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Ca^{2+}- and Myosin Phosphorylation–independent Relaxation by Halothane in K^+-depolarized Rat Mesenteric Arteries

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Background: **Volatile anesthetics inhibit vascular smooth muscle contraction, but the mechanisms responsible are uncertain. In this study, the effects of halothane on Ca^{2+} signaling and Ca^{2+} activation of contractile proteins were examined in high K^+-depolarized smooth muscle from rat mesenteric resistance arteries.**

Methods: **Vessels were cannulated and held at a constant transmural pressure (40 mmHg). Image analysis and microfluorimetry were used to simultaneously measure vessel diameter and smooth muscle intracellular [Ca^{2+}] concentration ([Ca^{2+}]$_i$). Myosin light chain (MLC) phosphorylation was measured using the Western blotting technique.**

Results: **Step increases in extracellular [Ca^{2+}] concentration (0–10 mM) in high K^+ (40 mM)–depolarized smooth muscle produced incremental increases in [Ca^{2+}]$_i$, MLC phosphorylation, and contraction. Halothane (0.5–4.5%) inhibited contraction in a concentration-dependent manner, but the decrease in [Ca^{2+}]$_i$ was small, and there was a marked shift in the [Ca^{2+}]$_i$–contraction relationship to the right, indicating an important Ca^{2+} desensitizing effect. Halothane (0.5–4.5%) did not affect MLC phosphorylation or the [Ca^{2+}]–MLC phosphorylation relationship, but the MLC phosphorylation–contraction relationship was also shifted rightward, indicating an "MLC phosphorylation" desensitizing effect. In contrast, control relaxations produced by the Ca^{2+} channel blocker nifedipine were accompanied by decreases in both [Ca^{2+}]$_i$ and MLC phosphorylation, and nifedipine had no affect on the [Ca^{2+}]$_i$–contraction, [Ca^{2+}]$_i$–MLC phosphorylation, and MLC phosphorylation–contraction relationships.**

Conclusions: **In high K^+-depolarized vascular smooth muscle, halothane relaxation is largely mediated by a Ca^{2+} and MLC phosphorylation desensitizing effect. These results suggest that the relaxing action of halothane is independent of the classic Ca^{2+}-induced myosin phosphorylation contraction mechanism.**

HALOTHANE and other volatile anesthetics are potent vasodilators at clinically relevant concentrations.[1,2] Direct inhibition of vascular smooth muscle contraction is an important factor in the vasodilating action[3]; however, the precise mechanisms whereby volatile anesthetics inhibit vascular smooth muscle contraction are not well understood. Contraction in vascular smooth muscle commences with an increase in the intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$), which activates the Ca^{2+}–calmodulin complex–dependent myosin light chain (MLC) kinase. Phosphorylation of the 20-kd regulatory MLC by MLC kinase promotes association of myosin with actin, which activates myosin Mg^{2+}–adenosine triphosphatase.[4–6] In addition to [Ca^{2+}]$_i$ increases, signaling pathways activated by endogenous vasoconstrictors may also produce "Ca^{2+} sensitization," a state in which [Ca^{2+}]$_i$ declines while MLC phosphorylation and contraction are maintained.[7] Finally, recent evidence suggests that contraction may also be maintained by mechanisms that are unrelated to, or act in parallel with, the classic Ca^{2+}-induced myosin phosphorylation–contraction mechanism. Phosphorylation of the thin filament–associated proteins calponin and caldesmon, for example, may facilitate the actin–myosin interaction and activate myosin adenosine triphosphatase, independent of changes in either [Ca^{2+}]$_i$ or MLC phosphorylation.[8–10]

Viewed in the context of these known mechanisms for smooth muscle contraction, the relaxing action of volatile anesthetics involves one or more of the following: inhibition of the Ca^{2+} signaling pathway(s) responsible for [Ca^{2+}]$_i$ increases; inhibition of Ca^{2+} sensitization mechanisms that maintain MLC phosphorylation independent of [Ca^{2+}]$_i$; and/or inhibition of contraction mechanisms that are unrelated to either Ca^{2+} signaling or the myosin phosphorylation mechanism. Interestingly, studies to date have implicated all three mechanisms, although the relative importance of each individual mechanism is unclear. Several studies have suggested that volatile anesthetic relaxation is related to decreases in [Ca^{2+}]$_i$.[11,12] Blocking effects on depolarization-activated Ca^{2+} channels[13] as well as inactivation of Ca^{2+} channels *via* potassium channel activation and membrane hyperpolarization have been reported.[14,15] In addition, a Ca^{2+} desensitizing effect appears to contribute significantly to the relaxing action of sevoflurane in high K^+- or norepinephrine-contracted mesenteric resistance arteries,[16] although it has not been determined whether the desensitizing action in resistance vessels is related to inhibition of MLC phosphorylation. The Ca^{2+} desensitizing effect of halothane in agonist-contracted airway smooth muscle is accompanied by MLC dephosphorylation,[17] apparently related to activation (or disinhibition) by halothane of the smooth muscle protein phosphatase that degrades phosphorylated MLC.[17,18] In contrast, a relaxing action of halothane in [Ca^{2+}]$_i$ clamped skinned vascular smooth muscle from rabbit pulmonary artery appears to be independent of changes in MLC phosphor-

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ylation, thus suggesting the involvement of a Ca^{2+}- and MLC phosphorylation–independent mechanism.[19]

The present study was designed to evaluate the mechanisms involved in the direct vascular smooth muscle relaxing action of halothane in high K^+-depolarized vascular smooth muscle from rat mesenteric resistance arteries.[20] Contraction, $[Ca^{2+}]_i$, and MLC phosphorylation were measured over a range of extracellular Ca^{2+} concentrations ($[Ca^{2+}]_o$) in high K^+-depolarized smooth muscle in the presence and absence of halothane. Using this approach, we were able to determine the effects of halothane in this model on the $[Ca^{2+}]_o$–$[Ca^{2+}]_i$ relationship, allowing an assessment of the effect on Ca^{2+} signaling; the $[Ca^{2+}]_i$–contraction and $[Ca^{2+}]_i$–MLC phosphorylation relationships, providing a measure of a Ca^{2+} desensitizing effect; and the MLC phosphorylation–contraction relationship, determining the potential involvement of MLC phosphorylation–independent relaxing mechanisms.

