Mechanisms of Direct Inhibitory Action of Isoflurane on Vascular Smooth Muscle of Mesenteric Resistance Arteries

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Background: Isoflurane has been shown to directly inhibit vascular reactivity. However, less information is available regarding its underlying mechanisms in systemic resistance arteries.

Methods: Endothelium-denuded smooth muscle strips were prepared from rat mesenteric resistance arteries. Isometric force and intracellular Ca2+ concentration ([Ca2+]i) were measured simultaneously in the fura-2-loaded strips, whereas only the force was measured in the β-escin membrane-permeabilized strips.

Results: Isoflurane (3–5%) inhibited the increases in both [Ca2+]i and force induced by either norepinephrine (0.5 μM) or KCl (40 mM). These inhibitions were similarly observed after depletion of intracellular Ca2+ stores by ryanodine. Regardless of the presence of ryanodine, after washout of isoflurane, its inhibition of the norepinephrine response (both [Ca2+]i and force) was significantly prolonged, whereas that of the KCl response was quickly restored. In the ryanodine-treated strips, the norepinephrine- and KCl-induced increases in [Ca2+]i were both eliminated by nifedipine, a voltage-gated Ca2+ channel blocker, whereas only the former was inhibited by niflumic acid, a Cl− channel blocker. Isoflurane caused a rightward shift of the Ca2+-force relation only in the fura-2-loaded strips but not in the β-escin-permeabilized strips.

Conclusions: In mesenteric resistance arteries, isoflurane depresses vascular smooth muscle reactivity by directly inhibiting both Ca2+ mobilization and myofilament Ca2+ sensitivity. Isoflurane inhibits both norepinephrine- and KCl-induced voltage-gated Ca2+ influx. During stimulation with norepinephrine, isoflurane may prevent activation of Ca2+-activated Cl− channels and thereby inhibit voltage-gated Ca2+ influx in a prolonged manner. The presence of the plasma membrane appears essential for its inhibition of the myofilament Ca2+ sensitivity.

ISOFLURANE produces systemic hypotension1 and significantly alters distribution of blood flow to various organs.5 Previous studies3–11 performed in a variety of vascular beds have suggested that isoflurane causes changes in vascular tone through its direct action on vascular smooth muscle (VSM) and/or endothelial cells.

In our previous study with isolated mesenteric resistance arteries,12 in the presence of endothelium, contractile response to norepinephrine (i.e., a neurotransmitter that plays a central role in sympathetic maintenance of vascular tone in vivo) was not inhibited during exposure to isoflurane. In addition, contractile response to KCl (i.e., contractile response mediated by voltage-gated Ca2+ channels [VGCCs] that play a crucial role in the regulation of vascular tone in vivo) was not inhibited during exposure to isoflurane.12 Therefore, we proposed that in subjects with intact endothelial function, the direct action of isoflurane on mesenteric resistance arteries may not contribute to systemic hypotension during isoflurane anesthesia. However, in the absence of endothelium, contractile responses to norepinephrine and KCl were both inhibited during exposure to isoflurane. Thus, we speculated that in subjects with impaired endothelial function, the direct (i.e., endothelium-independent) inhibitory action of isoflurane on mesenteric arterial VSMCs (VSMCs) may contribute to systemic hypotension during isoflurane anesthesia. Interestingly, only the norepinephrine response, not the KCl response, was significantly inhibited for a while (15 min or more) after washout of isoflurane in either the presence or absence of endothelium, indicating that its endothelium-dependent inhibitory action on contractile response to norepinephrine is prolonged.12 We thus proposed that the direct inhibitory action of isoflurane on norepinephrine response might contribute to the previously observed prolonged systemic hypotension after isoflurane anesthesia.13

Previous studies using isolated aorta and cultured aortic VSMCs4,5 have suggested that the direct inhibitory action of isoflurane on VSM is a result of both reduction of the intracellular Ca2+ concentration ([Ca2+]i) and inhibition of the myofilament Ca2+ sensitivity. However, less information is available regarding its underlying mechanisms in systemic resistance arteries, which are different from conduit arteries in many of their properties, including Ca2+-mobilization processes and responsiveness to pharmacological agents.14–16 We previously proposed that the direct inhibitory action of isoflurane on VSM is primarily a result of reduction of the [Ca2+]i in mesenteric resistance arteries.8 However, our proposal was based on the results obtained in experiments performed at approximately 22°C in the cell membrane-permeabilized condition,8 in which we might have failed to detect significant effects of isoflurane on myofilament Ca2+ sensitivity that require the intact cell membrane. In addition, little information is available regarding the mechanisms behind the aforementioned prolonged inhibitory action on norepinephrine response in mesen-

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teric resistance arteries. Furthermore, although there is direct evidence to indicate that isoflurane influences Ca\(^{2+}\) mobilization from the intracellular stores in VSMCs of isolated resistance arteries, no direct evidence is currently available to indicate that isoflurane inhibits plasmalemmal Ca\(^{2+}\) influx in VSMCs of systemic resistance arteries.

