

Effects of Halothane on the Sarcoplasmic Reticulum Ca^{2+} Stores and Contractile Proteins in Rabbit Pulmonary Arteries

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Background: The authors' purpose of this study was to elucidate the mechanisms of direct effects of halothane on the contractile proteins and Ca^{2+} release from the sarcoplasmic reticulum Ca^{2+} stores using isolated skinned strips (sarcolemma permeabilized with saponin) from rabbit pulmonary arteries.

Methods: The sarcoplasmic reticular Ca^{2+} stores were examined by immersing the skinned strips sequentially in solutions to load Ca^{2+} into and release Ca^{2+} from the sarcoplasmic reticulum using caffeine, inositol 1,4,5-trisphosphate, or halothane. The contractile proteins were assessed by activating the strips with Ca^{2+} followed by administration of halothane (with or without protein kinase C inhibitors). Tension, fura-2 fluorescence activated by Ca^{2+} release, and phosphorylation of myosin light chains were measured.

Results: Halothane (0.07–3.00%) increased Ca^{2+} , tension, and phosphorylation of myosin light chains in a dose-dependent manner. Halothane decreased accumulation of Ca^{2+} in the sarcoplasmic reticulum and enhanced the caffeine-induced tension transients. In strips pretreated with caffeine or inositol 1,4,5-trisphosphate, halothane-induced tension transients were reduced but Ca^{2+} was not. In strips activated by $1 \mu\text{M}$ Ca^{2+} , halothane (0.5–3.0%) decreased 20–45% of the activated force at 15 min. Halothane (3%) transiently increased the force (20%) associated with increases in Ca^{2+} and phosphorylation of myosin light chains. The increased force was abolished and the subsequent relaxation was enhanced by the protein kinase C inhibitor bisindolylmaleimide but not by indolocarbazole Gö-6976.

Conclusions: In skinned pulmonary arterial strips, halothane, at clinical concentrations, inhibits uptake of Ca^{2+} by and induces release of Ca^{2+} from intracellular stores possibly shared by caffeine and inositol 1,4,5-trisphosphate, which are

regulated by phosphorylation of myosin light chains. The time-dependent inhibition of the contractile proteins by halothane may be mediated by Ca^{2+} -independent protein kinase C. (Key words: Caffeine; fura-2 fluorescence; inositol 1,4,5-trisphosphate; myosin light chains; protein kinase C.)

IN whole animals or humans, halothane has been shown to induce vasoconstriction or vasodilation in different vascular beds,¹ in part by its direct effect on vascular smooth muscle (VSM). In isolated intact arterial (endothelium-denuded) preparations from various vascular beds and animal species,^{2–6} halothane has been shown to cause relaxation. In contrast, halothane induces contraction in isolated pulmonary arterial rings, which is correlated with release of Ca^{2+} from intracellular stores.⁷ The mechanism(s) of this halothane-induced contraction or relaxation of the VSM is not known.

The primary contractile process in VSM is elevation of cytosolic free Ca^{2+} , from either influx of Ca^{2+} through the sarcolemma or release of Ca^{2+} from intracellular stores (the sarcoplasmic reticulum [SR]) by various neurohumoral transmitters. This binds calmodulin, resulting in activation of myosin light chain (MLC) kinase and phosphorylation of MLCs (MLC-p). This increase in MLC-p activates myosin adenosine triphosphatase (AT-Pase), resulting in actin-myosin interactions and force generation.⁸ The release of Ca^{2+} from the SR occurs through channels that contain receptors specific to either ryanodine or inositol 1,4,5-trisphosphate (IP_3).⁹ Whether these two receptor types govern separate Ca^{2+} stores is not clear, nor is it known if halothane affects them equally. In contractile proteins, Ca^{2+} -independent pathways have been demonstrated to be regulated either by MLC-p¹⁰ or by the thin filament-associated proteins calponin and caldesmon.^{11,12} These multiple regulatory mechanisms in VSM contraction may contribute to the diversity of vascular responses to halothane.

At the intracellular sites of the contractile process, we have shown in saponin-skinned aortic strips that halothane enhances caffeine-induced tension tran-

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HALOTHANE EFFECT ON SKINNED PULMONARY ARTERIAL STRIPS

sients.³ This suggests that halothane causes release of Ca^{2+} from the SR or increases the sensitivity of the contractile proteins to Ca^{2+} . In cultured VSM cells, direct release of Ca^{2+} from the intracellular Ca^{2+} stores¹³ and inhibition of inositol phosphate formation¹⁴ by halothane have been observed. Therefore, these multiple effects of halothane on intracellular signaling would result in opposing effects. In isolated intact bovine pulmonary arterial rings treated with SR ATPase inhibitor (cyclopiazonic acid) or an SR Ca^{2+} release channel inhibitor (ryanodine), halothane and caffeine decrease contraction,⁷ which suggests halothane-induced release of Ca^{2+} from the caffeine-releasable SR Ca^{2+} . Whether halothane affects VSM contraction by releasing Ca^{2+} , either directly or indirectly, from IP_3 -releasable stores is not clear.

At the contractile protein level, halothane has been shown to decrease submaximum and maximum Ca^{2+} -activated force of the contractile proteins in sarcolemma-permeabilized rat mesenteric arterial strips.⁵ Recently, the inhibition of protein kinase C (PKC) activator-induced contraction by halothane has been observed in isolated rat coronary arteries,¹⁵ which could result in decreased influx of Ca^{2+} via the sarcolemma¹⁶ or depress force development by a Ca^{2+} -independent pathway.¹⁰⁻¹² The mechanisms of the Ca^{2+} -independent effect of halothane are not clear, however.

Accordingly, in this study, we examined intracellular mechanisms (either SR or the contractile proteins) of action of halothane using saponin-skinned (sarcolemma-permeabilized) arterial strips³ from rabbit pulmonary artery. This skinned strip preparation allowed us to examine the function of each organelle and to measure tension, free Ca^{2+} concentrations, and MLC isoforms simultaneously.

Materials and Methods

Skinned Arterial Strips

New Zealand male White rabbits (2.0–2.5 kg) were killed using a captive bolt pistol (approved by the Institutional Animal Care Committee of the University of Washington, Seattle, WA), followed by exsanguination. Right or left pulmonary arteries were rapidly and carefully isolated and kept on ice until experimentation.

