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Halothane Inhibits Agonist-Induced Inositol Phosphate and Ca^{2+} Signaling in A7r5 Cultured Vascular Smooth Muscle Cells

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SUMMARY

Halothane, an anesthetic with marked depressant effects on the circulation, was studied for its ability to inhibit inositol phosphate and Ca^{2+} signaling evoked by the vasoactive hormone arginine vasopressin (AVP) and Ca^{2+} responses elicited by platelet-derived growth factor and by thapsigargin in cultured A7r5 vascular smooth muscle cells. Changes in apparent $[Ca^{2+}]_i$ were measured using the indicator indo-1 and flow cytometry, whereas inositol phosphate levels were determined using *myo*-[^{3}H]inositol and column chromatography. Preincubation with clinically relevant concentrations of halothane resulted in dose-dependent depression of $[Ca^{2+}]_i$ responses evoked on stimulation with AVP. Halothane (2.0%) inhibited the increases in $[Ca^{2+}]_i$ by 34–45%. In cells incubated in Ca^{2+}-free medium plus 0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, the halothane effect was more marked, with 1.5% halothane inhibiting the responses by approximately 53–61%. However, when Ca^{2+} influx was stimulated by addition of 5 mM Ca^{2+} in the continued presence of the agonist, the $[Ca^{2+}]_i$ response was inhibited by only 15%, suggesting that release of Ca^{2+} rather than Ca^{2+} influx is more sensitive to inhibition by the anesthetic. The effects of halothane on Ca^{2+} homeostasis are not explained solely by anesthetic-induced depletion of Ca^{2+} from intracellular stores, because the anesthetic inhibited increases in $[Ca^{2+}]_i$ elicited by thapsigargin in cells suspended in Ca^{2+}-free medium by only 31%. Halothane inhibited inositol phosphate formation elicited by AVP, suggesting an additional means by which the anesthetic may alter agonist-induced Ca^{2+} responses. The current results also demonstrate that halothane actions are not specific solely to responses evoked by AVP, which acts via a guanine nucleotide-binding protein-linked signaling pathway, but include responses stimulated by platelet-derived growth factor, an agonist that elevates $[Ca^{2+}]_i$ via receptor-latent tyrosine kinase activity. The current results demonstrate that, in vascular smooth muscle cells, halothane alters Ca^{2+} homeostasis, an action that may underlie the *in vivo* vasodilator effects of the anesthetic.

Volatile anesthetics such as halothane not only abolish consciousness but also depress the circulation, in part by a direct action on arteries and arterioles (1, 2). Despite important clinical consequences, which include both decreased arterial blood pressure and increased blood flow to vital organs, including the heart (2) and brain (3), little is known concerning the mechanisms of the vascular smooth muscle effects of halothane.

Theories of general anesthesia suggest that lipid-soluble volatile anesthetics have actions predominantly at cell surface and intracellular membranes, rather than at sites within the cytoplasm (3, 4). Although halothane has been suggested to disorder membrane bulk lipid bilayers, current opinion favors more precise sites of action, with proteins, lipid-protein interfaces, and hydrophobic pockets on sensitive proteins within membranes being possible targets (4, 5). Consequently, anesthetics may alter the functions of regulatory proteins, enzymes, and ion channels and interfere with cell signaling (5). Evidence, although limited, exists to support this prediction. In neurons, halothane alters conductance through Ca^{2+}-sensitive K^+ channels (6) and inhibits increases in $[Ca^{2+}]_i$ due to receptor-operated and voltage-gated Ca^{2+} influx (7). In cardiac myocytes, halothane may alter sarcolemmal muscarinic receptor G protein function (8), inhibit voltage-gated Ca^{2+} influx (9), and impair Ca^{2+} storage in the sarcoplasmic reticulum (10). In contrast, the effects of halothane on vascular smooth muscle cells remain ill defined.

In the current experiments, halothane was investigated for its ability to inhibit inositol phosphate and Ca^{2+} responses stimulated by AVP. Many vasoactive hormones, including AVP, which has an important role in regulating peripheral vascular resistance (11), interact with receptors linked via G proteins to phosphoinositide-specific phospholipase C (12).

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; AVP, arginine vasopressin; PDGF, platelet-derived growth factor; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; HEPES, 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid; DMEM, Dulbecco's modified Eagles medium; MEM, minimum essential medium; DMSO, dimethyl sulfoxide.

This enzyme hydrolyzes membrane-bound phosphatidylinositol 4,5-bisphosphate to release Ins(1,4,5)P_3 and diacylglycerol (13). Whereas diacylglycerol regulates cell activity via protein kinase C, Ins(1,4,5)IP_3 interacts with receptors on intracellular storage sites to release Ca^{2+} from portions of these stores. Pressor hormones, including AVP (14), in addition promote extracellular Ca^{2+} entry into cells via channels in cell surface membranes (15).

Specifically, the current study sought first to determine whether halothane (a) altered resting $[Ca^{2+}]_i$ in quiescent cells, (b) attenuated increases in $[Ca^{2+}]_i$ evoked by AVP, (c) decreased release of Ca^{2+} from internal stores, (d) attenuated Ca^{2+} influx via cell surface membranes, and (e) inhibited AVP-induced inositol phosphate formation. Secondly, because halothane is believed to decrease Ca^{2+} availability for contraction in cardiac muscle, in part by depleting Ca^{2+} from the sarcoplasmic reticulum (16), the effects of halothane on Ca^{2+} storage in vascular smooth muscle cells were addressed by stimulating cells with thapsigargin. This probe discharges Ca^{2+} from Ins(1,4,5)P_3-sensitive intracellular stores (17), with the magnitude of the $[Ca^{2+}]_i$ response representing the amount of releasable Ca^{2+} present in the stores. Finally, in order to exclude a halothane effect specific to AVP, which elevates $[Ca^{2+}]_i$ via a G protein-linked signaling system (12), experiments were also performed by stimulating the cells with PDGF. This agonist, in contrast to AVP, evokes an increase in $[Ca^{2+}]_i$ via receptor dimerization, autophosphorylation, and uncovering of latent receptor tyrosine kinase activity, with possible direct stimulation of phospholipase C (18).

