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Effects of Halothane on Sarcoplasmic Reticulum Calcium Release Channels in Porcine Airway Smooth Muscle Cells

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Background: **Volatile anesthetics relax airway smooth muscle (ASM) by altering intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The authors hypothesized that relaxation is produced by decreasing sarcoplasmic reticulum Ca^{2+} content *via* increased Ca^{2+} "leak" through both inositol trisphosphate (IP_3) and ryanodine receptor channels.**

Methods: **Enzymatically dissociated porcine ASM cells were exposed to acetylcholine in the presence or absence of 2 minimum alveolar concentration (MAC) halothane, and IP_3 levels were measured using radioimmunoreceptor assay. Other cells were loaded with the Ca^{2+} indicator fluo-3 and imaged using real-time confocal microscopy.**

Results: **Halothane increased IP_3 concentrations in the presence and absence of acetylcholine. Inhibition of phospholipase C blunted the IP_3 response to halothane. Exposure to 2 MAC halothane induced a transient $[Ca^{2+}]_i$ response, suggesting depletion of sarcoplasmic reticulum Ca^{2+}. Exposure to 20 μM Xestospongin D, a cell-permeant IP_3 receptor antagonist, resulted in a 45 ± 13% decrease in the $[Ca^{2+}]_i$ response to halothane compared with halothane exposure alone. In permeabilized cells, Xestospongin D or 0.5 mg/ml heparin decreased the $[Ca^{2+}]_i$ response to halothane by 65 ± 13% and 68 ± 22%, respectively, compared with halothane alone. In both intact and permeabilized cells, 20 μM ryanodine blunted the $[Ca^{2+}]_i$ response to halothane by 32 ± 13% and 39 ± 21%, respectively, compared with halothane alone. Simultaneous exposure to Xestospongin D and ryanodine completely inhibited the $[Ca^{2+}]_i$ response to halothane.**

Conclusions: **The authors conclude that halothane reduces sarcoplasmic reticulum Ca^{2+} content in ASM cells *via* increased Ca^{2+} leak through both IP_3 receptor and ryanodine receptor channels. Effects on IP_3 receptor channels are both direct and indirect *via* elevation of IP_3 levels.**

VOLATILE anesthetics such as halothane produce bronchodilation, partly by decreasing intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which plays an important role in development and maintenance of force in airway smooth muscle (ASM) cells. For example, halothane has been shown to decrease the elevation in $[Ca^{2+}]_i$ produced by agonists such as acetylcholine.[1–3] These effects of halothane are partly attributable to an inhibition of Ca^{2+} influx.[3–5] Other studies in vascular smooth muscle[3] and cardiac tissue[6] have indicated that halothane depletes Ca^{2+} stores (content) of the sarcoplasmic reticulum (SR) by increasing Ca^{2+} "leakage." In a recent study, Yamakage *et al.*[7] demonstrated that volatile anesthetic–induced depletion of SR Ca^{2+} also occurs in canine tracheal smooth muscle. In other cell types, such anesthetic-induced SR, Ca^{2+} leak has been shown to occur *via* both inositol 1,4,5-trisphosphate (IP_3) and/or ryanodine receptor (RyR) channels.[8,9] Whether a similar mechanism is involved in ASM cells remains to be determined.

In ASM cells, agonist-induced elevation of $[Ca^{2+}]_i$ involves Ca^{2+} influx as well as SR Ca^{2+} release through IP_3 receptor channels.[10] More recently, it has also been demonstrated that agonist activation in ASM cells involves the novel second messenger system cyclic adenosine diphosphate ribose (cADPR) and Ca^{2+} release through RyR channels.[11–13] In a recent study, we showed that clinically relevant concentrations of halothane inhibit acetylcholine-induced $[Ca^{2+}]_i$ oscillations that are initiated by Ca^{2+} release through IP_3 receptor channels and are sustained by repetitive SR Ca^{2+} release through RyR channels.[14] Our results indicated that halothane inhibits $[Ca^{2+}]_i$ oscillations by decreasing SR Ca^{2+} content. Therefore, the purpose of the current study was to characterize the mechanisms by which halothane decreases SR Ca^{2+} content. We hypothesized that halothane increases SR Ca^{2+} leakage through both IP_3 receptor and RyR channels. To remove the confounding effects of halothane on Ca^{2+} influx and efflux, two ASM cell preparations were used: intact cells in which influx and efflux were blocked and β-escin permeabilized cells.

Materials and Methods

Cell Preparation

After obtaining porcine tracheae from a local abattoir, the smooth muscle layer was dissected, and ASM cells were dissociated using previously described techniques.[11,12,14,15] Briefly, the endothelial layer was removed, and the smooth muscle layer was excised and minced thoroughly in Hank's balanced salt solution (HBSS) buffered with 10 mM HEPES (pH 7.4; Life Technologies, Rockville, MD). The minced tissue was incubated for 2 h in Earle's balanced salt solution containing 20 U/ml papain and 2000 U/ml DNase (Worthington

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Biochemical Corp., Lakewood, NJ), and subsequently at 37°C with 1 mg/ml type IV collagenase (Worthington Biochemical Corp.) for another hour. The cells were then gently triturated, centrifuged, and resuspended in minimum essential medium containing 10% fetal calf serum. The isolated cells were split into two batches for IP_3 measurements and confocal imaging.

Inositol Triphosphate Measurements

Measurements of IP_3 in ASM samples were performed using a radioreceptor assay.[16,17] ASM cell suspensions (10^6 cells/ml) were placed in test tubes and aerated with 95% O_2 and 5% CO_2. The test tubes were placed on ice to minimize protein degradation. Cells were taken through one of the following protocols: (1) HBSS (vehicle control) for 2 min; (2) 1 μM acetylcholine in HBSS for 2 min; (3) 2 minimum alveolar concentration (MAC) halothane for 2 min; (4) 1 μM acetylcholine and 2 MAC halothane for 2 min; (5) 10 μM U73122 (Sigma Chemicals, St. Louis, MO), an inhibitor of phospholipase C (PLC), for 5 min, followed by 1 μM acetylcholine for 2 min; (6) 10 μM U73122 for 5 min followed by 2 MAC halothane; and (7) 10 μM U73122 for 5 min. A high concentration of U73122 was used to ensure maximum inhibition of PLC. One set of control experiments was performed to determine whether halothane by itself interfered with the IP_3 assay. HBSS with no ASM cells was bubbled with halothane, and the solution was processed as for the ASM cells.