Skinned strips were prepared in a manner reported previously for aorta.³ The strips (0.1–0.15 mm wide) were mounted on photodiode transducers, stretched to a 50-mg tension from the resting length and then

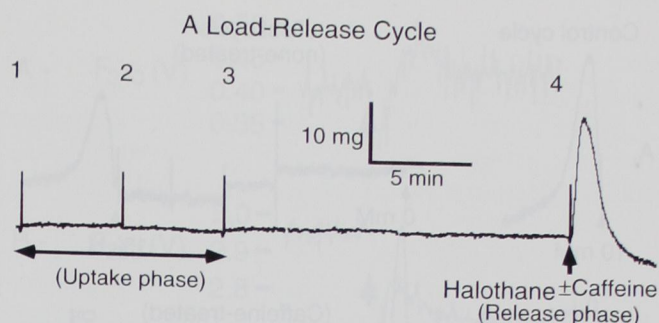


Fig. 1. A typical 10 mM caffeine-induced tension transient showing the experimental procedure for a load-release cycle. Numbers 1–4 are solution artifacts. The uptake phase represents the presence of halothane in solutions 1–3, and the release phase represents the presence of halothane (0.1–3.0%) in solution 4 with or without 10 mM caffeine. Duration of immersion is 5 min for solutions 1 and 2, 15 min for solution 3, and for solution 4 until steady state of the tension transient is reached. Three cycles were sequentially performed: a control (10 mM caffeine with no halothane), a test (halothane in the uptake phase or halothane with or without caffeine in the release phase), and finally another control.

immersed in a skinning solution (a relaxing solution containing 0.3 mg/ml saponin) for 5 min. Isometric tension of the skinned strips was recorded on a computer (Quadra 950; Apple Computer, Inc., Cupertino, CA) with a customized LabVIEW software program interfaced with a multifunction I/O board with 16-bit resolution (NB-MIO-16XL; National Instrument, Austin, TX). The experiments were performed at room temperature (20°C–23°C).

Study of Uptake or Release of Ca^{2+} from the Sarcoplasmic Reticulum

The skinned strips were immersed sequentially in four solutions to load Ca^{2+} into and to release Ca^{2+} from the SR (a load-release cycle; fig. 1). These four solutions contained the same ionic concentration (35 mM K^+ , 35 mM Na^+ , 15 mM creatine phosphate, 2 mM MgATP^{2-} , 0.1 mM Mg^{2+} , 80 mM methanesulfonate, and 50 mM piperazine-N,N'-bis[2-ethanesulfonic acid dipotassium; ionic strength, 0.15; pH 7.00), except that concentrations of EGTA and Ca^{2+} varied as follows. Solution 1 (no added Ca^{2+} , 7 mM EGTA) was used to wash away saponin in the strips or caffeine in subsequent load-release cycles; solution 2 (0.316 μM Ca^{2+} , 7 mM EGTA) was used to load Ca^{2+} rapidly into the SR; solution 3 (0.1 μM Ca^{2+} , 0.05 mM EGTA) was used to reduce EGTA in the strips; and solution 4 (same as solution 3 plus 10 mM caffeine for controls and various concentrations of

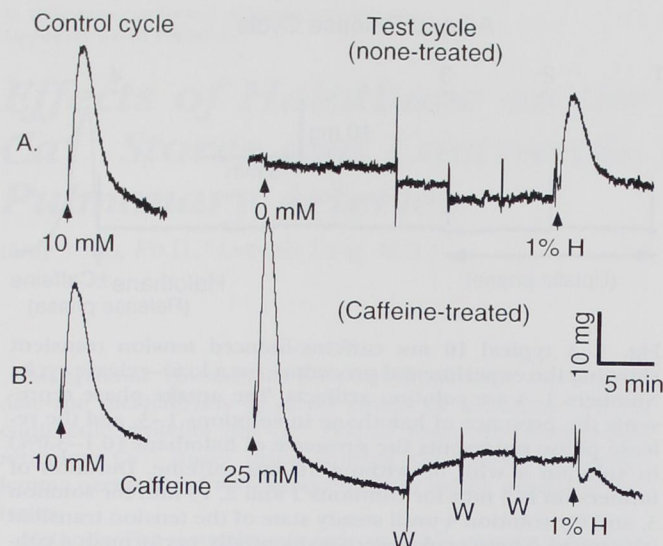


Fig. 2. Tracings showing experimental protocol for the study of Ca^{2+} stores. Two load-release cycles were performed sequentially in each study: a control cycle (10 mM caffeine in solution 4) and a test cycle. The test cycle was the same as the control cycle except that solution 4 contained 25 mM caffeine (or 30 μM inositol 1,4,5-trisphosphate [IP_3]; pretreatment) for 15 min. This was then followed by three washes in solution 3 (W), at 5 min each without reloading, and finally solution 4, containing various concentrations of halothane (test, 1% halothane [1%H]) until the tension transient reached a steady state. The test result was expressed as a percent of the control (10 mM caffeine). (A) Tracings showing the skinned strip without pretreatment (time control). (B) Tracings showing the skinned strip pretreated with 25 mM caffeine (test). We found that a tension transient induced by 1% halothane was reduced greatly in skinned strips pretreated with caffeine (1%H, test cycle, B) compared with that of the time control (1%H, test cycle, A).

halothane \pm caffeine for tests) was used to induce release of Ca^{2+} from the SR as measured with a tension transient or Ca^{2+} fura-2 fluorescence (described later). The duration of immersion was 5 min for solutions 1 or 2 and 15 min for solution 3, and the strips remained in solution 4 until the generated tension transient had returned to its steady-state baseline value (fig. 1).

Halothane was present in two different aspects of the study. In the first study, halothane was used either to modulate the magnitude of caffeine-induced release of Ca^{2+} (fig. 1) or to stimulate release of Ca^{2+} from the SR directly in the absence of caffeine. In the second study, the direct Ca^{2+} release effect of halothane was examined again in the absence of caffeine, but the Ca^{2+} stores in the SR were manipulated by prior treatment with caffeine or IP_3 (fig. 2).

Effect of Halothane on Uptake or Release of Ca^{2+} from the Sarcoplasmic Reticulum. Three load-release cycles were performed in each skinned strip: a control cycle (Ca^{2+} releasing solution 4 contained 10 mM caffeine), a test cycle (solutions contained halothane), and finally another control cycle. The test cycle consisted of one of the following conditions: (1) the uptake phase, in which halothane was present during Ca^{2+} loading into the SR in solutions 1-3; or (2) the release phase, in which halothane was present during release of Ca^{2+} from the SR in solution 4 with or without caffeine. The area of tension transient in the test cycle was expressed as a percent of that of the mean of the bracketing controls in each preparation.