Experiments were performed using a vascular smooth muscle cell line derived originally from rat aorta (19), which exhibits well characterized responses to vasoactive hormones including AVP (20). The cells were preincubated with halothane concentrations corresponding to those used in clinical practice, i.e., 0.5–2.0% in the gas phase.

Experimental Procedures

Materials and chemicals. AVP and thapsigargin were obtained from Calbiochem (San Diego, CA). Human recombinant PDGF β chain homodimer was purchased from Bachem Inc. (Torrance, CA). Halothane was purchased from Ayerst Laboratories (New York, NY). Indo-1 pentaacetoxymethyl ester was purchased from Molecular Probes (Eugene, OR) and digitonin from the Sigma Chemical Company (St. Louis, MO). *myo*-[^{3}H]Inositol was purchased from Amersham Corp. (Arlington Heights, IL). DMEM, MEM (no Ca^{2+}), inositol-free medium (medium 199), HEPES, and trypsin were obtained from GIBCO. Fetal bovine serum was acquired from Hyclone (Logan, VT).

Cell culture. The A7r5 cell line was obtained from the American Type Culture Collection (Rockville, MD), (ATCC number CRL 1444). The cells were grown in flasks containing DMEM with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37° in a humidified 95% air/5% CO_2 atmosphere. Experiments were performed with cells passaged once every 5–7 days for not more than 12 weeks.

Estimation of $[Ca^{2+}]_i$. The cultured cells were incubated with 4 μM indo-1/acetoxymethyl ester plus 0.1% pluronic acid for 35–40 min at 37° (20) (indo-1/acetoxymethyl ester is hydrolyzed in the cell to the cell-impermeant acidic form). After washing with phosphate-buffered saline, the cells were gently dissociated with 0.05% trypsin for 5 min, centrifuged, washed, and resuspended in DMEM plus 10% fetal bovine serum containing 25 mM HEPES. The cell suspension was centrifuged, and the concentrate was added to DMEM, with fetal bovine serum and buffered with HEPES, that had been aerated with or without halothane. Aeration with halothane was performed before addition of the cells, by passage of 95% O_2/5% CO_2 gas mixture containing the anesthetic, delivered by a calibrated vaporizer at 150 ml/min for 20 min, through 25 ml of medium at 37°, in a semiclosed container. Halothane concentration in the aerating gas was adjusted using an anesthetic analyzer (Siemens 120, Solna, Sweden), which itself had been calibrated using commercially available gas mixtures of known concentration (Scott Medical, Plumstead PA) and by using a mass spectrometer (Perkins Elmer 1100, Pomona, CA). The medium was aspirated into airtight glass syringes to prevent evaporation of the anesthetic. Control experiments were performed using medium treated in an identical manner but in the absence of the anesthetic. Appropriate halothane concentrations in the medium were confirmed by extracting the anesthetic from 400 μl of medium into 2 ml of hexane and analyzing for halothane content by gas chromatography with electron capture (Hewlett Packard 5880A), in argon/methane carrier gas. During the period of the experiment, a close correlation was achieved between desired anesthetic concentrations and actual concentrations measured in the culture medium. (Halothane levels in the medium were expressed as gas phase concentrations predicted using known partition coefficients for distribution between aqueous and gas phases.) Anesthetic concentrations in the aerating gas of 0.5, 1.25, 1.5, and 2.0% resulted in concentrations in the culture medium that corresponded to 0.69 ± 0.04, 1.09 ± 0.07, 1.62 ± 0.05, and 2.08 ± 0.09%, respectively.

The cells were preincubated at room temperature in medium with or without halothane for 10 min, to allow anesthetic uptake and equilibration. Apparent $[Ca^{2+}]_i$ was measured by passing cells through a flow cytometer (20) (FACStar-Plus; Beckton Dickinson, Mountain View, CA) (adapted for this purpose) (21), where a stream of cells (300–500 cells/sec) was excited with 351–364-nm light emitted from an argon laser. Fluorescence was measured simultaneously at 480–520 nm and 385–395 nm, using appropriate band pass filters in front of photomultiplier tubes and fluorescence ratios calculated for each cell. The cells were stimulated by addition of 10^{-9} M or 10^{-7} M AVP, 3.3×10^{-9} M PDGF, or 5×10^{-5} M thapsigargin directly into the cell suspension, without interruption of the cell flow. [The AVP dose range was chosen because it both stimulates well characterized $[Ca^{2+}]_i$ responses in A7r5 cells (20) and contracts isolated blood vessels. The PDGF dose was chosen because it evokes marked $[Ca^{2+}]_i$ responses in Swiss 3T3 cells. The thapsigargin dose was chosen after pilot experiments.] In some experiments, cells were suspended in Ca^{2+}-free MEM plus 0.5 mM EGTA and stimulated with AVP or with thapsigargin, in order to evoke increases in $[Ca^{2+}]_i$ due to Ca^{2+} discharge from intracellular stores. In other experiments, Ca^{2+} influx from extracellular sources was investigated by stimulation of cells with 10^{-7} M AVP while they were suspended in Ca^{2+}-free MEM plus 0.5 mM EGTA. After completion of the initial $[Ca^{2+}]_i$ transient, 5 mM $CaCl_2$ was added in the continued presence of AVP, resulting in a second, sustained, $[Ca^{2+}]_i$ response (14, 15).

The immediate effects of acute addition of halothane solution on $[Ca^{2+}]_i$ in quiescent unstimulated cells were assessed by addition of halothane dissolved in culture medium directly into the suspension, without interruption of the flow of the cells, to obtain levels corresponding to 1% and 2% gas phase concentrations. (The halothane solution was prepared by dissolving 25 μl of liquid halothane in 975 μl of DMSO and then diluting this solution further in predetermined volumes of medium. DMSO at this concentration had no independent effect on $[Ca^{2+}]_i$.) The gas phase above the cell suspension was flushed with O_2/CO_2 mixture containing 1% or 2% halothane, in order to prevent evaporation of the anesthetic. Appropriate halothane concentrations in the aqueous phase were confirmed using gas chromatography.

