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Volatile Anesthetics and Agonist-induced Contractions in Porcine Coronary Artery Smooth Muscle and Ca^{2+} Mobilization in Cultured Immortalized Vascular Smooth Muscle Cells

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Background: **These experiments addressed four specific questions. Do isoflurane and halothane (0.5–3.0% in the gas phase) inhibit contractions evoked in isolated porcine coronary artery rings (without endothelium) by the specific Ca^{2+} mobilizing agonists serotonin, endothelin-1, and F^-? Are contractions evoked by phorbol-activated protein kinase C inhibited by the anesthetics? In a well-characterized vascular smooth muscle cell culture model (A7r5 and A10), do the anesthetics attenuate serotonin- and endothelin-induced Ca^{2+} mobilization? Do the anesthetics inhibit intracellular Ca^{2+} mobilization *via* facilitated cAMP formation?**

Methods: **Tension was measured in rings suspended in organ chambers. Apparent intracellular Ca^{2+} was estimated in cells using indo-1 and flow cytometry. Cyclic AMP was measured by radioimmunoassay.**

Results: **At the anesthetic concentrations examined, isoflurane attenuated contractions evoked by serotonin and F^- but not those induced by endothelin-1 or phorbol dibutyrate. In cells, isoflurane 2% attenuated 3×10^{-5} M serotonin-induced Ca^{2+} mobilization by about 26%, whereas Ca^{2+} responses evoked by endothelin 10^{-8} M were more resistant to anesthetic inhibitory effect. Halothane attenuated contractions in rings evoked by serotonin, endothelin, and F^- but lacked effect on phorbol-induced responses. In cells, halothane 2% inhibited Ca^{2+} mobilization induced by serotonin by about 43% and that induced by endothelin by about 31%. Neither anesthetic facilitated cAMP formation.**

Conclusions: **Isoflurane and halothane variably attenuated contractions evoked by Ca^{2+} mobilizing agonists—by a cellular action beyond the receptor level—but did not inhibit phorbol activated protein kinase C. Serotonin- and endothelin-induced Ca^{2+} mobilization was inhibited by isoflurane and halothane—but the mechanism does not depend upon increased cAMP. (Key words: Anesthetics, volatile: halothane; isoflurane. Arteries, coronary: pig. Cell culture, smooth muscle: A10; A7r5. Ions: calcium; fluoride. Nucleotides: cyclic adenosine monophosphate. Pharmacology: endothelin; fluoride; phorbol dibutyrate; serotonin.)**

VOLATILE anesthetics attenuate intraoperative increases in vascular resistance and arterial blood pressure—two common and potentially adverse consequences of surgical stimulation. They do so, in part, by depressing autonomic vasoconstrictor outflow and in part by attenuating blood vessel responsiveness to vasoconstrictor stimuli. The latter, the direct inhibitory actions of anesthetics at the level of the blood vessel wall, have been extensively demonstrated in isolated vascular ring preparations where isoflurane and halothane variably inhibit contractions evoked by a range of constrictor agonists.[1–11] Smooth muscle rather than the endothelium[12] is regarded as the major site of anesthetic relaxant action but mechanisms underlying anesthetic effect remain ill defined. However, in common with cardiac muscle, Ca^{2+} mobilization is a likely target.

Vascular smooth muscle contraction is initiated by an abrupt agonist-induced increase in intracellular Ca^{2+} (Ca^{2+}_i).[13] The contractile response is sustained despite a decrease in this initial Ca^{2+}_i increase, in part, by the persistent action of protein kinase C[14]—an enzyme that phosphorylates proteins with roles in the contractile process. Current studies were designed to assess isoflurane and halothane actions on agonist-induced vascular smooth muscle responses. Four specific questions were posed.

Do isoflurane and halothane, in clinically meaningful gas phase concentrations, inhibit contractions evoked by "Ca^{2+} mobilizing agonists"? Serotonin, endothelin-1 (endothelin), and F^- were studied using isolated porcine coronary arteries—vessels of this species cho-

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sen for the claimed physiologic resemblance to human vessels. Rings were used without endothelium to exclude potential anesthetic-induced effects at this site[1,12] and to isolate responses to only vascular smooth muscle. Serotonin and endothelin were investigated because both have putative roles in regulating human vascular tone in health and disease.[15] In addition the contractile[16–20] and $Ca^{2+}{}_i$ mobilizing effects[20–26] of the two agonists are relatively well characterized. Specifically, at the cellular level, serotonin and endothelin interact with receptors of the seven–transmembrane-domain configuration linked *via* intermediary guanosine triphosphate–binding proteins (G-proteins) to the enzyme phospholipase C, which generates two well-recognized second messenger molecules: inositol trisphosphate, involved in Ca^{2+} mobilization, and diacylglycerol, responsible for protein kinase C activation.[27] Fluoride ions (generated by NaF/AlF_3) were studied because they interact in isolated porcine arteries with G-proteins linked to contraction.[18] In this way, studies of F^--induced contractions permitted assessment of anesthetic effects at a level beyond receptor stimulation.

Do isoflurane and halothane attenuate contractions elicited *via* activated protein kinase C? Responses were stimulated in coronary artery rings with the phorbol ester phorbol dibutyrate (PDBu). This pharmacological probe is structurally analogous to diacylglycerol, the natural second messenger molecule responsible for activating protein kinase C.[28] PDBu substitutes for diacylglycerol, eliciting contractions in the absence of receptor, G-protein, phospholipase-C activation and without changes in $Ca^{2+}{}_i$.[29]

Is the $Ca^{2+}{}_i$ component of vascular smooth muscle cell signal transduction a target for anesthetics? A cell culture model was used. Cells of the A7r5 and A10 lines, derived originally and simultaneously from the same fetal rat aorta,[30] were used in lieu of smooth muscle cells of porcine coronary artery origin. Primary cultures of cells from porcine vessels do not readily remain phenotypically stable in culture, losing differentiated properties and $Ca^{2+}{}_i$ signaling characteristics in an unpredictable manner. In contrast, A7r5, A10 cells are phenotypically stable in continuous culture, resulting in their widespread acceptance and adoption as cell models for the study of signal transduction *via* the phospholipase C-$Ca^{2+}{}_i$ axis.[22–26,31,32] Cells of the A7r5 line express 5-hydroxytryptamine$_2$ receptors,[22] the subtype responsible for contractions in pig coronary arteries.[16,17] Cells of the A10 line express receptors for endothelin.[25,26] This cell line was used by the research group originally responsible for identifying endothelin and its vascular smooth muscle cell signaling mechanisms.[25]

Volatile anesthetics have been suggested to increase vascular smooth muscle cyclic adenosine monophosphate[3] (cAMP), a second messenger that decreases $Ca^{2+}{}_i$, and causes vascular relaxation. Consequently, do isoflurane and halothane attenuate agonist-induced $Ca^{2+}{}_i$ mobilization in cultured vascular smooth muscle cells *via* a cAMP dependent mechanism?