Materials and Methods

Preparation

All experiments were performed in compliance with the policy of the National Institutes of Health, and the Washington University Animal Studies Committee approved all protocols. The vessel isolation and cannulation methods were similar to those previously described.[20] Mesenteric tissue was removed from halothane-anesthetized adult male Long Evans rats (weight, 200–250 g), and fourth order branches of mesenteric arteries were carefully dissected from surrounding tissue in a dissection dish filled with HEPES-buffered physiologic saline solution (PSS) (at < 20°C).

The vessels were transferred to a water-jacketed vessel bath containing PSS at 37°C, and vessels were cannulated with glass pipettes (tip outer diameter [OD], $\approx$50–75 μm). The vessel bath was then transferred to the stage of a Diaphot inverted microscope (Nikon, Tokyo, Japan). The intravascular system was closed, and transmural pressure was incrementally raised to 40 mmHg and maintained with a pressure servo system. A micrometer attached to one of the cannula was used to gently elongate the vessel to remove any redundancy in the vessel wall, and the vessel was then allowed to equilibrate for 60 min. During this initial equilibration period, the vessel was checked several times for leaks (*i.e.*, a drop in pressure and diameter with the pressure servo system turned off). Vessels that exhibited a significant leak after pressurization were not studied further.

The vessel bath perfusion system used for these studies has been described previously.[20,21] This system consists of a series of glass reservoirs with valves to select the reservoir that is used to perfuse the vessel bath. To prevent leak of anesthetic gasses from the system, the reservoirs are tightly sealed at the top with a polytetrafluoroethylene (Teflon) stopper. Equilibration gas consisting of either air or air plus halothane (at a constant flow rate of 2 l/min) was directed to both the bubbler in the glass reservoir and the vessel bath cover. Halothane was added using a calibrated temperature and flow-compensated vaporizer (Ohio Medical, Columbus, OH). Gas chromatography of extracted perfusate samples, taken from both the reservoir and the vessel bath, confirmed that halothane equilibrated rapidly ($\leq$ 2 min) and remained constant throughout the perfusion system, as described previously.[20] Water jacketing of both the vessel bath and the bath reservoirs was used to maintain a constant temperature of 37°C throughout the system.

After equilibration, the functional integrity of the vascular smooth muscle and endothelium was tested by application of phenylephrine (10 μM) followed by the addition of acetylcholine (1 μM). Vessels were considered "healthy" if they exhibited sustained constriction with phenylephrine that was uniform along the length of the vessel and full relaxation after the addition of acetylcholine. Vessels that did not respond in this manner were considered damaged and were not studied further.

After the initial assessment of smooth muscle and endothelial integrity, the endothelium was selectively removed utilizing a modified version of the air perfusion technique described by Emerson and Segal.[22] Air bubbles (10 μl) were introduced at the proximal cannula and then driven at an elevated transmural pressure (60 mmHg) through the vessel lumen and out the distal cannula. Preliminary experiments indicated that 10 bubbles completely eliminated the relaxation produced by supermaximal acetylcholine (10 μM) without affecting phenylephrine contractions, and this procedure was then used in all subsequent experiments.

Measurement of Smooth Muscle Contraction

Contraction and relaxation of smooth muscle were determined by continuous measurement of vessel diameter using a real-time image analysis system described previously.[20,21] In brief, images of the vessel were directed to the video port of the inverted microscope equipped with a digital video camera. The camera was interfaced to a programmable video board, and the image was processed using "thresholding" techniques, which yielded pixel values corresponding to the inside and outside "edges" of the vessels on each video line. Inside diameter (ID) and OD were than determined from the calibrated μm/pixel values for the objective being used. The ID and OD obtained on each video line were then averaged, and the average ID and OD at each time point were displayed and recorded for analysis.

Measurement of Smooth Muscle $[Ca^{2+}]_i$

Measurement of vascular smooth muscle $[Ca^{2+}]_i$ was accomplished using the fluorescent Ca^{2+} indicator fura-2 and a photomultiplier-based microfluorimetry system. After removal of the endothelium, the smooth muscle was

"loaded" with fura-2, using a stock solution of fura-2 acetoxymethyl ester (Molecular Probes) diluted to 10 μM in PSS containing bovine serum albumin, 0.2%. Before loading, measurements of fura-2 before fluorescence were made for background subtraction, after which the fura-2 loading solution was placed in the vessel bath for 2 h at room temperature. After loading, the tissue was washed with PSS, and the bath temperature was increased to 37°C. The microfluorimetry system consisted of a xenon arc lamp that generated excitation light, which was directed *via* liquid light guides through a two-sector servo-controlled monochromatic 340-/380-nm excitation filter wheel (Omega Optical, Brattleboro, VT) and into the fluorescence port of the microscope. The alternating 340- and 380-nm excitation light was reflected (DM400 dichroic mirror; Nikon) onto the vessel (10X fluor objective [N.A. = 0.5]; Nikon). The fura-2–emitted light (centered at 510 nm) was separated from the greater than 600-nm light used for image analysis by a 570-nm dichroic mirror, filtered (510 ± 30 nm), and collected by the photomultiplier (model 9893/350B; Thorn-EMI, La Madera, NM). The photomultiplier signal was processed by an amplifier–discriminator (Thorn-EMI model C604AF), and an ECL to TTL pulse converter (Thorn-EMI model 605F). TTL pulses were then counted on 9513A time counters of a LabMaster DMA A/D board (Scientific Solutions, Inc., Cupertino, CA). Counting interrupts triggered by an analog signal from the filter wheel indicating the filter wheel position were used to separate the 340- and 380-nm signals. After subtraction of the background fluorescence, the ratio of the fura-2 fluorescence emission intensities at the 340- and 380-nm excitation wavelengths was calculated for each filter wheel cycle (10 Hz). The formula described by Grynkiewicz *et al.*[23] was used to calculate $[Ca^{2+}]_i$ from the F340/F380 ratio, using a dissociation constant for fura-2 of 224 nM. The ratio of maximum fluorescence (4.3) to minimum fluorescence (0.27) in our system was determined using commercial calibration solutions (Molecular Probes, Inc., Cupertino, CA).