In this study, using the fura-2 fluorometry and thereby measuring force and \([\text{Ca}^{2+}]_i\) simultaneously, we further investigated the mechanisms behind the direct inhibitory action of isoflurane on VSM in membrane-intact mesenteric resistance arteries. Specifically, we tested two major hypotheses, i.e., (1) the hypothesis that isoflurane inhibits myofilament Ca\(^{2+}\) sensitivity of VSMCs in the presence of intact cell membrane in mesenteric resistance arteries and (2) the hypothesis that isoflurane directly inhibits plasmalemmal Ca\(^{2+}\) influx in VSMCs of mesenteric resistance arteries. Our experiments to test the latter hypothesis were performed in the presence of ryanodine, which has been shown to deplete the intracellular Ca\(^{2+}\) stores of mesenteric arterial VSMCs used in this study. We also tested the hypothesis that in mesenteric resistance arteries, isoflurane causes the prolonged inhibition of contractile response to norepinephrine by inhibiting Ca\(^{2+}\) mobilization in VSMCs. In this study, we also demonstrate a novel possibility that in VSMCs, isoflurane prevents activation of Ca\(^{2+}\)-activated Cl\(^{-}\) channels and thereby inhibits norepinephrine-induced Ca\(^{2+}\) influx in a prolonged manner.

Materials and Methods

Tissue Preparation

With approval from the Kyushu University Animal Care and Use Committee (Fukuoka, Japan), by use of the method previously detailed, endothelium-denuded strips were prepared from the third- or fourth-order branches of male Sprague-Dawley rat (250–350 g, 7–10 W) mesenteric arteries. These branches are known to contribute to the systemic vascular resistance. One strip was prepared from one animal.

Force and Ca\(^{2+}\) Measurements

Isometric force was measured by attaching the strip to a strain-gauge transducer as previously detailed. Briefly, the strip was mounted horizontally in a chamber attached to the stage of a microscope, and the resting tension was adjusted to obtain a maximal response to KCl. The solution was changed by infusing it into one end while aspirating simultaneously from the other end. Removal of endothelium was verified by the inability of acetylcholine (10 \(\mu\)M) to cause significant (10% or more) relaxation during contractions induced by norepinephrine (10 \(\mu\)M).

In the first series of experiments, changes in the \([\text{Ca}^{2+}]_i\) were measured simultaneously with those in force by use of fura-2, a fluorescent Ca\(^{2+}\)-indicator dye.

Our method on the fura-2 fluorometry was also detailed previously. Briefly, to allow loading of the fura-2 into the VSMCs, the strips were incubated in normal physiologic salt solution (PSS) containing 10 \(\mu\)M acetoxyethyl ester of fura-2 (fura-2/AM) and 2% albumin for approximately 2 h at approximately 35°C. After this period, the solution containing fura-2/AM was washed out with normal PSS for approximately 1 h to ensure sufficient esterification of fura-2/AM in the cells and to equilibrate the strips before the measurements. Changes in the fluorescence intensity of the fura-2–Ca\(^{2+}\) complex were measured by a fluorometer equipped with a dual-wavelength excitation device (CAM-230; Japan Spectroscopic, Tokyo, Japan) connected to the microscope with optical fibers. The VSM tissue was illuminated with ultraviolet light at wavelengths of 340 and 380 nm alternatively limited to a frequency of 1000 Hz. The fura-2 fluorescence signals induced by excitation at 340 and 380 nm were collected through the 10× objective lens (Plan Fluor; Nikon, Tokyo, Japan) and measured through a 500-nm filter with a photomultiplier. The background fluorescence as excited by 340- and 380-nm ultraviolet light was obtained after completion of each experiment by breaking the cell membranes with Triton X-100 (1%) and subsequently quenching the fura-2 fluorescence signals with MnCl\(_2\) (20 mM). The ratio (F\(_{340}/380\)) of fura-2 fluorescence intensities excited by 340 nm (F\(_{340}\)) to those excited by 380 nm (F\(_{380}\)) was calculated after the background fluorescence had been subtracted. None of the agents used during Ca\(^{2+}\) measurements influenced the fluorescence signals. In all the Ca\(^{2+}\) measurements, as we showed previously, changes in F\(_{340}\) and F\(_{380}\) were constantly in opposite directions. All experiments with the fura-2-loaded strips were performed during the period in which constant vascular responses were obtained, i.e., for approximately 3 h.

In the next series of experiments, only isometric force was measured in the non–fura-2–loaded endothelium-denuded strips, the smooth muscle membrane of which was permeabilized with \(\beta\)-escin. To achieve the membrane permeabilization, the strips were incubated with \(\beta\)-escin (50 \(\mu\)M for 25 min) at room temperature (approximately 22°C) in relaxing solution after steady contractions induced by 40 mM K\(^{+}\) had been measured. Lonomycin (0.3 \(\mu\)M) was present throughout the \(\beta\)-escin-permeabilized muscle experiments to eliminate the influence of intracellular Ca\(^{2+}\) stores.

To prevent early deterioration of the thin vascular strips, the aforementioned experiments with membrane-intact and \(\beta\)-escin membrane-permeabilized strips were performed at 35°C and room temperature (approximately 22°C), respectively, as done previously.

Solutions and Drugs

The ionic concentrations of the normal PSS were as follows (mM): NaCl 138, KCl 5.0, MgCl\(_2\) 1.2, CaCl\(_2\) 1.5,
HEPES 10, and glucose 10. The pH was adjusted with NaOH to 7.35 at 22°C. The high-K+ solutions were prepared by replacing NaCl with KCl isosmotically and adding guanethidine (3 μM)24 to prevent norepinephrine outflow from the sympathetic nerve terminals. The Ca2+-free solution was prepared by removing CaCl2 with or without adding EGTA.