A halothane and N_2 mixture was delivered through a Verni-Trol vaporizer (Ohio Medical Product, Madison, WI) for test solutions, and control solutions were saturated with 100% N_2 . The halothane concentrations in the final solutions were assayed by gas chromatography and expressed in partial pressure as a percent of one atmosphere.¹⁷ The calculated concentration of halothane in the gas was 0.41 mM for 1% vapor concentration, or 7.6 mmHg partial pressure of one atmosphere (760 mmHg at sea level) based on $n/V = P/RT$, where n/V is the molar concentration of halothane, R (gas constant) is 0.082, T ($^\circ\text{K}$) (absolute temperature) is 296 $^\circ\text{K}$ at 23 $^\circ\text{C}$, and P is partial pressure of halothane expressed as a fraction of atmospheric pressure. Assuming a solution/gas ratio of 1.5 at 23 $^\circ\text{C}$, the halothane concentration in the solution was 0.6 mM.

Effect of Halothane on Release of Ca^{2+} from Inositol 1,4,5-Trisphosphate- or Caffeine-Releasable Stores. In this study, we examined whether halothane releases Ca^{2+} from specific intracellular stores: the IP_3 - or caffeine-releasable store.

The experimental protocol consisted of two load-release cycles in the following sequence: (1) a control cycle (solution 4 contained 10 mM caffeine), and (2) a test cycle to deplete the Ca^{2+} stores (solution 4 with 25 mM caffeine or 30 μM IP_3 for 15 min followed by three washes in solution 3 of 5 min each), followed by 0.3%, 1.0%, or 3.0% halothane (in solution 3) to release residual Ca^{2+} (fig. 2). The test results were expressed as a percent of that of 10 mM caffeine in the control cycle.

Measurement of Fura-2 Fluorescence. Fura-2, a synthetic fluorescent dye,¹⁸ was selected to measure free Ca^{2+} concentration during muscle contraction because of several advantages including (1) sensitivity to $[\text{Ca}^{2+}]$ in the physiologic range, *i.e.*, $>0.01 \mu\text{M}$; (2) selec-

HALOTHANE EFFECT ON SKINNED PULMONARY ARTERIAL STRIPS

tivity for Ca^{2+} ; (3) the low concentration of fura-2 required (μM range) reduces the possibility of calcium buffering by the indicator; and (4) the ratio of fluorescence intensities from 340 (F_{340}) to 380 nm (F_{380}) excitation wavelength for $[\text{Ca}^{2+}]$ calculation eliminates variabilities of instrument efficiency, dye concentration, and effective cell thickness along the optical beam.

The validity of the measurement of fura-2 initially was confirmed by titrating 10 mM Ca^{2+} -EGTA into 10 mM EGTA buffer (Calcium Calibration Buffer Kit 1, C-3008; Molecular Probes, Inc., Eugene, OR). A dissociation constant for fura-2/ Ca^{2+} complex (K_d) of 173 nM was obtained in our laboratory using a modified calcium analyzer (CAF-100; Japan Spectroscopic, Tokyo, Japan), which is of a similar magnitude (145 nM) to that reported by Molecular Probes. Next, the calibration of Ca^{2+} was performed in the presence of the skinned strip during our experimental condition by equilibrating with 0.05 mM EGTA buffer (solution 3 in the load-release cycle) containing various concentrations of Ca^{2+} plus 2 μM fura-2. Based on the equation of Ca^{2+} (nM) = $K_d \times (S_{f2}/S_{b2}) \times [(R_i - R_{\min})/(R_{\max} - R_i)]$,¹⁸ the apparent K_d value was calculated from the best linear fit with $\log (S_{f2}/S_{b2}) \times [(R_i - R_{\min})/(R_{\max} - R_i)]$ as the y axis, and $\log [\text{Ca}^{2+}]$ (M) as the x axis. S_{f2} is the fluorescence emitted at F_{380} at 0 Ca^{2+} during which fura-2 was in free form, S_{b2} is the F_{380} at 0.1 mM Ca^{2+} during which fura-2 was in Ca^{2+} bound form, R_i is the F_{340} -to- F_{380} ratio at various concentrations of free Ca^{2+} , R_{\min} is the F_{340} -to- F_{380} ratio at 0 Ca^{2+} , and R_{\max} is the F_{340} -to- F_{380} ratio at 0.1 mM Ca^{2+} . The calibrated data were (mean \pm SEM [$n = 5$]) K_d , 252.2 ± 12.2 nM; S_{f2} , 1.23 ± 0.1 ; S_{b2} , 0.48 ± 0.04 ; R_{\min} , 0.06 ± 0.003 ; and R_{\max} , 0.56 ± 0.049 .

The experimental protocol was the same as described for tension measurement except that (1) all bathing solutions contained 2 μM fura-2 free acid, and fura-2 fluorescence was measured before (baseline) and during release of Ca^{2+} ; (2) halothane was prepared in a polyethylene centrifuge tube containing solution 3, and the concentration of halothane in the solution was assayed by gas chromatography.¹⁷ Simultaneous measurements of tension and fura-2 fluorescence (fig. 3) were performed by placing the skinned strip into a 150- μl quartz tissue bath containing solution 3 as baseline fluorescence (described later). Next, a releasing solution was injected (solution 4 of the control cycle or the test solution of the test cycle).

The fluorescence of fura-2 (F_{340} and F_{380}) was measured continuously by exposing the skinned strip alternately, at 50 Hz, to 340 ± 10 and 380 ± 10 nm excitation

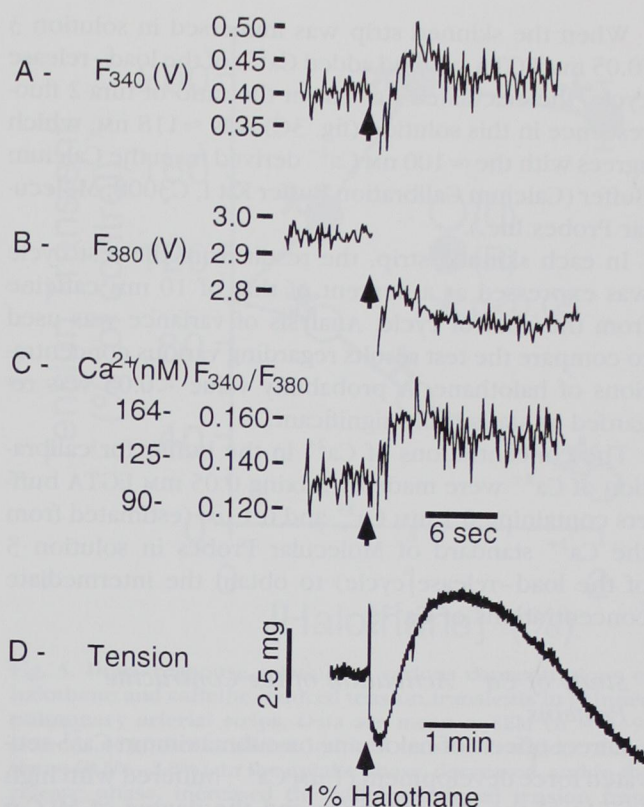


Fig. 3. Tracings showing simultaneous measurement of halothane-induced tension transients and release of Ca^{2+} from the SR in a skinned pulmonary arterial strip from a simple load-release cycle. Halothane at 1% increased F_{340} (A) and decreased F_{380} (B), thus increasing the F_{340} -to- F_{380} ratio (F_{340}/F_{380}) equivalent to 47.7 nM Ca^{2+} (C), which was followed by a tension transient (D). Ca^{2+} (nM) = calculated Ca^{2+} in nM; F_{340} (V) = voltage of fura-2 fluorescence from 340 nm excitation wavelength; F_{380} (V) = voltage of fura-2 fluorescence from 380 nm excitation wavelength.