Absence of nonspecific halothane effects on indo-1 fluorescence, reported by others (22), was reassessed by measuring indo-1 fluorescence ratios in cells treated with 2 μM digitonin, in the presence and absence of the anesthetic. Peak fluorescence ratios were controls, 0.62 ± 0.01; 1.5% halothane, 0.60 ± 0.01.

All protocols were repeated using batches of cells grown during a

minimum of three separate periods. Each individual experiment was accompanied by a control. Indo-1 fluorescence ratios were converted to $[Ca^{2+}]_i$ values by exposing cells acutely permeabalized with 10 μM digitonin to different concentrations of extracellular Ca^{2+} and then, using an indo K_D of 250 nM and measuring and calculating the ratio of fluorescence of Ca^{2+}-free indicator and Ca^{2+}-bound indicator, S_f/S_b (2.5), maximum fluorescence ratio (1.1), and minimum fluorescence ratio (0.1), solving the equation described by Grynkiewicz (23) and used by others (21).

Measurement of inositol phosphates. Cells were grown in 35-mm culture dishes for 5–7 days, to reach confluence, and were labeled for 21 hr with *myo*-[^{3}H]inositol (18.2 Ci/mmol in inositol-free medium without fetal bovine serum, buffered with 10 mM HEPES. They were washed with balanced salt solution containing 3.0 mM HEPES, pH 7.4, and then harvested, using 0.05% trypsin for 10 min, and centrifuged.

Experiments were performed after addition of the cell suspension to balanced salt solution, with or without halothane, in a closed container at 37°. Addition of halothane was performed before addition of the cells, by preparing halothane in DMSO (described above) and balanced salt solution and then adding predetermined volumes of this stock solution. After preincubation for 10 min and addition of 20 mM LiCl to prevent inositol phosphate breakdown, 0.5 ml of cell suspension was transferred to test tubes in a water bath at 37° in a semiclosed chamber. Halothane loss by evaporation was prevented by continuously passing air-containing halothane through the chamber. Anesthetic concentrations were monitored and adjusted as described above. Anesthetic concentrations of 1% and 2% in the aerating gas resulted in concentrations in the balanced salt solution of 1.01 ± 0.02% and 2.16 ± 0.03%, respectively.

Inositol phosphate formation was stimulated by addition of 10^{-7} M AVP to the test tubes, and the effect was terminated at predetermined times by lysing of the cells with 120 μl of 0.22 N HCl and 900 μl of chloroform/methanol (1:2). Chloroform/water (1:1) (1.8 ml) was added in order to extract inositol phosphates into the aqueous phase and, after centrifugation, the aqueous phase was removed, diluted 8-fold in water, and applied to anion exchange columns containing approximately 0.5 ml of mixed bed resin (Dowex AG 1-×8, 200–400 mesh, in the formate form; Bio-Rad, Irvine, CA). The columns were washed with 20 ml of 60 mM sodium formate, 5 mM sodium borate, before total inositol phosphates were eluted with 5 ml of 1.2 M ammonium formate, 100 mM formic acid. Radioactivity in the eluate was determined by liquid scintillation counting (Aquassure scintillation solution; DuPont, and Beckman LS 5000 TD counter).

Data analysis. Results are expressed as mean ± standard error. Changes in indo-1 fluorescence signal ratio are expressed as apparent $[Ca^{2+}]_i$. Integrated areas under time-response curves were obtained using apparent $[Ca^{2+}]_i$ values. Inositol phosphate levels are expressed as emission cpm. The n value represents the number of individual experiments. Data were analyzed using Student's t testing and p values of <0.05, using two-tailed analysis, were considered significant.

Results

Acute effects of halothane on $[Ca^{2+}]_i$ in quiescent cells. Addition of halothane solution directly into quiescent cell suspensions, to achieve anesthetic concentrations corresponding to 1% and 2%, produced immediate but very small and brief (approximately 50 sec.) increases in $[Ca^{2+}]_i$. The responses could barely be distinguished from the background effect of turbulence caused by addition of control or anesthetic solutions. Direct addition of halothane solution to cells in Ca^{2+}-free medium plus EGTA also resulted in immediate but also very small and brief increases in $[Ca^{2+}]_i$ (Table 1).

Effects of preincubation with halothane on $[Ca^{2+}]_i$ in quiescent cells. In a separate experiment, base-line $[Ca^{2+}]_i$ in unstimulated quiescent cells preincubated with halothane for 10 min was slightly lower than that in control untreated cells. Small depressant effects of the anesthetic were observed at both 1.25 and 2.0% halothane concentrations but not after 0.5% halothane. Preincubating cells in Ca^{2+}-free medium plus EGTA with 1.5% halothane also decreased resting $[Ca^{2+}]_i$ (Tables 2 and 3). Base-line $[Ca^{2+}]_i$ varied with each batch of cells, but the effects of halothane remained consistent.

TABLE 1

Immediate effects of acute addition of halothane solution on $[Ca^{2+}]_i$ in quiescent cells

Apparent $[Ca^{2+}]_i$ was calculated using the ratios of light emitted at dual wavelengths from indo-1-loaded A7r5 cells imaged using flow cytometry. Halothane dissolved in cell culture medium or control solution (no halothane) was acutely injected into the cell suspension without interruption of the flow of cells.

Halothane concentration[a]	n	Amplitude of the increases in $[Ca^{2+}]_i$	
		Control solution[b]	Halothane solution
%		nM	
1	8	3 ± 1	13 ± 3[c]
2	8	8 ± 2	21 ± 4[c]
2	8 (cells suspended in Ca^{2+}-free medium plus 0.5 mM EGTA)	0 ± 0	6 ± 1[c]

[a] Final concentration in the cell suspension.
[b] Increases in $[Ca^{2+}]_i$ observed after injection of control solution (no halothane) may represent the effect of turbulence.
[c] $p < 0.01$, in comparison with control.