After one of the aforementioned exposures, reactions were terminated by addition of equal volume of ice-cold 1 M trichloroacetic acid. The trichloroacetic acid was extracted from the medium using trioctylamine and trichloro-trifluoroethane in a 1:3 ratio. The cell-free extract was then used to measure IP_3 concentrations, using a commercially available radioreceptor assay (NEN Research Products, Boston, MA).[16,17] The measurement technique is similar to that used by other investigators.[7] A Lowry protein assay was used to measure protein concentrations for normalization of IP_3 concentrations.[18]

Real-time Confocal Imaging

Freshly dissociated cells were plated on collagen-coated glass coverslips and incubated in 5% CO_2 at 37°C for 1–2 h. Exclusion of trypan blue was used to assess cell viability (>90% of all cells). After incubation in minimum essential medium, each coverslip was washed with HBSS. The coverslip was then transferred to HBSS containing 5 μM of the cell-permeant form of the fluorescent Ca^{2+} indicator fluo-3 AM (Molecular Probes, Eugene, OR) and incubated for 30–45 min at 37°C. The coverslip was then washed in HBSS and mounted on an open slide chamber (RC-25F; Warner Instruments, Hamden, CT). Cells were perfused at 2–3 ml/min and maintained at room temperature.

The technique for real-time confocal imaging of ASM cells has been previously described in detail.[11,12,15] Briefly, cells were visualized using an Odyssey XL real-time confocal system (Noran Instruments, Middleton, WI) equipped with an Ar-Kr laser and attached to a Nikon Diaphot microscope. A Nikon 40X/1.3 oil-immersion objective lens (Melville, NY) was used for Ca^{2+} imaging. Image size was set to 640 × 480 pixels, and pixel area was calibrated using a stage micrometer (0.063 μm^2/pixel). The optical section thickness for the 40× lens was set at 1 μm by controlling the slit size on the confocal system. The 488-nm laser line was used to excite fluo-3, and emissions were collected using a 515-nm long-pass filter and a high-sensitivity photomultiplier tube. Based on previous experience,[11,12,15] a fixed combination of laser intensity (20% of maximum) and photomultiplier gain (1,700 from a maximum of 4,096) was used to ensure that pixel intensities within cells ranged between 25 and 254 gray levels. At these settings, laser power output at 3 mW varied less than 1% across a 15-min period, and only intermittent laser exposure of fluo-3 was required (<5 min), causing little detectable photobleaching (<1%). Measurements of $[Ca^{2+}]_i$ were obtained by defining a large region of interest around an entire cell. Software limitations allowed measurement from a maximum of eight regions of interest within a field.

Ca^{2+} Calibrations

Although fluo-3 is a nonratiometric Ca^{2+} indicator, several studies have used *in vitro* calibrations where fluorescence levels are measured at known Ca^{2+} concentrations; however, the dissociation constant (K_d) of the fluorescent dye differs *in vitro versus in vivo* (see Takahashi *et al.*[19] for a review). Therefore, as in previous studies,[15,20] we used an empirical *in vivo* calibration technique based on measurement of intracellular fluorescence levels at known $[Ca^{2+}]_i$ levels. Based on previous experience, a fixed combination of laser intensity (20% of maximum) and photomultiplier gain (1,700 from a maximum of 4,096) was set *a priori* to ensure pixel intensities between 25 and 255 gray levels. ASM cells loaded with 5 μM fluo3 AM were sequentially exposed to solutions containing 10 known Ca^{2+} concentrations (0 nM to 1.25 μM; Calcium Calibration Buffer Kit, Molecular Probes) and 10 μM of the Ca^{2+} ionophore A-23187. This technique allowed equilibration of $[Ca^{2+}]_i$ and extracellular $[Ca^{2+}]$.

Previous studies have used the equation:

$$[Ca^{2+}]_i = K_d \frac{F - F_{min}}{F_{max} - F}$$

to calculate $[Ca^{2+}]_i$ levels from fluorescence values (F), where F_{min} is the fluorescence at minimal $[Ca^{2+}]_i$ concentrations (0 nM in this study) and F_{max} is the fluorescence at saturating concentrations, determined using a

buffer and ionophore technique similar to the one described above.[19] Using the F_{min}, F_{max}, and gray level values from our measurements in ASM cells as previously described, we calculated the apparent K_d for fluo-3 in our system to be 455 ± 89 nM (mean of 10 calibrations), which is comparable to the 400 nM reported in previous studies.[19,20]

Administration of Halothane

A calibrated online vaporizer was used to deliver halothane (Wyeth-Ayerst Laboratories, St. Davids, PA) to the aerating gas mixture (95% O_2, 5% CO_2). The vaporizer setting produced aqueous concentrations of halothane in the HBSS equivalent to 2 MAC at room temperature. The halothane concentration in the perfusion chamber (in aqueous solution) was determined by gas chromatography from anerobically obtained samples using an electron capture detector (Hewlett-Packard 5880A, Palo Alto, CA), as described previously.[21] In the solutions used to examine the effects of halothane, the concentrations for 2 MAC halothane were 0.47 ± 0.03 mM.

Experimental Protocols

Effect of Halothane on Intracellular Ca^{2+} Concentration. In the first set of experiments, intact ASM cells were preexposed to zero-Ca^{2+} HBSS and 1 mM lanthanum chloride (Sigma) to nonspecifically inhibit both Ca^{2+} influx and efflux across the plasma membrane.[14,15] During these conditions in which the SR was effectively isolated, cells were exposed to 2 MAC halothane, and the $[Ca^{2+}]_i$ response was recorded.

In a second set of experiments, ASM cells were first permeabilized with 25 μM β-escin (Sigma) as described previously.[11,14] This permeabilization procedure allows for entry of large-molecular-weight compounds into the cytosolic compartment and for control of cytosolic Ca^{2+} concentrations *via* externally applied solutions of known Ca^{2+} concentration (pCa, negative logarithm of Ca^{2+} concentration). However, the confounding effects of Ca^{2+} fluxes across the cell membrane are removed. Furthermore, unlike Triton-X permeabilization, β-escin does not destroy receptor and G-protein structures in the plasma membrane and allows for agonist stimulation if necessary. The pCa solutions were prepared as described by Fabiato,[22] with stabilization constants described by Godt and Lindley.[23]

After β-escin permeabilization, cells were exposed to 9.0 pCa for 2 min, and the SR was then loaded by exposure to 6.3 pCa for 15 min. The cells were then exposed to 2 MAC halothane in 6.3 pCa, and the $[Ca^{2+}]_i$ response was recorded.

Effect of Inhibition of Inositol Triphosphate Receptor Channels. Intact ASM cells were first exposed to 2 MAC halothane in zero-Ca^{2+} HBSS and lanthanum chloride as previously described. The cells were then washed for 10 min and then for an additional 5 min with medium containing 20 μM Xestospongin D (XeD; Calbiochem, San Diego, CA), a potent cell-permeant inhibitor of IP_3 receptor channels.[24] In the continued presence of XeD, cells were reexposed to 2 MAC halothane. In control experiments, cells were exposed twice to halothane with an intervening 15-min wash period.