wavelengths. The fluorescence emitted at the 500 ± 20 nm wavelength was detected using a modified calcium analyzer (CAF-100; Japan Spectroscopic, Tokyo, Japan). The fluorescence emitted from excitation wavelengths of 340 and 380 nm were recorded, at a sampling rate of 10 Hz, on a PowerMac 7100 (Apple Computer, Inc.) using a customized LabVIEW software program interfaced with a multifunction I/O board with 16-bit resolution (NB-MIO-16XL; National Instrument).

Using these data from Ca^{2+} calibration in skinned strips and the R_i between baseline (solution 3 of the load-release cycle in the presence of a skinned strip) and the peak (3 s after administration of halothane; fig. 3), the amount of free Ca^{2+} released from the SR was estimated from the difference in Ca^{2+} between the baseline and the peak.

When the skinned strip was immersed in solution 3 (0.05 mM EGTA with no added Ca^{2+}) of the load-release cycle, the calculated Ca^{2+} from the ratio of fura-2 fluorescence in this solution (fig. 3C) was ≈ 118 nM, which agrees with the ≈ 100 nM Ca^{2+} derived from the Calcium Buffer (Calcium Calibration Buffer Kit I, C3008; Molecular Probes Inc.).

In each skinned strip, the result from the test cycle was expressed as a percent of that of 10 mM caffeine from the control cycle. Analysis of variance was used to compare the test results regarding various concentrations of halothane. A probability value <0.05 was regarded as statistically significant.

The concentrations of Ca^{2+} in the buffer for calibration of Ca^{2+} were made by mixing 0.05 mM EGTA buffers containing 0.1 mM Ca^{2+} and 0.1 μM (estimated from the Ca^{2+} standard of Molecular Probes in solution 3 of the load-release cycle) to obtain the intermediate concentrations of Ca^{2+} .

Study of Ca^{2+} Activation of the Contractile Proteins

Direct effects of halothane on submaximum Ca^{2+} -activated force development (1 μM Ca^{2+} , buffered with high EGTA) of the skinned strips and the degree of MLC-p were investigated. When the force reached a steady state, the same solution containing a specific concentration of halothane was injected into a tissue bath and the effects of halothane were observed for 15 min (fig. 4). The peak (within 1 min) force development and that at 15 min after administration of halothane were expressed as a percent of the control (before the administration of halothane). Parallel experiments were performed as time controls by administration of solution without halothane.

In different experiments, skinned strips were tested with (test group) or without (time control group) halothane (described previously), and at 1 min or 15 min the strips were quickly frozen in freon ($-130 \pm 30^\circ\text{C}$) cooled with liquid N_2 for quantification of MLC isoforms.

Quantification of Myosin Light Chain Isoforms by Two-dimensional Electrophoresis and Immunoblotting

The method of extraction and separation of MLC isoforms from VSM was the same as that described by Kitazawa *et al.*¹⁹ Myosin light chain proteins were extracted from the frozen strips. This was followed by separation of MLC isoforms by two-dimensional poly-

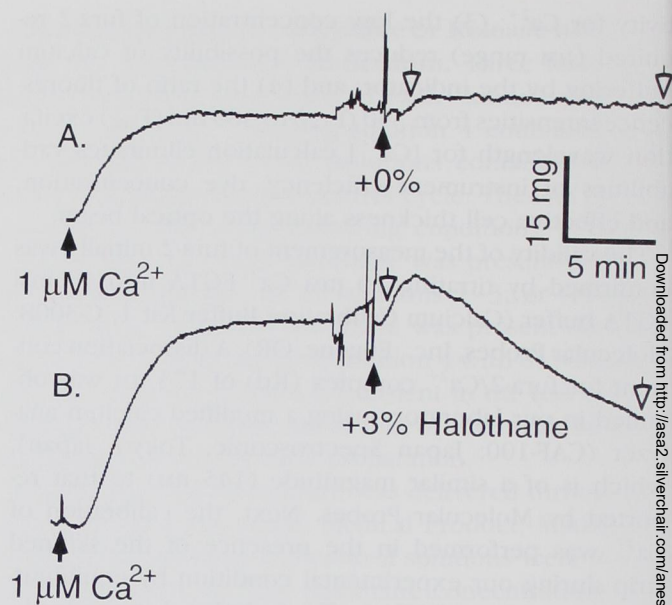


Fig. 4. Tracings showing the experimental procedure for studying the effects of halothane on submaximum Ca^{2+} -activated (1 μM) tension development, measurements of fura-2 fluorescence, and myosin light chain isoforms. (A) Time course of control experiment without halothane. (B) Test experiment with halothane. One micromole per liter Ca^{2+} was buffered with 0.05 mM EGTA; +0% = 1 μM Ca^{2+} + 0% halothane; +3% halothane = 1 μM Ca^{2+} + 3% halothane. The skinned strip was activated by 1 μM Ca^{2+} until force development reached steady state. The strip was then transferred to a quartz tissue bath, and 150 μl of the same solution saturated with various concentrations of halothane (0–3%) was injected. The ratio of fura-2 fluorescence was simultaneously recorded within 5 s and at various time intervals thereafter. For quantification of myosin light chain isoforms, the skinned strips were frozen 1 min and 15 min after administration of halothane (open arrows). This figure showed that 3% halothane caused a biphasic response of the skinned strip—an initial increase followed by a decrease in force compared with that of the time control group (+0% halothane, A).

acrylamide gel electrophoresis for its high resolution and sensitivity,²⁰ by their charge using isoelectric focusing polyacrylamide gel electrophoresis, and then by their mass (molecular weight, 20 kDa) using sodium dodecyl sulfate polyacrylamide gel electrophoresis in the second dimension.

Immunoblotting was performed as described by Hathaway and Haerberle²¹ by transferring sodium dodecyl sulfate gel onto nitrocellulose membranes by transblot electrophoresis, and MLC isoforms were specifically labeled with polyclonal affinity-purified rabbit anti-MLC antibody (supplied by Dr. Susan Gunst of the Department of Physiology, Indiana University, Indianapolis, IN). This was followed by exposure to a second antibody labeled with horseradish

HALOTHANE EFFECT ON SKINNED PULMONARY ARTERIAL STRIPS

peroxidase (antirabbit immunoglobulin G peroxidase conjugate; Sigma Chemical Co., St. Louis, MO) to react with the immobilized protein antigen (MLC), which was then detected on nitrocellulose membranes by autoradiography.

