The Action of Sevoflurane on Vascular Smooth Muscle of Isolated Mesenteric Resistance Arteries (Part 2)

Mechanisms of Endothelium-independent Vasorelaxation

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Background: The precise mechanisms behind the direct inhibitory action of sevoflurane on vascular smooth muscle have not been fully elucidated.

Methods: Endothelium-denuded smooth muscle strips were prepared from rat small mesenteric arteries. Isometric force and intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) were measured simultaneously in the fura-2–loaded strips. In another series of experiments, only isometric force was measured in the β-escin-membrane-permeabilized strips.

Results: Sevoflurane (3–5%) inhibited the increases in both the [Ca\(^{2+}\)]\(_i\) and the force induced by either norepinephrine (0.5–10 μM) or 40 mM K\(^+\). Sevoflurane still inhibited the increase in [Ca\(^{2+}\)]\(_i\) induced by norepinephrine after depletion of intracellular Ca\(^{2+}\) stores with ionomycin, although it little influenced the increase in [Ca\(^{2+}\)]\(_i\) induced by norepinephrine after treatment with verapamil. In the fura-2–loaded membrane-intact muscle, sevoflurane caused a rightward shift of Ca\(^{2+}\)-force relation during force development to stepwise increment of extracellular Ca\(^{2+}\) concentration during 40-mM K\(^+\) depolarization in either the presence or the absence of norepinephrine. In contrast, sevoflurane did not influence Ca\(^{2+}\)-activated contraction in the β-escin–permeabilized muscle, in which α-adrenergic receptor coupling was not retained.

Conclusions: The inhibitory effects of sevoflurane on both norepinephrine- and potassium chloride (KCl)–induced contractions are caused by reduction of [Ca\(^{2+}\)]\(_i\) in vascular smooth muscle and inhibition of the myofilament Ca\(^{2+}\) sensitivity. The [Ca\(^{2+}\)]\(_i\)-reducing effect of sevoflurane observed in both the norepinephrine- and the K\(^+\)-stimulated muscle is mainly caused by inhibition of voltage-gated Ca\(^{2+}\) influx. The inhibitory effect of sevoflurane on Ca\(^{2+}\) activation of contractile proteins seems to be mediated by the cell membrane or by some diffusible substances that are lost in the β-escin–permeabilized cells. (Key words: Halogenated volatile anesthetics; intracellular free calcium concentration; myofilament Ca\(^{2+}\) sensitivity; fura-2 fluorometry; membrane permeabilization; β-escin.)

In another article in this issue of Anesthesiology, we showed, as previously observed with halothane, isoflurane, and enfurane, that anesthetic concentrations of sevoflurane have direct (i.e., endothelium-independent) inhibitory effects on vascular smooth muscle (VSM) of isolated mesenteric resistance arteries. However, its precise mechanisms have not been fully clarified.

In our previous preliminary experiments in β-escin-membrane-permeabilized VSM cells, sevoflurane did not significantly influence Ca\(^{2+}\)-induced activation of contractile proteins, suggesting that sevoflurane relaxes VSM mainly by reducing the intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)) in the absence of receptor stimulation. However, we might have failed to see the inhibitory action of sevoflurane on myofilament Ca\(^{2+}\) sensitivity of VSM cells in those experiments. Although the membrane permeabilization can be a valuable tool in investigating mechanisms of VSM contraction by directly controlling cytoplasmic solute composition and by using cell membrane-impermeable agents, one major limitation is the loss of diffusible endogenous cofactors or regulators of contractile proteins. For example, β-escin (i.e., saponin ester) has been reported to make the cell membrane permeable to higher molecular weight (MW) substances.

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to significantly modulate the myofilament Ca\(^{2+}\) sensitivity. Therefore, if sevoflurane directly damaged by the chemical detergent used to permeabilize VSM cell membrane. Therefore, if sevoflurane exerts its inhibitory action on myofilament Ca\(^{2+}\) sensitivity of VSM cells through effects on such regulatory mechanisms that are impaired by the membrane permeabilization, the action will not be observed under the membrane-permeabilized condition. Therefore, the action of sevoflurane on the myofilament Ca\(^{2+}\) sensitivity does not seem to be fully understood. In addition, the precise mechanisms of the sevoflurane-induced reduction of the [Ca\(^{2+}\)]i have not been elucidated.

In this study, to address these issues, we investigated the direct (i.e., endothelium-independent) inhibitory action of sevoflurane on fura-2-loaded membrane-intact and \(\beta\)-escin membrane-permeabilized mesenteric arterial VSM cells, in the former of which isometric force and intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]i) both were measured simultaneously.

**Materials and Methods**

**Tissue Preparation**

With approval from the Kyushu University Animal Care and Use Committee, endothelium-denuded strips were prepared from the distal branches of Sprague-Dawley (male) rat mesenteric arteries using the same methods as described in Izumi et al.\(^1\) in this issue.