In a second set of experiments, β-escin–permeabilized cells were exposed to 2 MAC halothane in 6.3 pCa. The cells were then washed for 10 min in 6.3 pCa, and for an additional 5 min in 20 μM XeD in 6.3 pCa. They were then reexposed to halothane. Other cells were exposed to 0.5 mg/ml heparin instead of XeD, and then to halothane. We previously demonstrated that heparin can enter β-escin–permeabilized cells and inhibits IP_3 receptor channels.[11,14] In the current study also, inhibition of IP_3 receptor channels by XeD or heparin was confirmed by a lack of an $[Ca^{2+}]_i$ response to 10 μM IP_3 (Sigma) at the end of the experimental protocol, after a reloading of the SR with 6.3 pCa. In corresponding control experiments, cells were exposed twice to exogenous IP_3 with just an intervening wash. In other control experiments, cells were exposed twice to halothane in 6.3 pCa with an intervening 15-min wash period.

In a third set of experiments, the direct effect of halothane on IP_3 receptor channels independent of elevation of IP_3 was examined. Intact ASM cells were first exposed to 2 MAC halothane in zero-Ca^{2+} HBSS and lanthanum chloride as previously described. The cells were then washed for 10 min and then for an additional 5 min with medium containing 10 μM U73122 (Sigma). In the continued presence of U73122, cells were reexposed to halothane. To further verify the direct effect of halothane on IP_3 receptor channels, cells were washed, exposed to U73122 as well as XeD, and then exposed for a third time to halothane.

In a fourth set of experiments, the interactions between halothane and exogenous IP_3 on SR Ca^{2+} release were examined in β-escin–permeabilized ASM cells. After the first exposure to 10 μM IP_3 in 6.3 pCa, cells were washed in 6.3 pCa and exposed simultaneously to 2 MAC halothane and IP_3.

Effect of Inhibition of Ryanodine Receptor Channels. Intact ASM cells were first exposed to 2 MAC halothane in zero-Ca^{2+} HBSS and lanthanum chloride and then washed for 10 min. The cells were then exposed for an additional 5 min to 20 μM ryanodine (Sigma) to inhibit RyR channels.[11,15] In the continued presence of ryanodine, cells were reexposed to 2 MAC halothane.

In a second set of experiments, β-escin–permeabilized cells were exposed to 2 MAC halothane in 6.3 pCa. The cells were then washed for 10 min in 6.3 pCa and then for an additional 5 min in 20 μM ryanodine in 6.3 pCa. The cells were then reexposed to halothane.

Effect of Simultaneous Inhibition of Inositol Triphosphate and Ryanodine Receptor Channels.

Intact ASM cells were first exposed to 2 MAC halothane in zero-Ca^{2+} HBSS and lanthanum chloride and then washed for 10 min. The cells were then exposed for an additional 5 min to 20 μM XeD and 20 μM ryanodine to simultaneously inhibit both IP_3 and RyR channels. In the continued presence of these inhibitors, cells were reexposed to 2 MAC halothane.

In a second set of experiments, β-escin–permeabilized cells were exposed to 2 MAC halothane in 6.3 pCa, washed for 10 min in 6.3 pCa, and then exposed for an additional 5 min to 20 μM XeD and 20 μM ryanodine in 6.3 pCa. The cells were then reexposed to halothane.

Statistical Analysis

Inositol triphosphate measurements were compared using the independent Student *t* test. In these studies, n refers to the number of samples. For Ca^{2+} imaging experiments, it was not possible to apply all of the experimental protocols to every cell or to cells obtained from every animal. In each experiment, at least three and up to five cells were analyzed from each coverslip. If not otherwise stated, comparisons before and after halothane and/or inhibitor exposure were made using paired *t* tests. Bonferroni correction was used for pairwise comparisons. A P value < 0.05 was considered statistically significant. In all studies relating to single cells, n refers to the number of cells. Cells were obtained from eight animals; however, no attempt was made to determine interanimal variability in Ca^{2+} imaging studies. Values are reported as mean ± SD.

Results

Inositol Triphosphate Measurements

In ASM cells that were exposed to HBSS only for 2 min (vehicle control), IP_3 concentrations were 1.92 ± 0.07 pmol/10^6 cells or 17.12 ± 11.56 pmol/mg protein (n = 5). Exposure to 1 μM acetylcholine resulted in an approximately twofold increase in IP_3 concentrations (fig. 1; $P < 0.05$). Preexposure to 2 MAC halothane alone for 2 min also resulted in significantly elevated IP_3 concentrations ($P < 0.05$) that were almost fourfold higher than that obtained with exposure to acetylcholine alone. Simultaneous exposure to both acetylcholine and halothane increased IP_3 concentrations beyond those observed with acetylcholine or halothane alone ($P < 0.05$). Inhibition of PLC by U73122 significantly blunted the acetylcholine-induced IP_3 response to approximately 25% of that observed with acetylcholine alone ($P < 0.05$). In contrast, halothane-induced elevation of IP_3 was decreased by U73122 to only approximately 95% of that observed with halothane alone (fig. 1; $P < 0.05$). In control experiments, U73122 alone slightly decreased IP_3 concentrations in unstimulated cells (97 ± 4%), but this decrease was not significant. Exposure of HBSS to halothane did not result in any detectable levels of IP_3 in the assay.

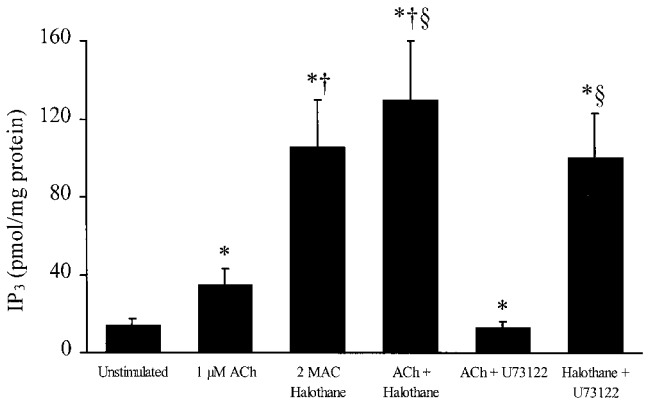

Fig. 1. Effect of halothane on inositol triphosphate (IP_3) concentrations in porcine airway smooth muscle cells. IP_3 concentrations were measured using radioimmunoreceptor assay. Compared with unstimulated control, IP_3 concentrations were significantly ($P < 0.05$) elevated by exposure to acetylcholine, 2 minimum alveolar concentration (MAC) halothane, and a combination thereof. The IP_3 response to simultaneous application of acetylcholine and halothane was significantly greater than that induced by either agent alone. Inhibition of phospholipase C by U73122 significantly blunted the IP_3 response to both acetylcholine and halothane, but the effect on halothane was considerably smaller. *Significant ($P < 0.05$) difference from unstimulated control. †Significant difference from acetylcholine exposure alone. §Significant difference from halothane exposure alone.