Determination of the extent of MLC-p in bands (immunoblots) was done with a multiresolution scanning imaging densitometer and the Molecular Analyst software program (model GS-700; Bio-Rad Laboratories, Hercules, CA).

Data Analysis

Areas of tension transients from load-release cycles or submaximum Ca^{2+} -activated force at peak or steady state were calculated. The test results were expressed as a percent of those of the control results for both the test and time control experiments. The amount of MLC-p was expressed as a percent of the total MLC.

The test results were compared with those of controls using Student's *t* test for unpaired data. Using the StatVIEW software program (BrainPower, Inc., Calabasas, CA), Student's *t* test for unpaired data was used to compare the results from the test experiments and the time controls within each concentration of halothane, and two-factorial analysis of variance was used to compare concentrations of halothane. Data are expressed as mean \pm SEM (n). A probability value <0.05 was considered significant.²²

Materials

Fura-2 pentapotassium salt and IP_3 were purchased from Molecular Probes. Thymol-free halothane was supplied by Halocarbon Laboratories (Hackensack, NJ). Antiserum for MLC was supplied by Dr. Susan Gunst (University of Indiana, Indianapolis, IN). Protein kinase C inhibitors (Gö-6976 and bisindolylmaleimide I-HCl) were purchased from Calbiochem (La Jolla, CA). Gö-6976 was made in 100% dimethylsulfoxide. Caffeine was purchased from Sigma Chemical Co., and other chemicals were analytical or reagent grade.

Results

Effects of Halothane on Uptake or Release of Ca^{2+} from the SR Measured with Caffeine-induced Tension Transients

We found that halothane (0.5%, 1.0%, and 2.0%) in the uptake phase (experimental protocol shown in fig. 1) decreased the caffeine-induced tension transients by

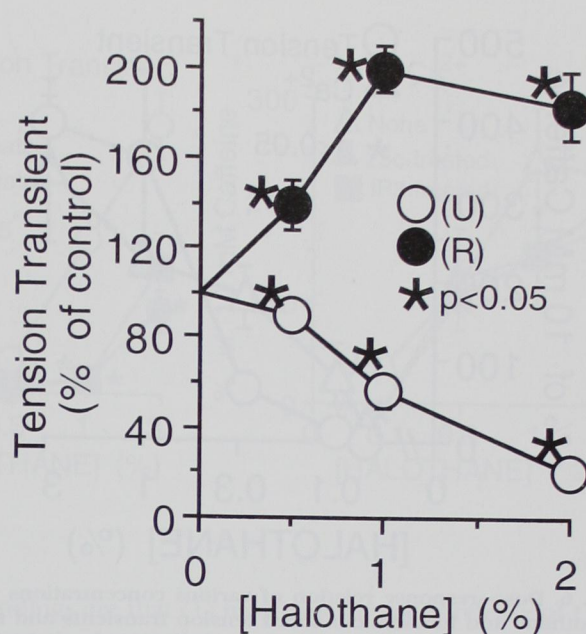


Fig. 5. Dose-response relation of various concentrations of halothane and caffeine-induced tension transients in skinned pulmonary arterial strips. Data are mean \pm SEM (n = 5–9) percent of control caffeine-induced tension transient. Halothane (0.5%–2.0%), in the uptake phase, decreased, and in the release phase, increased the caffeine-induced tension transients in a dose-dependent manner. (R) = halothane present in the caffeine-induced Ca^{2+} release phase; (U) = halothane present in the uptake phase.

10–80% in a dose-dependent fashion (fig. 5). In contrast, when halothane was present in the release phase (experimental protocol shown in fig. 1) the caffeine-induced tension transients significantly increased 40–100% and reached a maximum at 1% halothane (fig. 5).

Direct Effect of Halothane on Tension, Ca^{2+} , and Phosphorylation of Myosin Light Chains

In skinned pulmonary arterial strips, 10 mM caffeine produced a peak Ca^{2+} concentration of 37.7 ± 5.3 nM (n = 24). This amount of Ca^{2+} release is in agreement with that observed in permeabilized rabbit mesenteric arterial strips.²³

When halothane (0.1%, 0.3%, 1.0%, and 3.0%, no caffeine) was used to stimulate release of Ca^{2+} , tension transients (experimental protocol shown in fig. 1, a typical tracing shown in figure 3D) were produced in a dose-dependent manner (fig. 6). The tension transients were correlated with increases in release of Ca^{2+} from the SR (tracings shown in figs. 3A, 3B, and 3C; average results shown in fig. 6). The Ca^{2+} transients, however,

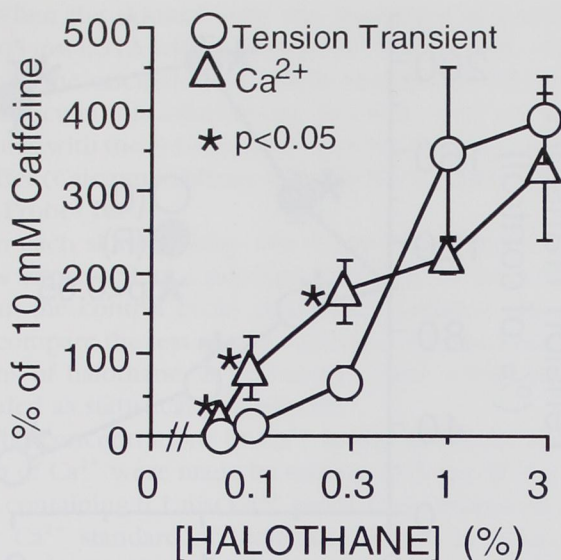


Fig. 6. Dose-response relation of various concentrations of halothane and halothane-induced tension transients and release of Ca^{2+} in skinned pulmonary arterial strips from simple load-release cycles. Data are mean \pm SEM ($n = 3-9$) percent of 10 mM caffeine-induced tension transient or release of Ca^{2+} . Halothane (0.1–3.0%) caused tension transients associated with increased Ca^{2+} in a dose-dependent manner.

appear to increase proportionally more at the lower concentrations of halothane and proportionally less at 3% halothane. This halothane-induced tension transient and release of Ca^{2+} were associated with increased MLC-p (30% of total MLC after 1 min of 1% halothane; fig. 7B) compared with the time control group, which showed no phosphorylation at 0% halothane (fig. 7B). After 7 min of 1% halothane, tension and myosin MLC-p had returned to baseline (fig. 7).