Materials and Methods

Porcine Coronary Artery Rings

After institutional approval had been obtained, hearts were obtained at time of death, from pigs slaughtered at local abattoirs. Left coronary arteries were dissected free and immersed in oxygenated cold Krebs–Ringer's bicarbonate solution of the following composition (millimolar): NaCl 119, KCl 4.8, $CaCl_2$ 2.6, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 4.1. On return to the laboratory (30 min) they were cleaned of adherent tissue and fat and cut into rings 5–6 mm in length. The intimal surface was rubbed to remove the endothelium. Each ring was connected to an isometric force transducer (Grass FT03) and suspended in an organ chamber containing 25 ml Krebs–Ringer's bicarbonate solution at 37°C, pH 7.4, aerated with 94% O_2–6% CO_2. The rings were allowed to stabilize. Optimal length for stress development (9–12 g) was determined for each ring by stretching until a maximum contraction occurred in response to 20 mM KCl. Rings were maintained at this tension. The absence of functioning endothelium was tested by observing absence of relaxation to 10^{-6} M bradykinin, an endothelium-dependent dilator, in rings precontracted with 40 mM KCl. Rings with functioning endothelium were replaced.

Anesthetics were added by vaporizer to the gas aerating half of the rings, while the remaining rings remained free of anesthetics and served as matched controls. Anesthetic concentrations in the gas were monitored and adjusted with an anesthetic analyzer (Siemens 120, Solna, Sweden), which was calibrated either with commercially available gas mixtures of known anesthetic concentration (Scott Medical, Plumstead, PA) or by mass spectrometry (Perkin Elmer, Pomona, CA). The concentration of anesthetics in the electrolyte solution bathing the rings was determined

by extracting the anesthetic from 400 μl samples of electrolyte solution into 4 ml hexane and then measuring concentrations using gas chromatography with (5880A, Hewlett-Packard, Avondale, PA). For clarity, concentrations measured in the bathing solution were converted to and are presented as gas phase values.

Eight rings were obtained from each pig heart. Two rings were aerated with 2% isoflurane; two served as matched controls; two were aerated with 2% halothane; and two rings served as matched controls. Cumulative increasing doses of serotonin 10^{-9} to 10^{-4} M or PDBu 10^{-11} to 10^{-4} M were added to the organ chambers to obtain dose-response relationships. Special care was taken to permit time for full development of force at each PDBu concentration because responses to this agent occur slowly. Separate pig hearts were used for each study with each agonist. Studies with serotonin were repeated in rings removed from additional pig hearts, to test the effects of 1% concentrations of the anesthetics. In a different series of experiments, the relaxant effects of sequentially increasing concentrations of isoflurane and halothane were examined in relation to sustained contractions. Contractions were obtained with endothelin 10^{-8} M, with 5 mM NaF plus 10 μM AlF_3 to generate F^- and with 10^{-5} M PDBu. Each agonist was examined using eight rings from separate pig hearts. In each experiment, four rings received isoflurane, and the other four served as matched controls. Arteries from different pig hearts were used to test halothane. Anesthetics were administered for 30 min at each concentration.

Cultured Vascular Smooth Muscle Cells

Cells were obtained from the American Type Culture Collection (A7r5 CRL 1444; A10 CRL 1476) and grown at 37°C in a 95% air–5% CO_2 atmosphere in Dulbecco's modified Eagles medium containing antibiotics and 10% fetal bovine serum, until they reached confluence (usually 5–7 days).

To estimate $Ca^{2+}{}_i$, confluent cells were incubated with the fluorescent probe indo-1 in the acetoxymethyl ester form (4 μM), plus 1% pluronic, for 35–40 min at 37°C. (Indo-1/acetoxymethyl ester is hydrolyzed in the cell to the cell-impermeant form.) After washing with phosphate-buffered saline, the cells were gently dissociated with 0.05% trypsin, washed and centrifuged and the supernatant discarded. Meanwhile, 25 ml buffer solution (Krebs–Ringer's solution plus 10% fetal bovine serum with 25 mM hydroxyethylpiperazineethanesulfonic acid) was aerated at 37°C in a semiclosed container, with 95% O_2–5% CO_2 gas mixture flowing at 150 ml/min, to which isoflurane or halothane was added using calibrated vaporizers. After equilibration with the anesthetic for 30 min, 6 ml of this buffer was transferred to gas-tight siliconized glass syringes along with 4 ml of the gas mixture and cells added. Gas chromatographic analysis of samples extracted in hexane confirmed the presence of appropriate anesthetic concentrations.[33] Cells were added and preincubated either with or without each anesthetic, for 20 min to permit anesthetic uptake and equilibration. Cells in suspension (0.5–1.0 ml) were passed at about 300–500 cells/s through a flow cytometer (FACStar plus®, Beckton Dickinson, Mountain View, CA). They were excited at 351–364 nm and emitted fluorescence was sampled simultaneously at 385–395 nm and 480–520 nm (Unlabelled cells and debris were excluded from analysis by using appropriate gating and intensity thresholds.) Increases in $Ca^{2+}{}_i$ were evoked by adding 3×10^{-5} M serotonin or 10^{-8} M endothelin to the cell suspension in the flow cytometer reservoir, without interrupting the continuous flow of cells through the instrument. Ratios of fluorescence wavelengths were converted to apparent $Ca^{2+}{}_i$ values by obtaining minimum (0.07) and maximum fluorescence ratios (1.10) in cells of both lines acutely permeabolized with 10 μM digitonin. Equations describing the relationships between ratio and $Ca^{2+}{}_i$ were solved using published, temperature corrected indo-1 K_d values (325 nM).[34,35]