The compositions of relaxing or activating solutions used in the β-escin-permeabilized muscle experiments were determined by solving multiequilibrium equations using a hydrogen ion activity coefficient of 0.75 and association constants for the various ions as detailed previously.8 The composition of the relaxing solution was 80 mM potassium methanesulfonate (KMS), 20 mM PIPES, 7 mM Mg(MS)2, 5 mM adenosine 5'-triphosphate, 10 mM creatinine phosphate, and 4 mM EGTA. The 4 mM EGTA–containing activating solution was prepared by adding a specific amount of Ca(MS)2 to obtain the desired concentration of free Ca2+ ions based on the calculations previously reported.8 The pH was adjusted with KOH to 7.00 at 22°C, and the ionic strength was kept constant at 0.2 mM by adjusting the concentration of KMS. Guanosine 5'-triphosphate (50 μM) was present throughout the experimental periods to minimize rundown of contractile responses in the β-escin-permeabilized strips.25

Nifedipine stock solution (1 mM) was prepared in 70% ethanol under conditions of reduced illumination, whereas niflumic acid was prepared as a stock solution (10 mM) in dimethyl sulfoxide.26

Adenosine 5'-triphosphate, creatinine phosphate, guanosine 5'-triphosphate, HEPES, ionomycin, β-escin, norepinephrine, acetylcholine, nifedipine, and niflumic acid were obtained from Sigma Chemical Co. EGTA, PIPES-K2, and methanesulfonic acid were obtained from Fluka Chemical AG. SKF-96365 was purchased from Calbiochem. Ryanodine was purchased from Agri Systems International. Fura-2/AM was purchased from Dojindo Laboratories. Isoflurane was obtained from Dainabot Co. All other reagents were of the highest grade commercially available.

**Experimental Design**

In experiments with the fura-2-loaded strips, we first examined the effects of isoflurane on increases in [Ca2+]i and force caused by norepinephrine or KCl, using protocols identical to those we used previously to examine the direct action of isoflurane on this artery.12 Because the sympathetic nervous system plays a central role in the maintenance of resting vascular tone in vivo, norepinephrine (0.5 μM [EC50]) was chosen as a test stimulant as in our previous study.12 Conversely, KCl (40 mM) was used as a tool to activate VGCCs. Each stimulant was applied for 3 or 5 min (3 min for KCl; 5 min for norepinephrine) at 7- or 17-min intervals (7 min for KCl; 17 min for norepinephrine) so as to obtain reproducible responses, and then isoflurane was applied for 5 or 15 min (5 min for KCl; 15 min for norepinephrine) before and during the subsequent applications of either stimulant until the steady-state effects were observed (for 15 min for KCl; for 59 min for norepinephrine). Our rationale to use these protocols was detailed previously.12,27

The underlying mechanisms of contractile response to either KCl or norepinephrine could be different between its initial phase (during development of force) and its sustained phase (during maintenance of force). Therefore, in some experiments, isoflurane was applied to the strip precontracted with KCl (40 mM) after the vascular response ([Ca2+]i, and force) to KCl had reached a plateau. However, isoflurane was not applied to the strips precontracted with 0.5 μM norepinephrine, because vascular response ([Ca2+]i and force) to 0.5 μM norepinephrine continued, gradually diminishing progressively after the maximum was reached, and a plateau was not shaped for some time.

In the above-described experiments, we investigated the vascular effects of 3 and 5% isoflurane, because these concentrations of isoflurane distinctly inhibited both norepinephrine- and KCl-induced contractions in our previous experiments with the non–fura-2-loaded, endothelium-denuded strips prepared from this artery.12 As detailed below, the aqueous concentrations produced by 3–5% isoflurane in our experiments would be considered as anesthetic (i.e., clinically relevant) concentrations.

To investigate mechanisms of the observed [Ca2+]i-reducing effects of isoflurane, we next attempted to characterize the vascular response to either norepinephrine or KCl by evaluating the effects of ryanodine (10 μM), nifedipine (0.01–10 μM), SKF-96365 (0.3–10 μM), and niflumic acid (3–100 μM) on the norepinephrine- or KCl-induced increases in [Ca2+]i and force. Ryanodine (10 μM, 20 min) was previously shown to deplete the intracellular Ca2+ stores (presumably sarcoplasmic reticulum, SR) in this mesenteric artery17 and thus was used to eliminate the influence of SR on the vascular responses in this study. Nifedipine is a selective blocker of the L-type VGCCs,28 and SKF-96365 was reported previously to block receptor-operated Ca2+ channels (ROCCs).29,30 Niflumic acid has been reported to selectively inhibit Ca2+-activated Cl− (Cl−) currents without influencing the VGCC activity in VSMCs.26,31 Preliminary experiments indicated that 5 min is sufficient for all of these channel blockers to exert their maximal effects on the response to norepinephrine or KCl. Thus, in the experiments with nifedipine, SKF-96365, or niflumic acid, the strips were incubated with each blocker for 5 min before and during subsequent application of either norepinephrine or KCl.