Effects of preincubation with halothane on increases in $[Ca^{2+}]_i$ evoked by AVP. Stimulation of untreated control cells with 10^{-9} M AVP produced a rapid increase in $[Ca^{2+}]_i$, followed by a gradual return towards base-line. Stimulation of control cells with 10^{-7} M AVP evoked a rapid and more marked increase in $[Ca^{2+}]_i$, followed by a rapid and more complete return towards base line. Preincubation with halothane resulted in inhibition of the responses evoked by AVP. Effects of the anesthetic on peak $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ at 180 sec after AVP are shown in Tables 2 and 3. Tracings from typical individual experiments during stimulation with 10^{-9} M and 10^{-7} M AVP are presented in Figs. 1 and 2, respectively. Dose-dependent percentage of inhibition of the responses evoked by AVP, expressed as integrated areas under individual time-response curves, are shown in Fig. 3.

Effects of preincubation with halothane on increases in $[Ca^{2+}]_i$ resulting from AVP-induced Ca^{2+} release from intracellular stores. The cells were suspended in Ca^{2+}-free medium plus EGTA, in order that increases in $[Ca^{2+}]_i$ evoked by AVP would result primarily from Ca^{2+} release from intracellular stores. Stimulation with 10^{-9} M and 10^{-7} M AVP caused responses that were of a lesser magnitude than those observed in the presence of extracellular Ca^{2+}. Pretreatment with 1.5% halothane attenuated the responses. The effects of halothane on peak $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ at 180 sec after AVP are shown in Tables 2 and 3. Inhibition of the $[Ca^{2+}]_i$ response, expressed as areas under the integrated time response curves, is shown in Fig. 3. Recordings from typical individual experiments are shown in Fig. 4.

Effects of preincubation with halothane on increases in $[Ca^{2+}]_i$ due to AVP-induced Ca^{2+} influx. The cells were suspended in Ca^{2+}-free MEM plus EGTA and stimulated with 10^{-7} M AVP and then, on completion of the initial Ca^{2+} transient, 5 mM Ca^{2+} was added in the continued presence of AVP. Ca^{2+} influx occurred, resulting in a second and sustained increase in $[Ca^{2+}]_i$ (14). This sustained plateau was assumed to

TABLE 2

Effects of preincubation with halothane upon base-line $[Ca^{2+}]_i$ and upon increases in $Ca^{2+}]_i$ evoked by 10^{-9} M AVP

The indo-1-loaded A7r5 cells were preincubated for 10 min at room temperature in culture medium, with or without halothane. Apparent $[Ca^{2+}]_i$ was obtained by calculation of the ratios of dual-wavelength light emitted during flow cytometry. AVP was injected into the cell suspension without interruption of the flow of cells.

Halothane concentration	n	Base-line $[Ca^{2+}]_i$[a]		Peak $[Ca^{2+}]_i$		$[Ca^{2+}]_i$ at 180 sec	
		Control	Halothane	Control	Halothane	Control	Halothane
%		nM		nM		nM	
0.5	7	54 ± 7	50 ± 7	289 ± 24	268 ± 29[b]	127 ± 5	119 ± 5
1.25	8	55 ± 5	45 ± 4[b]	259 ± 13	199 ± 13[b]	125 ± 6	90 ± 4[c]
2.0	12	45 ± 2	40 ± 2[b]	273 ± 8	178 ± 11[c]	123 ± 4	84 ± 5[c]
1.5	8 (cells suspended in Ca^{2+}-free medium plus 0.5 mM EGTA)	31 ± 4	20 ± 1[b]	154 ± 7	58 ± 3[c]	54 ± 8	30 ± 1[b]

[a] Base-line $[Ca^{2+}]_i$ varied with each batch of cells. A minimum of three batches of cells were used in each experiment.
[b] $p < 0.01$, in comparison with control.
[c] $p < 0.0001$, in comparison with control.

TABLE 3

Effects of preincubation with halothane upon base-line $[Ca^{2+}]_i$ and upon increases in $Ca^{2+}]_i$ evoked by 10^{-7} M AVP

Halothane concentration	n	Base-line $[Ca^{2+}]_i$[a]		Peak $[Ca^{2+}]_i$		$[Ca^{2+}]_i$ at 180 sec	
		Control	Halothane	Control	Halothane	Control	Halothane
%		nM		nM		nM	
0.5	7	44 ± 2	41 ± 4	668 ± 36	628 ± 17	97 ± 5	82 ± 5[b]
1.25	9	46 ± 4	43 ± 4	926 ± 99	714 ± 97[c]	112 ± 8	83 ± 8[c]
2.0	11	57 ± 3	50 ± 2[b]	876 ± 44	604 ± 38[c]	109 ± 7	66 ± 4[c]
1.5	13 (cells suspended in Ca^{2+}-free medium plus 0.5 mM EGTA)	31 ± 1	21 ± 2[b]	451 ± 29	178 ± 19[c]	39 ± 3	24 ± 2[b]

[a] Base-line $[Ca^{2+}]_i$ varied with each batch of cells. A minimum of three batches of cells were used in each experiment.
[b] $p < 0.01$, in comparison with control.
[c] $p < 0.0001$, in comparison with control.