Cyclic AMP levels were estimated in washed cells that were incubated with 10^{-4} M isobutylmethylxanthine to prevent degradation of cyclic AMP by phosphodiesterases and with 10^{-5} M indomethacin to inhibit production of prostanoids. Anesthetics were prepared in dimethyl sulphoxide (25 μl liquid anesthetic per 975 μl dimethyl sulphoxide), which was then added to Krebs–Ringer's solution to obtain a stock solution. Stock solution was added to cell suspension to obtain final desired anesthetic concentrations. The gas phase above the cell suspension (2×10^6 cells/well) was continuously flushed with air containing anesthetic, delivered by a vaporizer, in order to minimize anesthetic loss by evaporation. Appropriate anesthetic concentrations in the suspension were confirmed by gas chromatography. Cells were incubated with and without anesthetics for 0, 2, 5, and 30 min. Samples were obtained at each time point and centrifuged; the cells lysed in acetone–dry ice with ethanol and centrifuged; and the supernatant dried under nitrogen at 55°C. Cyclic AMP was assayed in triplicate using a kit (Amer-

Table 1. Active Tension Generated by Each Group of Rings before Introduction of Anesthetics

Experiment	Agonist	Maximal Developed Tension (g) before Anesthetic Intervention			
		Control	Before Isoflurane	Control	Before Halothane
Dose–response to serotonin					
1% anesthetic	60 mM KCl	9.4 ± 0.9	11.8 ± 0.9	11.1 ± 1.8	12.2 ± 2.7
2% anesthetic	60 mM KCl	10.9 ± 1.3	10.2 ± 1.0	8.1 ± 1.3	10.5 ± 1.3
Sustained contraction evoked by endothelin	10^{-8} endothelin	11.7 ± 1.0	10.6 ± 0.7	10.8 ± 0.4	8.8 ± 1.0
Sustained contraction evoked by F^-	5 mM NaF + 10 μM AlF_3	8.9 ± 1.4	10.4 ± 1.7	6.4 ± 1.0	6.4 ± 1.3
Dose–response to PDBu	60 mM KCl	9.5 ± 1.2	9.4 ± 1.5	11.2 ± 0.6	10.0 ± 1.1
Sustained contraction evoked by PDBu	10^{-5} M PDBu	11.6 ± 0.9	13.4 ± 1.3	12.3 ± 1.0	11.6 ± 1.0

Each agonist was assessed using separate groups of coronary artery rings. Each group of rings was obtained from n = 5 to 7 pig hearts.
PDBu: phorbol dibutyrate.

sham, Arlington Heights, IL) in which cyclic AMP in the cell lysate and [^{3}H]cAMP provided in the kit compete for a binding protein. Protein-bound cyclic AMP was separated from the unbound nucleotide by absorption of the free nucleotide on charcoal. [^{3}H]cAMP in the eluant was estimated by liquid scintillation counting. (Opti-fluor scintillation solution, Packard, Meriden, CT, and LS 5000 TD counter, Beckman, Fullerton, CA). Additional experiments were performed to determine if preincubation with isoflurane or halothane for 20 min would result in elevations in cAMP when the cells were stimulated with serotonin or with endothelin. (Neither agonist was expected to increase cAMP.) Finally, positive control experiments were performed to show that when stimulated with isoproterenol 10^{-6} M, cells demonstrated appropriate increases in cAMP.

Materials and Drugs

Serotonin, bradykinin, and PDBu were obtained from the Sigma Chemical Company (St. Louis, MO). Porcine endothelin-1 was obtained from Peninsula Laboratories (Belmont, CA). Indo-1 pentaacetoxy-methyl ester was purchased from Molecular Probes (Eugene, OR). Dulbecco's modified Eagles medium, hydroxyethylpiperazineethanesulfonic acid, and trypsin were obtained from GIBCO (Grand Island, NY). Fetal bovine serum was acquired from Hyclone (Logan, VT). Halothane was purchased from Ayerst Laboratories (New York, NY) and isoflurane from Anaquest (Madison). NaF and $AlCl_3$ were obtained from Aldrich (Milwaukee, WI).

Statistical Analysis

Results are expressed as mean ± standard error. In experiments with coronary artery rings, "n" refers to the number of pig hearts from which vessels were obtained. When dose-response relationships for serotonin and PDBu were obtained, contractions were expressed as percent of responses to a standard challenge with 60 mM KCl—before anesthetic or agonist administration. When sustained contractions to endothelin, F^- and PDBu were studied, relaxations were expressed as percent of the initial plateau contraction. Tension did not differ between matched control rings and rings exposed to the anesthetics. In experiments involving estimation of Ca^{2+}_i, n refers to the number of individual experiments performed, each consisting of control immediately followed by anesthetic treated cells. Cells used in studying each agonist were grown in a minimum of five separate batches. When cAMP was measured, n refers to the number of occasions the experiment was repeated. Data were analyzed using analysis of variance. Student's *t* test for paired observations was used to determine individual points of significance. $P < 0.05$ was considered significant.

Results

Experiments with Isoflurane

Isoflurane and Coronary Artery Rings. Experiments were designed to determine if isoflurane attenuated agonist-induced contractions in porcine coronary artery rings without endothelium. Baseline contractile responses, before aeration with isoflurane, are shown in table 1. Force of contractions did not differ between control rings and rings designated for anesthetic treatment.

Contractions were elicited with two Ca^{2+} mobilizing vasopressor hormones, serotonin and endothelin. Se-

rotonin 10^{-9}–10^{-4} M evoked responses that were maximal at 10^{-5} M and that were followed by relaxations—resulting in a biphasic contractile pattern typical for this agonist. Isoflurane 1% was without apparent inhibitory effect on this dose–tension relationship, whereas 2% isoflurane attenuated the contractions (fig. 1, left). It was not possible to obtain meaningful dose-response curves with endothelin because contractions were very slow in onset. Instead, sustained contractions induced by a single endothelin 10^{-8} M concentration were studied. Sequentially increasing concentrations of isoflurane from 0.5 to 2.5% were without apparent relaxant effect (fig. 1, right).

Rings were stimulated with F^- (generated by 5 mM NaF plus 10 μM AlF_3) to activate G-proteins, including those linked to phospholipase C. Sustained contractions were obtained that were less vigorous and less enduring than those evoked by endothelin. Sequentially increasing concentrations of isoflurane from 0.5 to 2.0% caused relaxations at all anesthetic concentrations tested (fig. 2).