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

All experiments were performed in the guanethidine-pretreated (3 \(\mu\)M) strips after confirming functional removal of endothelium. Our techniques of isometric force recording, method for confirming the removal of endothelium, and rationale for using guanethidine have already been described by Izumi et al.\(^1\)

In the first series of experiments, force and [Ca\(^{2+}\)]i both were simultaneously measured in the strips loaded with a fluorescent Ca\(^{2+}\)-indicator dye, fura-2.\(^14\) To enable loading of fura-2 into the smooth muscle cells, the strips, mounted on the microscope, were incubated in normal physiologic salt solution (PSS) containing 10 \(\mu\)M acetoxyethyl ester of fura-2 (fura-2/AM) and stock solution in dry dimethyl sulfoxide and 2% bovine 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.\(^15\)

The position of the strip was adjusted to the center of the field, with a mask placed in an intermediate image plane to reduce background fluorescence. Changes in the fluorescence intensity of the fura-2-Ca\(^{2+}\) complex were measured using a fluorimeter equipped with a dual-wavelength excitation device (CAM-230; Japan Spectroscopic, Tokyo, Japan) connected to the microscope with optical fibers. The microscope was focused on the smooth muscle layers and the vascular tissue was illuminated with ultraviolet lights of the wavelengths of 340 and 380 nm alternatively limited to a frequency of 1,000 Hz. The fura-2 fluorescence signals induced by excitation at 340 nm and 380 nm were collected through the 10–20\(\times\) objective lens (Plan Fluor; Nikon, Tokyo, Japan) and measured through a 500-nm filter with a photomultiplier. The background fluorescence (including autofluorescence of the strip) as excited by 340 and 380 nm ultraviolet light was obtained after completion of each experiment by breaking the cell membranes with a detergent, Triton-X-100 (1%; Nacalai Tesque, Kyoto, Japan) and subsequently quenching the fura-2 fluorescence signals with MnCl\(_2\) (20 mM), as reported previously.\(^16–19\)

In preliminary experiments, the background fluorescence obtained after completion of each experiment was identical to that obtained in fura-2 unloaded strips (i.e., before loading fura-2). During these conditions, the background fluorescence was approximately 10–15% of the fura-2 signals in smooth muscle strips at either excitation wavelength. The ratio of fura-2 fluorescence intensities excited by 340 nm (\(F_{340}\)) to those excited by 380 nm (\(F_{380}\)) was calculated after subtracting the background fluorescence.

In the Ca\(^{2+}\) measurement experiments, as shown in figure 1, changes in \(F_{340}\) and \(F_{380}\) were constantly in opposite directions as far as the strip was tightly fixed between one end of the chamber and the transducer under an isometric condition. According to the theory of
the fura-2 fluorometry, this strongly suggests that the changes in $F_{340}$ and $F_{380}$ observed in our experiments reflect changes in $[Ca^{2+}]_i$ but not in motion artifacts. Although it was previously shown that applications of stimulants such as high $K^+$ significantly increased $F_{340}$ and $F_{380}$ in a parallel manner in fura-2–unloaded muscle,\textsuperscript{16,18} none of the agents or solutions used in our study caused any significant shifts of either $F_{340}$ or $F_{380}$ signals in the fura-2–unloaded strips in control experiments. The movement artifacts or changes in fluorescence of endogeneous substances thus seem to be negligible in our experimental setting.

All experiments with the fura-2–loaded strips were performed during the period in which constant vascular responses (force and fura-2 signals both) were obtained; \textit{i.e.}, for approximately 3 h after contractile responses to the stimulants became constant (fig. 1).

In another series of experiments, only isometric force was measured in the fura-2–nonloaded endothelium-denuded strips, the smooth muscle membrane of which was permeabilized with $\beta$-escin (saponin ester). To achieve the membrane permeabilization, the smooth muscle strips were incubated with $\beta$-escin (50 $\mu$M for 25 min) at room temperature ($\approx 22^\circ$C) in relaxing solution after measuring steady contractions induced by 40 mM $K^+$\textsuperscript{2,20} Ionomycin (0.3 $\mu$M) was present throughout the

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β-escin-permeabilized muscle experiments to eliminate the influence of intracellular Ca$^{2+}$ stores. As previously reported, all experiments with β-escin-membrane-permeabilized muscle strips were performed at room temperature (=22°C) to prevent early deterioration of the thin vascular strips, whereas all other experiments were performed at 35°C.

**Solutions and Drugs**

The ionic concentrations and pH of either normal PSS or high K$^+$ solution buffered with 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) were described by Izumi et al. in this issue. The Ca$^{2+}$-free solution was prepared by removing CaCl$_2$ with or without adding 2 mM ethyleneglycol-bis-(β-amino ethyl ether)N,N,N',N'-tetraacetic acid (EGTA). The Ca$^{2+}$-free solution without EGTA was used to eliminate the low-affinity extracellular bound Ca$^{2+}$, whereas the Ca$^{2+}$-free solution containing EGTA was used to eliminate both low- and high-affinity extracellular bound Ca$^{2+}$. The composition of the relaxing solution was 80 mM potassium methansulfonate, 20 mM piperazine-1,4-bis-(2-ethanesulfonic acid) (PIPES), 7 mM Mg(MS)$_2$, 5 mM adenosine triphosphate (ATP), 10 mM creatinine phosphate (CP), 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 Ca$^{2+}$ ions based on the calculations previously reported. The pH was adjusted with KOH to 7.00 at 22°C, and the ionic strength was kept constant at 0.2 M by adjusting the concentration of potassium methansulfonate. Guanosine 5’-triphosphate (50 μM) was present throughout the experimental periods to minimize rundown of contractile responses in the β-escin-permeabilized strips.