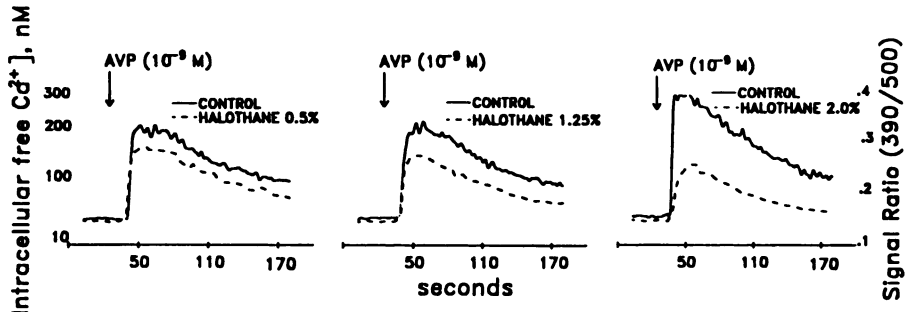

Fig. 1. Effects of preincubation with halothane on increases in $[Ca^{2+}]_i$ evoked by 10^{-9} M AVP in cultured A7r5 vascular smooth muscle cells. The indo-1-loaded cells were incubated with either 0.5, 1.25, or 2.0% halothane for 10 min or incubated without the anesthetic (controls). Cell suspension was passed through a flow cytometer at 300–500 cells/sec. AVP was added without interruption of the flow of cells. Fluorescence, at dual wavelengths, was used to calculate apparent $[Ca^{2+}]_i$. Results from typical individual experiments are shown.

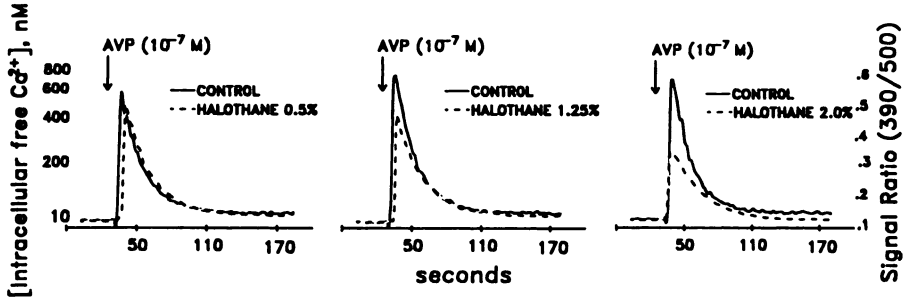

Fig. 2. Effects of preincubation with halothane on increases in $[Ca^{2+}]_i$ evoked by 10^{-7} M AVP in cultured A7r5 vascular smooth muscle cells. The indo-1-loaded cells were incubated with either 0.5, 1.25, or 2.0% halothane for 10 min or incubated without the anesthetic (controls). Cell suspension was passed through a flow cytometer at 300–500 cells/sec. AVP was added without interruption of the flow of cells. Fluorescence, at dual wavelengths, was used to calculate apparent $[Ca^{2+}]_i$. Results from typical individual experiments are shown.

result from receptor-operated Ca^{2+} influx, because it was not inhibited by 10^{-6} M diltiazem, a voltage-operated Ca^{2+} channel blocker. Sustained phase $[Ca^{2+}]_i$ was 183 ± 27 nM in control cells and 174 ± 24 nM in the presence of diltiazem. In addition, pretreatment with 1 nM La^{3+}, an inorganic nonspecific Ca^{2+} channel blocker, decreased the sustained phase from 184 ± 28 nM to 45 ± 19 nM, suggesting that the plateau phase was caused by Ca^{2+} entry. The contribution of nonspecific Ca^{2+} entry was small, because addition of 5 nM Ca^{2+} in the absence of AVP increased $[Ca^{2+}]_i$ by only 49 ± 11 nM.

Preincubation with 1.5% halothane resulted in marked inhibition of the Ca^{2+} release transient but had only modest inhibitory effects on the second sustained response resulting from Ca^{2+} influx. The effects of halothane on the Ca^{2+} influx plateau are shown in Table 4. The results of typical individual experiments are presented in Fig. 5. Comparisons of integrated areas under the time-response curves indicated that halothane inhibited the response due to Ca^{2+} release by 53 ± 4% ($p < 0.0001$), whereas the subsequent influx response was inhibited by only 15 ± 4% ($p < 0.01$).

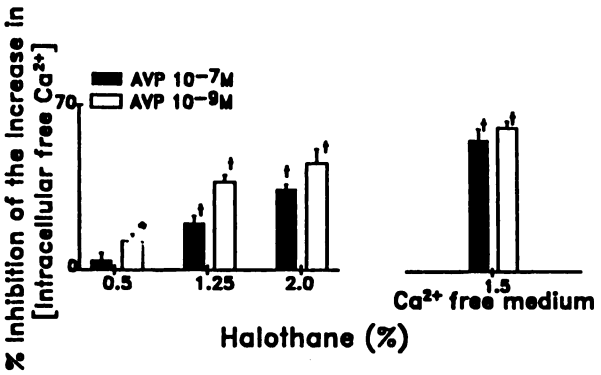

Fig. 3. Percentage of inhibition by halothane of increases in $[Ca^{2+}]_i$ induced by 10^{-9} and 10^{-7} M AVP in cultured A7r5 vascular smooth muscle cells. *Left*, cells were incubated in DMEM, with or without 0.5, 1.25, or 2.0% halothane, for 10 min and then stimulated with AVP. *Right*, cells were incubated with or without halothane in Ca^{2+}-free MEM plus 0.5 mM EGTA, to decrease extracellular $[Ca^{2+}]$. (On stimulation with AVP, the increase in $[Ca^{2+}]_i$ was due to Ca^{2+} release from intracellular stores.) Integrated areas under time-response curves were used to indicate changes in $[Ca^{2+}]_i$. n = 8–13 pairs for each experiment. *, $p < 0.01$; †, $p < 0.0001$.