PDBu was used to induce contractile responses *via* protein kinase C activation. Cumulative increasing concentrations of PDBu from 10^{-11} to 10^{-4} M elicited slowly developing contractions. Isoflurane 2% was without detectable inhibitory effect. Dose-response curves obtained in the presence and absence of anesthetics were so similar that they could be virtually superimposed (fig. 3, left). Sustained stable contractions elicited with 10^{-5} M PDBu remained unaffected by concentrations of isoflurane sequentially increased from 0.5 to 3.0% (fig. 3, right).

Isoflurane and Ca^{2+}_i Mobilization in Cultured Cells. Experiments were designed to determine if isoflurane attenuated agonist-induced Ca^{2+} mobilization in cultured smooth muscle cells of fetal rat aorta origin. A7r5 cells, which possess well-characterized 5-hydroxytryptamine$_2$ receptors,[22] were preincubated for 20 min with 2% isoflurane or without the anesthetic (controls). Serotonin 3×10^{-5} M was added and evoked an abrupt increase in Ca^{2+}_i, followed by a plateau phase in which Ca^{2+}_i gradually decreased, despite the continued presence of the agonist. Isoflurane 2% attenuated the increases in Ca^{2+}_i. Data are shown in table 2, and a typical individual study is illustrated in figure 4 (left).

Cultured A10 cells, which possess well-characterized endothelin receptor responses,[25,26] were preincubated for 20 min with and without (controls) 1, 2, or 3% isoflurane. Endothelin 10^{-8} M was added and evoked a rapid, transient increase in Ca^{2+}_i followed by a decrease in Ca^{2+}_i. (Increases in Ca^{2+}_i were greater than those induced by serotonin.) Both 1 and 2% isoflurane were without statistically significant inhibitory effect on the increase in Ca^{2+}_i evoked by endothelin. However, 3% isoflurane attenuated the Ca^{2+}_i response. Data are shown in table 2 and results of typical individual experiments are presented in figure 4 (right).

Isoflurane and Cyclic Adenosine Monophosphate Concentrations in Cultured Cells. Studies were designed to determine if isoflurane directly stimulated increases in cAMP levels in A7r5 and A10 cells, in this way providing an explanation for the anesthetic's inhibitory effects on Ca^{2+} mobilization. Isoflurane was added directly to quiescent cells in suspension to achieve a final anesthetic concentration of 2%. Cyclic AMP levels were unaffected by isoflurane during the 2, 5, and 30 min time course of the experiment. Isoflurane was also without effect on cAMP levels when cells were challenged with serotonin 3×10^{-5} M or endothelin 10^{-8} M. In the absence of anesthetics the ability of cells to increase cAMP when appropriately stimulated was tested by adding 10^{-6} M isoproterenol to activate adenylate cyclase. Data are shown in table 3.

Experiments with Halothane

Halothane and Coronary Artery Rings. Experiment were designed to determine if halothane attenuated agonist-induced contractions in porcine coronary artery rings without endothelium. Studies followed the same protocols used in isoflurane investigations (above). Before exposure to halothane, the contractile characteristics of rings used in the anesthetic studies did not differ from those used as controls (table 1).

Halothane 1 and 2% attenuated contractions evoked by serotonin (fig. 5, left). Halothane had a significant but modest relaxant effect on sustained contractions evoked by 10^{-8} M endothelin, but only at 2 and 2.5% anesthetic concentrations. Washout of halothane in the final stage of the experiment was accompanied by partial restoration of contractile force (fig. 5, right). Halothane, at all concentrations tested, attenuated contractions induced by F^- (fig. 6). The anesthetic was without inhibitory effects on responses elicited with PDBu, even when halothane concentrations were increased to 3%, a dose exceeding that commonly used in clinical practice (fig. 7).

Halothane and Ca^{2+} Mobilization in Cultured Cells. Experiments were performed in a manner similar to that described for isoflurane (above). Ca^{2+} mobilization evoked by 3×10^{-5} M serotonin in A7r5 cells

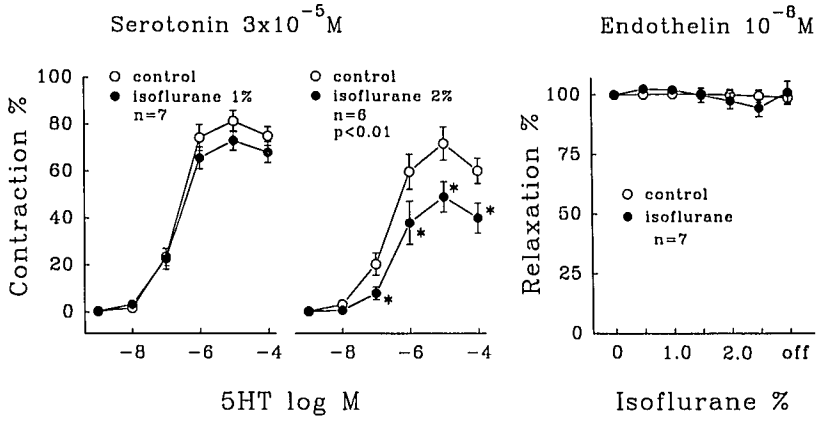

Fig. 1. (*Left*) The effects of isoflurane 1 and 2% on contractions evoked by cumulative sequentially increasing concentrations of serotonin (5-HT) in isolated porcine coronary artery rings without endothelium. Isoflurane was administered for 30 min before and throughout the challenge with serotonin. Control rings were obtained from the same coronary artery of the same pig heart and were studied in parallel. Contractions are expressed as a percent of the maximal response obtained with 60 mM KCl before anesthetic or serotonin administration. (*Right*) The effects of sequentially increasing doses of isoflurane on sustained contractions elicited with 10^{-8} M porcine endothelin-1. The anesthetic was administered for 30 min at each time point except for final 30 min for isoflurane wash-out. Contractions are expressed as a percent of maximal response to 10^{-8} M endothelin before isoflurane administration. Data are mean ± SE; n = number of pig hearts. *P* values indicate differences from controls. Points of individual significance are denoted; *$P < 0.05$.