To investigate the effects of isoflurane on norepinephrine-induced plasmalemmal Ca2+ influx, we examined the effects of isoflurane (3–5%) on the norepinephrine (0.5 μM)-induced increases in [Ca2+]i, and force after treatment with ryanodine (10 μM, 20 min).17

To investigate the effects of isoflurane on myofilament Ca2+ sensitivity, we examined its effects on increases in

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[Ca\(^{2+}\)]_i and force evoked by stepwise incremental increases in the extracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]_e) from 0 to 5 mM during 40 mM K\(^+\) depolarization or those evoked by stepwise incremental increases in the [Ca\(^{2+}\)]_e from 0 to 1.5 mM during stimulation with norepinephrine (0.5 μM) in the fura-2-loaded strips. Our rationale to use these protocols has been detailed previously.\(^{25,33}\) In our previous experiments\(^{12}\) as well as in the above-described experiments, 5 and 37 min were considered sufficient for isoflurane to exert its maximal (i.e., steady-state) effects on the responses to KCl (40 mM) and norepinephrine (0.5 mM), respectively. Therefore, the strips were incubated for 10 and 40 min before and during subsequent applications of Ca\(^{2+}\) in these experiments with 40 mM K\(^+\) depolarization and norepinephrine stimulation, respectively. In the above-described experiments, both 3 and 5% isoflurane distinctly inhibited the KCl response, whereas only 5% isoflurane consistently produced distinct inhibition of the norepinephrine response. Thus, the effects of both 3 and 5% isoflurane were examined in the experiments with 40 mM K\(^+\) depolarization, whereas only the effects of 5% isoflurane were examined in the experiments with norepinephrine.

Finally, to further investigate the effects of isoflurane on myofilament Ca\(^{2+}\) sensitivity, we also examined the effects of isoflurane on the Ca\(^{2+}\)-force relation in the β-escin-permeabilized, non–fura-2-loaded strips in the absence of norepinephrine; α-adrenergic receptor coupling was not retained in the β-escin-permeabilized strips prepared from this artery, as reported previously.\(^{25,35}\)

**Isoflurane Delivery and Analysis**

Isoflurane was delivered *via* a calibrated isoflurane vaporizer (Forawick; Muraco Medical Co., Tokyo, Japan) in line with the air gas aerating the HEPES-buffered solutions. Each solution was equilibrated with isoflurane for at least 15 min before introduction to the chamber, which was covered with thin glass plates to prevent the equilibration gas from escaping into the atmosphere. Using gas chromatography, we previously reported concentrations of isoflurane in the PSS produced by 0.5, 1.0, 1.5, and 3.0% isoflurane under exactly the same experimental conditions,\(^{8,34}\) and the values obtained were within 92% (91.8–98.5%) of theoretical values predicted by the partition coefficient of isoflurane in Krebs solution at 37°C (0.55). Excellent linear relationship was obtained between the aqueous concentrations of isoflurane (γ) and its concentrations (vol%) in the gas mixture (x): γ = −0.0068 + 0.21x, r = 0.998.\(^{34}\) Therefore, the concentrations produced by 1, 2, 3, and 5% isoflurane in the PSS can be predicted as 0.21, 0.42, 0.63, and 1.05 mM, respectively. The aqueous concentration produced by 3% isoflurane (i.e., 0.65 mM) is almost equal to a previously reported concentration of isoflurane (0.65 mM)\(^{35}\) in blood sampled from this rat under steady-state anesthesia with 1.5% (1 minimum alveolar concentration [MAC] in this rat\(^{35}\)) isoflurane. Accordingly, the aqueous concentration produced by 5% isoflurane would correspond to its blood concentration in this rat under steady-state anesthesia with 2.5% (1.67 MAC in this rat\(^{35}\)) isoflurane. Because its partition coefficients in blood and Krebs solution at 37°C are 1.43 and 0.55, respectively, isoflurane is much more (approximately 2.6 times more) soluble in blood than in the buffer solution. On the basis of calculation using the blood/gas partition coefficient of isoflurane (i.e., 1.43), its blood concentration would reach to 1.0 mM during steady-state anesthesia with 1.8% (approximately 1.5 MAC in human) isoflurane. Usually, 1.5 to 2.0 times MAC is required to maintain anesthesia with only a single inhalational agent and to suppress cardiovascular response to incision.\(^{36}\) We thus believe that the aqueous concentrations produced by 3–5% isoflurane in our experiments can be considered anesthetic (i.e., clinically relevant) concentrations.

**Calculation and Data Analysis**

Although absolute values of [Ca\(^{2+}\)]_i could be calculated on the basis of the fura-2 fluorescence ratio and the dissociation constant of fura-2 for Ca\(^{2+}\) binding obtained in *vitro*,\(^{21}\) the dissociation constant of fura-2 for Ca\(^{2+}\) binding in cytoplasm is significantly different (threelfold to fourfold increase) from that measured in the absence of protein, because more than half of the fura-2 molecules in cytoplasm are protein-bound.\(^{37}\) Therefore, we used the ratio of F\(_{340}\) to F\(_{380}\) (R\(_{340/380}\)), which was calculated after the background fluorescence had been subtracted, as an indicator of [Ca\(^{2+}\)]_i.

Changes in the R\(_{340/380}\) and force were expressed as the percentage value of the reference (i.e., values before application of isoflurane). The basal values in normal PSS were assumed to be 0% in all experiments. In experiments in which the strips were pretreated with isoflurane, its effects on the response to norepinephrine or KCl were evaluated 3 or 5 min (3 min for KCl; 5 min for norepinephrine) after application of each stimulant. In experiments in which isoflurane was applied to the strips precontracted with KCl, the effects of isoflurane were evaluated 20, 60, 120, and 180 s after its application.