Intracellular Ca^{2+} Concentration Measurements

Effect of Halothane on Intracellular Ca^{2+} Concentration. In intact ASM cells where both Ca^{2+} influx and efflux across the plasma membrane were nonspecifically inhibited by preexposure to zero-Ca^{2+} HBSS and lanthanum chloride, basal $[Ca^{2+}]_i$ ranged from 90 to 130 nM (108 ± 49 nM; n = 12). These values were not significantly different from $[Ca^{2+}]_i$ when cells were perfused with normal HBSS (80–125 nM; 94 ± 42 nM). During these conditions, exposure to 2 MAC halothane induced a transient $[Ca^{2+}]_i$ response after an approximately 10-s delay (fig. 2A). The profile of the $[Ca^{2+}]_i$ response to halothane was comparable to that observed with halothane in previous studies from our laboratory.[14] The rate of increase of the $[Ca^{2+}]_i$ response (measured over a 1-s interval) was 20 ± 17 nM/s, peak amplitude was 500 ± 42 nM, and rate of decrease (also measured over 1 s) was 75 ± 14 nM/s. After washout, reexposure to halothane produced another transient $[Ca^{2+}]_i$ response with a profile that was not significantly different from the first response (fig. 2A), with a rate of increase of 23 ± 17 nM/s, peak amplitude of 485 ± 45 nM, and rate of decrease of 82 ± 17 nM/s.

In β-escin–permeabilized cells, exposure to 2 MAC halothane in 6.3 pCa also resulted in a transient $[Ca^{2+}]_i$ response that was qualitatively similar in profile to that observed in intact cells (fig. 2B). As in previous studies,

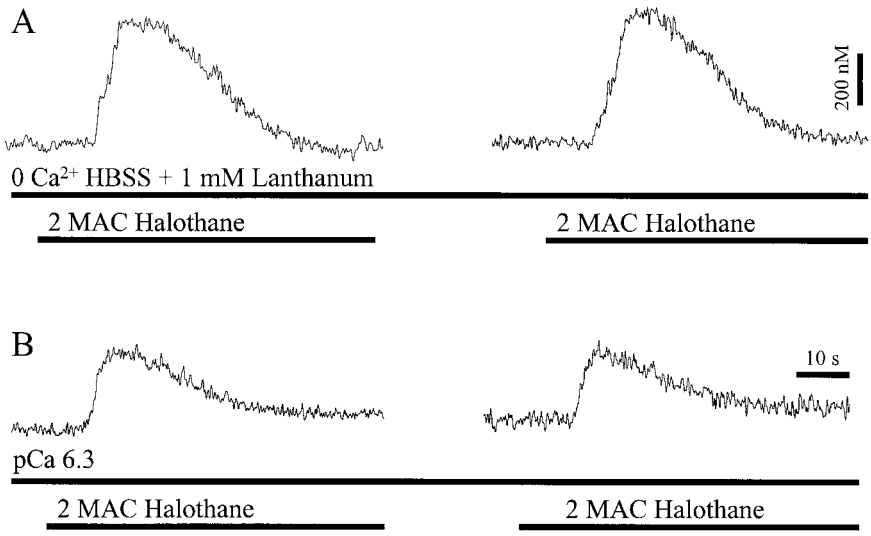

Fig. 2. Representative examples of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) response of intact (*A*) and β-escin–permeabilized (*B*) airway smooth muscle cells to 2 minimum alveolar concentration (MAC) halothane. In intact cells, Ca^{2+} influx and efflux across the plasma membrane were nonspecifically blocked using zero extracellular Ca^{2+} and lanthanum chloride. During these conditions, exposure to halothane induced a transient $[Ca^{2+}]_i$ response that was reproducible after an intervening washout. A similar effect was elicited with cells in which plasma membrane effects were eliminated by permeabilization. Amplitude bar for (*A*) only.

we did not attempt to quantify the amplitude of the $[Ca^{2+}]_i$ response in permeabilized cells because of indeterminate amounts of fluo-3 leakage after exposure to β-escin.[14] To ensure that there was minimal continued leakage of dye through the course of the protocol, control experiments were performed in which the cells were exposed to the same agent (*e.g.*, halothane) twice with an intervening washout period (fig. 2B). In these studies, we found the amplitude of the second $[Ca^{2+}]_i$ response to be 94 ± 9% of the first response (n = 10). Furthermore, in subsequent protocols, comparisons of anesthetic–drug effects were made only within a cell and not across cells.

Effect of Inhibition of Inositol Triphosphate Receptor Channels. In intact ASM cells with blocked Ca^{2+} influx and efflux, exposure to 2 MAC halothane produced a transient $[Ca^{2+}]_i$ response as previously described. Subsequent exposure to 20 μM XeD did not significantly alter resting $[Ca^{2+}]_i$ (110 ± 46 nM; n = 11). However, in the continued presence of XeD, exposure to 2 MAC halothane produced a transient $[Ca^{2+}]_i$ response that was significantly slower (rate of increase, 136 ± 30%; rate of decrease, 154 ± 36% control; $P < 0.05$) and smaller (amplitude, 61 ± 20% control; $P < 0.05$) in profile compared with the first response (fig. 3A).

In β-escin–permeabilized cells (n = 9) first exposed to 2 MAC halothane in 6.3 pCa, subsequent exposure to halothane in the presence of 20 μM XeD also significantly blunted the second $[Ca^{2+}]_i$ response (fig. 3B; 56 ± 22% control). In other cells exposed to 0.5 mg/ml heparin instead of XeD (n = 5), the second exposure to halothane also resulted in a diminished $[Ca^{2+}]_i$ response (54 ± 31% control). In both protocols, exposure to 10 μM IP_3 at the termination of the protocol did not produce a significant elevation in $[Ca^{2+}]_i$, confirming complete inhibition of IP_3 receptor channels by XeD or heparin. In