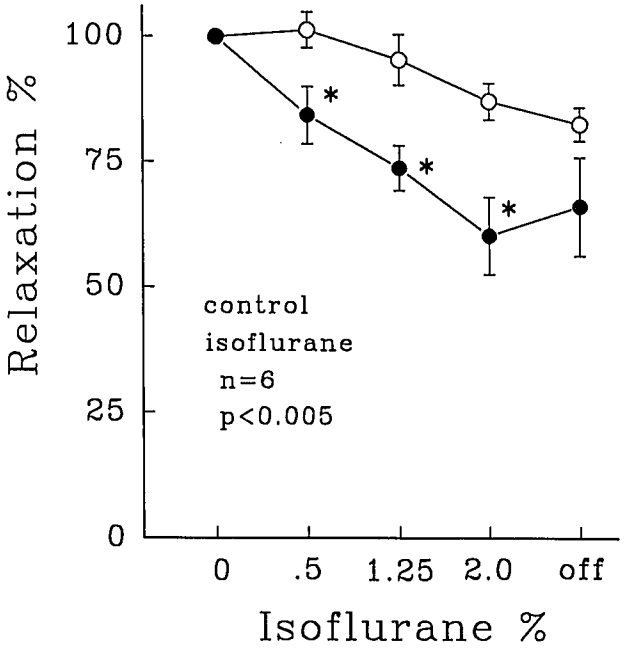

Fig. 2. The effects of sequentially increasing doses of isoflurane on sustained contractions evoked by 5 mM NaF plus 10 μM AlF_3 (to generate F^-) in porcine coronary artery rings without endothelium. Isoflurane was administered for 30 min at each time point except for final 30 min for isoflurane wash-out. Contractions are expressed as percent of maximal response to 5 mM NaF plus 10 μM AlF_3 before anesthetic administration. Data are mean ± SE; n = number of pig hearts; $P < 0.05$.

and by 10^{-8} M endothelin in A10 cells was inhibited by halothane at all anesthetic concentrations tested. Results are shown in table 2. Tracings from typical individual experiments are presented in figure 8.

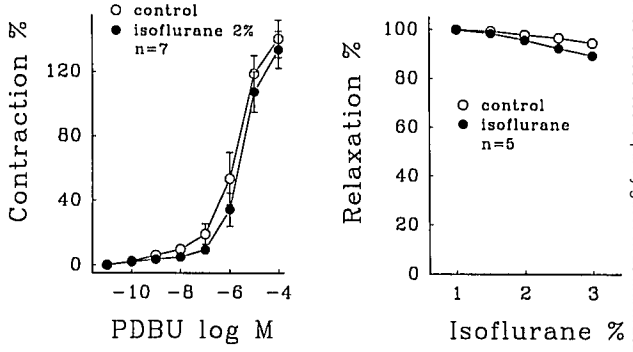

Fig. 3. (*Left*) The effects of 2% isoflurane on contractions evoked by cumulative sequentially increasing concentrations of phorbol dibutyrate (PDBu) in isolated porcine coronary artery rings without endothelium. Isoflurane was administered for 30 min before and throughout the challenge with PDBu. Contractions are expressed as a percent of the maximal response obtained with 60 mM KCl before anesthetic or PDBu administration. (*Right*) The effects of sequentially increasing doses of isoflurane on sustained contractions elicited with 10^{-5} M PDBu. The anesthetic was administered for 30 min at each time point. Contractions are expressed as a percent of maximal response to 10^{-5} M PDBu before isoflurane administration. Data are mean ± SE; n = number of pig hearts; *$P < 0.05$.

Table 2. Effects of Anesthetics on Ca_i^{2+} in Cultured Vascular Smooth Muscle Cells Stimulated with 3×10^{-5} M Serotonin or with 10^{-8} M Endothelin-1

Anesthetic	Agonist	Baseline Ca_i^{2+} (nM)		Amplitude of the Increase in Ca_i^{2+} (nM)		% Inhibition of the Increase in Ca_i^{2+} by the Anesthetic
		Control	With Anesthetic	Control	With Anesthetic	
Isoflurane 2%	Serotonin	65 ± 2	57 ± 1	230 ± 20	167 ± 12†	26 ± 3
Isoflurane 1%	Endothelin	60 ± 4	60 ± 5	285 ± 24	284 ± 25	—
Isoflurane 2%	Endothelin	67 ± 4	68 ± 4	254 ± 37	355 ± 27	—
Isoflurane 3%	Endothelin	65 ± 4	66 ± 4	391 ± 14	332 ± 19*	14 ± 4
Halothane 2%	Serotonin	65 ± 3	49 ± 1	239 ± 19	124 ± 7†	43 ± 4
Halothane 1%	Endothelin	76 ± 3	77 ± 3	407 ± 30	359 ± 34*	13 ± 3
Halothane 2%	Endothelin	78 ± 2	71 ± 3	366 ± 20	230 ± 21†	31 ± 5
Halothane 3%	Endothelin	65 ± 4	74 ± 7	391 ± 14	241 ± 25†	41 ± 6

n = 10 to 16 experiments, each paired with matched control.

* $P < 0.005$.

† $P < 0.0001$.

Halothane and Cyclic Adenosine Monophosphate Concentrations in Cultured Cells. Halothane was added directly to quiescent cells in suspension to achieve a final anesthetic concentration of 2%. Halothane was without effect on cAMP levels, which remained unchanged during the 2, 5 and 30 min time course of the experiment. Halothane was without effect on cAMP levels when cells were challenged with either serotonin 3×10^{-5} M or endothelin 10^{-8} M. Data are shown in table 3.

Discussion

Isoflurane attenuated contractions evoked by serotonin, an agent that acts *via* phospholipase C, Ca^{2+} mobilization and protein kinase C activation. This finding was expected as the anesthetic is known to inhibit contractions in the isolated vessels of various species[2,8,16] and vasoconstriction in intact pigs[9]—mediated *via* serotonin. Isoflurane attenuated contractions evoked by F^-. This finding indicates isoflurane must act at a level of signal transduction beyond ligand-receptor interactions because F^- ions interact with G-proteins specifically associated with phospholipase C, Ca^{2+} mobilization, protein phosphorylation and contraction.[18] These data raise the possibility that G-protein mediated cell signaling *via* phospholipase C is a target of anesthetic action. However, isoflurane cannot be considered a non-specific inhibitor of cellular events mediated *via* this axis because the anesthetic did not attenuate con-

