Comparison of Volatile Anesthetic Actions on Intracellular Calcium Stores of Vascular Smooth Muscle

Investigation in Isolated Systemic Resistance Arteries

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Background: Volatile anesthetic actions on intracellular Ca^{2+} stores (*i.e.*, sarcoplasmic reticulum [SR]) of vascular smooth muscle have not been fully elucidated.

Methods: Using isometric force recording method and fura-2 fluorometry, the actions of four volatile anesthetics on SR were studied in isolated endothelium-denuded rat mesenteric arteries.

Results: Halothane ($\geq$ 3%) and enflurane ($\geq$ 3%), but not isoflurane and sevoflurane, increased the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in Ca^{2+}-free solution. These Ca^{2+}-releasing actions were eliminated by procaine. When each anesthetic was applied during Ca^{2+} loading, halothane ($\geq$ 3%) and enflurane (5%), but not isoflurane and sevoflurane, decreased the amount of Ca^{2+} in the SR. However, if halothane or enflurane was applied with procaine during Ca^{2+} loading, both anesthetics increased the amount of Ca^{2+} in the SR. The caffeine-induced increase in $[Ca^{2+}]_i$ was enhanced in the presence of halothane ($\geq$ 1%), enflurane ($\geq$ 1%), and isoflurane ($\geq$ 3%) but was attenuated in the presence of sevoflurane ($\geq$ 3%). The norepinephrine-induced increase in $[Ca^{2+}]_i$ was enhanced only in the presence of sevoflurane ($\geq$ 3%). Not all of these anesthetic effects on the $[Ca^{2+}]_i$ were parallel with the simultaneously observed anesthetic effects on the force.

Conclusions: In systemic resistance arteries, the halothane, enflurane, isoflurane, and sevoflurane differentially influence the SR functions. Both halothane and enflurane cause Ca^{2+} release from the caffeine-sensitive SR. In addition, both anesthetics appear to have a stimulating action on Ca^{2+} uptake in addition to the Ca^{2+}-releasing action. Halothane, enflurane, and isoflurane all enhance, while sevoflurane attenuates, the Ca^{2+}-induced Ca^{2+}-release mechanism. However, only sevoflurane stimulates the inositol 1,4,5-triphosphate–induced Ca^{2+} release mechanism. Isoflurane and sevoflurane do not stimulate Ca^{2+} release or influence Ca^{2+} uptake.

IN vascular smooth muscle (VSM), Ca^{2+} is stored intracellularly in sarcoplasmic reticulum (SR), which contains at least two types of Ca^{2+}-release channels: inositol 1,4,5-triphosphate (IP_3) receptor–Ca^{2+}-release channel and ryanodine receptor (RyR)–Ca^{2+}-release channel.[1,2] The IP_3-receptor channel is believed to play a primary physiologic role in Ca^{2+} mobilization.[3] The Ca^{2+}-induced Ca^{2+} release (CICR) may occur through the RyR channel, although its physiologic role is not fully understood.[1,2,4,5] However, it is generally agreed that the SR plays a pivotal role in the regulation of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, a major determinant of vascular tone, not only as a supply of the activator Ca^{2+} but also as a buffer against VSM activation by Ca^{2+}.[3,6]

Evidence is accumulating that volatile anesthetics at clinical concentrations impair functional integrity of the SR and thereby alter vascular homeostasis.[7–16] Halothane, enflurane, or isoflurane appeared to stimulate Ca^{2+} release or leakage from either ryanodine-sensitive[9–18] or IP_3-sensitive SR,[17,19,20] causing transient or sustained increases in vascular tone. The anesthetic-induced Ca^{2+} release from ryanodine-sensitive SR may be caused by activation of the RyR–Ca^{2+}-release channels.[7,8,14,16] In addition, these anesthetics may inhibit Ca^{2+} uptake by the SR, decreasing the amount of Ca^{2+} in SR (*i.e.*, Ca^{2+} availability for VSM contraction).[7,8,12,14–16] In cultured VSM cells, halothane and isoflurane appeared to attenuate inositol phosphate formation and thereby inhibit the IP_3-induced Ca^{2+} release.[19,20] Furthermore, halothane was recently shown to have a stimulating action on the Ca^{2+} uptake in addition to its Ca^{2+}-releasing action.[12]

Previous studies have investigated volatile anesthetic actions on the SR of VSM by measuring changes in either force or $[Ca^{2+}]_i$.[7–9,12–17] However, it is not always straightforward to interpret results from the force experiments, in which changes in force do not necessarily indicate changes in the $[Ca^{2+}]_i$, because of the possible anesthetic effects on myofilament Ca^{2+} sensitivity and the nonlinear relation between force and $[Ca^{2+}]_i$. Some investigators have already examined the anesthetic actions on the SR of conduit arteries by measuring the $[Ca^{2+}]_i$.[9,13,16,17] However, the anesthetic actions on SR in small arteries, the major sites of regulation of vascular resistance and thus of tissue perfusion and of arterial blood pressure, have not yet been studied in the $[Ca^{2+}]_i$ measurement experiments. In addition, less is known about the action of sevoflurane on the SR.[15] We thus investigated the actions of volatile anesthetics, including

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sevoflurane, on the SR of VSM in isolated systemic resistance arteries, using fura-2 fluorometry.

Materials and Methods

Force and Ca^{2+} Measurements

With approval from the Kyushu University Animal Care and Use Committee (Fukuoka, Japan), an endothelium-denuded strip was prepared from the third-order branch of male Sprague-Dawley rat (250–350 g) mesenteric artery ($\approx$ 150–200 μm in diameter), and isometric force was measured by attaching the strip to a strain gauge transducer as previously detailed.[11,21] Briefly, the strip was horizontally mounted 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. Functional removal of endothelium was verified by lack of acetylcholine (10 μM)-induced vasorelaxation, as previously described.[22]

In most of the experiments, changes in $[Ca^{2+}]_i$ were measured simultaneously with those in force, using fura-2, a fluorescent Ca^{2+}-indicator dye.[23] Our method on the fura-2 fluorometry was also previously detailed.[24,25] Briefly, to enable loading of the fura-2 into the VSM cells, the strips were incubated in normal physiologic salt solution (PSS) containing 10 μM acetoxymethyl 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.[24,25] Changes in the fluorescence intensity of the fura-2–Ca^{2+} complex were measured by 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 VSM layers, and the 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 and 380 nm were collected through the 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 Triton-X-100 (1%) and subsequently quenching the fura-2 fluorescence signals with $MnCl_2$ (20 mM) as reported previously.[25–27] The ratio ($R_{340/380}$) 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.

None of the agents used during Ca^{2+} measurements, except procaine, influenced the fluorescence signals. High concentrations ($\geq$ 3 mM) of procaine, as previously reported in fluo-3-fluorometry,[28] quenched both the F_{340} and F_{380} signals and thereby decreased the $R_{340/380}$ in fura-2 (2 μM)-containing Ca^{2+} free solution or normal PSS (regardless of the presence of the strips). Therefore, the changes in $R_{340/380}$ during application of the high concentrations of procaine were not evaluated. In all of the measurements except those with procaine, as we recently showed,[25] changes in F_{340} and F_{380} were constantly in opposite directions. Therefore, the observed changes in F_{340} and F_{380} would reflect changes in the $[Ca^{2+}]_i$ but not motion artifacts. All experiments with the fura-2–loaded strips were conducted during the period in which constant vascular responses were obtained, i.e., for approximately 3 h.[25]

Solutions and Drugs

The ionic concentrations of HEPES-buffered PSS were as follows: 138 mM NaCl, 5.0 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose. The pH was adjusted with NaOH to 7.35 at 35°C. The Ca^{2+}-free solution was prepared by removing $CaCl_2$ with or without adding 2 mM EGTA.