Adenosine triphosphate, creatinine phosphate, guanosine-5’-triphosphate, HEPES, ionomycin, β-escin, norepinephrine, and acetylcholine were obtained from Sigma Chemical (St. Louis, MO). EGTA, PIPES-K$_2$, and methanesulfonic acid were obtained from Fluka Chemie AG (Buchs, Switzerland). Fura-2/AM was purchased from Dojindo Laboratories (Kumamoto, Japan). Sevoflurane was obtained from Kodama Pharmaceutical (Osaka, Japan). All other reagents were of the highest grade commercially available.

**Experimental Design**

In experiments with the fura-2-loaded strips, we evaluated the effects of sevoflurane on increases in [Ca$^{2+}$]i and force caused by norepinephrine (0.5 [EC$_{50}$] and 10 μM [maximum]) or 40 mM K$^+$, using protocols identical to those used and detailed in the article by Izumi et al. Briefly, each stimulus (norepinephrine or high K$^+$) was applied for 3–5 min (5 min for 40 mM K$^+$ and 10 μM norepinephrine; 5 min for 0.5 μM norepinephrine) at 7- or 17-min intervals (7 min for 40 mM K$^+$ and 10 μM norepinephrine; 17 min for 0.5 μM norepinephrine) to obtain reproducible responses, and then sevoflurane was applied for 5 min before and during the subsequent applications of either stimulant. The concentrations of sevoflurane used in these experiments with the fura-2-loaded strips were 3 and 5%, concentrations at which sevoflurane distinctly inhibited norepinephrine- and high K$^+$-induced contractions in the fura-2-nonloaded strips. In some of these experiments, sevoflurane was applied to the strip precontracted with 40 mM K$^+$ after vascular response ([Ca$^{2+}$]i and force) to 40 mM K$^+$ reached a plateau. However, sevoflurane was not applied to the strips precontracted with 10 μM norepinephrine, because, with this protocol, sevoflurane little influenced the contractile response to 10 μM norepinephrine in the fura-2-nonloaded strips in preliminary experiments. In addition, this protocol could not be used in experiments with a low concentration (0.5 μM) of norepinephrine because vascular responses ([Ca$^{2+}$]i and force) to 0.5 μM norepinephrine continued, gradually diminishing progressively after the maximum was reached and a plateau was not shaped for a while.

To investigate mechanisms of the observed [Ca$^{2+}$]i-reducing effect of sevoflurane in the presence of norepinephrine, we first characterized the vascular response to norepinephrine by evaluating the effects of ionomycin, of removal of the extracellular Ca$^{2+}$, and of voltage-gated Ca$^{2+}$ channel blockers (i.e., verapamil, diltiazem, and nifedipine) on the increases in [Ca$^{2+}$]i and force caused by norepinephrine. We then evaluated the effects of sevoflurane (5%) on the increases in [Ca$^{2+}$]i and force caused by norepinephrine in the presence of either ionomycin or verapamil.

Finally, to investigate the effects of sevoflurane on myofilament Ca$^{2+}$ sensitivity, we evaluated the effects of sevoflurane on increases in force and [Ca$^{2+}$]i evoked by stepwise incremental increases in the extracellular Ca$^{2+}$ concentrations ([Ca$^{2+}$]$^e$) from 0 to 10 mM during 40 mM K$^+$ depolarization, either in the presence of norepinephrine or in its absence in the fura-2-loaded strips. We also
evaluated the effects of sevoflurane on Ca\(^{2+}\)-force relation in the β-escin-permeabilized, fura-2-nonloaded strips in the absence of norepinephrine; α-adrenergic receptor coupling was not retained in the β-escin-permeabilized strips prepared from this artery, as previously reported.\(^{1,5,22}\)

**Sevoflurane Delivery and Analysis**

The information regarding the sevoflurane delivery system and concentrations of sevoflurane in the PSS determined by gas chromatography already is provided by Izumi et al.\(^{1}\) in this issue of Anesthesiology.