The concentration–response data for the effects of nifedipine, SKF-96365, and niflumic acid and the Ca\(^{2+}\)-force relation in the β-escin-permeabilized strips were fitted according to a four-parameter logistic model described by De Lean *et al.*\(^{38}\) The 50% inhibitory concentration (IC\(_{50}\)) and the 50% effective concentration (EC\(_{50}\)) were derived from the least-squares fit by use of the above-described model. Because the relationship between the R\(_{340/380}\) value (nonphysiologic value) and the [Ca\(^{2+}\)]_i is not theoretically linear, the R\(_{340/380}\) values were not transformed to a logarithmic scale on the x axis in the representation of the R\(_{340/380}\)-force relationship, and attempts were not made to fit the data for

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the $R_{340/380}$–force relation according to the above-described logistic model. Because the relationship between actual concentrations of isoflurane in the solutions and anesthetic concentrations (vol%) in the gas mixture is theoretically linear, the anesthetic concentrations on the x axis are displayed as vol% for the isoflurane concentration–response relationships, as done previously.8,27

Statistics

All results are expressed as mean ± SD. The term n denotes the number of strips. Data were analyzed by one- or two-factor ANOVA, Scheffé F test, contrast, Student t test, and Welch’s t test. Comparisons among groups were performed by two-factor ANOVA for repeated measures. When overall differences were detected, individual comparisons among groups at each time or concentration were performed by either Scheffé F test (for multiple comparisons), two-tailed, unpaired Student t test (for comparison between two groups with homogeneous population variances), or Welch’s t test (for comparison between two groups with heterogeneous population variances). Comparisons within each group were made by one-factor ANOVA for repeated measures, and post hoc comparisons were made by use of the contrast for multiple comparisons. All other necessary comparisons between two groups, including comparisons between the present and our previous data25 in the Discussion, were made by either the Student t test or Welch’s t test. As previously detailed,25 statistical analysis was not made in the overall comparison of the R$_{340/380}$–force relations. Alternatively, we attempted to find data points in the isoflurane-treated group where the increases in R$_{340/380}$ are not significantly different from those of certain data points in the control group and then compare the force levels at the certain R$_{340/380}$ levels between the control and isoflurane-treated groups. A value of $P < 0.05$ was considered significant.

Results

Effects of Isoflurane on Increases in R$_{340/380}$ and Force Induced by Norepinephrine or KCl

Norepinephrine (0.5 μM) produced increases in both R$_{340/380}$ and force in the normal PSS (fig. 1). The ryanodine treatment slightly but significantly inhibited the norepinephrine-induced increases in both R$_{340/380}$ and force ($P < 0.05$; n = 10; R$_{340/380}$: 79.3 ± 6.9% of control; force, 96.7 ± 4.4% of control). The norepinephrine-induced increases in both R$_{340/380}$ and force were inhibited during application of isoflurane (3–5%) in either the presence or absence of ryanodine (fig. 1).

Fig. 1. Effects of isoflurane (ISO) on norepinephrine (NE)–induced increases in force and R$_{340/380}$ in either the absence (–ryanodine) or presence (+ryanodine) of ryanodine. (A) An example of time-dependent effects of isoflurane on the norepinephrine response. The norepinephrine responses before application of isoflurane (control), 59 min after application of isoflurane, 15 min after washout of isoflurane, and 59 min after washout of isoflurane. (B) Examples of steady-state (i.e., maximal) effects of isoflurane on the norepinephrine response in either the absence (left) or presence (right) of ryanodine. (C) Analyzed data (left, in the absence of ryanodine; right, in the presence of ryanodine) (n = 5). *$P < 0.05$ versus control. **$P < 0.01$ versus control.

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These inhibitions were still observed 15 min after washout of isoflurane from the chamber (table 1), and it took more than 15 min for VSMCs to recover from the inhibitions (fig. 1).

KCl (40 mM) also produced increases in both R_{340/380} and force in the normal PSS (fig. 2). The KCl-induced increase in force was inhibited by both 3 and 5% isoflurane; however, the KCl-induced increase in R_{340/380} was inhibited only by 5% isoflurane but not by 3% isoflurane (figs. 2 and 3). The ryanodine treatment did not significantly influence either the KCl-induced increases in both R_{340/380} and force or the isoflurane-induced inhibitions of the KCl-induced increases in both R_{340/380} and force (n = 5) (fig. 2).

Isoflurane (3–5%) did not influence the basal levels of either R_{340/380} or force; however, in the strips precontracted with KCl, it produced small transient increases in R_{340/380} and force, which were followed by sustained decreases in R_{340/380} and force, respectively (fig. 3). Such transient increases in R_{340/380} and force caused by 5% isoflurane were totally eliminated by the ryanodine treatment (n = 5).

### Effects of Nifedipine, SKF-96365, and Niclumic Acid on Increases in [Ca^{2+}]_{i} (R_{340/380}) Induced by Norepinephrine

In the ryanodine-treated strips, both nifedipine and SKF-96365 significantly inhibited both the norepinephrine- and KCl-induced increases in R_{340/380} (figs. 4 and 5), whereas niclumic acid (30–100 μM) inhibited only the norepinephrine-induced, not KCl-induced, increase in R_{340/380} (fig. 6). There was no concentration range in which SKF-96365 selectively inhibited the norepinephrine-induced increase in R_{340/380} (fig. 5).