HEPES, norepinephrine, acetylcholine, and albumin (bovine) were obtained from Sigma Chemical Co. (St. Louis, MO). Caffeine and EGTA were obtained from Nacalai Tesque (Kyoto, Japan). Fura-2–AM was purchased from Dojindo Laboratories (Kumamoto, Japan). Ryanodine was purchased from Agri Systems International (Wind Gap, PA). Halothane and sevoflurane were obtained from Takeda Pharmaceutical Co. (Osaka, Japan) and Kodama Pharmaceutical Co. (Osaka, Japan), respectively. Enflurane and isoflurane were obtained from Dainihon Pharmaceutical Co. (Osaka, Japan). All other reagents were of the highest grade commercially available.

Experimental Design

The effects of halothane, enflurane, isoflurane, and sevoflurane at 1–5% on the SR were examined in both fura-2–nonloaded and –loaded strips, in the latter of which isometric force and $[Ca^{2+}]_i$ were simultaneously measured. All experiments were conducted at 35°C to prevent early deterioration of the strips, as previously described.[11,21]

We first characterized the SR of this artery by examining the effects of ryanodine on increases in $R_{340/380}$ caused by either caffeine or norepinephrine in the Ca^{2+}-free solution. We also investigated this issue by consecutively applying caffeine and norepinephrine in the Ca^{2+}-free solution in the fura-2–loaded strips.

We next examined the ability of those anesthetics to cause Ca^{2+} release from the SR in both the fura-2–

nonloaded and –loaded strips. In this series of experiments, after loading the SR with Ca^{2+} by incubating the strips in PSS (1.5 mM Ca^{2+}) for 8 min, each anesthetic and caffeine were consecutively applied in the Ca^{2+}-free solution at a 5-min interval. In some experiments, procaine, previously suggested to inhibit CICR,[4,28,29] was applied before and during application of either halothane or enflurane.

We then examined the effects of those anesthetics on Ca^{2+} uptake by the SR. For these experiments, after depletion of the SR by caffeine, each anesthetic was applied during Ca^{2+} loading (1.5 mM Ca^{2+}, 8 min), and the amount of Ca^{2+} in SR was estimated from an increase in either force or $[Ca^{2+}]_i$ caused by caffeine 2 min after removal of extracellular Ca^{2+}. In some experiments, halothane or enflurane was applied during Ca^{2+} loading with or without procaine, which inhibited the Ca^{2+} releasing action of these anesthetics in the aforementioned experiments.

We finally examined the actions of those anesthetics on either CICR or IP_3-induced Ca^{2+} release (IICR) mechanism by examining the effects of each anesthetic on an increase in $[Ca^{2+}]_i$ evoked by either caffeine or norepinephrine; after the Ca^{2+} loading, each anesthetic was applied for 5 min before and during the application of either caffeine or norepinephrine in the Ca^{2+}-free solution.

Volatile Anesthetic Delivery and Analysis

Volatile anesthetics were delivered *via* calibrated agent-specific vaporizers in line with the air gas aerating the solutions. Each solution was equilibrated with each anesthetic for at least 15 min before introduction to the chamber, which was covered with a thin glass plate to prevent the equilibration gas from escaping into the atmosphere. Using gas chromatography, we previously reported concentrations of each anesthetic in the PSS produced by multiple concentrations of each anesthetic during the same experimental condition; the obtained values were within 90% of theoretical values predicted by the partition coefficient of each anesthetic in Krebs solution or water.[11,30] An excellent linear relation was obtained between the aqueous concentrations of each anesthetic (y) and its concentrations (volume percent) in the gas mixture (x): halothane, y = 0.0033 + 0.30x, r = 0.999; enflurane, y = 0.0019 + 0.29x, r = 0.997; isoflurane, y = −0.0068 + 0.21x, r = 0.998; sevoflurane, y = 0.0028 + 0.13x, r = 0.999.[11,30] Therefore, the concentrations produced by 1–5% halothane, enflurane, isoflurane, and sevoflurane in the PSS can be predicted as 0.3–1.5, 0.29–1.45, 0.21–1.05, and 0.13–0.67 mM, respectively. Recently reported concentrations of halothane, isoflurane, and sevoflurane in blood sampled from this rat during steady state anesthesia with 1 minimum alveolar concentration of each anesthetic (1.0, 1.5, and 2.8% for halothane, isoflurane, and sevoflurane, respec-

tively[31,32]) were 0.70, 0.65, and 0.66 mM, respectively.[33] Therefore, the aqueous concentrations of these three anesthetics examined in our study can be considered as clinical concentrations. To our knowledge, no information is available regarding the concentration of enflurane in blood sampled from this rat during steady state anesthesia with enflurane. However, the theoretical blood concentration of enflurane in this rat during anesthesia with 1 minimum alveolar concentration (2.45%[34]) enflurane calculated using the blood–gas partition coefficient (1.91) is approximately 1.84 mM, which is larger than the concentration produced by 5% enflurane in the PSS in this study, *i.e.*, 1.45 mM. Therefore, the concentrations produced by 3–5% enflurane in the PSS in this study also can be considered as clinical concentrations.

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*,[23] the dissociation constant of fura-2 for Ca^{2+} binding in cytoplasm appears to be significantly different from that measured in the absence of protein because more than half of the fura-2 molecules in cytoplasm are protein-bound.[35] Therefore, the ratio of F_{340} to F_{380} ($R_{340/380}$), calculated after subtracting the background fluorescence, was used as an indicator of $[Ca^{2+}]_i$, as previously described.[24–27]

The concentration–response data for the anesthetic-induced increase in either force or $R_{340/380}$ were fitted according to a logistic model described by De Lean *et al.*,[36] and the EC_{50} values were derived from the least-squares fit using the aforementioned model. Because the relation between actual concentrations of volatile anesthetics in the solutions and anesthetic concentrations (volume percent) in the gas mixture is theoretically linear, the anesthetic concentrations on the x-axis are displayed as volume percent for the anesthetic concentration–response relations as previously described.[11,12,25]

Statistics

All results are expressed as the mean ± SD. The n denotes the number of preparations (= the number of animals). Data were analyzed using analysis of variance, the Scheffé F test, and the Student *t* test. Comparisons among groups were performed by two-factor analysis of variance for repeated measures. When overall differences were detected, individual comparisons among groups at each concentration were performed by the Scheffé F test. Comparisons within each group were made by one-factor 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 by the Student *t* test. $P < 0.05$ was considered significant.