**Calculation and Data Analysis**

Although absolute values of [Ca\(^{2+}\)]i have been calculated based on the fura-2 fluorescence ratio and the dissociation constant of fura-2 for Ca\(^{2+}\) binding obtained in vitro,\(^{14}\) the dissociation constant of fura-2 for Ca\(^{2+}\) binding in cytoplasm (i.e., in the living cells) is known to be significantly different (three- 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.\(^{23}\) Therefore, we used the ratio of F\(_{340}\) to F\(_{380}\) (R\(_{340/380}\)), which was calculated after subtracting the background fluorescence, as an indicator of [Ca\(^{2+}\)]i, as previously.\(^{15-19}\)

Changes in the R\(_{340/380}\) and force were expressed as the percent value of the reference. The basal values in normal PSS were assumed to be 0% in all experiments. In experiments in which the strips were pretreated with sevoflurane, the maximum values of control (preanesthetic) responses to either norepinephrine or potassium chloride (KCl) were assumed to be 100%, whereas, in experiments in which sevoflurane was applied to the strips precontracted with KCl, the values immediately before application of sevoflurane were assumed to be 0%. Because the responses to 0.5 \(\mu\)M norepinephrine consisted of two distinct components, i.e., an initial phasic and a subsequent tonic component, the effects of sevoflurane, applied before the application of 0.5 \(\mu\)M norepinephrine, were assessed for both the phasic and the tonic responses to 0.5 \(\mu\)M norepinephrine. However, because the phasic response was not necessarily distinct in the response to either 40 mM KCl or 10 \(\mu\)M norepinephrine, the effects of sevoflurane on the response to 40 mM KCl or 10 \(\mu\)M norepinephrine were evaluated mainly for the tonic response. The effect of sevoflurane on the tonic response was assessed 3 or 5 min (5 min for KCl and 10 \(\mu\)M norepinephrine; 5 min for 0.5 \(\mu\)M norepinephrine) after application of each stimulant.

The concentration–response data for the relation between extracellular Ca\(^{2+}\) concentration and force in the fura-2-loaded strips and the Ca\(^{2+}\)-force relation in the fura-2-nonloaded, β-escin-permeabilized strips were fitted according to a four-parameter logistic model described by De Lean et al.\(^{24}\) and the EC\(_{50}\) (the concentration that produced 50% of the maximal response) values were derived from the least-squares fit using the aforementioned model. Because the relation between the R\(_{340/380}\) value (nonphysiologic value) and [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 relation, and attempts were not made to fit the data for the R\(_{340/380}\)-force relation according to this logistic model. Rather, attempts were made to either linearly or polynomially fit the data for the R\(_{340/380}\)-force relation, using the least-squares methods, as previously.\(^{16,25}\) Because the relation between actual concentrations of sevoflurane in the solutions and anesthetic concentrations (vol %) in the gas mixture is theoretically linear, the anesthetic concentrations on the \(x\)-axis are displayed as the vol % for the sevoflurane concentration–response relations.

**Statistics**

All results are expressed as the mean ± SEM. The n denotes the number of preparations. The statistical assessment of the data was made by one- or two-factor analysis of variance, the Scheffé F test, and the Student \(t\) test, where appropriate. Comparisons among (or between) the groups (e.g., control vs. sevoflurane-treated groups) were performed by two-factor (e.g., time, concentration) analysis of variance for repeated measures. When overall differences were detected, individual comparisons among groups at each time or concentration were performed using the Scheffé F test (for multiple comparisons) or by the unpaired Student \(t\) test (for comparison between two groups). Comparisons within each group were made using one-factor (concentration, R\(_{340/380}\), or time) analysis of variance for repeated measures, and post hoc comparisons were made using the Scheffé F test for multiple comparisons. All other necessary comparisons between two groups were made using the Student \(t\) test (paired or unpaired).

We could not find appropriate statistical methods to compare the Ca\(^{2+}\)-force relation obtained in the fura-2-loaded strips, in which each data point accompanies two error bars on both the \(x\)- (R\(_{340/380}\)) and \(y\)- (force) axes, among the groups (i.e., control vs. sevoflurane-treated groups). In addition, no statistical analyses were made to

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compare the Ca$^{2+}$–force relation obtained in fura-2–loaded muscle in many of previous studies in which the protocols used were identical to ours.$^{16,18,19,26-28}$ Therefore, in this study, statistical analysis was not made in the overall comparison of the R340/380–force relations. Alternatively, we attempted to find data points in the sevoflurane-treated group in which the increases in [Ca$^{2+}$]$_i$ (R340/380) were not significantly different from those of certain data points in the control group, and then compare the force levels at the certain [Ca$^{2+}$]$_i$ (R340/380) levels between the control and sevoflurane-treated groups (see Results). $P < 0.05$ was considered significant.

**Results**

**Effects of Sevoflurane on Increases in [Ca$^{2+}$]$_i$ (R340/380) and Force Induced by Potassium Chloride or Norepinephrine**

The tonic increases in R$_{340/380}$ and force caused by either KCl (40 mM) or norepinephrine (0.5 and 10 μM) were inhibited ($P < 0.05$) in the strips pretreated with sevoflurane (3–5%; fig. 2). However, the phasic increases in R$_{340/380}$ (and force) caused by either 10 μM norepinephrine or 0.5 μM norepinephrine were not inhibited by the pretreatment with sevoflurane (3–5%; $P > 0.05$; fig. 2). In the strips precontracted with KCl, 3 and 5% sevoflurane both attenuated ($P < 0.05$) the force; however, only 5%, but not 3%, sevoflurane reduced ($P < 0.05$) the R$_{340/380}$ (fig. 3).