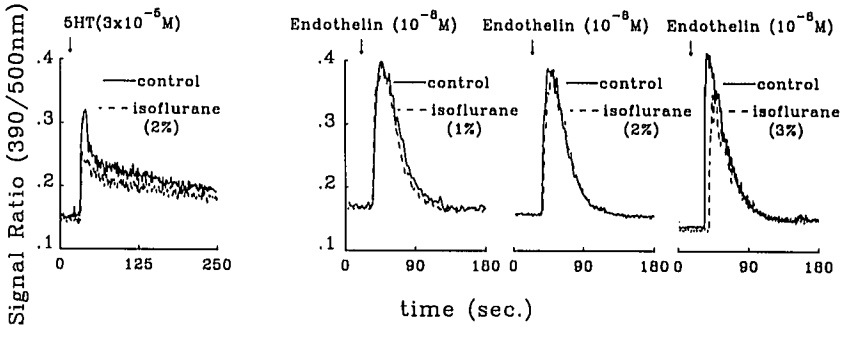

Fig. 4. Typical tracings from individual experiments illustrating the effects of isoflurane on $Ca^{2+}{}_i$ mobilization evoked by serotonin (5-HT) (*left*) and porcine endothelin-1 (*right*) in cultured vascular smooth muscle cells. The A7r5 (*left*) and A10 lines (*right*), derived originally and simultaneously from fetal rat aorta were used. Changes in apparent $Ca^{2+}{}_i$ were detected using the fluorescent cellular probe indo-1 in the AM form and with flow cytometric analysis. Cells were preincubated with isoflurane for 20 min before addition of the agonists. Dual emitted fluorescence was detected at ~390 and 500 nm. Each individual experiment was accompanied by a sequential individual matched control. Data were obtained from about 100,000 individual cells/tracing.

Table 3. cAMP Levels in Cultured Vascular Smooth Muscle Cells

Intervention	cAMP pmol/2 × 10^6 A7r5 Cells (×10)				cAMP pmol/2 × 10^6 A10 Cells (×10)			
	0 Min	2 Min	5 Min	30 Min	0 Min	2 Min	5 Min	30 Min
Isoflurane added to quiescent cells	6 ± 1	5 ± 1	4 ± 1	4 ± 1	6 ± 1	5 ± 1	5 ± 1	6 ± 1
Time control: no anesthetic	4 ± 1	4 ± 1	6 ± 1	4 ± 1	6 ± 1	5 ± 1	6 ± 1	6 ± 1
Halothane added to quiescent cells	6 ± 1	5 ± 1	6 ± 1	6 ± 1	5 ± 1	6 ± 1	5 ± 1	6 ± 1
Cells stimulated with serotonin								
Isoflurane pretreatment	7 ± 1	6 ± 1						
Halothane pretreatment	5 ± 2	7 ± 3						
Controls	7 ± 1	7 ± 1						
Cells stimulated with endothelin								
Isoflurane pretreatment					5 ± 2	5 ± 1		
Halothane pretreatment					4 ± 1	9 ± 3		
Controls					7 ± 1	12 ± 3		
Cells stimulated with isoproterenol:								
no anesthetics	4 ± 1	237 ± 31*			5 ± 2	146 ± 30*		

Anesthetics = 2%, serotonin, 3 × 10^{-5} M; endothelin-1, 10^{-8} M; isoproterenol, 10^{-6} M. n = 4 separate experiments for each study, each performed in triplicate.
* $P < 0.05$.

tractions evoked by endothelin, a Ca^{2+} mobilizing agonist known to also act, at least in part, *via* a G-protein, phospholipase-C signaling axis.

Current results do not reveal reasons for the relative resistance of endothelin-induced contractions to isoflurane. Contractions evoked by endothelin, along with those induced by F^- and serotonin, share a similar (but not identical) biochemical basis in which $Ca^{2+}{}_i$ and activated protein kinase C act in concert to sustain the response.[14] However, endothelin induces contractions with a vigorous and sustained character.[18,19] Sustained contractions induced by endothelin may be particularly

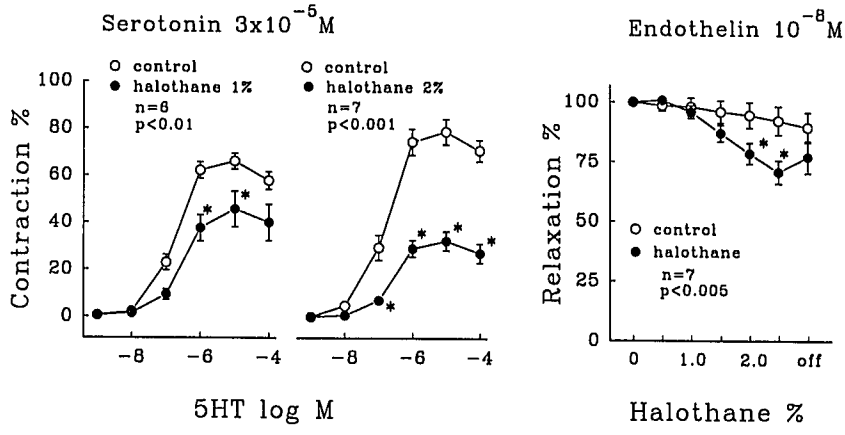

Fig. 5. (*Left*) The effects of halothane 1 and 2% on contractions evoked by cumulative sequentially increasing concentrations of serotonin (5-HT) in isolated porcine coronary artery rings without endothelium. Halothane was administered for 30 min before and throughout the challenge with serotonin. Control rings were obtained from the same coronary artery of the same pig heart and were studied in parallel. Contractions are expressed as a percent of the maximal response obtained with 60 mM KCl before anesthetic or serotonin administration. (*Right*) The effects of sequentially increasing doses of halothane on sustained contractions elicited with 10^{-8} M porcine endothelin-1. The anesthetic was administered for 30 min at each time point except for final 30 min for halothane wash-out. Contractions are expressed as a percent of maximal response to 10^{-8} M endothelin before halothane administration. Data are mean ± SE; n = number of pig hearts. *P* values indicate differences from controls. Points of individual significance are denoted; *$P < 0.05$.

