongly suggest that volatile anesthetics attenuate VSM tone in hypertensive subjects primarily by modulation of the elevated level of the sympathetic neural control that exists in the neurogenic SHR model. Therefore, we concluded that *in situ* differences in anesthetic effect on VSM E_m (and tone) between the SHR and WKY do not involve the neurally independent vasodilator mechanisms that were the focus of the present study. Evidence exists in support of intrinsic differences in potassium channel function between WKY and SHR,[13] as well as an altered NO-dependent control of VSM tone in hypertensive subjects.[14] Results of the present study suggest that none of these neurally independent mechanisms account for the hemodynamic instability characteristic of anesthesia in the hypertensive condition.[3] It is possible that differential effects of volatile anesthetics on vascular control in hypertensive *versus* normotensive subjects may involve mechanisms not addressed in the present study (*e.g.*, altered sensitivity of contractile proteins to volatile anesthetics). Clearly, the SHR is only one model of human essential hypertension and does not precisely manifest all of the possible causes of this disease.[5] Similar studies using other models may produce different results.

One possibility that was not addressed in our previous studies was the lack of a complete local sympathetic denervation of the vessel preparation by the 6-hydroxydopamine pretreatment. If so, subsequent administration of isoflurane (either locally or systemically) potentially may not produce neurally independent effects, but rather merely a completion of a partial denervation. To address this issue, in preliminary studies we compared the effect of local denervation on VSM E_m in normotensive Sprague-Dawley animals using 300 mg/ml 6-hydroxydopamine *versus* 1 mg/ml tetrodotoxin. We observed no differences in the isoflurane-mediated hyperpolarization after inhibition of sympathetic neural input with either of these two agents (data not shown). Tetrodotoxin is a well-established antagonist of sodium channels in nerve and an effective agent for removal of sympathetic control of VSM tone.[42] Thus, we concluded that the sympathetic neural denervation by 6-hydroxydopamine was effective and complete.

Another question in the present study is the effectiveness of the inhibitors of the potassium channels and of the analogs and inhibitors of the second messenger pathways. The concentrations of the K^+ channel inhibitors (iberiotoxin and glybenclamide) used were 10 times greater than those reported to produce effective half-block of K_{ATP} and K_{Ca} channels, respectively. Because of the selectivity of these K^+ channel inhibitors, crossover effects to other channels is unlikely.[9] It is unclear why either iberiotoxin or glybenclamide alone were capable of virtually eliminating the isoflurane-mediated hyperpolarization in the present study. It might be expected that either inhibitor alone would produce only partial attenuation of the response, and complete blockade would require both agents simultaneously. However, the current data (as well as previous results)[11] do not support this and instead suggest a possible interaction between K_{Ca} and K_{ATP} channels (assuming that each inhibitor is specific as reported for the concentrations used).[9] The existence and possible mechanisms of such a potential interaction remain to be clarified.

Finally, in the present study we did not control for the possible isoflurane-mediated enhancement of at least two other endogenous vasodilators in the SHR and WKY vessels (prostacyclin and endothelium-derived hyperpolarizing factor [EDHF]). Evidence exists to indicate that prostacyclin vasodilates by enhancing potassium channel activity through the cAMP-PKA pathway.[43] However, very little is currently known about the effects of anesthetics on this mechanism. Similarly, the identity and

mechanisms of action of EDHF have not been elucidated, although a variety of substances have now been identified as having potential EDHF activity.[44] The effect of anesthetics on these EDHF-related mechanisms is even less clear. Although not studied in detail, initial evidence suggests that any effect that anesthetics may have on release of EDHF appears to be inhibitory.[44,45] Such an effect would not explain the isoflurane-mediated hyperpolarization observed in the present study. Further studies are needed to establish the relative importance of both of these substances (prostacyclin and EDHF) in mediating anesthetic-induced VSM hyperpolarization and vasodilatation.

In summary, the results of the present study indicate that 1.0 minimum alveolar concentration–superfused isoflurane hyperpolarizes VSM and attenuates control of VSM tone in mesenteric small resistance-regulating arteries and capacitance-regulating veins equally in SHR and WKY by enhancing the activity of membrane-bound K_{Ca} and K_{ATP} channels. The elimination of isoflurane-induced VSM hyperpolarization by inhibition of cAMP synthesis or PKA activation suggests that at least a portion of this anesthetic action results from an enhanced activity of VSM membrane-bound and intracellular components of the cAMP-mediated second messenger system. Isoflurane-induced VSM hyperpolarization appears to be independent of endothelial-derived NO and the related cGMP pathway. The lack of a difference between SHR and WKY suggests that these neurally independent effects of isoflurane (and presumably other volatile anesthetics) on control of the VSM do not account for the hemodynamic instability characteristic of anesthetic administration in hypertensive subjects. Based on previous data, it appears that such differences are the result of alterations in the level of sympathetic vascular control.

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