**Effects of Sevoflurane on Increases in [Ca$^{2+}$]$_i$ (R$_{340/380}$) and Force Induced by Potassium Chloride or Norepinephrine after Treatment with Either Ionomycin or Verapamil**

Caffeine (20 mM, maximum) and norepinephrine (10 μM, maximum) both produced transient phasic increases in R$_{340/380}$ and force in Ca$^{2+}$-free, 2 mM EGTA solution. The maximal increases in R$_{340/380}$ caused by caffeine (20 mM) and norepinephrine (10 μM) in the Ca$^{2+}$-free solution were 114.3 ± 23.3% (n = 9) and 32.8 ± 13.4% (n = 4), respectively, of that caused by KCl (40 mM) in normal PSS. Similarly, the maximal increases in force caused by caffeine (20 mM) and norepinephrine (10 μM) in the Ca$^{2+}$-free solution were 69.9 ± 18.8% (n = 9) and 103.7 ± 19.4% (n = 4), respectively, of that caused by KCl (40 mM) in normal PSS. Treatment with ionomycin (0.3 μM, 25 min) eliminated these phasic increases in

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Effects of Sevoflurane on Ca$^{2+}$ (R$_{340/380}$)–Force Relation in Membrane-depolarized, Fura-2–loaded Muscle

In the fura-2–loaded, K$^+$-membrane-depolarized strips, as shown in figure 1, the stepwise increment of [Ca$^{2+}$]e (0–10 mM) produced concentration-dependent increases in R$_{340/380}$ and force. Sevoflurane (3–5%) inhibited ($P < 0.05$) the increases in R$_{340/380}$ and force induced by higher [Ca$^{2+}$]e (≥1.5 mM) during the K$^+$ depolarization; however, sevoflurane inhibited only the increases in force, not the increases in R$_{340/380}$ induced by the lower [Ca$^{2+}$]e (0.3–1.5 mM) during K$^+$ depolarization (fig. 5). This suggests that inhibition of the myofilament Ca$^{2+}$ sensitivity is involved in the observed sevoflurane-induced inhibition of contraction. Indeed, the obtained R$_{340/380}$ (Ca$^{2+}$)–force relation was shifted to the right in the presence of sevoflurane (fig. 5C). Despite the observed identical ($P > 0.05$) increases in R$_{340/380}$, the increase in force caused by 10 mM extracellular Ca$^{2+}$ after exposure to 3% sevoflurane was smaller ($P < 0.05$) than that caused by 5 mM extracellular Ca$^{2+}$ before exposure to sevoflurane (fig. 5). Similarly, regardless of the similar ($P > 0.05$) increases in R$_{340/380}$, the increase in force caused by 5 mM extracellular Ca$^{2+}$ after exposure to 5% sevoflurane was smaller ($P < 0.05$) than that produced by 1.5 mM extracellular Ca$^{2+}$ before exposure to sevoflurane (fig. 5). These findings also support the idea that sevoflurane inhibits the myofilament Ca$^{2+}$ sensitivity.

In the norepinephrine-stimulated (0.5 μM), fura-2–loaded strips, the stepwise increment of [Ca$^{2+}$]e (0–10 mM) during K$^+$ depolarization also produced concentration-dependent increases in R$_{340/380}$ and force (fig. 6). The increases in R$_{340/380}$ in response to the stepwise increment of [Ca$^{2+}$]e in the presence of norepinephrine were similar to those in its absence; however, the increases in force evoked by the stepwise increment of [Ca$^{2+}$]e in the presence of norepinephrine were far...
more than those in its absence (fig. 6), indicating that norepinephrine caused the well-recognized increase in myofilament Ca\(^{2+}\) sensitivity.\(^4\) Sevoflurane (5%) again inhibited (\(P < 0.05\)) the increases in R\(_{340/380}\) and force caused by the stepwise increment of [Ca\(^{2+}\)]\(_e\) in the presence of 0.5 \(\mu\)M norepinephrine (fig. 6). The R\(_{340/380}\) (Ca\(^{2+}\)–force relation in the presence of 5% sevoflurane shifted downward and right from that in the absence of sevoflurane (fig. 6C). Despite the obtained identical (\(P > 0.05\)) increases in R\(_{340/380}\), the increases in force caused by 1, 1.5, 5, and 10 mM extracellular Ca\(^{2+}\) after exposure to sevoflurane (5%) were smaller (\(P < 0.05\)) than those caused by 0.5, 1.0, 1.5, and 3.0 mM extracellular Ca\(^{2+}\) before exposure to sevoflurane, respectively (fig. 5), suggesting that sevoflurane inhibits the myofilament Ca\(^{2+}\) sensitivity in the K\(^+\)-depolarized, norepinephrine-stimulated strips.