Results

Characterization of the Sarcoplasmic Reticulum Ca^{2+} Stores

Caffeine and norepinephrine both produced transient increases in $R_{340/380}$ and force in the Ca^{2+}-free solution in the fura-2–loaded strips. The maximal increases in $R_{340/380}$ and force caused by caffeine (20 mM, maximum) were 158.3 ± 13.2% and 65.5 ± 7.2%, respectively, of that caused by KCl (40 mM) in normal PSS (n = 4). The maximal increases in $R_{340/380}$ and force caused by norepinephrine (10 μM, maximum) were 136.9 ± 19.5% and 109.1 ± 18.1%, respectively, of that caused by KCl (40 mM) in normal PSS (n = 4). Treatment with ryanodine (10 μM, 20 min) consistently eliminated these increases in $R_{340/380}$ and force caused by either caffeine or norepinephrine in the Ca^{2+}-free solution (n = 4). In addition, when caffeine (20 mM) and norepinephrine (10 μM) were consecutively applied in the Ca^{2+}-free solution, caffeine consistently eliminated the increases in $R_{340/380}$ and force caused by subsequently applied norepinephrine (n = 4).

Ability of Volatile Anesthetics to Stimulate Ca^{2+} Release from the Sarcoplasmic Reticulum

When each anesthetic and caffeine were consecutively applied in the Ca^{2+}-free solution in the fura-2–nonloaded strips, both halothane and enflurane, but not isoflurane and sevoflurane, caused transient contractions and inhibited the contractions caused by subsequently applied caffeine (fig. 1). Similarly, when each anesthetic and caffeine were consecutively applied in the Ca^{2+}-free solution in the fura-2–loaded strips, both halothane and enflurane, but not isoflurane and sevoflurane, caused transient increases in $R_{340/380}$ and force and inhibited increases in $R_{340/380}$ and force caused by subsequently applied caffeine (fig. 2). In some strips, 5% isoflurane caused a very small increase in $R_{340/380}$ in the Ca^{2+}-free solution (fig. 2); however, the effect was not statistically significant. No significant differences were observed in the vasoconstricting actions of both halothane and enflurane at 3 and 5% between the fura-2–nonloaded and –loaded strips. Significant differences were observed in the vasoconstricting effects in both the fura-2–nonloaded and –loaded strips and also in the Ca^{2+}-releasing effects in the fura-2–loaded strips between halothane and enflurane, and the order of potency on all of these actions was halothane > enflurane (> isoflurane, sevoflurane) (figs. 1 and 2).

When procaine was applied before and during application of halothane (5%) or enflurane (5%) in the experiments in which each anesthetic and caffeine were consecutively applied in the Ca^{2+}-free solution in either the fura-2–nonloaded or –loaded strips, the increases in either force or $R_{340/380}$ induced by each anesthetic were inhibited strongly or eliminated, as were the anesthetic
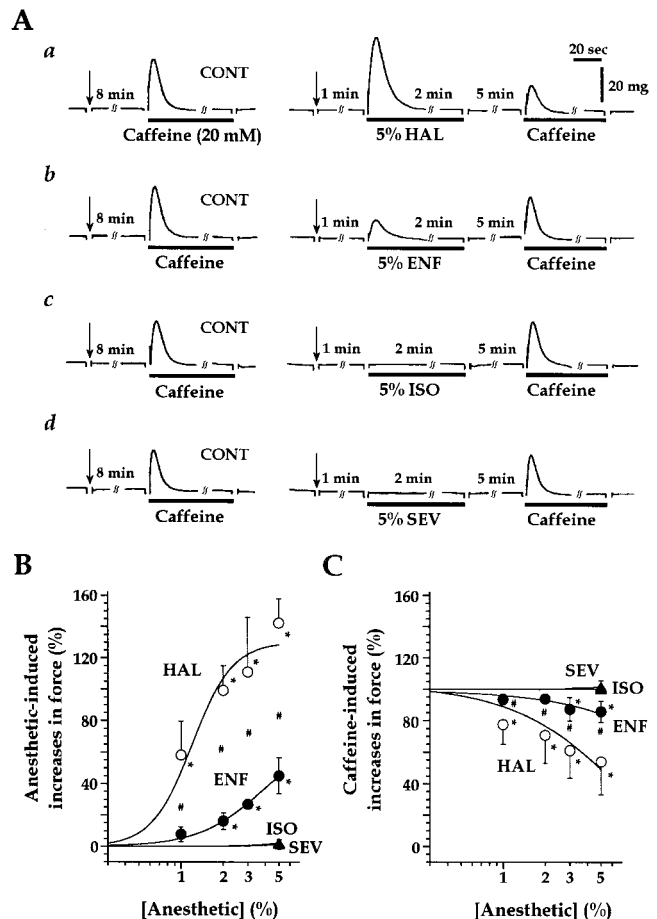
Fig. 1. Contractions in the fura-2–nonloaded strips produced by consecutive applications of either halothane (HAL), enflurane (ENF), isoflurane (ISO), or sevoflurane (SEV) and caffeine in the Ca^{2+}-free solution (n = 4). (A) Examples. (Left) Control caffeine contractions (CONT). (Right) Anesthetic effects. Arrows indicate the time points when the extracellular Ca^{2+} was removed after the Ca^{2+} load. (B and C) Concentration–response data for the consecutive applications of each anesthetic (B) and caffeine (C). The contractions induced by each anesthetic or caffeine were normalized to the control caffeine contraction (100%). The EC_{50} values for the halothane and enflurane contractions were 1.20 and 3.88%, respectively. *$P < 0.05$ versus control within each group. #$P < 0.05$ between halothane and enflurane groups at each concentration.