Differentiation of Possible Ca^{2+} Stores in the Sarcoplasmic Reticulum Released by Halothane and its Mechanisms of Action

This study was performed to determine further whether the halothane-induced release of Ca^{2+} from the SR was from a specific SR store (caffeine- or IP_3 -releasable store). In strips pretreated with either caffeine or IP_3 without reloading of Ca^{2+} into the SR (experimental protocol shown in fig. 2), the halothane-induced tension transients were reduced greatly (averages of 80–90%, 90%, and 50–70% for 0.3%, 1.0%, and 3.0% halothane, respectively, fig. 8A) compared with those in strips without pretreatment (fig. 8A). The reduction was similar between caffeine and IP_3 -treated strips (fig. 8A). In contrast, the degree of Ca^{2+} released by halothane

was not significantly changed during either condition nor in all three concentrations of halothane tested (fig. 8B).

Effects of Halothane on Submaximum Ca^{2+} Activation of the Contractile Proteins

At $1 \mu\text{M}$ Ca^{2+} , skinned pulmonary arterial strips generated a steady-state force of 35.2 ± 2.4 ($n = 10$) of the maximum tension. The initial effect of halothane was no change in force except at 3% halothane, at which a transient increase (20%) in force was observed (tracings shown in fig. 4; average results shown in fig. 9). Within 15 min of halothane exposure, the submaximum Ca^{2+} -activated force was decreased in a dose dependent manner (average of 22–44%, fig. 9A).

The initial increase in force produced by 3% halothane was accompanied by increases in concentrations of Ca^{2+} (data not shown) and MLC-p (fig. 9B). In contrast, the halothane-induced decrease in force at 15 min was not associated with changes in MLC-p (fig. 9B).

The role of PKC in this halothane-induced relaxation

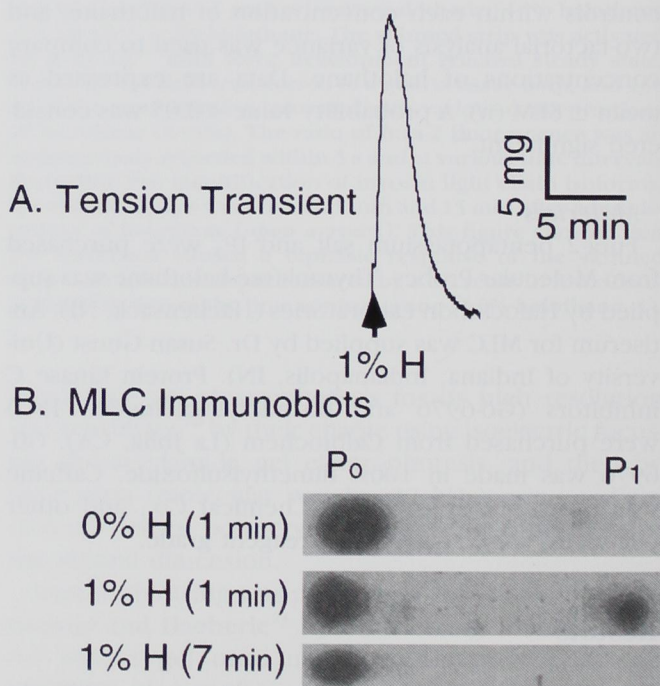
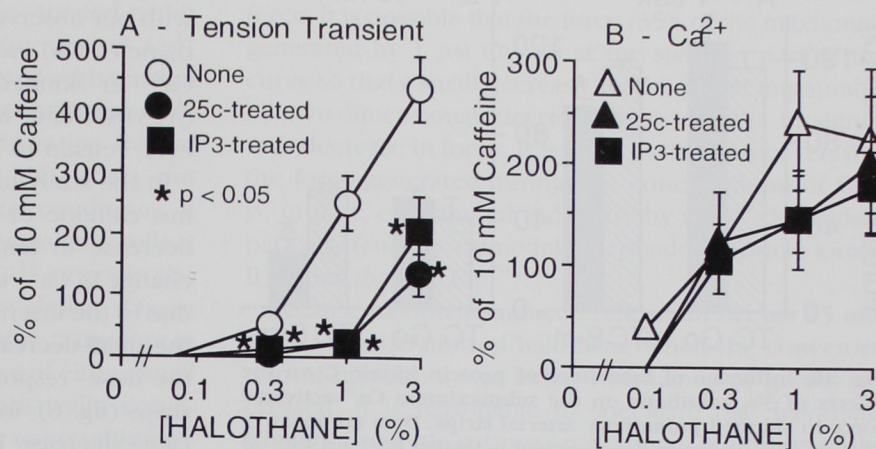


Fig. 7. Halothane-induced tension transient (A) and its time course of myosin light chain (MLC) phosphorylation (B). Halothane (1%) induced a tension transient (A) accompanied by increased phosphorylated MLCs (1% halothane [H], 1 min, B) compared with the time control (0% H, 1 min, B) or after the tension returned to baseline (1% H, 7 min, B). P_0 = unphosphorylated MLC; P_1 = one phosphorylated MLC.

HALOTHANE EFFECT ON SKINNED PULMONARY ARTERIAL STRIPS

Fig. 8. Effects of halothane on caffeine- or inositol 1,4,5-trisphosphate (IP_3)-releasable SR Ca^{2+} stores in skinned pulmonary arterial strips. Data are mean \pm SEM ($n = 3-14$). Halothane-induced tension transient or Ca^{2+} release (nm) is expressed as percent that of caffeine. Halothane, in a dose-dependent manner, induced tension transients (*open circles, A*) associated with increased Ca^{2+} (*open triangles, B*), except there was no further increase in Ca^{2+} with 3% halothane. In skinned strips pretreated with either 25 mM caffeine (25c-treated) or IP_3 (IP_3 -treated), the halothane-induced tension transients (*filled circles and squares, A*) were markedly reduced to a similar degree without significant changes, however, in increased Ca^{2+} (*filled triangles and squares, B*). None = time control without pretreatment. * $P < 0.05$ compared with control cycle.



of the submaximum Ca^{2+} -activated force was investigated further using inhibitors of PKC. We found that Go-6976, a specific inhibitor of the Ca^{2+} -dependent PKC isozymes α and β I,²⁴ did not affect the initial increase or subsequent decrease in force by 3% halothane (fig. 10). The initial increase was blocked, however, and the subsequent decrease in force was enhanced by bisindolylmaleimide I-HCl (fig. 10), an inhibitor of the α , β I, β II, γ , and ϵ isozymes of PKC.²⁵