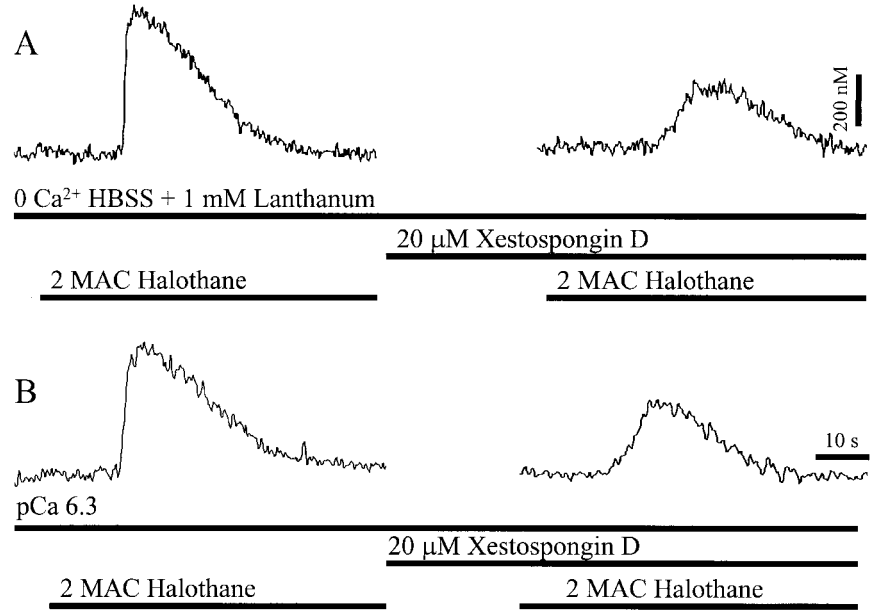

Fig. 3. Effect of halothane on inositol triphosphate (IP_3) receptor channels in intact (*A*) and β-escin–permeabilized (*B*) airway smooth muscle (ASM) cells. During conditions of blocked Ca^{2+} influx and efflux, halothane induced a transient intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) response even when IP_3 receptor channels were blocked by a membrane permeant inhibitor, Xestospongin D (XeD). These data indicate that halothane increases sarcoplasmic reticulum Ca^{2+} release also through ryanodine receptor (RyR) channels. The decrease in the $[Ca^{2+}]_i$ response to halothane during these conditions is consistent with figure 3, indicating the IP_3 receptor component. Amplitude bar for (*A*) only. MAC = minimum alveolar concentration.

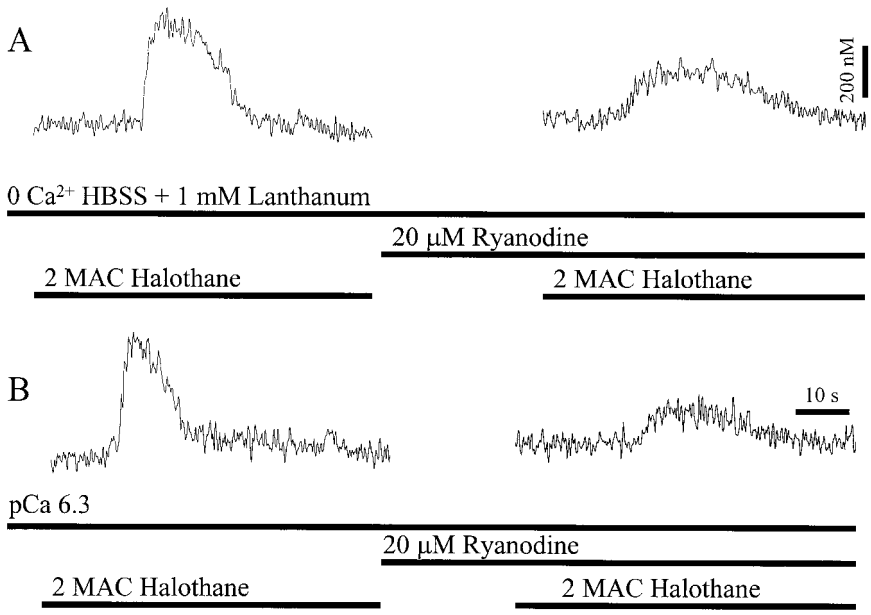

Fig. 4. Effect of halothane on ryanodine receptor channels in intact (*A*) and β-escin–permeabilized (*B*) airway smooth muscle cells. During conditions of blocked Ca^{2+} influx and efflux, halothane induced a transient intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) response even when ryanodine receptor channels were blocked by high concentrations of ryanodine. These data indicate that halothane increases sarcoplasmic reticulum Ca^{2+} release through inositol triphosphate receptor channels. The decrease in the $[Ca^{2+}]_i$ response to halothane during these conditions suggests a ryanodine receptor component. Amplitude bar for (*A*) only. MAC = minimum alveolar concentration.

control experiments, where cells were exposed twice to IP_3 with just an intervening wash, there was no significant difference in the two $[Ca^{2+}]_i$ responses (second exposure was 95 ± 8% of first exposure). These control data are consistent with our previous study.[11,14]

In a third set of experiments, the direct effect of halothane on IP_3 receptor channels was examined in the presence of U73122, which inhibited PLC. During these conditions, the $[Ca^{2+}]_i$ response to halothane was 80 ± 15% of control (first exposure to halothane; $P < 0.05$). Further inhibition of IP_3 receptor channels with XeD, in the continued presence of U73122, resulted in a response to halothane that was 58 ± 10% of the first exposure ($P < 0.05$) but was not significantly different from the response in the presence of XeD alone (61 ± 20%; see above).

In a fourth set of experiments, where the interaction between halothane and exogenous IP_3 on SR Ca^{2+} release was examined, the $[Ca^{2+}]_i$ response to simultaneous application of IP_3 and halothane was 126 ± 14% of the response to IP_3 alone ($P < 0.05$).

Effect of Inhibition of Ryanodine Receptor Channels. In intact ASM cells with inhibited Ca^{2+} influx and efflux, exposure to 20 μM ryanodine slightly elevated basal $[Ca^{2+}]_i$ (150 ± 11 nM; $P < 0.05$; n = 13). In the continued presence of ryanodine, exposure to 2 MAC halothane resulted in transient $[Ca^{2+}]_i$ response that was also considerably slower (rate of increase, 146 ± 43%; rate of decrease, 175 ± 50% control; $P < 0.05$) and smaller (amplitude, 43 ± 29% control; $P < 0.05$) compared with the first response in the absence of ryanodine (fig. 4A). Compared with the $[Ca^{2+}]_i$ response to halothane in the presence of XeD, the response in the presence of ryanodine was significantly smaller when expressed as percentage of control ($P < 0.05$). However, it must be emphasized that these experiments were conducted in separate cells.

In β-escin–permeabilized cells first exposed to 2 MAC halothane in 6.3 pCa, addition of 20 μM ryanodine also resulted in a significantly smaller $[Ca^{2+}]_i$ response on reexposure to halothane (fig. 4B; 50 ± 24% control; $P < 0.05$; n = 8).

Simultaneous Inhibition of Inositol Triphosphate and Ryanodine Receptor Channels. In intact ASM cells (n = 11) where Ca^{2+} influx and efflux were inhibited and a $[Ca^{2+}]_i$ response to halothane was first verified, simultaneous addition of 20 μM XeD and 20 μM ryanodine completely abolished the subsequent $[Ca^{2+}]_i$ response to halothane (fig. 5).