inhibitions of the increases in either force or $R_{340/380}$ induced by subsequently applied caffeine (fig. 3). The halothane contractions were consistently eliminated by procaine (10 mM) in both the fura-2–nonloaded (n = 4) and –loaded (n = 5) strips (fig. 3). The amplitude of caffeine contraction after previous treatment with halothane + procaine in the fura-2–nonloaded strips was 101.9 ± 7.46% ($P > 0.05$, n = 4) of control; the caffeine-induced increases in $R_{340/380}$ and force after previous treatment with halothane + procaine in the fura-2–loaded strips were 92.7 ± 5.3% ($P < 0.05$, n = 5) and 98.3 ± 13.7% ($P > 0.05$, n = 5), respectively, of control. The enflurane-induced increases in $R_{340/380}$ and force in the presence of procaine (1 mM) in the fura-2–loaded strips were 19.5 ± 14.4% ($P < 0.05$, n = 5) and 5.7 ±
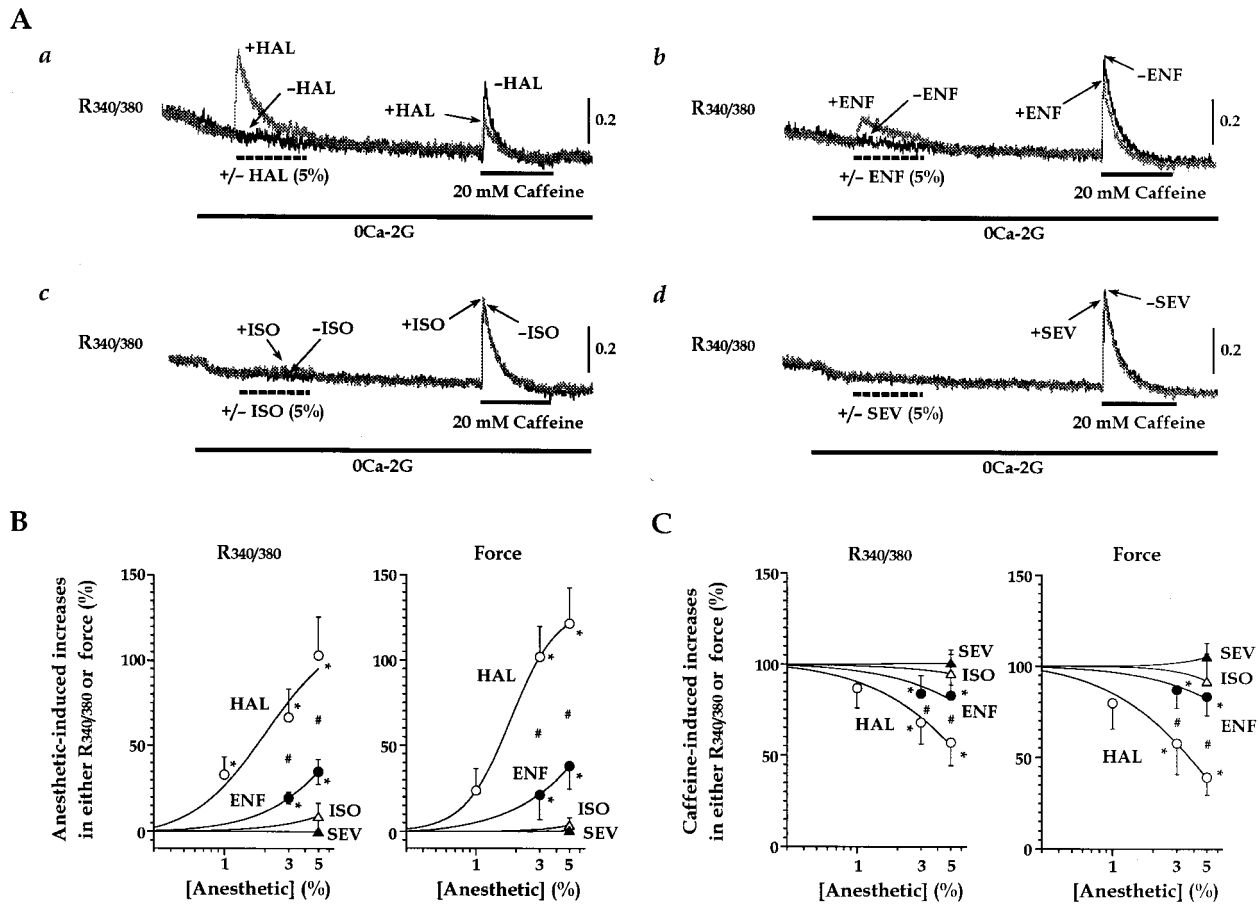
Fig. 2. Changes in $R_{340/380}$ and force in the fura-2–loaded strips produced by consecutive applications of either halothane (HAL), enflurane (ENF), isoflurane (ISO), or sevoflurane (SEV) and caffeine in the Ca^{2+}-free solution (n = 5 or 6). (*A*) Examples: the responses with (+) or without (−) (control) the previous treatment with each anesthetic (5%) are depicted with gray and black lines, respectively. Because the changes in force observed in this experiment were identical to those shown in the figure 1, only the changes in $R_{340/380}$ are shown. 0Ca-2G = Ca^{2+}-free, 2-mM EGTA solution. Arrows indicate the peaks of responses. (*B* and *C*) Concentration–response data for the consecutive applications of each anesthetic (*B*) and caffeine (*C*). The increases in $R_{340/380}$ and force induced by each anesthetic or caffeine were normalized to control (no anesthetic treatment) caffeine-induced increases in $R_{340/380}$ and force, respectively. The EC_{50} values for the halothane-induced increases in $R_{340/380}$ and force were 1.82 and 2.06%, respectively. *P < 0.05 *versus* control within each group. #P < 0.05 between halothane and enflurane groups at each concentration.

12.8% (P < 0.05, n = 5), respectively, of control, while the caffeine-induced increases in $R_{340/380}$ and force after previous treatment with enflurane + procaine (1 mM) were 94.3 ± 9.3% (P > 0.05, n = 5) and 103.1 ± 11.4% (P > 0.05, n = 5), respectively, of control.

Effects of Volatile Anesthetics on Ca^{2+} Uptake by the Sarcoplasmic Reticulum

Halothane (≥ 3%), applied during Ca^{2+} loading, inhibited the caffeine-induced increases in $R_{340/380}$ and force in either the fura-2–nonloaded or –loaded strips, indicating a decrease in amount of Ca^{2+} in the SR (fig. 4). Enflurane (5%), applied during Ca^{2+} loading, also inhibited the caffeine-induced increases in $R_{340/380}$ in the fura-2–loaded strips but did not inhibit the caffeine-induced increases in force in either the fura-2–nonloaded or –loaded strips (fig. 4). Neither isoflurane (5%) nor sevoflurane (5%), applied during Ca^{2+} loading, influenced the caffeine-induced increases in $R_{340/380}$ and

force in either the fura-2–nonloaded or –loaded strips (fig. 4).

Both halothane (≥ 3%) and enflurane (5%), applied during Ca^{2+} loading with procaine, enhanced the caffeine-induced increases in $R_{340/380}$ and force in either the fura-2–loaded or –nonloaded strips (fig. 4). In control experiments, procaine (1 and 10 mM), applied during the Ca^{2+} loading phase, did not influence the caffeine-induced increases in $R_{340/380}$ and force in either the fura-2–nonloaded or –loaded strips (fig. 4); the caffeine-induced increases in force after treatment with 1 and 10 mM procaine in the fura-2–nonloaded strips were 104.1 ± 3.7% and 99.5 ± 5.2%, respectively, of control (P > 0.05, n = 4).

Effects of Volatile Anesthetics on Caffeine- or Norepinephrine-induced Ca^{2+} Release Mechanisms