**Effects of Sevoflurane on Ca\(^{2+}\)–Force Relation in \(\beta\)-escin-membrane–permeabilized Muscle**

In the \(\beta\)-escin–treated muscle strips, the stepwise increment of [Ca\(^{2+}\)] \((0.1-100 \mu\)M) in the bath solution produced concentration-dependent increases in force (fig. 7). Although the Ca\(^{2+}\)–activated "maximal" contraction slightly deteriorated (\(\approx 20\%\)) during three successive Ca\(^{2+}\) applications (figs. 7A and C), the Ca\(^{2+}\)–force relation assessed after the Ca\(^{2+}\)-activated maximal contraction in each Ca\(^{2+}\) application normalized to 100% was well-preserved for the three successive Ca\(^{2+}\) applications with the EC\(_{50}\) values of approximately 2.5 \(\mu\)M (fig. 7C). In addition, the Ca\(^{2+}\)–force relation constructed from the numerical average of the first and third Ca\(^{2+}\) applications was almost perfectly overlapped with that at the second Ca\(^{2+}\) application (fig. 7C). Therefore, the Ca\(^{2+}\)–force relation constructed from the average of the first and third Ca\(^{2+}\) applications was considered as the control for the effect of sevoflurane on the Ca\(^{2+}\) sensitivity in experiments in which sevoflurane was present 15 min before and during the second Ca\(^{2+}\) application (figs. 7B and C). The Ca\(^{2+}\)–force relation in the presence of 5% sevoflurane was not significantly different from the control Ca\(^{2+}\)–force relation (figs. 7B and C). In addition, the Ca\(^{2+}\)–force relation assessed after the normalization of Ca\(^{2+}\)-activated maximal contraction to 100% (i.e., assessed by the EC\(_{50}\) values for the Ca\(^{2+}\)–force relation) was also not significantly influenced by sevoflurane (\(P > 0.05\); \(n = 4\); not shown).
Discussion

The results obtained in the fura-2–loaded muscle indicate that the sevoflurane-induced inhibitions of norepinephrine- and high K+–induced contractions both are caused by suppression of myofilament Ca2+ sensitivity and reduction of [Ca2+]i in VSM cells. Although the low concentration (3%) of sevoflurane caused notable inhibition of force development, its reduction of R340/380 was either relatively small or negligible (figs. 2 and 3). This would suggest that the suppression of myofilament Ca2+ sensitivity predominates over the reduction of [Ca2+]i in the direct vasorelaxation caused by 3% sevoflurane. In contrast, the reduction of R340/380 produced by 5% sevoflurane was also notable, suggesting that the suppression of myofilament Ca2+ sensitivity and reduction of [Ca2+]i both are significantly involved in the direct vasorelaxation caused by 5% sevoflurane.

The ability of sevoflurane to inhibit the norepinephrine-induced increase in R340/380 after depletion of the intracellular Ca2+ stores with ionomycin suggests that the Ca2+-reducing effect of sevoflurane in the presence of norepinephrine is caused, at least in part, by inhibition of the transmembrane Ca2+ influx. The elimination of norepinephrine-induced increases in R340/380 by removal of the low-affinity extracellular bound Ca2+ or by voltage-gated Ca2+ channel (VGCC) blockers in the ionomycin-treated strips further suggest that the norepinephrine-induced Ca2+ influx is caused by activation of the VGCCs. The previously reported threshold concentrations of norepinephrine for membrane depolarization were 0.3–1 μM in small mesenteric arteries.29–31 We believe that norepinephrine, even at the low concentration of 0.5 μM, causes significant membrane depolarization and thereby activates the VGCCs in this artery. Alternatively, the low concentrations of norepinephrine might cause significant changes in some properties of

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the VGCCs themselves (e.g., voltage-sensitivity), thereby enabling them to open at more negative potentials or to have a longer open time. Our idea is consistent with a recent proposal in rat mesenteric arteries that membrane potential–independent, receptor-operated Ca\(^{2+}\) channels play only a minor, if any, role in the transmembrane Ca\(^{2+}\) entry during norepinephrine contractile response.

Depletion of the intracellular Ca\(^{2+}\) stores with ionomycin eliminated the norepinephrine-induced phasic increase in R\(_{340/380}\) whereas it only modestly inhibited the norepinephrine-induced tonic increase in R\(_{340/380}\). In addition, norepinephrine produced only a phasic increase in R\(_{340/380}\) after removal of the extracellular Ca\(^{2+}\). Finally, verapamil had little effect on the norepinephrine-induced phasic increase in R\(_{340/380}\) but strongly inhibited the norepinephrine-induced tonic increase in R\(_{340/380}\). These findings suggest that the norepinephrine-induced phasic response is caused by Ca\(^{2+}\) release from the intracellular stores, whereas its tonic response results from an interplay between Ca\(^{2+}\) influx and Ca\(^{2+}\) release from the intracellular stores. The lack of effect of sevoflurane on the norepinephrine-induced phasic response therefore suggests that sevoflurane does not influence the norepinephrine-induced Ca\(^{2+}\) release from the intracellular stores. This idea was also supported by the observed lack of effect of sevoflurane on the norepinephrine-induced increase in R\(_{340/380}\) after exposure to verapamil. These results also suggest that sevoflurane does not reduce [Ca\(^{2+}\)]i by stimulating transmembrane Ca\(^{2+}\) extrusion or Ca\(^{2+}\) uptake by the intracellular stores. We therefore conclude that the sevoflurane-in-