In β-escin–permeabilized cells (n = 7) first exposed to

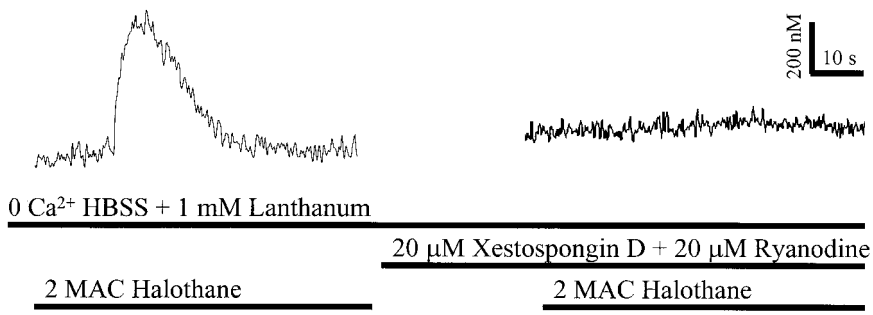

Fig. 5. Effect of simultaneous inhibition of both inositol triphosphate (IP_3) and ryanodine receptor (RyR) channels on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) response of intact airway smooth muscle (ASM) cells to halothane. In the presence of blocking concentrations of both Xestospongin D and ryanodine, halothane did not elevate $[Ca^{2+}]_i$, indicating that the effect of halothane on the sarcoplasmic reticulum is mediated entirely *via* the two receptor channels. MAC = minimum alveolar concentration.

2 MAC halothane in 6.3 pCa, addition of 20 μM XeD and 20 μM ryanodine also completely abolished the $[Ca^{2+}]_i$ response to a subsequent reexposure to halothane (not shown). The effects of various inhibitors and their interactions with halothane in intact ASM cells are summarized in figure 6.

Discussion

The results of the current study demonstrate that a clinically relevant concentration of halothane affects $[Ca^{2+}]_i$ in ASM cells by decreasing SR Ca^{2+} content *via* increased leak through Ca^{2+} channels in the SR. Even in the absence of agonist activation, halothane increases IP_3 concentrations, partly by activating PLC in the plasma membrane. However, independent of this effect on IP_3, halothane also increases SR Ca^{2+} leak through IP_3 receptor channels, which contributes to the depletion of SR Ca^{2+} content. Additional leak is induced by halothane effects on RyR channels.

Methodologic Issues

We used freshly dissociated ASM cells to examine the effects of halothane on $[Ca^{2+}]_i$ regulation. Variations in cell dissociation and dye loading, and inherent cellular differences, may introduce potential variability in the observed $[Ca^{2+}]_i$ responses between cells and/or animals. However, in previous studies we determined that there were no significant differences in the coefficient of variation of $[Ca^{2+}]_i$ responses of ASM cells within or across animals.[14] Furthermore, in the current experimental design, each cell served as its own control. Therefore, cellular variability was not considered to be a confounding issue.

A potential concern with the use of a nonratiometric Ca^{2+} indicator such as fluo-3 is that dye compartmentalization or bleaching may affect the observed $[Ca^{2+}]_i$ responses. Furthermore, the apparent K_d of the dye may differ *in vitro versus in vivo* and furthermore may be cell-specific. Therefore, it was essential to perform an empiric calibration using ASM cells and the confocal microscope used in the current study. The reliability of the calibration technique is indicated by relatively small variations in basal $[Ca^{2+}]_i$ across cells obtained on different days.

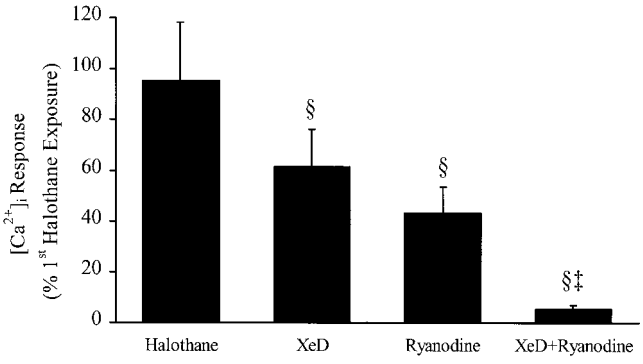

Fig. 6. Summary of the effects of halothane on inositol triphosphate *versus* ryanodine receptor channels. Statistical comparisons were made to the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) response to the first exposure to halothane alone. Accordingly, the control for these interactions was a second exposure to halothane alone (first bar). § Significant ($P < 0.05$) difference from first halothane exposure. ‡ Significant difference from exposure to Xestospongin D or ryanodine exposure alone.

The current study focused only on halothane effects at the level of the SR, while recognizing that additional effects on mechanisms such as Ca^{2+} influx and efflux are possible. Indeed, there is already considerable evidence in the literature that the decrease in $[Ca^{2+}]_i$ by halothane involves inhibition of Ca^{2+} influx[1–3] through voltage-gated L-type Ca^{2+} channels.[3,5] However, these effects could not have contributed to the observed $[Ca^{2+}]_i$ responses because all experiments were conducted during conditions of blocked influx and efflux.[3,5]

Effect of Halothane on Inositol Triphosphate Levels

In ASM cells, muscarinic receptors are coupled to G proteins that activate plasma membrane PLC, which catalyzes hydrolysis of membrane-associated phosphatidylinositol bisphosphate to IP_3 and diacylglycerol. The elevation in IP_3 levels after acetylcholine stimulation and its inhibition by U73122 are therefore consistent with activation of PLC. Agonist-induced IP_3 is metabolized *via* specific phosphatases. Metabolism of IP_3 may be regulated by other mechanisms such as protein kinase C,[26] which has been thought to inhibit agonist-induced IP_3 either by inhibition of PLC or activation of phosphatases, or by protein kinase A[26] acting in a similar fashion. After elevation of IP_3, release of Ca^{2+} occurs *via* IP_3-gated receptor channels of the SR. Previous studies have shown that the IP_3 receptor channel displays a bell-shaped dependence on the level of $[Ca^{2+}]_i$ itself,[27,28] such that at a fixed concentration of IP_3, Ca^{2+} conductance is low when $[Ca^{2+}]_i$ is also low, but conductance increases with increasing $[Ca^{2+}]_i$ to a point. Accordingly, during unstimulated conditions, there is only a low, background level of Ca^{2+} release through IP_3 receptor channels.

Exposure to halothane resulted in marked elevation of IP_3 concentrations in ASM cells. In this regard, it is of significance that inhibition of PLC by U73122, which should theoretically blunt any halothane effects on PLC *per se*, resulted in an extremely small effect on the halothane-induced elevation of IP_3 concentrations. These data suggest that, in addition to an effect on PLC itself, halothane may also influence the activity of other regulatory mechanisms such as phosphatases, thus altering the time course of IP_3 formation and degradation. For example, previous studies have demonstrated that halothane can inhibit the effects of PKC in smooth muscle. Accordingly, PKC-modulated degradation of IP_3 may be

delayed in the presence of halothane, resulting in continued elevation of IP_3 concentrations even when PLC is inhibited by U73122. Whether halothane affects these specific intracellular regulatory mechanisms remains to be determined.

The increase in IP_3 concentrations induced by halothane may have a complex effect on $[Ca^{2+}]_i$ regulation in the cell. On the one hand, elevated IP_3 concentrations may induce SR Ca^{2+} release, thus partially accounting for the transient $[Ca^{2+}]_i$ response observed in single ASM cells. On the other hand, increased IP_3 concentrations will lead to faster inactivation of the IP_3 receptor channel, especially if $[Ca^{2+}]_i$ is also somewhat elevated, as with concurrent acetylcholine stimulation (see review by Taylor[28] on the interaction between $[Ca^{2+}]_i$ and IP_3 receptor function). Such inactivation would inhibit subsequent SR Ca^{2+} release, resulting in ASM relaxation.