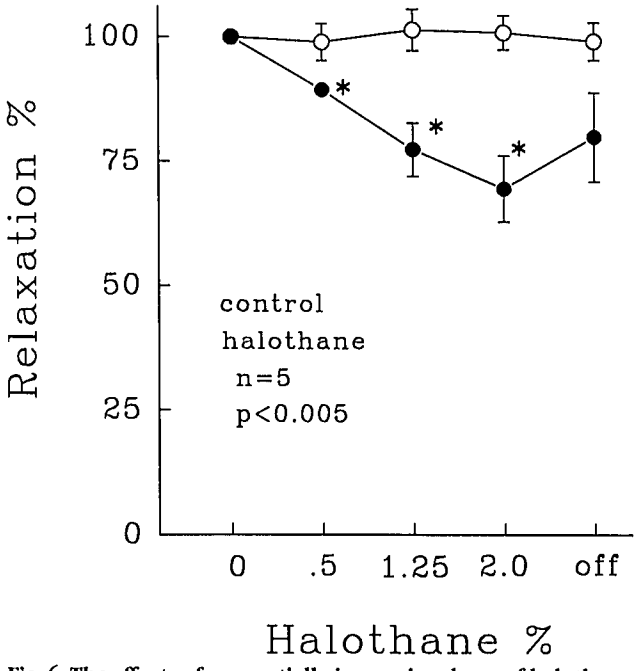

Fig. 6. The effects of sequentially increasing doses of halothane on sustained contractions evoked by 5 mM NaF plus 10 μM AlF_3 (to generate F^-) in porcine coronary artery rings without endothelium. Halothane was administered for 30 min at each time point except for final 30 min for halothane wash-out. Contractions are expressed as percent of maximal response to 5 mM NaF plus 10 μM AlF_3 before anesthetic administration. Data are mean ± SE; n = number of pig hearts; $P < 0.05$.

dependent upon persistent protein kinase C activity,[14,36,37] perhaps in part because protein kinase C can alter Ca^{2+} sensitivity of contraction—an agonist specific phenomenon[18]—perhaps *via* a caldesmon mediated system.[38] The direct effects of isoflurane on the contractile actions of activated protein kinase C were probed in the current studies using the phorbol ester PDBu.

Isoflurane was without apparent inhibitory effect on contractions elicited by PDBu. Plots of the PDBu dose-tension relationships in the presence and absence of isoflurane could be virtually superimposed, arguing against a major anesthetic effect at the activated protein kinase C locus. However, this conclusion must be tempered by the presence of unavoidable limitations in experimental design. First, protein kinase C exists in multiple isoforms with variable tissue distribution,[39] precluding conclusions obtained in porcine coronary arteries being rigorously applied to all tissues. Second, contractions were elicited with PDBu, which, although structurally analogous to diacylglycerol, is not a natural second messenger. Nevertheless, results suggest that, within the confines of the current experiment, differences in magnitude of isoflurane action in relation to serotonin, endothelin, and F^- are unlikely to be explicable in terms of direct anesthetic action at the level of activated protein kinase C.

In the current experiments, isoflurane effects upon the $Ca^{2+}{}_i$ limb of signal transduction were studied using a cell culture model. Choice of cell model posed a problem because porcine coronary artery smooth muscle cells do not necessarily retain stable Ca^{2+} signaling characteristics in primary culture, considerable and unpredictable phenotypic differences occurring between separate cultures and at each passage level during culture. Cells freshly obtained by dispersal provide an alternative to culture methods,[21,40] but numbers of cells obtained by this technique are insufficient for flow cytometric and biochemical studies. In lieu of an adequate porcine cell culture model, cells of the A7r5 and A10 lines were used. Both lines possess key characteristics common to vascular smooth muscle, *i.e.*, receptor G-protein systems linked to phospholipase C, inositol trisphosphate–sensitive Ca^{2+} stores, and receptor- and voltage-gated plasmalemmal Ca^{2+} influx channels.[22–26,31,32] Consequently, both lines have gained a degree of acceptance as models for studying signaling *via* $Ca^{2+}{}_i$. However, no culture model can

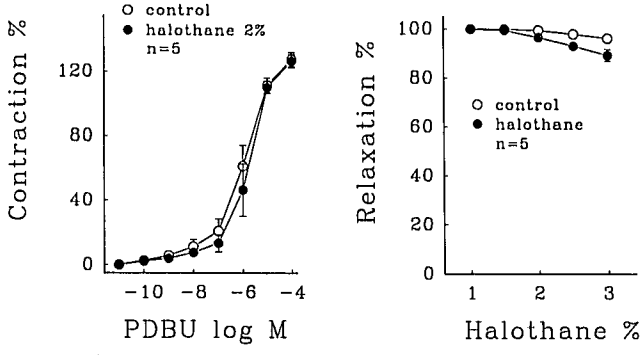

Fig. 7. (*Left*) The effects of 2% halothane on contractions evoked by cumulative sequentially increasing concentrations of phorbol dibutyrate (PDBu) in isolated porcine coronary artery rings without endothelium. Halothane was administered for 30 min before and throughout the challenge with PDBu. Contractions are expressed as a percent of the maximal response obtained with 60 mM KCl before anesthetic or PDBu administration. (*Right*) The effects of sequentially increasing doses of halothane on sustained contractions elicited with 10^{-5} M PDBu. The anesthetic was administered for 30 min at each time point. Contractions are expressed as a percent of maximal response to 10^{-5} M PDBu before halothane administration. Data are mean ± SE; n = number of pig hearts; *$P < 0.05$.

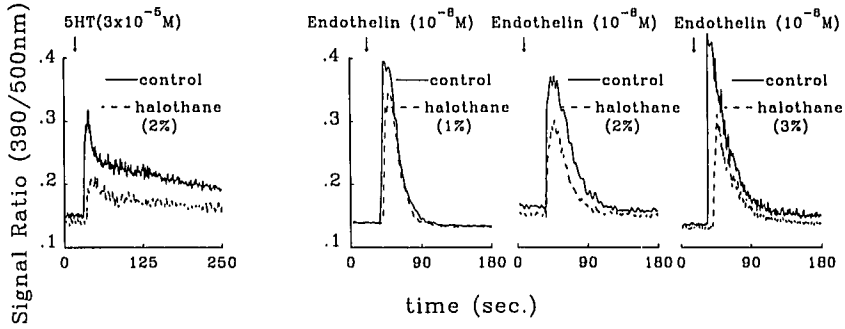

Fig. 8. Typical tracings from individual experiments illustrating the effects of halothane on Ca^{2+}_i mobilization evoked by serotonin (5-HT) (*left*) and porcine endothelin-1 (*right*) in cultured vascular smooth muscle cells. The A7r5 (*left*) and A10 lines (*right*) derived originally and simultaneously from fetal rat aorta were used. Changes in apparent Ca^{2+}_i were detected using the fluorescent cellular probe indo-1 in the AM form and with flow cytometric analysis. Cells were preincubated with halothane for 20 min before addition of the agonists. Dual emitted fluorescence was detected at ~390 and 500 nm. Each individual experiment was accompanied by a sequential individual matched control. Data were obtained from about 100,000 individual cells per tracing.