For most experiments, the effects of halothane on smooth muscle contraction and $[Ca^{2+}]_i$ were measured simultaneously over a range of halothane concentrations in high K^+-depolarized vessels in which the $[Ca^{2+}]_o$ was incrementally raised from 0 to 10 mM. The incremental increases in $[Ca^{2+}]_o$ resulted in stepwise (*i.e.*, Ca^{2+} concentration–dependent) increases in both $[Ca^{2+}]_i$ and contraction. This approach was used to determine the concentration-dependent effects of halothane on the relationship between $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ (*i.e.*, Ca^{2+} signaling) and the relationship between $[Ca^{2+}]_i$ and contraction (*i.e.*, Ca^{2+} sensitivity).

Measurement of MLC Phosphorylation in Vascular Smooth Muscle

Phosphorylation of MLC in smooth muscle was measured in identically treated vessels using the method of Kamm and Stull.[24] Two to three vessel segments were used for each measurement of MLC phosphorylation. At the designated time, vessel segments were rapidly fixed by the addition of cold (0°C) trichloroacetic acid solution (final trichloroacetic acid concentration, 50%) to the chamber. The entire sample was then dropped immediately into liquid nitrogen and stored at −80°C before analysis. Tissue samples were subsequently thawed, placed in trichloroacetic acid, 10%, containing 0.5 M dithiotreitol and acetone, and centrifuged at 11,000*g* for 1 min. The pellet was resuspended, washed three times with acetone, and allowed to air dry. A urea–glycerol polyacrylamide gel was prepared and preelectrophoresed for 60 min at 400 V in a minigel apparatus. The air-dried pellet was resuspended in 60 μl urea sample buffer containing 0.5 M dithiotreitol and sucrose (10%), homogenized, and subjected to urea–glycerol polyacrylamide gel electrophoresis for 1 h. The proteins were then transferred to nitrocellulose and subjected to immunoblotting. The nitrocellulose membrane was blocked with a buffer containing powdered milk, 5%, and incubated overnight at room temperature with rabbit polyclonal antibody to MLC (obtained from Dr. James T. Stull, Ph.D., Professor and Chairman, Department of Physiology, University of Texas Southwestern Medical Center, Dallas, TX). The membranes were then washed and incubated with goat anti-rabbit, horseradish peroxidase–conjugated, antibody (Chemicon International, Temecula, CA) and developed with the "Renaissance" Western blot chemiluminescence reagent (Dupont NEN, Wilmington, DE). The nonphosphorylated and phosphorylated MLC isoforms were visualized by autoradiography and quantified by laser densitometry. The percentage phosphorylation was then determined directly from the relative intensities of the bands representing the phosphorylated and nonphosphorylated MLC isoforms.[24] The percentage phosphorylation data were correlated with the $[Ca^{2+}]_i$ and contraction data that were obtained at the same $[Ca^{2+}]_o$ to determine the $[Ca^{2+}]_I$–MLC phosphorylation and MLC phosphorylation–contraction relationships.

Solutions

The HEPES-buffered PSS has the following composition (in mM): NaCl, 135; HEPES, 10; $NaHCO_3$, 2.6; KCl, 5; $CaCl_2$, 1.6; KH_2PO_4, 0.44; $MgSO_4$, 1.17; Na_2HPO_4, 0.34; EDTA, 0.025; glucose, 5.5. The buffer pH was adjusted to 7.35 (37°C). The 40 mM "high" K^+ solution was prepared by isotonic replacement of NaCl with KCl. Ca^{2+}-free PSS solution was made by equimolar substitution of $MgCl_2$ for $CaCl_2$ and the addition of 2 mM EGTA. Solutions were equilibrated with air or air plus halothane.

Drugs

Halothane was obtained from Ayers Laboratories, Inc. (Philadelphia, PA). Fura-2 was obtained from Molecular

Probes. HEPES, epinephrine, nifedipine, guanethidine, acetylcholine hydrochloride, and EGTA were obtained from Sigma Chemical Company (St. Louis, MO). All the solutions contained 5 μM guanethidine to prevent norepinephrine release from residual sympathetic nerve terminals in the preparation. Nifedipine was solubilized in ethyl alcohol (99%) with a final concentration of less than 0.1% to avoid alcohol-related effects. All solutions containing nifedipine were covered with foil to prevent inactivation of nifedipine by light.

Statistical Analysis

The results are expressed as mean ± SD (n = the number of vessels, which equals the number of rats used). Percentage contraction was calculated from the reference baseline ID using the following equation:

$$\% \text{ contraction} = [(D_{BL} - D_M)/D_{BL}] \times 100, \quad (1)$$

where D_{BL} is the baseline diameter at the start of the experiment and D_M is the measured diameter. As ID was sometimes difficult to accurately measure in tightly constricted vessels, ID was calculated when necessary from OD as described previously[21] using the following equation:

$$ID = (OD^2 - 4/\pi \times WCSA)^{-2}, \quad (2)$$

where WCSA is the vessel wall cross-sectional area calculated from OD and ID in the relaxed state.

Statistical comparisons of each of the measured responses between control and halothane- or nifedipine-treated groups (*i.e.*, percentage contraction, $[Ca^{2+}]_i$, and MLC phosphorylation) were accomplished using two-way ANOVA followed by *post hoc* comparisons (*i.e.*, at each level of $[Ca^{2+}]_o$) using the Bonferroni-corrected Student *t* test. $P < 0.05$ was considered significant.

The $[Ca^{2+}]_o$–$[Ca^{2+}]_i$, $[Ca^{2+}]_i$–contraction, $[Ca^{2+}]_i$–MLC phosphorylation, and MLC phosphorylation–contraction relationships were described using the Hill equation:

$$Y = b/[1 + (K/X)^n], \quad (3)$$

where Y is the measured or dependent response (*i.e.*, $[Ca^{2+}]_i$, contraction, or MLC phosphorylation) at each level of the independent variable X (*i.e.*, $[Ca^{2+}]_o$, $[Ca^{2+}]_i$, or MLC phosphorylation). The maximum response (b), half-maximal response value (*K*) (*i.e.*, EC_{50}), and the Hill coefficient (n) were obtained by the best fit to the response data with the latter equation using a nonlinear least-squares method.