Table 1. Norepinephrine (0.5 μM)-induced Increases in R_{340/380} and Force 15 Minutes after Washout of Isoflurane (3–5%) from the Chamber in Either the Presence or Absence of Ryanodine

<table>
<thead>
<tr>
<th>Concentration of Isoflurane</th>
<th>−Ryanodine (n = 4)</th>
<th>+Ryanodine (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R_{340/380}</td>
<td>Force</td>
</tr>
<tr>
<td>3%</td>
<td>55.2 ± 35.8</td>
<td>42.9 ± 40.4*</td>
</tr>
<tr>
<td>5%</td>
<td>46.7 ± 20.7**</td>
<td>36.8 ± 30.7*</td>
</tr>
</tbody>
</table>

Values are expressed as the percent value of the control (before application of isoflurane). * P < 0.05 vs. control (100%), ** P < 0.01 vs. control (100%). −Ryanodine = in the absence of ryanodine; +Ryanodine = in the presence of ryanodine.
**Effects of Isoflurane on Ca^{2+}–Force Relation in β-Escin-Membrane-permeabilized Muscle**

In the β-escin–treated strips, the stepwise increment of [Ca^{2+}]_i in the bath solution produced concentration-dependent increases in force (fig. 9). Isoflurane (5%) did not significantly influence the Ca^{2+}–force relation (fig. 9).

**Discussion**

Using fura-2 fluorometry, this study, for the first time, provides direct evidence to indicate that isoflurane inhibits myofilament Ca^{2+} sensitivity and plasmalemmal Ca^{2+} influx during stimulation with either norepinephrine.
isoflurane on the R_{340/380}-force relation suggest that the depressed contractile response to either KCl or norepinephrine during exposure to 5% isoflurane is a result of both reduction of the [Ca^{2+}], and inhibition of the myofilament Ca^{2+} sensitivity.

In this artery, treatment with ryanodine (10 μM) depletes not only the caffeine/ryanodine-sensitive SR but also the norepinephrine/inositol 1,4,5-triphosphate-sensitive SR. 17,52 Thus, the norepinephrine-induced increase in R_{340/380} observed after ryanodine treatment is presumably because of plasmalemmal Ca^{2+} influx. The effects of nifedipine on either norepinephrine- or KCl-induced increases in R_{340/380} observed after ryanodine treatment suggest that not only the KCl-induced but also the norepinephrine (0.5 μM)-induced Ca^{2+} influxes are attributable exclusively to activation of the VGCCs. This is consistent with the previously reported threshold concentrations of norepinephrine for membrane depolarization in small mesenteric arteries (i.e., 0.3–1 μM) 50–41 and the recent recognition that VGCCs play a major role, whereas ROCCs play only a minor role, in the norepi-

![Diagram](image-url)

**Fig. 5.** Effects of SKF-96365 on norepinephrine (NE)- and KCl-induced increases in force and R_{340/380} in the presence of ryanodine (+ryanodine). (A) Examples of steady-state (i.e., maximal) effects of SKF-96365 on the responses to either norepinephrine (left) or KCl (right). (B) Analyzed data (left, force; right, R_{340/380}) (n = 4). \( P < 0.05 \) versus control (100%) within each group. **\( P < 0.01 \) versus control (100%) within each group. The IC_{50} values for the inhibitions of norepinephrine-induced increases in force and R_{340/380} were 1.8 and 2.5 μM, respectively, whereas those for the inhibitions of KCl-induced increases in force and R_{340/380} were 1.3 and 5.3 μM, respectively.

![Diagram](image-url)

**Fig. 6.** Effects of nifedipine acid on norepinephrine (NE)- and KCl-induced increases in force and R_{340/380} in the presence of ryanodine (+ryanodine). (A) Examples of steady-state (i.e., maximal) effects of nifedipine acid on the responses to either norepinephrine (left) or KCl (right). (B) Analyzed data (left, force; right, R_{340/380}) (n = 4). \( P < 0.05 \) versus control (100%) within each group. **\( P < 0.01 \) versus control (100%) within each group. The IC_{50} values for the inhibitions of norepinephrine-induced increases in force and R_{340/380} were 15.7 and 22.5 μM, respectively, whereas the IC_{50} value for the inhibitions of KCl-induced increases in force was 19.5 μM. Note that the KCl-induced increases in R_{340/380} were not significantly inhibited.
Fig. 7. Effects of isoflurane on increases in $R_{340/380}$ (A) and force (B) caused by incremental increases in the extracellular Ca$^{2+}$ concentration ($[Ca^{2+}]_e$) during KCl (40 mM) depolarization in the fura-2–loaded strips. After the control responses to KCl (40 mM) had been recorded, the strips were treated with isoflurane for 10 min before and during subsequent application of various concentrations of Ca$^{2+}$ during the depolarization. In these analyses, the maximal increase in either $R_{340/380}$ or force induced by KCl before exposure to isoflurane was assumed to be 100% and their basal values in normal physiologic salt solution 0%.

* $P < 0.05$ versus control at each Ca$^{2+}$ concentration. n = 4. (C) Effects of isoflurane on the $R_{340/380}$–force relation. The $R_{340/380}$–force relation either in the absence or in the presence of isoflurane was constructed from the data shown in A and B. $\alpha P < 0.05$ versus control group. $\beta P < 0.05$ versus a in the control group. $\gamma P < 0.05$ versus c in the control group.

Fig. 8. Effects of isoflurane on increases in $R_{340/380}$ (A) and force (B) caused by incremental increases in the extracellular Ca$^{2+}$ concentration ($[Ca^{2+}]_e$) during stimulation with norepinephrine (0.5 μM) in the fura-2–loaded strips. After the control responses to KCl (40 mM) had been recorded, the strips were treated with isoflurane for 40 min before and during subsequent application of various concentrations of Ca$^{2+}$ in the presence of norepinephrine. In these analyses, the maximal increase in either $R_{340/380}$ or force induced by KCl before exposure to isoflurane was assumed to be 100% and their basal values in normal physiologic salt solution 0%.