Halothane (≥ 1%), enflurane (≥ 1%) and isoflurane (≥ 3%) all enhanced, although only sevoflurane (≥ 3%)
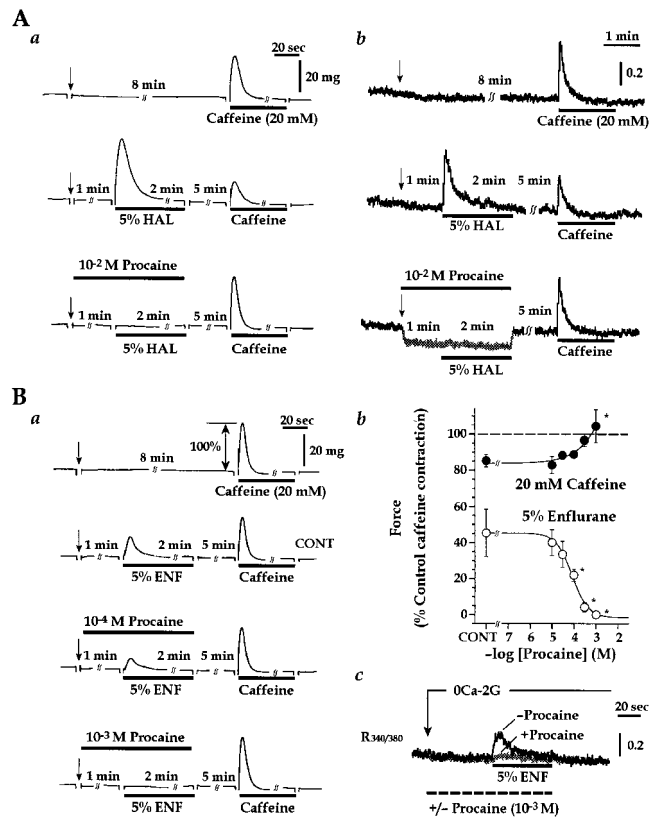
Fig. 3. Effects of procaine applied with either halothane (HAL, *A*) or enflurane (ENF, *B*) on responses to consecutively applied each anesthetic and caffeine (20 mM) in the Ca^{2+}-free solution. (*A-a*) An example of the effect of procaine on contractions induced by consecutive application of halothane and caffeine in the fura-2–nonloaded strips. Identical results were obtained in the fura-2–loaded strips (not shown). (*A-b*) A typical example of the effect of procaine on changes in $R_{340/380}$ induced by consecutive application of halothane and caffeine in the fura-2–loaded strips. The changes in $R_{340/380}$ during application of procaine do not reflect those in the $[Ca^{2+}]_i$ because of its quenching effect on the fluorescence (see Methods). (*B-a, b*) Examples (*a*) and the analyzed data (*b*; n = 3) on the effects of procaine (applied with enflurane) on contractions induced by the consecutive application of enflurane and caffeine in the fura-2–nonloaded strips. The contractions induced by enflurane (open circles) or caffeine (closed circles) were normalized to a control caffeine-induced contraction (100%; see the uppermost trace of *B-a*). CONT = control (no procaine) responses to the consecutive application of enflurane and caffeine. The IC_{50} value for the procaine-induced inhibition of the enflurane contraction was 82.1 μM. *$P < 0.05$ *versus* control within each group. (*B-c*) An example of the inhibition by procaine of the enflurane-induced increase in $R_{340/380}$ in the fura-2–loaded strips. +/− procaine = in the presence (+, gray) or absence (−, black) of procaine. All arrows indicate the time points when the extracellular Ca^{2+} was removed after the Ca^{2+} load. 0Ca-2G = Ca^{2+}-free, 2-mM EGTA solution.

attenuated, the caffeine-induced increase in $R_{340/380}$ and force (fig. 5).

The four anesthetics influenced the norepinephrine response variously. Halothane ($\geq$ 3%) inhibited the norepinephrine-induced increases in both $R_{340/380}$ and force (fig. 6). However, enflurane did not influence the norepinephrine-induced increases in both $R_{340/380}$ and force (fig. 6). Isoflurane ($\geq$ 3%) enhanced only the norepi-

nephrine-induced increase in force but not its increase in $R_{340/380}$ (fig. 6). Sevoflurane ($\geq$ 3%) enhanced the norepinephrine-induced increases in both $R_{340/380}$ and force (fig. 6).

Discussion

Using fura-2 fluorometry, this study provides direct evidence that halothane, enflurane, isoflurane, and sevoflurane at clinical concentrations differentially influence the SR functions in isolated systemic resistance arteries. Presumably because of the volatile anesthetic effects on myofilament Ca^{2+} sensitivity and the nonlinear relation between $[Ca^{2+}]_i$ and force, not all of the observed anesthetic effects on $R_{340/380}$ ($[Ca^{2+}]_i$) were parallel with the simultaneously observed anesthetic effects on force, as specifically discussed below.

The Ca^{2+}-releasing Action of Volatile Anesthetics

Results from the experiments in which each anesthetic and caffeine were consecutively applied in the Ca^{2+}-free solution indicate that both halothane and enflurane stimulate Ca^{2+} release from the ryanodine–caffeine-sensitive SR, as previously suggested in both conduit and resistance arteries.[8–12,15,16,18] This study confirms the procaine sensitivity of the Ca^{2+}-releasing action of halothane that we previously proposed based on results from contraction experiments.[12] In addition, this study, for the first time, demonstrates that the Ca^{2+}-releasing action of enflurane is also sensitive to procaine. Neither isoflurane nor sevoflurane caused any significant increases in $R_{340/380}$ in the Ca^{2+}-free solution, indicating that both anesthetics (5%, 35°C) do not stimulate Ca^{2+} release from the SR. However, previous experiments performed at 22–23°C[11,14] showed that high concentrations ($\geq$ 2%) of isoflurane cause ryanodine-sensitive, endothelium-independent contractions. Isoflurane may stimulate Ca^{2+} release from the ryanodine-sensitive SR if its aqueous concentration is high or the tissue temperature is low.

Previous studies suggested that halothane and isoflurane may stimulate Ca^{2+} leakage from the IP_3-sensitive SR.[17,19,37] As the norepinephrine-induced increases in $R_{340/380}$ in the Ca^{2+}-free solution were eliminated by previous treatment with either ryanodine or caffeine, the IP_3-sensitive SR appears to be overlapped with the ryanodine-sensitive SR in this artery, consistent with previous studies suggesting a structural overlap between the both types of SR in smooth muscle.[2,38] Therefore, the anesthetic effects on the ryanodine–caffeine-sensitive SR are undistinguishable from those on the norepinephrine–IP_3-sensitive SR in this study.

In our previous experiments,[12] procaine (in millimolar concentrations), proposed to inhibit the CICR,[4,28,29] also
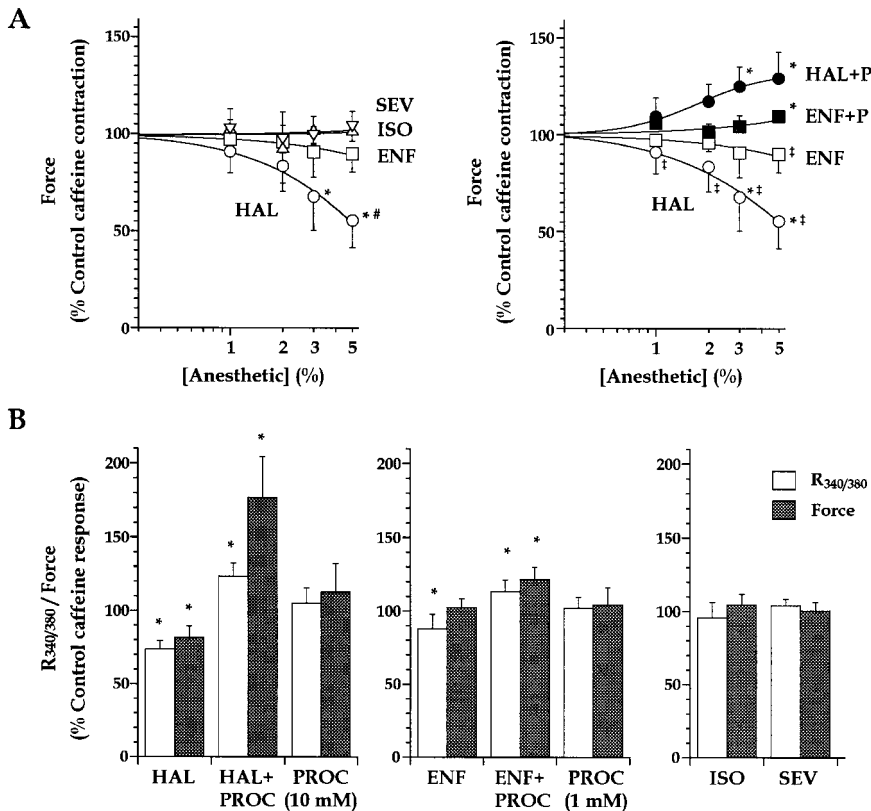
A