Results

Vessel Preparation and Endothelial Cell Removal

The vessels (n = 42) used in current study had an initial resting OD (*i.e.*, at 40-mmHg transmural pressure) of 158.0 ± 8.8 μm, ID of 121.5 ± 8.0 μm, wall thickness of 29.7 ± 1.8 μm, and length of ≈1.5 mm. Before endothelial removal, the vessels had responses to phenylephrine and acetylcholine that indicated acceptable endothelial and smooth muscle function as described under Preparation in Materials and Methods. The air bubbles introduced into the cannula for endothelial removal passed rapidly (< 1 s) through the vessels at the 60-mmHg driving pressure. After treatment with 10 bubbles, acetylcholine-mediated vasorelaxation was completely eliminated along the entire length of all the vessels used in this study. The air treatment had minimal effect on the resting diameter of the vessels (< 10%), and the responses to phenylephrine were slightly increased.

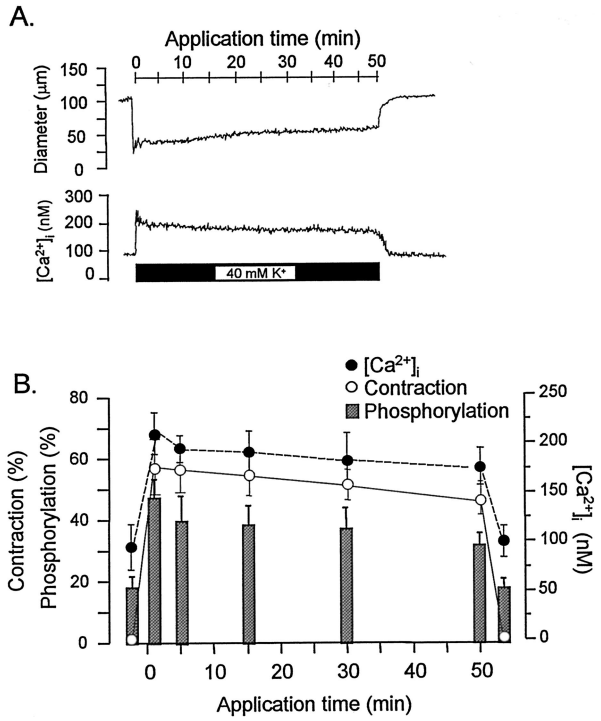

Fig. 1. Contraction, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and myosin light chain (MLC) phosphorylation measured during prolonged (50 min) application of high K^+ (40 mM) physiologic saline solution (1.6 mM extracellular $[Ca^{2+}]$ concentration). (*A*) *Data traces* showing vessel inner diameter and $[Ca^{2+}]_i$ recorded during the 50-min application of high K^+. Both traces were measured simultaneously in the same preparation. (*B*) Mean data for measurements of contraction, $[Ca^{2+}]_i$, and MLC phosphorylation at various times over the 50-min application of high K^+. Data at each time point shown represent the mean of four to five observations ± SD.

Time-dependent Changes in Contraction, $[Ca^{2+}]_i$, and MLC Phosphorylation in High K^+-depolarized Vascular Smooth Muscle

The effects of high K^+ (40 mM) PSS (1.6 mM $[Ca^{2+}]_o$) on contraction, $[Ca^{2+}]_i$, and MLC phosphorylation are shown in figure 1. Application of high K^+ produced sustained elevations in contraction, $[Ca^{2+}]_i$, and MLC phosphorylation (fig. 1). After an initial peak and decline

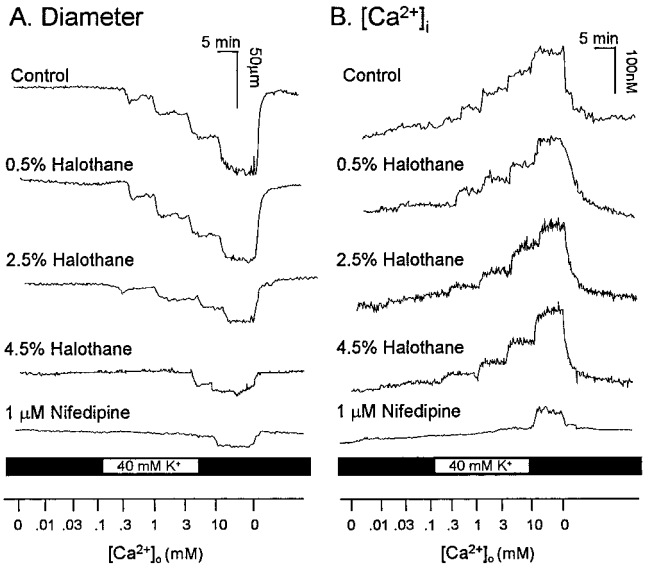

Fig. 2. ***Data traces*** **showing typical responses in (*A*) vessel inner diameter and (*B*) intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) during the stepwise increases in extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) in high K^+ (40 mM)–depolarized, endothelium-denuded mesenteric resistance arteries. Control responses are shown at the top, followed by responses during application of halothane (0.5–4.5%) or nifedipine (1 mM). Halothane and nifedipine additions were made to the bath 10 min before and then continuously throughout the stepwise increases in $[Ca^{2+}]_o$. Five minutes was allowed for equilibration after each step increase in $[Ca^{2+}]_o$.**

during the first 5 min, the elevations in contraction, $[Ca^{2+}]_i$, and MLC phosphorylation remained relatively stable, with only small declines (15–22% from the 5-min values) during the remainder of the 50-min high K^+ application period (n = 4 to 5).

Effects of Halothane on $[Ca^{2+}]_i$ Increases and Contraction in High K^+-depolarized Vascular Smooth Muscle

In figure 2, the changes in vessel diameter (ID) and $[Ca^{2+}]_i$ produced by stepwise increases in $[Ca^{2+}]_o$ (from 0 to 10 mM) are shown in the high K^+-treated, fura-2–loaded vessels before and after treatments with halothane (0.5–4.5%) or nifedipine (1 μM).