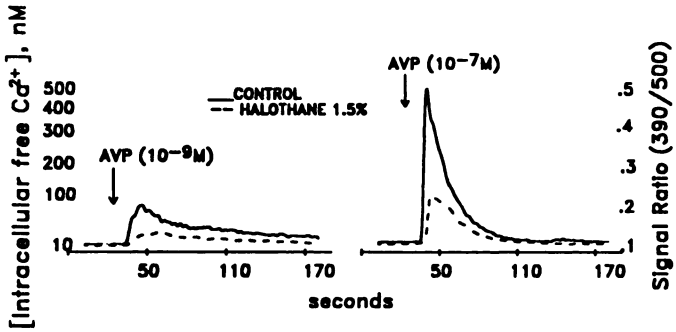

Fig. 4. Effects of preincubation with halothane on increases in $[Ca^{2+}]_i$ evoked by AVP (*left*, 10^{-9} M; *right*, 10^{-7} M in A7r5 cultured vascular smooth muscle cells. The indo-1-loaded cells were incubated either with or without 1.5% halothane in Ca^{2+}-free MEM plus 0.5 mM EGTA, to decrease extracellular $[Ca^{2+}]$. Apparent $[Ca^{2+}]_i$ was measured using dual-wavelength sampling from cells imaged during flow cytometry. The increase in $[Ca^{2+}]_i$ represents Ca^{2+} release from intracellular stores evoked by AVP. *Tracings* from typical experiments are presented.

Effects of preincubation with halothane on increases in $[Ca^{2+}]_i$ evoked by thapsigargin. The effects of preincubation with halothane on the Ca^{2+} content of intracellular stores was assessed by suspending the cells in Ca^{2+}-free medium plus EGTA and stimulating them with thapsigargin. [This agent discharges Ca^{2+} from $Ins(1,4,5)P_3$-sensitive stores, with the magnitude of the Ca^{2+} response reflecting the amount of releasable Ca^{2+} present (17).] Thapsigargin induced a gradual increase in $[Ca^{2+}]_i$, with maximum levels occurring at approximately 200 sec, followed by a gradual return towards base line. The amplitude of the increase in $[Ca^{2+}]_i$ was 59 ± 3 nM in control cells and decreased to 41 ± 2 nM in cells preincubated with 1.5% halothane ($p < 0.0001$). Comparisons of integrated areas under time-response curves indicated that halothane inhibited the response by 31 ± 5% ($p < 0.001$). Tracings from typical individual experiments are shown in Fig. 6.

Effects of preincubation with halothane on increases in $[Ca^{2+}]_i$ evoked by PDGF. Stimulation of untreated cells with 3.3×10^{-9} M PDGF resulted in increases in $[Ca^{2+}]_i$ with maximum levels occurring at approximately 100 sec, followed by return towards base line. The responses were inhibited in cells preincubated with 1.5% halothane. Base-line, peak, and 250-sec $[Ca^{2+}]_i$ levels are shown in Table 5. Comparison of integrated areas under the time-response curves indicated halothane decreased the PDGF-induced $[Ca^{2+}]_i$ responses by 43 ± 5% ($p < 0.0001$). Tracings from typical individual experiments are presented in Fig. 7.

Effects of preincubation with halothane on increases in total inositol phosphates evoked by AVP. The aim was to determine whether preincubation with halothane inhibited increases in inositol phosphates evoked by 10^{-7} M AVP. Total inositol phosphate levels were first measured 2 min before addition of AVP when results demonstrated that resting inositol phosphate levels in quiescent cells did not differ between cells treated with halothane and untreated cells. AVP was then added and induced a sustained increase in total inositol phosphate levels. (Degradation of inositol phosphates was inhibited due to the presence of LiCl.) The response was attenuated after pretreatment of cells with both 1 and 2% halothane. Levels of inositol phosphates in cells untreated with either AVP or halothane remained stable during the experiment and provided time control data. Comparisons of integrated areas under the time-response curves, calculated using time-control measurements as base-line, indicated that 1% halothane inhibited the AVP-induced increase in inositol phosphates by 46 ± 15% ($p < 0.02$) and that 2% halothane inhibited the response by 61 ± 19% ($p < 0.02$). The results are displayed in Fig. 8.

Discussion

The mechanisms of action of anesthetics are not understood. Current theories predict that the anesthetic state and related side effects such as vasodilatation may arise from interference with cell signaling (5). Results from the current experiments suggest that agonist-induced increases in $[Ca^{2+}]_i$ and in inositol phosphates, two uniquely important steps in cell signaling, are inhibited by halothane. Preincubation with clinically relevant concentrations of halothane resulted in attenuation of the

TABLE 4

Effects of preincubation with halothane upon Ca^{2+} release from intracellular stores and upon Ca^{2+} influx, both evoked by 10^{-7} M AVP

The indo-1-loaded cells were preincubated for 10 min, with or without halothane, in Ca^{2+}-free culture medium plus 0.5 mM EGTA. Apparent $[Ca^{2+}]_i$ was obtained using flow cytometry. AVP was injected into the cell suspension without interruption of the flow of cells. After completion of the initial transient, which represents Ca^{2+} release from intracellular stores, 5 mM Ca^{2+} was added to the cell suspension in the continued presence of AVP. The second response represents Ca^{2+} influx.

Halothane concentration	n	Peak $[Ca^{2+}]_i$ due to Ca^{2+} release		Peak $[Ca^{2+}]_i$ due to Ca^{2+} influx		$[Ca^{2+}]_i$ at 250 sec during Ca^{2+} influx	
		Control	Halothane	Control	Halothane	Control	Halothane
%		nM		nM		nM	
1.5	12	449 ± 28	184 ± 18[a]	188 ± 24	150 ± 17[b]	152 ± 9	132 ± 15[b]

[a] $p < 0.0001$, in comparison with control.
[b] $p < 0.01$, in comparison wtih control.

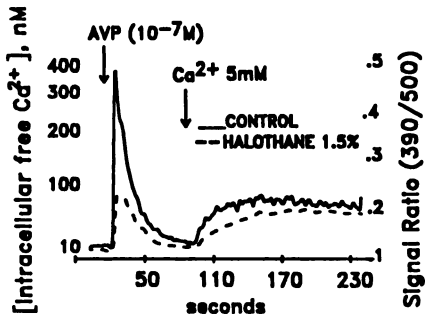

Fig. 5. Effects of preincubation with 1.5% halothane on increases in $[Ca^{2+}]_i$ due to Ca^{2+} release and Ca^{2+} influx in A7r5 cultured vascular smooth muscle cells stimulated with 10^{-7} M AVP. The indo-1-loaded cells were incubated either with or without halothane in Ca^{2+}-free MEM plus 0.5 mM EGTA, to decrease extracellular $[Ca^{2+}]$. Apparent $[Ca^{2+}]_i$ was measured using dual-wavelength sampling of light emitted from cells during flow cytometry. The initial peak (*left*) represented $[Ca^{2+}]_i$ increase due to Ca^{2+} release from intracellular stores. The second sustained increase occurred when 5 mM Ca^{2+} was added after completion of the initial transient, in the continued presence of AVP, and represented Ca^{2+} influx. *Tracings* from typical individual experiments are shown.