* $P < 0.05$ versus control at each Ca$^{2+}$ concentration. n = 4. (C) Effects of isoflurane on the $R_{340/380}$–force relation in the presence of norepinephrine. The $R_{340/380}$–force relation either in the absence or in the presence of isoflurane was constructed from the data shown in A and B. $\alpha P < 0.05$ versus control group. $\beta P < 0.05$ versus a in the control group. $\gamma P < 0.05$ versus c in the control group.
nephrine-induced Ca$^{2+}$ influx in rat mesenteric arteries.\textsuperscript{42}

The ability of nimodipine, a Ca$^{2+}$ channel blocker,\textsuperscript{26,31} to selectively inhibit the nephrine-induced increase in R$^{340/380}$ in the presence of ryanodine indicates that activation of the Ca$^{2+}$ channels is also involved in the nephrine-induced Ca$^{2+}$ influx. In VSM, activation of the Ca$^{2+}$ channels would result in membrane depolarization and hence activation of VGCCs, because the Ca$^{2+}$ equilibrium potential (i.e., approximately $-20$ to $-30$ mV) is more positive than both the resting membrane potential (i.e., approximately $-50$ to $-70$ mV) and the threshold potential for opening VGCCs (i.e., approximately $-60$ to $-45$ mV).\textsuperscript{43} Thus, as has been proposed in some vascular preparations,\textsuperscript{26,44,45} nephrine presumably activates the Ca$^{2+}$ channels and thereby depolarizes the cell membrane, leading to the opening of VGCCs and hence contraction. In contrast, KCl directly depolarizes the cell membrane and thereby activates VGCCs. Previous studies have suggested that during stimulation with nephrine, Ca$^{2+}$ released from SR activates the Ca$^{2+}$ channels.\textsuperscript{44} However, we speculate that the Ca$^{2+}$ channels were presumably activated by Ca$^{2+}$ entering the cells through the nonselective cation channels during stimulation with nephrine in our experiments with the ryanodine-treated strips.

The ability of isoflurane to inhibit both the nephrine- and KCl-induced increases in R$^{340/380}$ in the presence of ryanodine indicates that isoflurane inhibits both the nephrine- and KCl-induced plasmalemmal Ca$^{2+}$ influxes, which are presumably through VGCCs, as discussed above. Isoflurane was previously shown to inhibit the whole cell L-type Ca$^{2+}$ currents in cerebral or coronary arterial cells.\textsuperscript{46,47} Thus, in the mesenteric arterial cells, isoflurane also may inhibit the activation of VGCCs and thereby depress both the nephrine- and KCl-induced Ca$^{2+}$ influx. However, the observed difference in recovery time course after washout of isoflurane between the nephrine- and KCl-induced increases in R$^{340/380}$ (after ryanodine treatment) indicates that the mechanisms behind the isoflurane-induced inhibition of the nephrine-induced Ca$^{2+}$ influx are at least in part different from those behind its inhibition of the KCl-induced Ca$^{2+}$ influx. Because activation of the Ca$^{2+}$ channels appears to be involved in the nephrine-induced but not the KCl-induced Ca$^{2+}$ influx, isoflurane may prevent the activation of Ca$^{2+}$ channels and hence the nephrine-induced Ca$^{2+}$ influx through VGCCs in a prolonged manner. In other words, isoflurane may act at some steps between receptor binding and activation of the Ca$^{2+}$ channels to cause a prolonged inhibition of the nephrine-activated signaling pathway. Because of its high lipophilicity, isoflurane may remain present in the VSMCs for some time after its removal from the extracellular space to exert such a prolonged effect.

Ryanodine strongly inhibited the initial phase of the nephrine-induced increase in R$^{340/380}$ but only modestly inhibited its tonic phase, as shown previously in this artery.\textsuperscript{25,32} This suggests that the nephrine-induced initial increase in $[Ca^{2+}]_{i}$ is attributable primarily to Ca$^{2+}$ release from SR, whereas its tonic increase in $[Ca^{2+}]_{i}$ is primarily a result of the plasmalemmal Ca$^{2+}$ influx. Because isoflurane does not seem to influence the nephrine-induced Ca$^{2+}$ release from SR in this artery,\textsuperscript{17} the isoflurane-induced inhibition of the nephrine-induced increase in $[Ca^{2+}]_{i}$ observed in the absence of ryanodine was
probably exclusively a result of its inhibition of the norepinephrine-induced Ca\textsuperscript{2+} influx.

The inability of ryanodine to influence the KCl-induced increase in R\textsubscript{340/380} suggests that the KCl-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i} is exclusively a result of activation of the plasmalemmal Ca\textsuperscript{2+} influx, consistent with our previous studies in this artery.\textsuperscript{8,23,32} Because isoflurane seems to enhance the caffeine-induced Ca\textsuperscript{2+} release (presumably Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release) from SR in this artery,\textsuperscript{17} if the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release mechanism operates significantly during the KCl response, the ryano dine treatment would enhance the isoflurane-induced inhibition of KCl-induced increase in R\textsubscript{340/380}. However, it was not influenced by the ryanodine treatment, suggesting that the isoflurane-induced inhibition of the KCl-induced increase in [Ca\textsuperscript{2+}]\textsubscript{i} is exclusively a result of its effect on the plasmalemmal Ca\textsuperscript{2+} influx. The Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release mechanism may play only a minor, if any, role in the KCl response in this artery.