Figure 3 shows the composite data from multiple experiments identical to those shown in figure 2. The concentration-dependent effects of halothane and the effects of 1 μM nifedipine on $[Ca^{2+}]_i$ and contraction are shown as a function of $[Ca^{2+}]_o$ (0–10 mM). As can be seen, the stepwise increases in $[Ca^{2+}]_o$ were accompanied by incremental increases in both $[Ca^{2+}]_i$ and contraction. Compared with the control responses, halothane produced a small concentration-dependent effect on the $[Ca^{2+}]_i$ increases as a function of $[Ca^{2+}]_o$, and the effect of halothane on the $[Ca^{2+}]_o$–$[Ca^{2+}]_i$ relationship was significant at the highest halothane concentration tested (4.5%). As shown in figure 3, *B*, halothane also produced concentration-dependent inhibition of the

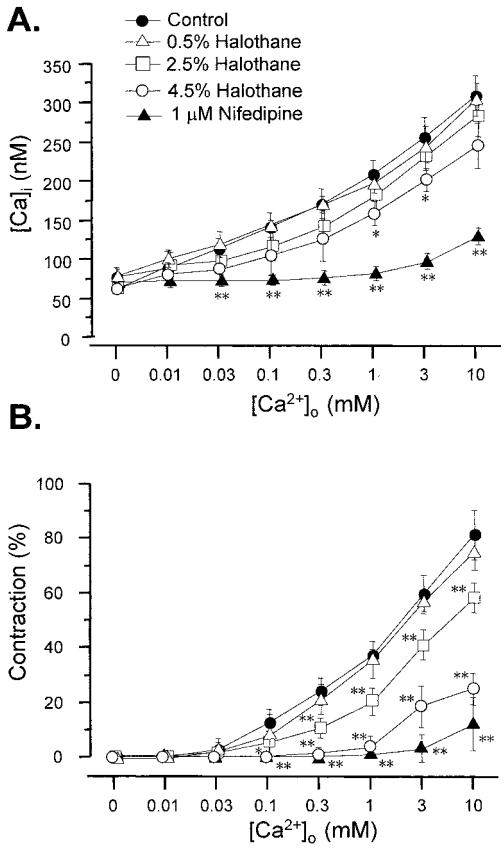

Fig. 3. Summary of the (*A*) intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and (*B*) contraction responses, plotted as a function of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$), in high K^+ (40 mM)–depolarized, endothelium-denuded mesenteric resistance arteries. Control responses are shown as *closed circles*, responses during halothane as *open symbols*, and responses during nifedipine as *closed triangles*. (*A*) Increases in $[Ca^{2+}]_i$ are strongly inhibited by nifedipine, whereas halothane has a comparatively small effect on $[Ca^{2+}]_i$ that was significant only at the highest concentration tested (4.5%) ($P < 0.05$ by ANOVA). (*B*) Contractions are significantly inhibited by halothane (0.5–4.5%) in a concentration-dependent manner, and by nifedipine (1 μM). Both contraction and $[Ca^{2+}]_i$ were measured simultaneously, and halothane and nifedipine were added 10 min before and continuously throughout the experiment, with 5 min for equilibration after each step increase in $[Ca^{2+}]_o$. Mean data from at least four measurements at each point are shown (± SD). *$P < 0.05$, **$P < 0.01$ *versus* corresponding control value.

contractions that occurred with the increases in $[Ca^{2+}]_o$ (fig. 3, *A*). As can be seen, the effects of 1 μM nifedipine and halothane, 4.5%, on contraction were comparable (fig. 3, *B*); however, nifedipine had a much greater effect on the $[Ca^{2+}]_i$ increases and the $[Ca^{2+}]_o$–$[Ca^{2+}]_i$ relationship than did halothane, 4.5%. Thus, although halothane, 4.5%, and 1 μM nifedipine produce comparable relaxation, halothane had comparably little effect on $[Ca^{2+}]_i$ signaling.

Effects of Halothane on Ca^{2+}-induced MLC Phosphorylation in High K^+-depolarized Vascular Smooth Muscle

In figure 4, *A*, immunoblots showing the two nonphosphorylated MLC isoforms and the two monophosphory-

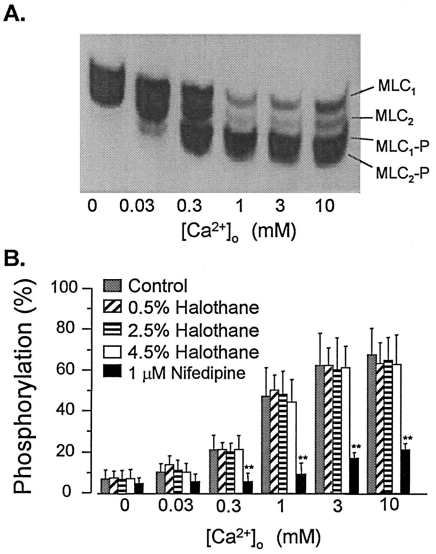

Fig. 4. Myosin light chain (MLC) phosphorylation in response to the stepwise increases in $[Ca^{2+}]_o$ in high K^+ (40 mM)–depolarized vascular smooth muscle is shown in the absence or presence of halothane, 0.5 − 4.5%. (*A*) Immunoblots showing the two nonphosphorylated MLC isoforms (MLC_1 and MLC_2) and the two phosphorylated isoforms (MLC_1-P and MLC_2-P) after separation by urea–glycerol polyacrylamide gel electrophoresis and visualization by chemiluminescence. As can be seen, the nonphosphorylated isoforms (MLC_1 and MLC_2) decreased, whereas the phosphorylated isoforms (MLC_1-P and MLC_2-P) increased as a function of increasing $[Ca^{2+}]_o$. (*B*) Phosphorylation responses in the control (*shaded bars*), halothane-treated (0.5%, *diagonal bars*; 2.5%, *horizontal bars*; 4.5%, *open bars*), or nifedipine-treated 1 μM, *closed bars*) vessels are shown. Halothane had no significant effect on MLC phosphorylation, whereas nifedipine significantly inhibited MLC phosphorylation. The percentage MLC phosphorylation was taken as the sum of the densities of the MLC_1-P and MLC_2-P bands divided by the total MLC, as described under Materials and Methods. Data are shown as mean ± SD; there are three to six measurements at each data point. **$P < 0.01$ *versus* corresponding control value.