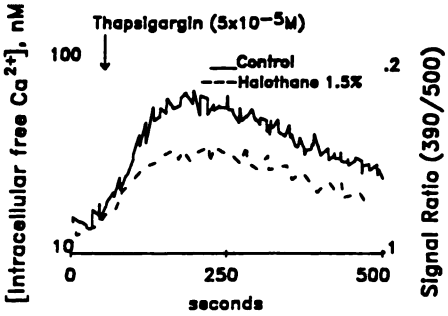

Fig. 6. Effects of preincubation with 1.5% halothane on increases in $[Ca^{2+}]_i$ elicited in cultured A7r5 vascular smooth muscle cells by 5×10^{-5} M thapsigargin. [Thapsigargin is believed to elevate $[Ca^{2+}]_i$ by discharging Ca^{2+} from Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} stores.] Cells were suspended in Ca^{2+}-free MEM plus 0.5 mM EGTA to decrease extracellular $[Ca^{2+}]$. Results from an individual experiments are shown.

$[Ca^{2+}]_i$ responses evoked in cultured vascular smooth muscle cells by the vasoactive hormone AVP. The anesthetic effect was dose dependent and the dose-response relationship was surprisingly steep. The effects of halothane on Ca^{2+} release were apparently much greater than those on Ca^{2+} influx. The actions of the anesthetic on Ca^{2+} homeostasis cannot be explained solely by depletion of Ca^{2+} from intracellular stores, because preincubation with halothane resulted in only a moderate inhibition of the $[Ca^{2+}]_i$ responses elicited by thapsigargin in cells suspended in Ca^{2+}-free medium. Halothane inhibited inositol phosphate formation elicited by AVP, suggesting an additional action by which the anesthetic may alter agonist-induced $[Ca^{2+}]_i$ responses. Halothane actions were not specific solely to responses elicited by AVP, which acts via a G protein-linked signaling system, because the anesthetic also inhibited those evoked by PDGF, an agonist that elevates $[Ca^{2+}]_i$ via receptor-latent tyrosine kinase activity.

Vasoactive hormones, including AVP, elevate inositol phosphates and $[Ca^{2+}]_i$ by activating a signaling system consisting of cell surface receptors that are presumed to possess a topography of seven membrane-spanning domains and are coupled to effector enzymes and ion channels via intermediary G proteins (12). Phosphoinositide-specific phospholipase C is the membrane-bound enzyme responsible for hydrolysis of the major substrate, phosphatidylinositol 4,5-bisphosphate, to form the cytosolic second messengers Ins(1,4,5)P_3 and diacylglycerol (13). The Ins(1,4,5)P_3 receptor on membranes enclosing intracellular Ca^{2+} stores has been purified and cloned, and its activation results in discharge of Ca^{2+} from portions of the endoplasmic reticulum. In some cells, Ins(1,4,5)P_3 is believed to act in conjunction with its metabolite inositol 1,3,4,5-tetrakisphosphate in regulating Ca^{2+} movement (13). In contrast to release of Ca^{2+}, an understanding of the mechanisms regulating Ca^{2+} influx has remained more elusive (15, 17).

Halothane had a marked inhibitory effect on increases in $[Ca^{2+}]_i$ caused by Ca^{2+} release. This action could be explained by interference with the signaling system responsible for Ca^{2+} release or, alternatively, by depletion of Ca^{2+} from intracellular stores. The current results suggest that both occur. In the current study, the effects of halothane upon Ca^{2+} content of intracellular storage pools were addressed by preincubating cells with the anesthetic and then stimulating them with thapsigargin. The increase in $[Ca^{2+}]_i$ elicited by this agent results from Ca^{2+} discharge from Ins(1,4,5)P_3-sensitive storage sites (17), with the magnitude of the response reflecting releasable Ca^{2+} remaining in the pools. Preincubation with halothane inhibited the response, suggesting that Ca^{2+} storage is impaired by the anesthetic. However, the magnitude of the depletion of Ca^{2+} was not sufficiently great to completely account for inhibition of the $[Ca^{2+}]_i$ responses evoked by AVP and by PDGF. The results are in agreement with observations made in cardiac tissue, where halothane is thought to deplete Ca^{2+} from the sarcoplasmic reticulum by decreasing uptake via the Ca^{2+}-ATPase pump (16, 24) or by discharging Ca^{2+} via caffeine-sensitive Ca^{2+}-release channels (25). Less is known concerning the actions of halothane in vascular smooth muscle, although halothane promotes Ca^{2+} loss from caffeine-sensitive stores in rabbit aortic strips (26). The current results indicate that Ins(1,4,5)P_3-sensitive Ca^{2+} stores in vascular smooth muscle cells are susceptible to depletion by halothane.

The absence of immediate and pronounced increases in $[Ca^{2+}]_i$ upon acute addition of halothane to quiescent cells suggests that the anesthetic does not discharge Ca^{2+} from stores in a rapid manner. This absence of immediate effect was surprising, because it contrasts sharply with responses reported

TABLE 5

Effects of preincubation with halothane upon base-line $[Ca^{2+}]_i$ and upon increases in $[Ca^{2+}]_i$ evoked by 3.3×10^{-9} M PDGF

Halothane concentration	n	Base-line $[Ca^{2+}]_i$		Peak $[Ca^{2+}]_i$		$[Ca^{2+}]_i$ at 250 sec	
		Control	Halothane	Control	Halothane	Control	Halothane
%		nM		nM		nM	
1.5	11	50 ± 1	46 ± 1[a]	390 ± 17	217 ± 19[b]	102 ± 5	78 ± 4[b]

[a] $p < 0.01$, in comparison with control.
[b] $p < 0.0001$, in comparison wtih control.