The halothane-induced elevation in IP_3 concentrations observed in the current study sharply contrasts with the findings of Yamakage *et al.*[7] In that study using canine ASM, halothane was found to decrease IP_3 concentrations in the presence of muscarinic stimulation with carbachol. The reasons underlying this discrepancy are not entirely clear but may be related either to species differences in the sensitivity of the IP_3 regulatory mechanisms to anesthetics, including PLC *versus* phosphatase activities, or to anesthetic concentrations (approximately 0.45 mM in the current study *vs.* 0.75–1.15 mM in the study by Yamakage *et al.*[7]). Furthermore, in the study by Yamakage *et al.*, the time course of examining IP_3 concentrations was also more extended compared with the current study. It is entirely possible that with prolonged exposure to halothane, IP_3 concentrations are indeed reduced. However, the focus of the current study was the immediate time period after acetylcholine exposure, which was consistent with time course of acetylcholine-induced $[Ca^{2+}]_i$ oscillations in our previous study.[14] Indeed, other studies have shown that halothane induces formation of IP_3 in neuroblastoma cells[29] and erythrocytes.[30] Furthermore, our comparisons have been performed both in the presence and absence of muscarinic stimulation.

Effect of Halothane on Intracellular Ca^{2+} Concentration

The transient $[Ca^{2+}]_i$ response of both intact and β-escin–permeabilized ASM cells to halothane is consistent with previous evidence from other tissues such as pituitary cells,[8] cardiac muscle,[6,31] vascular smooth muscle,[32,33] and our recent study on ASM.[14] Because all of the experiments in this study were performed under conditions where both Ca^{2+} influx and efflux across the plasma membrane were blocked, the results clearly indicate that the elevation in $[Ca^{2+}]_i$ is caused by SR Ca^{2+} release. The current study demonstrates that the decrease in SR Ca^{2+} content is mediated by increased Ca^{2+} leak through both IP_3 receptor and RyR channels.

In a recently published study, we demonstrated that clinically relevant concentrations of halothane affect acetylcholine-induced $[Ca^{2+}]_i$ oscillations in ASM cells.[14] We had previously established that acetylcholine-induced $[Ca^{2+}]_i$ oscillations represent repetitive SR Ca^{2+} release and reuptake, where initiation of oscillations is dependent on Ca^{2+} release through IP_3 receptor channels, but sustenance of oscillations occurs through Ca^{2+}-induced Ca^{2+} release mechanisms *via* RyR channels.[11,12,15] The amplitude of $[Ca^{2+}]_i$ oscillations thus represented SR Ca^{2+} content, and the frequency represented Ca^{2+}-induced Ca^{2+} release sensitivity. We found that halothane decreases both the amplitude and frequency of the oscillations and therefore decreases SR Ca^{2+} content and reduces the sensitivity for Ca^{2+}-induced Ca^{2+} release. However, the mechanisms underlying halothane effects on the SR were not examined.

The current study demonstrates a direct effect of halothane on Ca^{2+} release through IP_3 receptors in ASM. This is supported by the interactions between PLC inhibition *via* U73122 and IP_3 receptor channel blockage with XeD. Clearly, part of the $[Ca^{2+}]_i$ response to halothane does occur because of elevated IP_3 concentrations alone, as indicated by the decreased $[Ca^{2+}]_i$ response in the presence of U73122. However, the fact that XeD produces further decrement in the $[Ca^{2+}]_i$ response indicates a direct effect on the IP_3 receptor channels. Halothane-induced SR Ca^{2+} release through IP_3 receptor channels has been previously demonstrated in pituitary cells.[8] On the other hand, other studies in different cell systems have found that halothane inhibits the $[Ca^{2+}]_i$ response to agonists known to work predominantly *via* the IP_3 mechanism.[34–36] These differing results may be related to a number of factors, including the type of IP_3 receptor channel involved and the relative sensitivities of the channel to IP_3 itself *versus* halothane (given the fact that IP_3 concentrations are also differentially affected).

Another major finding in the current study was the effect of halothane on SR Ca^{2+} release through RyR channels, resulting in decreased SR Ca^{2+} content. These data are also consistent with our previous study on halothane effects on acetylcholine-induced $[Ca^{2+}]_i$ oscillations,[14] which involve repetitive release through these channels.[11,15] Our data are also consistent with studies in cardiac muscle,[6,9,31] skeletal muscle,[37] and vascular smooth muscle[33,34] demonstrating that volatile anesthetics increase SR Ca^{2+} leak through RyR channels. Therefore, it is likely that halothane-induced Ca^{2+} release through RyR channels also contributes to ASM relaxation *via* decreased SR Ca^{2+} content.

In addition to a direct effect on the RyR channel itself, halothane may have effects on upstream $[Ca^{2+}]_i$ regulatory mechanisms. In ASM cells, acetylcholine stimulation

also leads to production of cADPR, which has been shown to be a major second messenger system in a number of cell types (see review by Lee[38]). In a recent study,[13] we demonstrated that cADPR indirectly releases SR Ca^{2+} through RyR channels in ASM cells. Further studies are required to determine the effects of halothane on acetylcholine-induced elevation of cADPR concentrations in ASM cells. These effects would only further contribute to decreased $[Ca^{2+}]_i$.

In summary, halothane affects $[Ca^{2+}]_i$ regulation in porcine ASM cells by decreasing SR Ca^{2+} content, mediated through increased Ca^{2+} leak through both IP_3 and RyR channels. These effects likely contribute to anesthetic-induced decrease in the ASM response to receptor stimulation.

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References

1. Jones KA, Housmans PR, Warner DO, Lorenz RR, Rehder K: Halothane alters cytosolic calcium transient in tracheal smooth muscle. Am J Physiol 1993; 265:L80–6

2. Jones KA, Lorenz RR, Morimoto N, Sieck GC, Warner DO: Halothane reduces force and intracellular Ca^{2+} in airway smooth muscle independently of cyclic nucleotides. Am J Physiol 1995; 268:L166–72

3. Yamakage M, Kohro S, Kawamata T, Namiki A: Inhibitory effects of four inhaled anesthetics on canine tracheal smooth muscle contraction and intracellular Ca^{2+} concentration. Anesth Analg 1993; 77:67–72

4. Warner DO, Jones KA, Lorenz RR: The effects of halothane pretreatment on manganese influx induced by muscarinic stimulation of airway smooth muscle. Anesth Analg 1997; 84:1366–71

5. Yamakage M, Hirshman CA, Croxton TL: Volatile anesthetics inhibit voltage-dependent Ca^{2+} channels in porcine tracheal smooth muscle cells. Am J Physiol 1995; 268:L187–91