Fig. 6. Effects of sevoflurane (5%) on increases in R\(_{340/380}\) and force caused by incremental increases in the extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_e\)) during 40-mM K\(^+\) depolarization in the fura-2-loaded, norepinephrine-stimulated (0.5 \(\mu\)M) strips. The protocol used in this experiment was identical to that shown in figure 1A; 5% sevoflurane was applied to the strips for 10 min before and during the cumulative application of various concentrations of Ca\(^{2+}\). (A, B) Analyzed data of the effects of sevoflurane on the increase in either R\(_{340/380}\) (A) or force (B) caused by the incremental increases in [Ca\(^{2+}\)]\(_e\). In these analyses, the maximal increase in either R\(_{340/380}\) or force induced by 40 mM K\(^+\) before exposure to sevoflurane was assumed to be 100%, whereas basal values in normal PSS were assumed to be 0%. *P < 0.05 versus control at each Ca\(^{2+}\) concentration (n = 4). (C) Effects of sevoflurane (5%) on the R\(_{340/380}\)-force relation in the presence of 0.5 \(\mu\)M norepinephrine and effects of norepinephrine (0.5 \(\mu\)M) on the myofilament Ca\(^{2+}\) sensitivity. The R\(_{340/380}\)-force relation in the norepinephrine-stimulated strips either in the absence (control, open circles) or the presence (closed circles) of sevoflurane was constructed from the data shown in A and B. The data were best fitted using the third-order polynomial least-squares regression curves. The "basal" R\(_{340/380}\)-force relation in the norepinephrine-unstimulated strips was shown with Xs; the data of the basal Ca\(^{2+}\) sensitivity were obtained in the experiments shown in figure 5.

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duced reduction of \([\text{Ca}^{2+}]_i\) in norepinephrine-stimulated muscle is mainly caused by inhibition of the \([\text{Ca}^{2+}]_i\) influx through VGCCs.

The depletion of intracellular \([\text{Ca}^{2+}]_i\) stores with ionomycin little influenced the KCl-induced increases in \([\text{Ca}^{2+}]_i\) and force, suggesting that the KCl contraction is exclusively caused by activation of transmembrane \([\text{Ca}^{2+}]_i\) influx. This finding is consistent with our previous observation in this artery that ryanodine did not have any significant effect on the KCl contraction.\(^2\) The sevoflurane-induced reduction of \([\text{Ca}^{2+}]_i\) in the KCl-stimulated muscle seems to be caused by inhibition of the voltage-gated \([\text{Ca}^{2+}]_i\) influx, as in the norepinephrine-stimulated muscle. Halothane and isoflurane both were previously shown to inhibit the whole cell L-type \([\text{Ca}^{2+}]_i\) channel currents in VSM cells.\(^3\) To our knowledge, sevoflurane has not been shown to have such an action; however, the L-type \([\text{Ca}^{2+}]_i\) channels might be a target of the volatile anesthetics. Although the exact mechanisms by which those anesthetics inhibit the L-type \([\text{Ca}^{2+}]_i\) channel activity also do not appear to have been clarified, the anesthetics may indirectly inhibit the L-type \([\text{Ca}^{2+}]_i\) channel activity by increasing cyclic nucleotide levels in VSM cells. Increases in cyclic \(3',5'\)-adenosine monophosphate