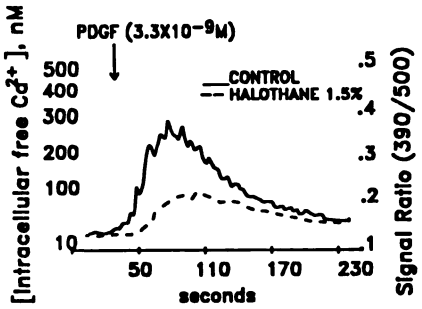

Fig. 7. Effects of preincubation with 1.5% halothane on increases in $[Ca^{2+}]_i$ in cultured A7r5 vascular smooth muscle cells stimulated with 3.3 × 10^{-9} M PDGF. The indo-1-loaded cells were incubated either with or without halothane for 10 min. Results from typical individual experiments are presented.

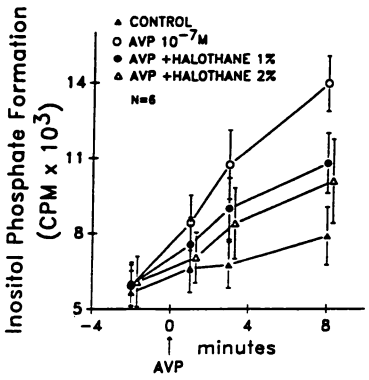

Fig. 8. Effects of halothane on inositol phosphate formation stimulated with 10^{-7} M AVP in cultured A7r5 vascular smooth muscle cells. The *myo*-[^{3}H]inositol-labeled cells were incubated either with or without 1% or 2% halothane, for 10 min, and then stimulated with AVP. (Control cells were exposed to neither halothane nor AVP.) Comparisons of responses evoked by AVP alone versus responses evoked by AVP after preincubation with halothane indicated statistically significant depressant effects at each anesthetic concentration (comparison of slopes, $p < 0.05$. Comparison of integrated areas, $p < 0.02$).

in many types of cells, including those originating from heart (10), liver (27), skeletal muscle (28), blood (22), and brain (29), where halothane acutely elevates $[Ca^{2+}]_i$. Increase in $[Ca^{2+}]_i$ is a marker of cell activation. In neuronal cells, increased $[Ca^{2+}]_i$ has been associated with activation of K^+ channels, and the resulting depression of excitability has served as a model of general anesthesia (6, 29). However, gradual depletion of Ca^{2+} from internal stores noted in the current experiments, rather than abrupt acute discharge of Ca^{2+} into the cytoplasm observed in other cell types, is more compatible with the *in vivo* vascular effects of halothane, which involve vasodilatation.

Attenuation of AVP-induced Ca^{2+} release may be also explained by the inhibitory effect of halothane on AVP-induced inositol phosphate formation, a finding that represents a previously unreported action of volatile anesthetics. The effect was present 45 sec after stimulation with AVP and persisted at 8 min when inositol phosphate formation was maximal (due to inhibited breakdown in the presence of LiCl). Although the experiments did not define the locus of the actions of halothane, the results may be explained by direct inhibition of phospholipase C, by depletion of phosphatidylinositol 4,5-bisphosphate as a substrate for phospholipase C, or by increased breakdown of inositol phosphates. Enzymes are not generally believed to be sensitive to volatile anesthetics; however, notable exceptions exist, with the most well characterized example being inhibition of the light-emitting enzyme firefly luciferase by halothane (5). In this instance, the anesthetic competes with the subtrate firefly luciferin for binding sites on luciferase. In the current study, the proposed relationship between inhibition of inositol phosphate formation and attenuation of Ca^{2+} release should be viewed with a degree of caution, because total inositol phosphates were measured rather than concentrations of $Ins(1,4,5)P_3$, the messenger responsible for Ca^{2+} release. However, because phosphatidylinositol 4,5-biphosphate degradation results in the formation of inositol phosphates via $Ins(1,4,5)P_3$, with only small amounts being formed by hydrolysis of other inositol phospholipids, the levels of total inositol phosphates should reflect concentrations of $Ins(1,4,5)P_3$ (30).

In contrast to the marked inhibitory effect of halothane on AVP-induced Ca^{2+} release, Ca^{2+} influx was apparently only modestly decreased by the anesthetic. This observation was surprising because, in many types of cells, Ca^{2+} release and Ca^{2+} influx are tightly coupled, with depletion of Ca^{2+} from intracellular stores serving to initiate Ca^{2+} entry (17). An alternative but related model of influx control suggests that activated receptors directly open cell membrane Ca^{2+} channels (15). The current divergent effects of halothane on release versus influx may suggest that direct opening of channels by receptors, followed by Ca^{2+} influx, is not sensitive to inhibition by the anesthetic. The current results differ from observations made in isolated ventricular myocytes, where halothane depressed the inward Ca^{2+} current (9); however, these responses were evoked by depolarization rather than by receptor activation.

Halothane inhibited increases in $[Ca^{2+}]_i$ elicited by PDGF, an agonist that acts via an intracellular effector system that differs from the signaling pathway activated by pressor hormones. Receptors for this growth factor possess a single transmembrane region and a cytoplasmic domain that, on receptor dimerization, exhibits tyrosine kinase activity, resulting in phosphorylation of phospholipase C, possibly without the involvement of G proteins (18). The results indicate that the actions of halothane are not restricted solely to responses evoked by AVP but include responses elicited via distinctly different signaling pathways.

In conclusion, it is tempting to speculate that, in humans undergoing anesthesia, the vasodilator effects of halothane result from a direct effect of the anesthetic on Ca^{2+} homeostasis in vascular smooth muscle cells. However, the cell culture model should be viewed with a degree of caution, because the closeness with which cell culture experiments represent *in vivo* physiological events always remains uncertain.

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