lated MLC isoforms are shown as a function of $[Ca^{2+}]_o$ in the high K^+-depolarized vessels. As can be seen, MLC phosphorylation increases as a function of increases in $[Ca^{2+}]_o$, and both of the nonphosphorylated isoforms undergo similar shifts in mobility with phosphorylation (fig. 4, *A*). Results of analysis of the $[Ca^{2+}]_o$–dependent MLC phosphorylation data for control, halothane-treated, and nifedipine (1 μM)–treated vessels are shown in figure 4, *B*. As can be seen, halothane (0.5–4.5%) had no effect on Ca^{2+}-dependent MLC phosphorylation in the high K^+-depolarized vessels, whereas MLC phosphorylation was markedly inhibited by nifedipine (1 μM).

Effects of Halothane on $[Ca^{2+}]_i$–Contraction, $[Ca^{2+}]_i$–MLC Phosphorylation, and MLC Phosphorylation–Contraction Relationships in High K^+-depolarized Vascular Smooth Muscle

As shown in figure 5, halothane (0.5–4.5%) shifted the $[Ca^{2+}]_i$–contraction relationship to the right in a concentration-dependent manner but had no evident effect on the $[Ca^{2+}]_i$–MLC phosphorylation relationship. As such, halothane has an evident Ca^{2+} desensitizing effect (fig. 5, *A*). However, the desensitizing action of halothane did not involve an effect on Ca^{2+}-induced MLC phosphorylation (fig. 5, *B*). As shown in figure 6, halothane also produced a concentration-dependent rightward shift of the MLC phosphorylation–contraction relationship, indicating an "MLC phosphorylation" desensitizing action.

Effects of "Acute" Application of Halothane on Contraction and $[Ca^{2+}]_i$ in High K^+-depolarized Vascular Smooth Muscle

In figure 7, the effects of "acute" application of halothane on contraction and $[Ca^{2+}]_i$ are shown in vessels precontracted with high K^+ at physiologic $[Ca^{2+}]_o$ (1.6 mM). As shown in the upper panel of figure 7, *A*, halothane (4.5%) or 1 μM nifedipine produces near-complete relaxation of the high K^+-contracted vessels. However, compared with nifedipine, halothane had only a small effect on $[Ca^{2+}]_i$ (fig. 7, *A, lower panel*). Contraction and $[Ca^{2+}]_i$ data from five identical experiments using this protocol at each halothane concentration tested (0.5–4.5 vol%) and at 1 μM nifedipine are shown in figure 7, *B* and *C*. The results demonstrate concentration-dependent effects of halothane on contraction (fig. 7, *B*)

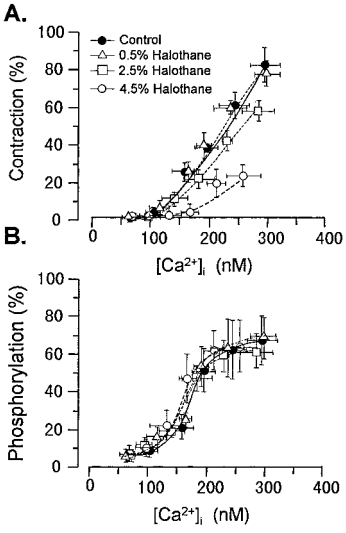

Fig. 5. Effects of halothane on the (*A*) intracellular $[Ca^{2+}]$ concentration ($[Ca^{2+}]_i$)–contraction and (*B*) $[Ca^{2+}]_i$–myosin light chain (MLC) phosphorylation relationships in high K^+–depolarized vascular smooth muscle. Data are shown in the absence (*closed circles*) or presence of halothane, 0.5%, 2.5%, and 4.5% (*open symbols*). (*A*) Halothane has a clearly evident inhibitory effect on the $[Ca^{2+}]_i$–contraction relationship as evidenced by the diminished contractile responses to the increases in $[Ca^{2+}]_i$. (*B*) By contrast, halothane has no effect on the $[Ca^{2+}]_i$–MLC phosphorylation relationship, indicating that halothane has no effect on Ca^{2+}-induced MLC phosphorylation. Thus, the apparent Ca^{2+} desensitizing effect of halothane evident in *A* is not related to a decrease in MLC phosphorylation. The curves for the effects of $[Ca^{2+}]_i$ on contraction and MLC phosphorylation were obtained by fitting the data points to the Hill equation as described in Materials and Methods. Data are shown as mean ± SD; there are three to six measurements per data point.

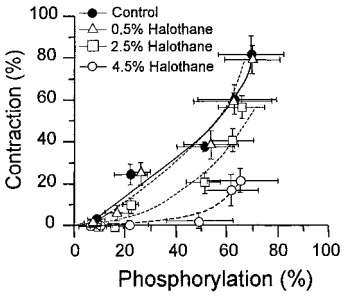

Fig. 6. Effects of halothane on the myosin light chain (MLC) phosphorylation–contraction relationship in high K^+–depolarized vascular smooth muscle. As can be seen, halothane (0.5–4.5%) has a clear concentration-dependent inhibitory effect on the phosphorylation–contraction relationship as evidenced by the diminished contractile responses to the increases in MLC phosphorylation. This "MLC phosphorylation" desensitizing effect is similar to the Ca^{2+} desensitizing effect of halothane evident in figure 5A and likely reflects an effect of halothane on contractile protein activation that is unrelated to MLC phosphorylation (see Discussion). As described in Materials and Methods, the curves shown represent best fits to the Hill equation using the nonlinear least-squares method. Data are shown as mean ± SD; there are three to six measurements per data point.

with a relatively small effect on $[Ca^{2+}]_i$ compared with nifedipine, which strongly inhibited both contraction and $[Ca^{2+}]$ (fig. 7, *C*). The results using "acute" application of halothane to precontracted vessels shown in figure 7 are thus identical to the results obtained utilizing the protocol shown in figure 2, in which halothane was applied before contraction.