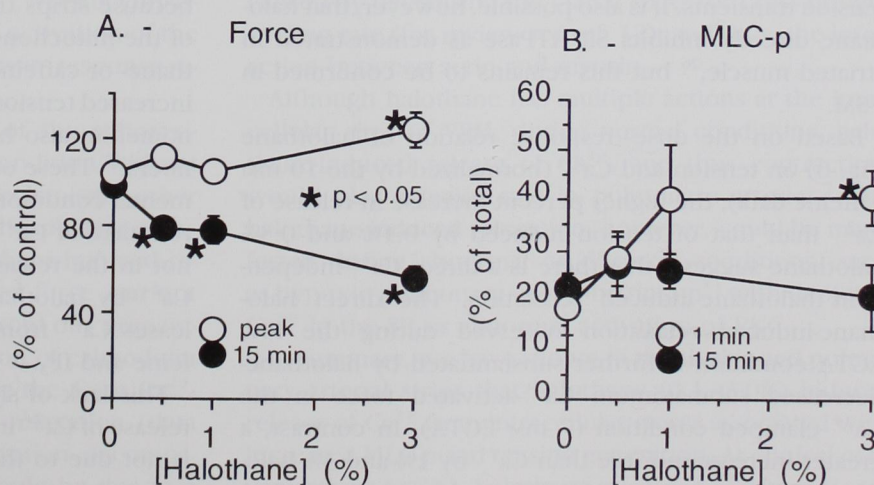
Discussion

This study shows that halothane has complex effects in skinned rabbit pulmonary arterial strips. The major

findings are that (1) halothane activates release of Ca^{2+} from the intracellular stores, although prerelease of intracellular stores with caffeine and IP_3 has a modest effect on this process; (2) all concentrations of halothane appear ultimately to cause a time-dependent depression of tension development that is not associated with decreased MLC-p; and (3) halothane at high concentrations induces a transient activation that may involve more than simple release of Ca^{2+} .

The direct halothane-induced tension transient is associated with increased release of Ca^{2+} from the SR. The increased cytosolic Ca^{2+} binds to calmodulin and activates Ca^{2+} /calmodulin-dependent MLC kinase. This activated MLC kinase phosphorylates the MLCs resulting

Fig. 9. Dose-response relation between concentrations of halothane and force or myosin light chain phosphorylation in skinned pulmonary arterial strips activated by $1 \mu M$ Ca^{2+} . Data are mean \pm SEM ($n = 4-27$). Submaximum Ca^{2+} -activated force development (*A*) is expressed as percent of control force before administration of halothane. Phosphorylated myosin light chain (MLC-p, *B*) is expressed as percent of total MLCs. Halothane (3%) significantly increased force development (*open circles, A*) and MLC-p (*open circles, B*) and was associated with an increased ratio of fura-2 fluorescence (not shown) 1 min after administration of halothane. In contrast, 15 min after administration of halothane (0.5–3.0%), force was decreased (*filled circles, A*) in a dose-dependent manner without changes in MLC-p (*filled circles, B*).



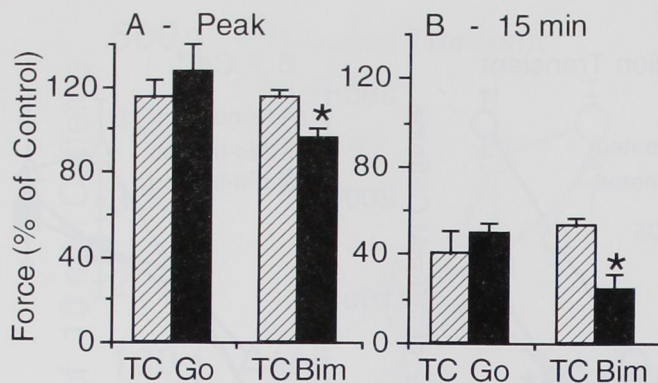


Fig. 10. Influence of inhibitors of protein kinase C on the effects of 3% halothane on the submaximum Ca^{2+} -activated force in skinned pulmonary arterial strips. Data are mean \pm SEM ($n = 4-7$). Force development activated by $1 \mu\text{M}$ Ca^{2+} at 1 min (A) and 15 min (B) after administration of halothane is expressed as percent of control force (before administration of halothane). The halothane-induced increases (time control [TC], A) in force were blocked by $10 \mu\text{M}$ bisindolylmaleimide HCl (Bim, an inhibitor of α and ϵ isozymes) but not by $10 \mu\text{M}$ Gö-6976 (Go, an α isozyme inhibitor) (A). In contrast, halothane-induced decreases (TC, B) in force were enhanced by Bim but not affected by Go (B). * $P < 0.05$ compared with TC.

in increased actomyosin ATPase activity, actin-myosin interaction, and finally force generation.⁸ The halothane-induced release of Ca^{2+} from the SR also is observed in isolated intact bovine pulmonary artery.⁷ The direct release of Ca^{2+} from the SR by halothane contributes, at least in part, to the enhancement of caffeine-induced tension transients when halothane is present in the release phase. Direct release of Ca^{2+} from the SR also could result in less accumulation of Ca^{2+} in the SR when halothane is present in the Ca^{2+} uptake phase and thus might explain the decreased caffeine-induced tension transients. It is also possible, however, that halothane directly inhibits SR ATPase as demonstrated in striated muscle,²⁶ but this remains to be confirmed in VSM.

Based on the dose-response relation of halothane (fig. 6) on tension and Ca^{2+} (normalized by the 10 mM caffeine data), the higher percent increase in release of Ca^{2+} than that of tension induced by 0.1% and 0.3% halothane suggests that there is a direct Ca^{2+} -independent halothane-induced relaxation. The direct halothane-induced relaxation observed during the low EGTA condition is further substantiated by halothane-decreased submaximum Ca^{2+} -activated force in the Ca^{2+} clamped condition (7 mM EGTA). In contrast, a greater increase in force than Ca^{2+} by 1% and 3% halothane (fig. 6) suggests that halothane increases the sensi-

tivity of the contractile proteins to Ca^{2+} , which agrees with our observations that high concentrations of halothane ($>5\%$) induced contracture with little change in Ca^{2+} in skinned strips treated with Ca^{2+} ionophore (A23187; data not shown). These speculations, however, remain to be proved.

In the study of Ca^{2+} stores pretreated with either 25 mM caffeine or IP_3 without reloading, the significant decrease in tension without a statistically significant change in Ca^{2+} caused by 1% and 3% halothane may be due to the insensitivity of fura-2 measurement to detect the small decrease in Ca^{2+} . This speculation is based on the dose-response relation of halothane in untreated strips (fig. 6), in which a small (not statistically significant) decrease in Ca^{2+} is accompanied by a large decrease in tension between 1.0% and 0.3% halothane. It is possible that in treated strips this curve is shifted to the right of that for untreated strips, so that the steepest part of the curve is now between 1% and 3% halothane. Moreover, there is a trend toward a decrease in release of Ca^{2+} by 1% and 3% halothane in caffeine- and IP_3 -treated strips compared with untreated strips. If this speculation is correct, the markedly decreased tension in caffeine- and IP_3 -treated strips could be the result of combined decreases in sensitivity of the contractile proteins and small decreases in release of Ca^{2+} by halothane. Nonetheless, the finding that there is no difference between caffeine- and IP_3 -treated strips suggests that halothane releases Ca^{2+} from both caffeine- and IP_3 -releasable stores.