B

Fig. 4. Effects of volatile anesthetics on Ca^{2+} uptake by SR. (*A*) Effects of halothane (HAL), enflurane (ENF), isoflurane (ISO), and sevoflurane (SEV), applied during the Ca^{2+} loading, on the caffeine (20 mM)-induced contraction, an estimate for the amount of stored Ca^{2+}, in either absence (*left*) or presence (*right*) of procaine (P; 10 mM for halothane, 1 mM for enflurane) in the fura-2–nonloaded strips (n = 4). *$P < 0.05$ *versus* control (100%) within each group. #$P < 0.05$ *versus* the enflurane group at each concentration. ‡$P < 0.05$ *versus* the procaine-treated group at each concentration in each anesthetic group. (*B*) Effects of halothane (HAL), enflurane (ENF), isoflurane (ISO), and sevoflurane (SEV), applied during Ca^{2+} loading, on the caffeine (20 mM)-induced increases in $R_{340/380}$ (open column) and force (gray column), estimates for the amount of stored Ca^{2+}, in either absence or presence of procaine (PROC; 10 mM for halothane, 1 mM for enflurane) in the fura-2–loaded strips. Effects of procaine alone, applied during Ca^{2+} loading phase, on the caffeine response are also shown (n = 4 or 5). *$P < 0.05$ *versus* control (100%).

inhibited the Ca^{2+}-releasing actions of phenylephrine and IP_3, suggesting that procaine is a nonspecific inhibitor of Ca^{2+} release. Therefore, we cannot discuss the mechanisms of the Ca^{2+}-releasing action of halothane or enflurane from their procaine sensitivity. Su et al.[7,8,14,16] previously showed that, in isolated conduit arteries, halothane, enflurane, and isoflurane all facilitated the ryanodine depletion of the SR. As ryanodine depletes the SR by binding to the RyR–CICR channels in an open state and then locking them open,[1] these anesthetics may enhance opening of the RyR–CICR channels.[7,8,14,16] Therefore, the ability of these anesthetics to release Ca^{2+} from the ryanodine-sensitive SR, universally observed in both conduit and resistance arteries,[8–12,15,16,18] may be a result of activation of the RyR–CICR channels. There is direct evidence to indicate that both halothane and enflurane activate the RyR–CICR channels of cardiac SR: in isolated SR vesicles from cardiac muscle, both anesthetics stimulated the [³H]ryanodine binding, an index for the open state probability of the RyR–CICR channels.[39,40] The order of potency for the Ca^{2+}-releasing effects of volatile anesthetics from the ryanodine-sensitive SR observed in this artery (*i.e.*, halothane > enflurane [>isoflurane]) is consistent with previous studies in which halothane and enflurane, but not isoflurane, stimulated the [³H]ryanodine binding to cardiac SR.[39,40] Although halothane stimulated IP_3 formation in erythrocyte membranes,[41] to our knowledge, no convincing evidence is presently available to indicate that volatile anesthetics

stimulate IP_3 formation and thereby cause the Ca^{2+} release in VSM cells.

The Actions of Volatile Anesthetics on Ca^{2+} Uptake

Both halothane and enflurane, applied during Ca^{2+} loading, inhibited the caffeine-induced increase in $R_{340/380}$ as an estimate for the amount of Ca^{2+} in SR, indicating that both anesthetics inhibit Ca^{2+} uptake by the SR. In contrast, neither isoflurane nor sevoflurane, applied during Ca^{2+} loading, influenced the caffeine-induced increase in $R_{340/380}$, indicating that these anesthetics do not influence the Ca^{2+} uptake. Interestingly, halothane and enflurane, applied during the Ca^{2+} loading with procaine at concentrations sufficient to block their Ca^{2+}-releasing action, conversely enhanced the caffeine-induced increase in $R_{340/380}$, indicating that these anesthetics enhance the Ca^{2+} uptake after blockade of their Ca^{2+}-releasing action. This suggests that both anesthetics have a stimulating action on Ca^{2+} uptake in addition to the Ca^{2+}-releasing action, and that their inhibitory effects on Ca^{2+} uptake in the absence of procaine were caused by their Ca^{2+}-releasing action but not by inhibition of SR Ca^{2+}–adenosine triphosphatase activity. These suggestions on halothane support previous findings in conduit arteries[7,15,16] and also our previous findings in contraction experiments with the same artery.[11,12] However, the action of enflurane on the Ca^{2+} uptake in systemic resistance artery was, for the first time, de
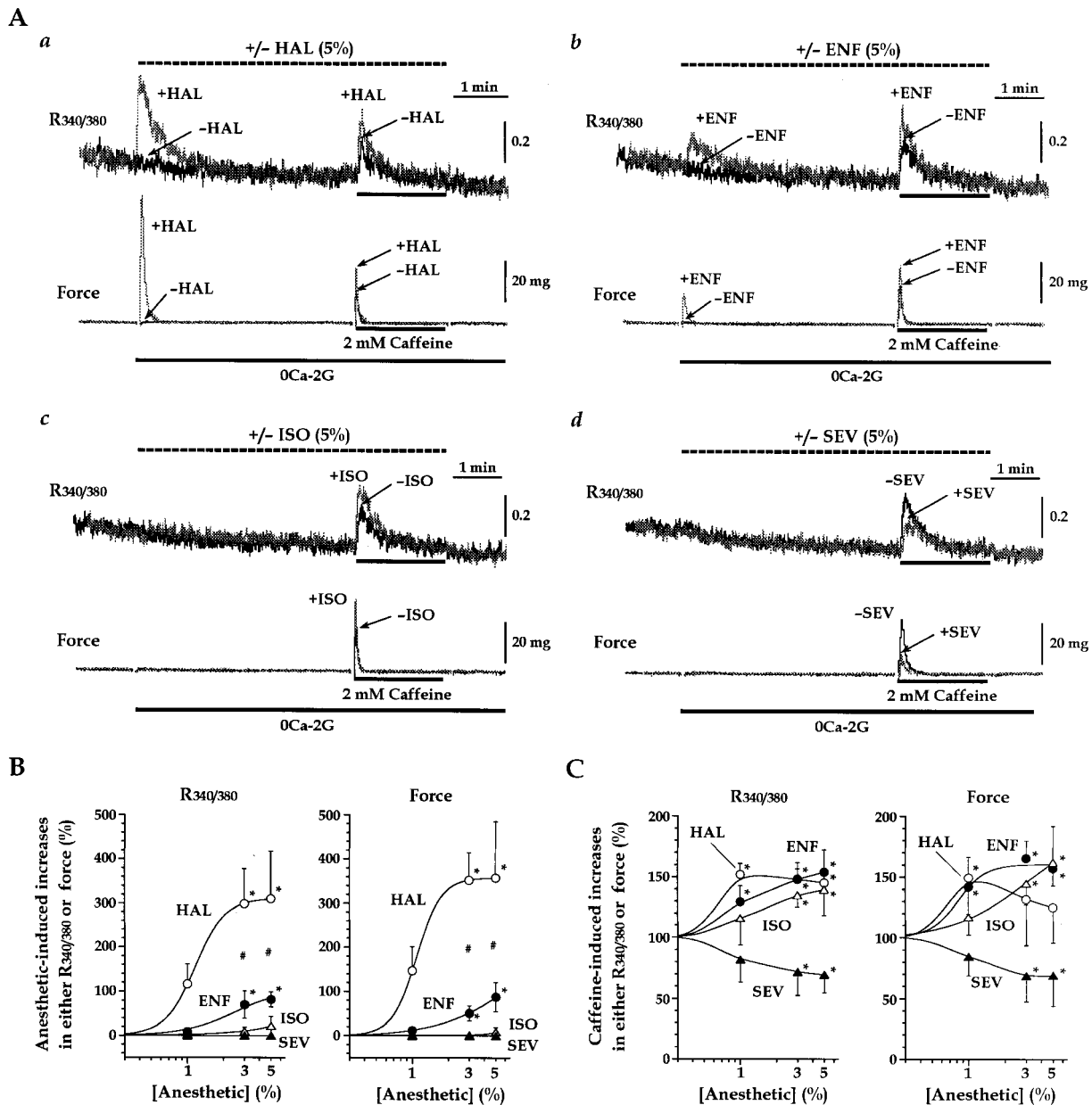
Fig. 5. Effects of halothane (HAL), enflurane (ENF), isoflurane (ISO), and sevoflurane (SEV) on the caffeine-induced increases in $R_{340/380}$ and force in the Ca^{2+}-free solution (n = 5). (A) Examples: the responses in either presence (+) or absence (−) of each anesthetic are depicted with gray and black lines, respectively. 0Ca-2G = Ca^{2+}-free, 2-mM EGTA solution. Arrows indicate the peaks of responses. (B and C) Concentration–response data for the anesthetic-induced increases in $R_{340/380}$ and force (B) and the caffeine-induced increases in $R_{340/380}$ and force in the presence of each anesthetic (C). The increases in $R_{340/380}$ and force induced by each anesthetic or caffeine were normalized to control caffeine-induced increases in $R_{340/380}$ and force, respectively. The EC_{50} values for the halothane-induced increases in $R_{340/380}$ and force were 1.16 and 1.20%, respectively. *$P < 0.05$ versus control within each group. #$P < 0.05$ between halothane and enflurane groups at each concentration.