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**Fig. 7.** Effects of sevoflurane on \([\text{Ca}^{2+}]_i\)-force relation in the \(\beta\)-escin membrane–permeabilized strips. (A) A typical example of changes in \([\text{Ca}^{2+}]_i\)-force relation (i.e., myofilament \([\text{Ca}^{2+}]_i\) sensitivity) progressively (i.e., time control data). Note the slight rundown of the \([\text{Ca}^{2+}]_i\)-induced maximal contraction during the three successive \([\text{Ca}^{2+}]_i\) applications (1–3); the analyzed data were shown in C (a–c). (B) A typical example of the effect of 5% sevoflurane on the \([\text{Ca}^{2+}]_i\)-force relation. Note that sevoflurane little influences the myofilament \([\text{Ca}^{2+}]_i\) sensitivity under the \(\beta\)-escin–permeabilized condition; the analyzed data were shown in C (d). (C) a) Comparison of the \([\text{Ca}^{2+}]_i\)-force relation among the three successive \([\text{Ca}^{2+}]_i\) applications (1–3). In this analysis, the amplitude of the \([\text{Ca}^{2+}]_i\)-activated maximal contraction at the first \([\text{Ca}^{2+}]_i\) application (1) was assumed to be 100% (n = 4). a) Comparison of the myofilament \([\text{Ca}^{2+}]_i\) sensitivity assessed after normalization of the \([\text{Ca}^{2+}]_i\)-activated maximal contraction in each \([\text{Ca}^{2+}]_i\) application to 100% among the three successive \([\text{Ca}^{2+}]_i\) applications (1–3). (C) b) Comparison of the \([\text{Ca}^{2+}]_i\)–force relation constructed from the average of the first (1) and third (3) \([\text{Ca}^{2+}]_i\) applications with that at the second \([\text{Ca}^{2+}]_i\) application. Also in this analysis, as in C (a), the amplitude of the \([\text{Ca}^{2+}]_i\)-activated maximal contraction at the first \([\text{Ca}^{2+}]_i\) application (1) was assumed to be 100%. (C) d) Comparison of the \([\text{Ca}^{2+}]_i\)-force relation in the presence of 5% sevoflurane, with the control \([\text{Ca}^{2+}]_i\)-force relation constructed from the average of the preanesthetic (C1) and postanesthetic (C2) values. Sevoflurane was applied for 15 min before and during subsequent application of various concentrations of \([\text{Ca}^{2+}]_i\). The amplitude of the \([\text{Ca}^{2+}]_i\)-activated maximal contraction at the first \([\text{Ca}^{2+}]_i\) application (C1; preanesthetic) was assumed to be 100% in this analysis (n = 4). C1 = preanesthetic control, C2 = postanesthetic control.
and cyclic 3′,5′-guanosine monophosphate levels have been proposed to inhibit the L-type Ca\(^{2+}\) channel activity in VSM cells\(^{34,35}\); and halothane and isoflurane both were previously shown to increase basal levels of these cyclic nucleotides in rat aorta\(^{36,35}\). However, sevoflurane did not increase the basal cyclic guanosine monophosphate level in the rat aorta\(^{38}\). Because small-resistance arteries are different from large-conductance arteries in many physiologic and pharmacologic properties\(^{39,40}\), the anesthetic effects on the cyclic nucleotide levels should be further investigated in the resistance arteries.

Despite the lack of effect on the Ca\(^{2+}\)-force relation in the β-escin-permeabilized muscle, sevoflurane shifted the R\(_{340/380}\) ([Ca\(^{2+}\)])–force relation to the right in the fura-2–loaded membrane-intact muscle in either the presence or the absence of norepinephrine stimulation. We therefore suggest that the sevoflurane-induced inhibition of the myofilament Ca\(^{2+}\) sensitivity observed only in the membrane-intact muscle is mediated by the intact plasma membrane or caused by an effect on some intracellular regulatory mechanisms of contractile proteins that are impaired in the β-escin-permeabilized strips. Because α-adrenergic receptor coupling was not retained in our β-escin-permeabilized strips,\(^{13,22}\) we could not specifically evaluate the effects of sevoflurane on the norepinephrine-induced increases in myofilament Ca\(^{2+}\) sensitivity. Although sevoflurane inhibited the myofilament Ca\(^{2+}\) sensitivity both in the presence and the absence of norepinephrine, it is thus unclear whether sevoflurane inhibits only the Ca\(^{2+}\)-activation of contractile proteins (i.e., basal Ca\(^{2+}\) sensitivity of contractile proteins) or inhibits both the basal Ca\(^{2+}\) sensitivity and the norepinephrine-induced Ca\(^{2+}\) sensitizing mechanisms. Additional studies, such as studies of α-toxin-membrane–permeabilized VSM in which the receptor G-protein coupling is retained, are needed to clarify this issue.

More than half, possibly as much as 85%, of fura-2 molecules in myoplasma appear to be in a protein-bound form.\(^{23}\) Such binding of fura-2 to intracellular proteins may influence functional integrity of the VSM cells, thereby altering their responsiveness or sensitivity to anesthetics. However, no significant differences were found in the steady state effects of sevoflurane on contractile responses to either norepinephrine or KCl between the fura-2–loaded and –nonloaded strips (according to comparison between our data from the fura-2–nonloaded strips presented in fig. 1 of the article by Izumi et al.\(^{1}\) in this issue of ANESTHESIOLOGY and the current data [by Student \(t\) test]). Therefore, the fura-2 loading, a nonphysiologic intervention as well, does not appear to significantly influence the action of sevoflurane on VSM cells.

In conclusion, the sevoflurane-induced inhibition of KCl- and norepinephrine-induced contractions both are probably caused by reduction of [Ca\(^{2+}\)]\(_i\) and suppression of the myofilament Ca\(^{2+}\) sensitivity of VSM cells. The [Ca\(^{2+}\)]\(_i\)-reducing action observed in both the norepinephrine- and the KCl-stimulated muscle is largely caused by inhibition of transmembrane Ca\(^{2+}\) influx through VGCCs, and sevoflurane does not seem to significantly influence the norepinephrine-induced, presumably inositol 1,4,5-triphosphate–induced, Ca\(^{2+}\) release from the intracellular stores. The inhibitory action of sevoflurane on Ca\(^{2+}\)-activation of contractile proteins seems to be mediated by some cell membrane-associated or intracellular regulatory mechanism of contraction that are impaired as a result of the membrane permeabilization.

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