The lack of change of halothane-induced Ca^{2+} transients in contrast to decreases in tension transients in strips treated with caffeine or IP_3 is not likely due to halothane-induced release of Ca^{2+} from mitochondria, because strips treated with sodium azide (an inhibitor of the mitochondria pump) do not show reduced halothane- or caffeine-induced tension transients but rather increased tension transients (data not shown). This phenomenon also has been shown in skinned myocardial fibers.²⁷ These observations suggest that, in our experimental conditions, mitochondria may play a role in the reuptake of Ca^{2+} released by halothane or caffeine but not in the release of Ca^{2+} . A localization of release of Ca^{2+} by halothane may reveal whether halothane releases Ca^{2+} from stores that are not releasable by caffeine and IP_3 .

This lack of significant change of halothane-induced release of Ca^{2+} in caffeine or IP_3 -treated strips, however, is not due to the lack of validity of the fura-2 fluorescence measurement as evidenced by the following.

HALOTHANE EFFECT ON SKINNED PULMONARY ARTERIAL STRIPS

First, the amount of Ca^{2+} release by 10 mM caffeine is in the same magnitude reported in β -escin-treated rabbit mesenteric arteries.²³ Second, halothane releases Ca^{2+} in a dose-dependent fashion, accompanied with tension transients (fig. 6). Third, in the same conditions, Ca^{2+} and tension transients are abolished in strips treated with Triton-X-100 (Sigma, St. Louis, MO) (data not shown). Therefore, in our experimental conditions, it is reasonable to assume that tension transients reflect the amount of Ca^{2+} release from the Ca^{2+} stores in the SR (fig. 6). Using this assumption, the similar degree of reduction of halothane-induced tension transients in caffeine- or IP_3 -pretreated strips, because of either small reductions of Ca^{2+} transients (not statistically significant, fig. 8B) or direct inhibition of the contractile proteins by halothane (discussed later), suggests that the SR stores are shared by ryanodine and IP_3 receptor channels. Thus, this release of Ca^{2+} from the SR by halothane from both caffeine- and IP_3 -releasable Ca^{2+} stores could be mediated either by activation of the ryanodine receptor SR Ca^{2+} release channel as observed in isolated cardiac SR²⁸ or by increases in SR membrane permeability to Ca^{2+} . The resulting increased cytosolic free Ca^{2+} also would enhance ryanodine depression of caffeine-induced tension transients as observed in striated muscle.^{3,29} This remains to be confirmed in VSM, however.

The 3% halothane-induced transient increase in submaximum Ca^{2+} -activated force (fig. 4) associated with increases in fura-2 fluorescence and MLC-p suggests that a large amount of Ca^{2+} (mainly buffered by 7 mM EGTA) is released by 3% halothane. Based on the $[\text{Ca}^{2+}]$ -tension relationship (data not shown), the increased fura-2 fluorescence by 3% halothane is not large enough to account for the force development. This increase in force is greater than the increase in Ca^{2+} , a result consistent with the direct halothane-induced activation of the contractile proteins suggested in the dose-response relation of halothane.

The halothane-induced depression of the submaximum force under the Ca^{2+} clamped condition without decreases in MLC-p suggests that mechanisms other than a decreased MLC kinase to MLC phosphatase activity ratio underlie its action. This halothane-induced relaxation in submaximum Ca^{2+} -activated force can not be due to leak of soluble calmodulin from our preparations, because exogenous administration of calmodulin (up to 0.1 mM) did not affect either the 1 μM Ca^{2+} -activated force or halothane-induced relaxation (data not shown). Halothane-induced relaxation accompanied with no change in MLC-p also could be due to a

hyperbolic relationship between MLC-p and isometric force. It is possible that the force (35% of the maximum) generated by 1 μM Ca^{2+} is at the steepest part of the curve so that a small decrease in MLC-p (not measurable by two-dimensional electrophoresis) results in significant decrease in force. It is also possible, however, that the force generated during low concentration of Ca^{2+} is, in part, contributed indirectly by a Ca^{2+} -dependent pathway (such as calmodulin-dependent protein kinase II) other than MLC-p.³⁰

Because halothane-induced relaxation occurs 15 min after administration of halothane when the concentration of halothane was either maintained or greatly decreased, it is reasonable to speculate that halothane triggers a cascade pathway resulting in inhibition of the contractile proteins. This cascade pathway could be triggered by a small release of Ca^{2+} by halothane or could be independent of Ca^{2+} . The blockade of initial increased force by 3% halothane and enhancement of halothane-induced delayed relaxation by the inhibitor of both Ca^{2+} -dependent and Ca^{2+} -independent isozymes (α , β I, β II, γ , and ϵ) of PKC but not by the inhibitor of specific Ca^{2+} -dependent PKC- α or PKC- β I suggests that inhibition of one or more of the Ca^{2+} -independent isozymes, possibly ϵ isozyme, may account for halothane-induced relaxation. The identification of PKC- α and PKC- ϵ , in collaboration with Liao *et al.*,³¹ (data not shown) in our preparation substantiates this speculation. Moreover, an inhibition of PKC by halothane also has been suggested in isolated intact rat coronary arterial preparations contracted by a PKC activator.¹⁵ Whether halothane directly inhibits PKC- ϵ and whether it is regulated by calponin³² remain to be examined. It is also possible that halothane-induced relaxation is caused by decreasing the "latch state" or cross-bridges cycling rate (for review see ref. 12), and thus, the interaction between actin and myosin.

Although halothane has multiple actions at the intracellular sites of VSM, during normal conditions, halothane-induced release of Ca^{2+} , and thus contraction, would play a major role in pulmonary arteries.⁷ The halothane-induced relaxation, however, could be manifested during abnormal or diseased conditions, such as hypoxic pulmonary vasoconstriction³³ with depleted Ca^{2+} in the SR or abnormal activation of PKC.

In summary, we have shown in rabbit skinned pulmonary arterial strips that halothane (0.1–3.0%) induces release of Ca^{2+} from intracellular stores associated with increased MLC-p and tension generation. At clinical concentrations ($<3\%$), halothane releases Ca^{2+} from stores

shared by both caffeine and IP_3 . During the submaximum Ca^{2+} activated state, halothane causes relaxation of the skinned strips by inhibition of Ca^{2+} -independent PKC.

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