Isoflurane has been suggested to increase vascular tone through its direct effects on SR,\textsuperscript{8,9} plasmalemmal Ca\textsuperscript{2+} influx,\textsuperscript{48} or the protein kinase C system.\textsuperscript{11–49} In this study, isoflurane caused small transient increases in R\textsubscript{340/380} and force during stimulation with KCl (but not in the resting state). The observed increases in R\textsubscript{340/380} and force were eliminated by the ryanodine treatment, indicating that the isoflurane-induced contraction results from the Ca\textsuperscript{2+} release from SR, consistent with the previous proposal in rat aorta.\textsuperscript{9} Because the amount of Ca\textsuperscript{2+} stored in SR would be increased during stimulation with KCl, which activates voltage-gated Ca\textsuperscript{2+} influx and thereby causes a continuous increase in the [Ca\textsuperscript{2+}]\textsubscript{i}, the inability of isoflurane to cause significant increases in R\textsubscript{340/380} in the resting state might be explained by possibly decreased amounts of Ca\textsuperscript{2+} in SR. In a recent study using membrane-permeabilized rabbit pulmonary arteries,\textsuperscript{49} isoflurane caused transient contraction similar to that observed in this artery. However, on the basis of sensitivity to protein kinase C inhibitors, the contraction in the pulmonary artery appeared to be caused by protein kinase C activation.\textsuperscript{49} The difference might be explained by the species or regional differences.

On the basis of sensitivity to SKF-96365, a putative inhibitor of ROCCs,\textsuperscript{29,30} isoflurane was previously proposed to enhance contractile response to phenylephrine by stimulating Ca\textsuperscript{2+} influx through ROCCs in rat aorta.\textsuperscript{48} However, our results on SKF-96365 indicate that SKF-96365 does not serve as a selective inhibitor of ROCCs, consistent with the recent recognition that selective inhibitors of ROCCs or nonselective cation channels are not currently available.\textsuperscript{50}

The observed difference in the effect of isoflurane on the Ca\textsuperscript{2+}-force relation between the fura-2-loaded membrane-intact and the β-escin-permeabilized conditions suggests that its inhibition of the Ca\textsuperscript{2+} activation of contractile proteins is presumably mediated by the intact plasma membrane or because of an effect on some intracellular regulatory mechanisms of contractile proteins that are impaired in the β-escin-permeabilized strips. It is currently unclear whether isoflurane inhibits only the Ca\textsuperscript{2+} activation of contractile proteins (i.e., basal Ca\textsuperscript{2+} sensitivity of contractile proteins) or inhibits both the basal Ca\textsuperscript{2+} sensitivity and the norepinephrine-induced Ca\textsuperscript{2+} sensitizing mechanism(s). Further investigations are necessary to clarify this issue.

More than half of the fura-2 molecules in myoplasma are in a protein-bound form,\textsuperscript{57} possibly influencing responsiveness or sensitivity of the VSMCs to isoflurane. However, no significant differences were found in its steady-state effects on contractile responses to either norepinephrine or KCl between the fura-2–loaded and nonloaded strips (according to comparison between our previous data\textsuperscript{12} and the present data). Thus, the fura-2 loading, a nonphysiologic intervention as well, does not seem to significantly influence the action of isoflurane on VSMCs.

The direct vascular action of isoflurane observed in this study was substantially identical to that of sevoflurane previously observed in this artery.\textsuperscript{23} In our experiments, the aqueous concentrations produced by 3% isoflurane (i.e., 0.63 mM) and 5% sevoflurane (i.e., 0.67 mM) are almost equal to the respective anesthetic concentrations in blood sampled from this rat under steady-state anesthesia at 1 MAC (1.5% for isoflurane,\textsuperscript{35} 2.8% for sevoflurane\textsuperscript{51}), i.e., 0.65 and 0.66 mM, respectively.\textsuperscript{13} We thus compared the effect of 3% isoflurane on contractile response to either norepinephrine or KCl with that of 5% sevoflurane observed in our previous study.\textsuperscript{23} No significant difference (P > 0.05) was observed in the inhibition of the norepinephrine (0.5 μM)-induced increase in either force or R\textsubscript{340/380} between 3% isoflurane and 5% sevoflurane, suggesting that the inhibitory action of isoflurane on the norepinephrine response is identical to that of sevoflurane compared at the equivalent MAC. However, a significant difference was found in the inhibition of the KCl response between 3% isoflurane and 5% sevoflurane: namely, although no significant difference was observed in the inhibition of the KCl-induced increase in force between 3% isoflurane and 5% sevoflurane (P > 0.05), only 5% sevoflurane, not 3% isoflurane, significantly inhibited the KCl-induced increase in R\textsubscript{340/380}. Thus, the mechanisms behind the depressed contractile response to KCl appear different between 3% isoflurane and 5% sevoflurane.

In conclusion, isoflurane directly inhibits VSM reactivity by inhibiting both Ca\textsuperscript{2+} mobilization and myofilament Ca\textsuperscript{2+} sensitivity in systemic resistance arteries. Isoflurane inhibits plasmalemmal Ca\textsuperscript{2+} influx through VGCCs during stimulation with either KCl or norepinephrine. However, the mechanisms behind its inhibition of the voltage-gated Ca\textsuperscript{2+} influx during stimulation with KCl are presumably at least in part different from those during stimulation with norepinephrine. During stimulation with norepinephrine, isoflurane may prevent activation of the Cl\textsuperscript{−}/Ca\textsuperscript{2+} channels and thereby inhibit the Ca\textsuperscript{2+} influx.
through VGCCs in a prolonged manner. The presence of the plasma membrane appears essential for isoflurane to exert its inhibitory action on the Ca$^{2+}$-induced activation of contractile proteins.

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References