scribed in this study. Our data on the effects of isoflurane or sevoflurane on Ca^{2+} uptake are consistent with the previous findings in contraction experiments at 37°C in isolated mesenteric arteries,[15] but not with those on isoflurane obtained in contraction experiments at 23°C in isolated aorta.[14] In the latter, isoflurane, like halothane and enflurane, also inhibited the Ca^{2+} uptake.[14] Because isoflurane caused Ca^{2+} release in the aorta,[14] the ob-

served inhibitory effect of isoflurane on Ca^{2+} uptake might also be caused by its Ca^{2+}-releasing action.

Effects of Volatile Anesthetics on Ca^{2+}-releasing Mechanisms

Although no direct evidence is presently available, as previously discussed, halothane, enflurane, and isoflurane all appear to activate the RyR–CICR channels in
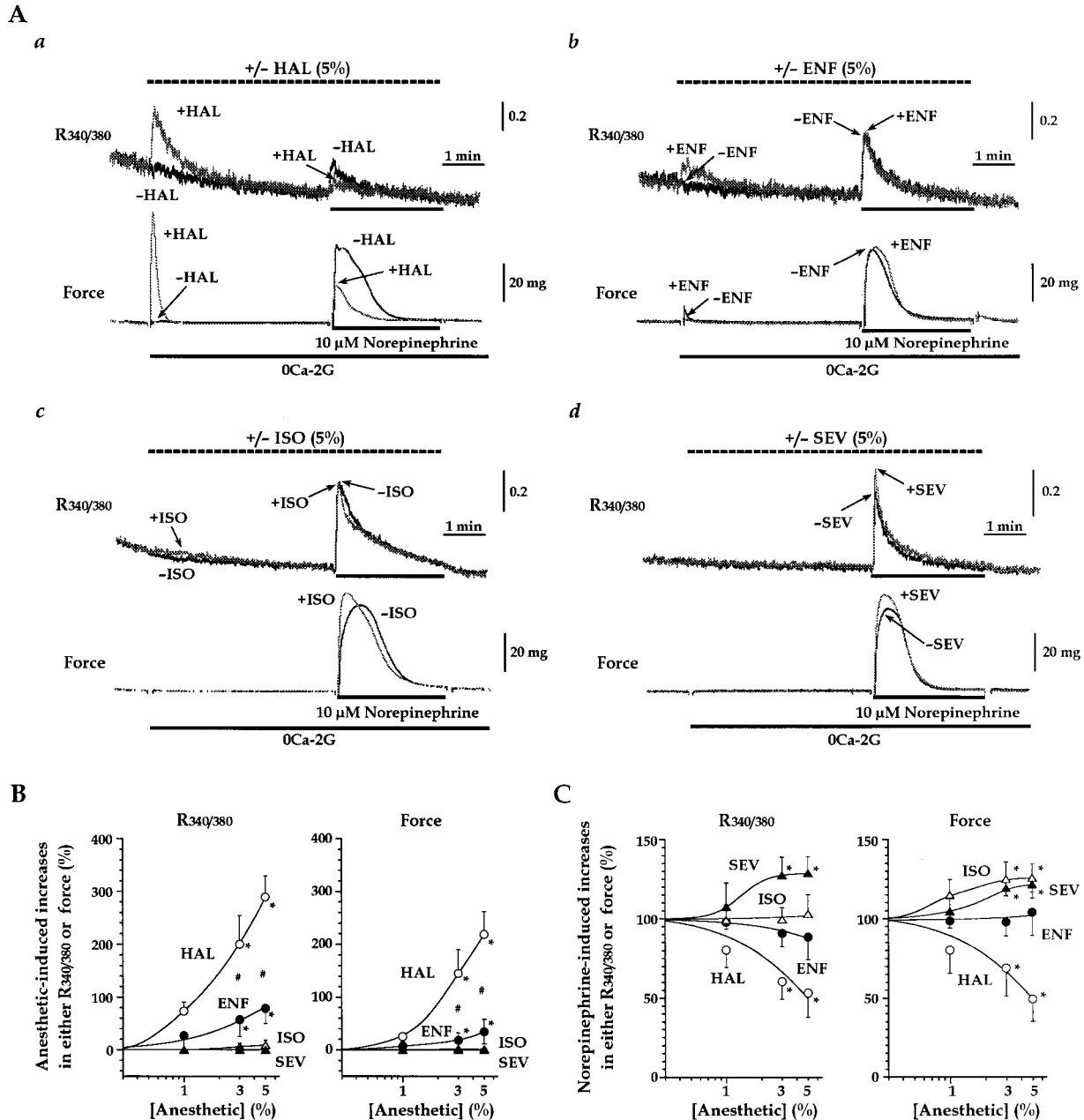
Fig. 6. Effects of halothane (HAL), enflurane (ENF), isoflurane (ISO), and sevoflurane (SEV) on the norepinephrine-induced increases in $R_{340/380}$ and force in the Ca^{2+}-free solution ($n = 4$ or 5). (*A*) Examples: the responses in either presence ($+$) or absence ($-$) of each anesthetic were depicted with gray and black lines, respectively. 0Ca-2G = Ca^{2+}-free, 2-mM EGTA solution. Arrows indicate the peaks of responses. (*B* and *C*) Concentration–response data for the anesthetic-induced increases in $R_{340/380}$ and force (*B*) and the norepinephrine-induced increases in $R_{340/380}$ and force in the presence of each anesthetic (*C*). The increases in $R_{340/380}$ and force induced by each anesthetic or caffeine were normalized to the control norepinephrine-induced increases in $R_{340/380}$ and force, respectively. *$P < 0.05$ *versus* control within each group. #$P < 0.05$ between halothane and enflurane groups at each concentration.