Discussion

Two important new findings are identified in this study. First, our results clearly indicate that a decrease in Ca^{2+} sensitivity, or "Ca^{2+} desensitization," is the principle effect that underlies the vascular smooth muscle relaxing action of halothane in high K^+-depolarized smooth muscle from rat mesenteric resistance arteries. Halothane relaxation in this model was accompanied by a decrease in $[Ca^{2+}]_i$, which may also have contributed to the relaxing action, but the $[Ca^{2+}]_i$ decrease was relatively small compared with the Ca^{2+} desensitizing effect, as evidenced by the marked rightward shift of the $[Ca^{2+}]_i$–contraction relationship. Second, halothane relaxation in this model occurred in the presence of sustained high levels of MLC phosphorylation. Halothane had no effect on the $[Ca^{2+}]_i$–MLC phosphorylation relationship, and a MLC phosphorylation desensitizing action was clearly evident in the concentration-dependent rightward shift of the MLC phosphorylation–contraction relationship.

Ca^{2+} signaling, *via* activation of voltage-dependent Ca^{2+} channels and/or release of Ca^{2+} from intracellular stores, followed by Ca^{2+}-induced MLC phosphorylation, is the principle mechanism responsible for activation of the contractile machinery in vascular smooth muscle.[5,6,25] MLC phosphorylation and contraction may also be maintained independent of $[Ca^{2+}]_i$ by agonist-induced activation of the protein kinase C and/or Rho kinase signaling pathways, which produce decreased activity of the smooth muscle protein phosphatase that degrades phosphorylated MLC.[5–7,26] Similarly, the actions of endogenous vasodilators have also been explained either by effects on Ca^{2+} signaling—*via* membrane hyperpolarization, Ca^{2+} channel block, or increased Ca^{2+} clearance—or by disinhibition (*i.e.*, activation) of smooth muscle protein phosphatase—*via* inhibition of protein kinase C– and/or Rho kinase–induced inhibition of smooth muscle protein phosphatase.[5–7,27–30] The observations in our study that the high K^+ contractions were accompanied by predictable increases in $[Ca^{2+}]_i$ and MLC phosphorylation are consistent with this classic myosin phosphorylation–contraction mechanism, as is the vasodilation produced by the Ca^{2+} channel blocker nifedipine or removal of extracellular Ca^{2+} (which were both accompanied by proportionate and predictable decreases in $[Ca^{2+}]_i$ and MLC phosphorylation). The fact that halothane relaxation in this model could not

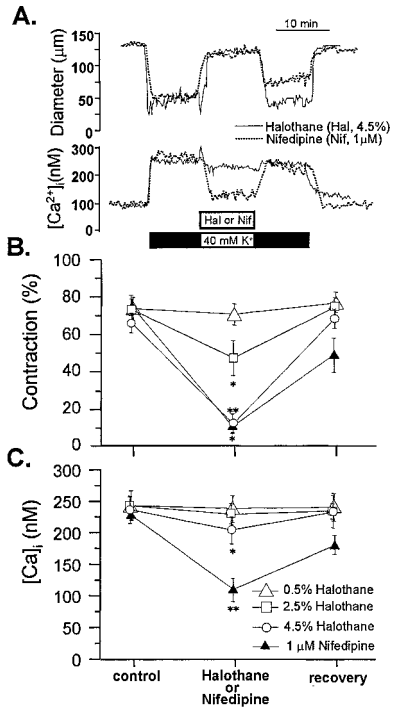

Fig. 7. Effects of "acute" application of halothane or nifedipine on vascular smooth muscle precontracted with high K^+ physiologic saline solution (1.6 mM extracellular $[Ca^{2+}]$ concentration) are shown. (*A*) *Raw data traces* demonstrate that halothane, 4.5%, and 1 μM nifedipine have comparable effects on contraction (*upper panel*), but the effects on intracellular $[Ca^{2+}]$ concentration ($[Ca^{2+}]_i$) differ considerably (*lower panel*). The concentration-dependent inhibitory effects of halothane (0.5–4.5%) or 1 μM nifedipine on (*B*) contraction and (*C*) $[Ca^{2+}]_i$ are shown using this protocol. The contrasting effects of nifedipine on $[Ca^{2+}]_i$ signaling and the Ca^{2+} desensitizing effect of halothane are again evident using this protocol. Mean data from five vessels are shown (± SD). *$P < 0.05$, **$P < 0.01$ *versus* corresponding control values.

be accounted for by changes in either $[Ca^{2+}]_i$ or MLC phosphorylation, however, indicates that the relaxing action of halothane does not involve the classic MLC phosphorylation mechanism. These findings further suggest that maintenance of MLC phosphorylation alone is not sufficient for maintenance of contraction and that some other mechanism—which is inhibited by halothane—is important in high K^+-depolarized vascular smooth muscle.

Previous studies have highlighted the importance of volatile anesthetic effects on Ca^{2+} signaling,[11–15] and the emphasis on the Ca^{2+} desensitizing effect in the study reported here represents a significant departure from that view. An effect on $[Ca^{2+}]_i$ was evident in our investigation; however, it was also clear from the Ca^{2+}–contraction relationship that the Ca^{2+} signaling effect could account only for a fraction of the relaxing action. Differences in the pharmacomechanical and electromechanical coupling mechanisms between the small resistance vessels used here and large conductance vessels used in earlier studies may account for some of this difference.[25] In addition, the effects of halothane on the $[Ca^{2+}]_i$–contraction relationship were not established in previous studies, and the potential involvement of a Ca^{2+} desensitizing effect could have been overlooked. Conversely, our experiments were specifically designed to permit assessment of effects on Ca^{2+} sensitivity, but perhaps our protocols also placed greater emphasis on Ca^{2+} desensitization. Nevertheless, it is clear from this investigation, and other recent studies, that Ca^{2+} signaling and Ca^{2+} desensitizing effects may both be involved in the smooth muscle relaxing action of volatile anesthetics.[16,31] Their relative importance may depend on the type of vessel studied, the experimental conditions employed, or the specific anesthetic agent used.