6. Connelly TJ, Coronado R: Activation of the Ca^{2+} release channel of cardiac sarcoplasmic reticulum by volatile anesthetics. ANESTHESIOLOGY 1994; 81:459–69

7. Yamakage M, Kohro S, Matsuzaki T, Tsuchida H, Namiki A: Role of intracellular Ca^{2+} stores in the inhibitory effect of halothane on airway smooth muscle contraction. ANESTHESIOLOGY 1998; 89:165–73

8. Hossain MD, Evers AS: Volatile anesthetic-induced efflux of calcium from IP_3-gated stores in clonal (GH3) pituitary cells. ANESTHESIOLOGY 1994; 80:1379–89

9. Lynch Cr, Frazer MJ: Anesthetic alteration of ryanodine binding by cardiac calcium release channels. Biochim Biophys Acta 1994; 1194:109–17

10. Coburn RF, Baron CB: Coupling mechanisms in airway smooth muscle. Am J Physiol 1990; 258:L119–33

11. Kannan MS, Prakash YS, Brenner T, Mickelson JR, Sieck GC: Role of ryanodine receptor channels in Ca^{2+} oscillations of porcine tracheal smooth muscle. Am J Physiol 1997; 272:L659–64

12. Sieck GC, Kannan MS, Prakash YS: Heterogeneity in dynamic regulation of intracellular calcium in airway smooth muscle cells. Can J Physiol Pharmacol 1997; 75:878–88

13. Prakash YS, Kannan MS, Walseth TF, Sieck GC: Role of cyclic ADP ribose in the regulation of $[Ca^{2+}]_i$ in porcine tracheal smooth muscle. Am J Physiol 1998; 274:C1653–60

14. Pabelick CM, Prakash YS, Kannan MS, Jones KA, Warner DO, Sieck GC: Effect of halothane on intracellular calcium oscillations in porcine tracheal smooth muscle cells. Am J Physiol 1999; 276:L81–9

15. Prakash YS, Kannan MS, Sieck GC: Regulation of intracellular calcium oscillations in porcine tracheal smooth muscle cells. Am J Physiol 1997; 272: C966–75

16. Challiss RA, Batty IH, Nahorski SR: Mass measurements of inositol(1,4,5)trisphosphate in rat cerebral cortex slices using a radioreceptor assay: Effects of neurotransmitters and depolarization. Biochem Biophys Res Commun 1988; 157:684–91

17. Bredt DS, Mourey RJ, Snyder SH: A simple, sensitive, and specific radioreceptor assay for inositol 1,4,5-trisphosphate in biological tissues. Biochem Biophys Res Commun 1989; 159:976–82

18. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265–75

19. Takahashi A, Camacho P, Lechleiter JD, Herman B: Measurement of intracellular calcium. Physiol Rev 1999; 79:1089–1125

20. Prakash YS, van der Heijden HF, Gallant EM, Sieck GC: Effect of beta-adrenoceptor activation on $[Ca^{2+}]_i$ regulation in murine skeletal myotubes. Am J Physiol 1999; 276:C1038–45

21. Van Dyke RA, Wood CL: Binding of radioactivity from 14 C-labeled halothane in isolated perfused rat livers. ANESTHESIOLOGY 1973; 38:328–32

22. Fabiato A: Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. Meth Enzymol 1988; 157:378–416

23. Godt RE, Lindley BD: Influence of temperature upon contractile activation and isometric force production in mechanically skinned muscle fibers of the frog. J Gen Physiol 1982; 80:279–97

24. Gafni J, Munsch JA, Lam TH, Catlin MC, Costa LG, Molinski TF, Pessah IN: Xestospongins: Potent membrane permeable blockers of the inositol 1,4,5-trisphosphate receptor. Neuron 1997; 19:723–33

25. Yang CM, Ong R, Chen YC, Hsieh JT, Tsao HL, Tsai CT: Effect of phorbol ester on phosphoinositide hydrolysis and calcium mobilization induced by endothelin-1 in cultured canine tracheal smooth muscle cells. Cell Calcium 1995; 17:129–40

26. Ding KH, Husain S, Akhtar RA, Isales CM, Abdel-Latif AA: Inhibition of muscarinic-stimulated polyphosphoinositide hydrolysis and Ca^{2+} mobilization in cat iris sphincter smooth muscle cells by cAMP-elevating agents. Cell Signal 1997; 9:411–21

27. Bezprozvanny I, Watras J, Ehrlich BE: Bell-shaped calcium-response curves of Ins(1,4,5)P3- and calcium-gated channels from endoplasmic reticulum of cerebellum. Nature 1991; 351:751–4

28. Taylor CW: Inositol trisphosphate receptors: Ca^{2+}-modulated intracellular Ca^{2+} channels. Biochim Biophys Acta 1998; 1436:19–33

29. Smart D, Smith G, Lambert DG: Halothane and isoflurane enhance basal and carbachol-stimulated inositol (1,4,5) triphosphate formation in SH-SY5Y human neuroblastoma cells. Biochem Pharmacol 1994; 47:939–45

30. Rooney TA, Hager R, Stubbs CD, Thomas AP: Halothane regulates G-protein-dependent phospholipase C activity in turkey erythrocyte membranes. J Biol Chem 1993; 268:15550–6

31. Wheeler DM, Katz A, Rice RT, Hansford RG: Volatile anesthetic effects on sarcoplasmic reticulum Ca content and sarcolemmal Ca flux in isolated rat cardiac cell suspensions. ANESTHESIOLOGY 1994; 80:372–82

32. Fehr DM, Larach DR, Zangari KA, Schuler HG: Halothane constricts bovine pulmonary arteris by release of intracellular calcium. J Pharmacol Exp Ther 1996; 277:706–13

33. Su JY, Zhang CC: Intracellular mechanisms of halothane's effect on isolated aortic strips of the rabbit. ANESTHESIOLOGY 1989; 71:409–17

34. Sill JC, Uhl C, Eskuri S, Van Dyke R, Tarara JG: Halothane inhibits agonist-induced inositol phosphate and Ca^{2+} signaling in A7r5 cultured vascular smooth muscle cells. Mol Pharmacol 1991; 40:1006–13

35. Loeb A: Alteration of calcium mobilization in endothelial cells by volatile anesthetics. Biochem Pharmacol 1993; 45:1137–42

36. Kohro S, Yamakage M: Direct inhibitory mechanisms of halothane on human platelet aggregation. ANESTHESIOLOGY 1996; 85:96–106

37. Blanck TJ, Peterson CV, Baroody B, Tegazzin V, Lou J: Halothane, enflurane, and isoflurane stimulate calcium leakage from rabbit sarcoplasmic reticulum. ANESTHESIOLOGY 1992; 76:813–21

38. Lee HC: Mechanisms of calcium signaling by cyclic ADP ribose and NAADP. Physiol Rev 1997; 77:1133–64