VSM. In support of this idea, in the current study, the increase in $R_{340/380}$ induced by caffeine, believed to activate the CICR, was enhanced in the presence of these anesthetics (despite the reduced amount of Ca^{2+} in the SR on application of caffeine because of their Ca^{2+}-releasing actions). This suggests that these anesthetics activate the CICR. However, sevoflurane inhibited the caffeine-induced increase in $R_{340/380}$, indicating

that sevoflurane attenuates the CICR (a novel finding of this study).

Previous studies have demonstrated that contractile response to caffeine was enhanced in the presence of halothane, enflurane, and isoflurane, suggesting that these anesthetics may enhance the CICR.[7,8,14,15] However, these anesthetics were shown to influence myofilament Ca^{2+} sensitivity in VSM.[11,17,18,25,42] In particular,

both enflurane and isoflurane appear to increase the myofilament Ca^{2+} sensitivity.[18,42] Therefore, the enhanced contractile responses to caffeine in the presence of these anesthetics could reflect the anesthetic effects on both the Ca^{2+} sensitivity and $[Ca^{2+}]_i$. In addition, because volatile anesthetics were simultaneously applied with caffeine in the previous studies,[7–9,14,15] the response to caffeine also could reflect the Ca^{2+}-releasing action of the anesthetics. This study, in which changes in $[Ca^{2+}]_i$ were measured and each anesthetic was applied before application of caffeine, for the first time, definitely proves that the three anesthetics enhance the caffeine–CICR mechanism in VSM.

Halothane appeared to attenuate inositol phosphate formation and thereby inhibit the Ca^{2+} release in the cultured VSM cells.[19] In this study, halothane inhibited the norepinephrine-induced increase in $R_{340/380}$, apparently suggesting that halothane attenuates the IP_3-induced Ca^{2+} release. However, because the Ca^{2+} amount in SR had been reduced on application of norepinephrine because of the previous treatment with halothane (which caused the Ca^{2+} release), the effect of halothane on norepinephrine-induced Ca^{2+} release mechanism was unclear from our data. Although a previous study[9] suggested that halothane may not influence the norepinephrine-induced increase in $[Ca^{2+}]_i$ in isolated aorta, the effect of halothane on the norepinephrine-induced Ca^{2+} release was also unclear in the previous study[9] because of the protocol used, *i.e.*, as halothane was applied simultaneously with norepinephrine in the study, the observed norepinephrine-induced increase in $[Ca^{2+}]_i$ could reflect not only the effect of halothane on norepinephrine-induced Ca^{2+} release but also the Ca^{2+}-releasing effect of halothane. Further investigation is needed to clarify this issue.

Isoflurane also appeared to attenuate inositol phosphate formation and thereby inhibit the Ca^{2+} release in the cultured aortic VSM cells.[20] However, in this study, isoflurane did not influence the norepinephrine-induced increase in $R_{340/380}$, suggesting that isoflurane does not influence the IICR. This appears to be consistent with a previous finding in isolated aorta.[9] The responsiveness of the cultured cells to isoflurane might be altered in the culture process.

Little is known about the effects of enflurane on the IICR. In this study, enflurane did not influence the norepinephrine-induced increase in $R_{340/380}$, suggesting that enflurane may not influence the IICR. However, as enflurane also caused Ca^{2+} release, the amount of Ca^{2+} in the SR had been reduced on application of norepinephrine because of the previous treatment with enflurane. Therefore, there is a possibility that enflurane stimulates the IICR and thereby counteracted the attenuated norepinephrine response caused by the reduced amount of Ca^{2+} in the SR. However, because the enflurane-induced increase in $R_{340/380}$ (*i.e.*, the amount of Ca^{2+} released

from SR by enflurane) was rather small, this possibility appears to be minor.

The observed effect of sevoflurane on the norepinephrine-induced increase in $R_{340/380}$ suggests that sevoflurane stimulates the IICR. This is contradictory to our recent suggestion that sevoflurane does not influence the IICR, which was based on its inability to influence the norepinephrine (0.5 μM)-induced increase in $R_{340/380}$ after treatment with verapamil, a Ca^{2+} channel blocker.[25] As verapamil was previously suggested to influence the Ca^{2+} release from intracellular stores,[43] the sensitivity of Ca^{2+} release mechanism to sevoflurane might be altered by the verapamil treatment in our previous study. Alternatively, sevoflurane may enhance only the Ca^{2+} release mechanism induced by higher concentrations of norepinephrine. We attempted to examine the effects of sevoflurane on the increase in $R_{340/380}$ caused by lower concentrations of norepinephrine (≤ 1 μM). However, as the increase in $R_{340/380}$ caused by the low concentrations of norepinephrine in the Ca^{2+} solution were rather small and somewhat unstable, we did not investigate this issue further.

Comparison of the Data between the Ca^{2+} Measurement and Force Recording Experiments

Not all of the observed anesthetic effects on $R_{340/380}$ were parallel with the simultaneously observed anesthetic effects on force. Enflurane (5%), applied during Ca^{2+} loading, inhibited the caffeine-induced increase in $R_{340/380}$ but not the caffeine-induced increase in force. Because the $[Ca^{2+}]_i$–force relation is hyperbolic, the enflurane-induced slight ($\approx 10\%$) decrease in $R_{340/380}$ might not cause any significant decrease in force. Alternatively, because enflurane appeared to increase myofilament Ca^{2+} sensitivity in VSM,[18] the possible prolonged effect of enflurane on the Ca^{2+} sensitivity might counteract its SR-depleting effect.

Isoflurane did not influence the norepinephrine-induced increase in $R_{340/380}$ but enhanced the norepinephrine-induced increase in force, suggesting that isoflurane enhances the myofilament Ca^{2+} sensitivity. This is consistent with previous observations, where isoflurane enhanced Ca^{2+}-activated contractions in skinned VSM.[42]

The changes in force in the presence of volatile anesthetics observed in the fura-2–nonloaded strips were identical to those observed in the fura-2–loaded strips, suggesting that fura-2 loading, a nonphysiologic intervention, does not significantly influence the volatile anesthetic actions on VSM cells.

Conclusion

In conclusion, in VSM cells of systemic resistance arteries, halothane, enflurane, isoflurane, and sevoflurane at clinical concentrations appear to influence either Ca^{2+} mobilization or Ca^{2+} removal from the cytoplasm through direct effects on the SR Ca^{2+} stores, *i.e.*, the

effects on amount of Ca^{2+} in the SR, Ca^{2+} uptake process, or Ca^{2+}-releasing mechanisms. Although both halothane and enflurane have opposing actions on the amount of Ca^{2+} in the SR (*i.e.*, Ca^{2+}-releasing action and a stimulating action on Ca^{2+} uptake), their overall effects appear to reduce the amount of Ca^{2+} in the SR, which could influence vascular tone or reactivity both by decreasing Ca^{2+} availability for VSM contraction and by enhancing the Ca^{2+}-buffering capacity of SR. In addition, all four of these anesthetics may significantly influence vascular reactivity through their effects on the CICR or IICR mechanism. Finally, the observed differences in the direct effects on the SR among the four anesthetics may underlie the differences in their circulatory effects.

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