There are few previous data concerning whether volatile anesthetic–induced Ca^{2+} desensitization results from decreased MLC phosphorylation or from the MLC phosphorylation desensitizing action reported here. The Ca^{2+} desensitizing action of halothane in agonist-contracted airway smooth muscle[32,33] is associated with decreased MLC phosphorylation,[17] resulting from an increase in smooth muscle protein phosphatase activity.[18] Thus, the Ca^{2+} desensitization by halothane in airway smooth muscle appears to be related to inhibition of agonist-induced Ca^{2+} sensitization *via* the classic MLC phosphorylation mechanism. In contrast, Su and Tang[19] described a slowly developing relaxation produced by halothane in Ca^{2+} clamped skinned pulmonary artery strips, which was not associated with any significant decrease in MLC phosphorylation. More recently, Su and Vo[34] demonstrated that this relaxing action is partially blocked by a CAM kinase II inhibitor, but, interestingly, they related the effect of the CAM kinase II inhibitor to decreased MLC phosphorylation *via* CAM kinase II–induced phosphorylation of MLC kinase.[35] Since the thin filament–associated protein caldesmon is the other well-known substrate for CAM kinase II and caldesmon phosphorylation has been implicated in MLC phosphorylation–independent contraction, not relaxation,[5,36] it is difficult to reconcile the effect of the CAM kinase II inhibitor with the MLC phosphorylation–independent relaxing action previously reported in that model. Nevertheless, as Su and Vo did not report on the effect of the CAM kinase II inhibitors on MLC phosphorylation in their recent study, we did not report on the effect of CAM kinase II inhibitors on relaxation in our study. Thus, neither study clarifies the role of CAM kinase II in the MLC phosphorylation–independent relaxing action of halothane. At present, the biochemical basis for the Ca^{2+}- and MLC phosphorylation–independent relaxing action of halothane in intact vascular smooth muscle reported here remains uncertain.

The MLC phosphorylation desensitizing action we observed could result from a direct effect on one of the downstream components of the MLC phosphorylation–contraction mechanism, such as the actin–myosin interaction or activation of the myosin adenosine triphosphatase, or this action could result from inhibition of some other fundamental process, such as actin monomer assembly. Arguing against such a mechanism, however, is the previously reported finding that the relaxing action of halothane and other volatile anesthetics is substantially attenuated in permeabilized vascular smooth muscle from these vessels.[37] Since contractions in permeabilized smooth muscle are dependent on the classic MLC phosphorylation mechanism,[38,39] a direct effect on one of the components downstream of MLC phosphorylation, or on a more fundamental process involved in contraction, would not be consistent with the attenuated relaxing action in the permeabilized model. On the other hand, however, it is well known that membrane permeabilization disrupts membrane-dependent or soluble messenger–mediated pathways, including pathways that modulate contraction independent of MLC phosphorylation.[40,41] Thus, the results for the skinned pulmonary artery strips notwithstanding, the observation that volatile anesthetic–induced relaxation is attenuated in permeabilized smooth muscle indicates that key components of the contractile machinery are probably not directly affected and that a soluble second messenger– or membrane-dependent pathway, which is lost after permeabilization, is involved in the MLC phosphorylation desensitizing action of halothane.

Recent studies indicate that, in addition to MLC phosphorylation, contraction mechanisms involving the actin-containing "thin filaments" may be important in maintaining contractions produced by both receptor agonists and high K^+.[5,7–10] Ca^{2+} binding and/or phosphorylation of the actin-associated proteins calponin and caldesmon— by signaling pathways involving protein kinase C, mitogen-activated protein kinases, or CAM kinase II— promotes actin–myosin association and increased myo-

sin adenosine triphosphatase activity, independent of MLC phosphorylation.[8–10] Although an obligatory role of thin filament regulation in maintaining contraction has not been established and the involvement of this mechanism in the relaxing action of halothane is unknown, investigation of the biochemical basis for halothane's MLC phosphorylation–independent relaxing action—*via* effects on calponin and caldesmon phosphorylation— could provide insight into both volatile anesthetic relaxation and smooth muscle contraction mechanisms.

Clearly, volatile anesthetics have a number of potentially important actions that could affect vascular tone *in vivo*. Effects on sympathetic tone, cardiovascular reflexes, and tissue metabolism may indirectly affect vasomotor tone,[42–44] whereas direct effects on vascular smooth muscle[11–16,20] and vascular endothelial cell function[45,46] may also have a significant impact. Moreover, the importance of any one of these effects may vary significantly depending on the clinical circumstance or the experimental conditions employed. Nevertheless, the potential significance of the MLC phosphorylation desensitization mechanism reported here should not be underestimated. High K^+-depolarized vascular smooth muscle is widely used to study basic Ca^{2+}-dependent smooth muscle contraction mechanisms. Moreover, this model is particularly analogous to the myogenic contraction mechanism that is primarily responsible under basal conditions for (1) autoregulation of blood flow and capillary hydrostatic pressure over the physiologic range of blood pressure, (2) providing sufficient tone to permit vasodilatory responses to endogenous mediators released in response to increased metabolism or flow, and (3) augmentation of responsiveness to neurohumoral vasoconstrictor substances.[30,47,48] Thus, although the high K^+-depolarized vascular smooth muscle model may not reflect a situation seen in the *in vivo* setting, relaxation in this model may translate to a significant effect on myogenic tone. The MLC phosphorylation desensitizing action identified in these studies could therefore have a considerable impact on vascular tone *in vivo*.

In summary, halothane produced vascular relaxation in high K^+-depolarized smooth muscle from resistance arteries by a desensitizing action that is largely independent of changes in either $[Ca^{2+}]_i$ or MLC phosphorylation. Previous studies have emphasized the potential importance of mechanisms that affect regulation of myosin phosphorylation, including effects on $[Ca^{2+}]_i$ signaling and Ca^{2+} sensitizing effects involving decreases in MLC kinase or increases in smooth muscle protein phosphatase activities. Our data, however, suggest that a novel MLC phosphorylation–independent desensitizing action plays an important role in the vascular smooth muscle relaxing action of halothane. Further investigation of the biochemical basis for this action should provide valuable insights into basic mechanisms of volatile anesthetic action and smooth muscle relaxation.

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