exactly replicate events occurring in vivo at the cellular level. For example, in regard to the current experiments, although endothelin presumably causes prolonged Ca^{2+}_i elevation in contracted vascular smooth muscle, such a plateau phase was not observed when A10 cells were stimulated with endothelin 10^{-8} M. (At this endothelin dose, Ca^{2+} influx and Ca^{2+} efflux are suggested to be matched, or alternatively, Ca^{2+} influx may be rapidly down-regulated resulting in an absent plateau phase.[21])

Isoflurane attenuated Ca^{2+} mobilization evoked by both serotonin and endothelin—extending observations previously made in relation to vasopressin[33] to two other Ca^{2+} mobilizing agonists. However, endothelin responses were relatively resistant to isoflurane effect, isoflurane 2% lacking an inhibitory action, whereas isoflurane 3% modified the response by a modest 14%. Why the magnitude of the isoflurane inhibitory effect differed between serotonin and endothelin is not known. The greater size of the endothelin-induced Ca^{2+}_i transients—in relation to those evoked by serotonin—is unlikely to provide an explanation because in pilot experiments isoflurane 2% failed to attenuate even small increases in Ca^{2+}_i induced with 10^{-9} M endothelin (data not shown). It is also unlikely that differences between A7r5 and A10 cells accounted for agonist dependency of isoflurane action as both lines were derived simultaneously from the same fetal rat aorta.[30] Their Ca^{2+}_i homeostasis mechanisms are very similar. In addition, in pilot studies, isoflurane 2% was without inhibitory effect on Ca^{2+}_i responses induced by endothelin in A7r5 cells (data not shown).

Mechanisms underlying attenuation of serotonin- and endothelin-induced Ca^{2+}_i mobilization by isoflurane are not known. However, isoflurane has previously been shown to inhibit vasopressin-induced inositiol phosphate formation and Ca^{2+} release in A7r5 cells.[33] (The anesthetic also attenuates depolarization-induced inward currents, presumably Ca^{2+}, in freshly dispersed individual canine coronary artery cells.[40]) An anesthetic effect at the level of phosphoinositide turnover would, at least in part, explain attenuated Ca^{2+}_i mobilization and is supported by a recent report that indicates volatile anesthetics potently inhibit agonist-induced, G-protein linked phospholipase C activity.[41]

Isoflurane's inhibitory effects on serotonin and endothelin-induced Ca^{2+} mobilization cannot be explained *via* anesthetic-induced elevations in cAMP levels. Isoflurane did not increase cAMP in resting A7r5 and A10 cells, nor did isoflurane increase cAMP when cells were stimulated with serotonin or with endothelin (serotonin can increase cAMP *via* receptors other than 5-hydroxytryptaimine$_2$ in some types of cells). Absence of anesthetic effect on cAMP levels was not due to an inability of the cells to generate cAMP as positive control experiments demonstrated elevation in this cyclic nucleotide when cells were stimulated with isoproterenol. Increased cAMP is not a requirement for isoflurane inhibitory effect on agonist-induced Ca^{2+} mobilization.

Studies with halothane were pursued using experimental designs similar to those employed for isoflurane. However, halothane and isoflurane were studied at equal gas phase concentrations, not at equivalent MAC values (in this way avoiding the application of the MAC

concept to cell lines of rodent fetal origin grown *ex vivo* in continuous culture). Consequently, as halothane is a substantially more potent anesthetic, results obtained with the two anesthetics cannot be directly compared. Nevertheless, in the current experiments, results obtained with halothane were not substantially qualitatively dissimilar to results obtained with isoflurane. In vascular rings, halothane attenuated contractions elicited with serotonin and F^-. However, halothane also attenuated contractions evoked with endothelin—although statistically significant inhibitory effects were observed only at higher halothane concentrations. Halothane lacked inhibitory effect on contractions evoked by PDBu—a surprising finding as halothane has recently been shown to inhibit rat brain protein kinase activity[42] and to attenuate agonist-induced migration of protein kinase C from cell surface membranes to the cytosol in canine tracheal smooth muscle.[43] Furthermore, inhibition of protein kinase C is implied to play a role in the state of halothane-induced general anesthesia.[44] Why halothane lacked inhibitory effects on PDBu-mediated contractions in the current experiments is not known, although mechanisms proposed to explain the similar lack of isoflurane action, *i.e.*, the use of PDBu, an agonist that substitutes for diacylglycerol but is not an endogenous second messenger and variability in expression of protein kinase C isoforms depending upon tissue studied,[39] might also serve to explain absence of halothane action.

In cultured cells, halothane attenuated increases in $Ca^{2+}{}_i$ evoked by serotonin. Halothane, at all concentrations tested, also inhibited $Ca^{2+}{}_i$ responses induced by endothelin. Together with previous data, halothane is now known to inhibit $Ca^{2+}{}_i$ mobilization evoked by three vasopressor hormones—vasopressin,[45] serotonin and endothelin in the A7r5-A10 cell culture model. The mechanism remains unknown. Inhibited phosphoinositide turnover,[33] perhaps by a halothane effect on agonist-activated G-protein phospholipase C signaling[41] would, in combination with inhibited Ca^{2+} influx at the plasmalemma,[40] provide an explanation, at least in part, for anesthetic effect on Ca^{2+} mobilization. (If halothane inhibits Ca^{2+} mobilization *via* an action at the agonist-activated G-protein, phospholipase C level,[41] then it might be anticipated that the anesthetic would also decrease diacylglycerol generation, causing indirect inhibition of contractions mediated *via* protein kinase C. Such an effect would not have been revealed in current vascular ring experiments where contractions were induced with PDBu as a substitute for diacylglycerol.) Alternatively, or in addition, halothane may deplete Ca^{2+} from caffeine-sensitive intracellular smooth muscle stores.[46] (In the A7r5 model [and A10 model; data not shown] halothane appears to somewhat decrease the amount of releasable Ca^{2+} present in inositol trisphosphate-sensitive stores when probed using thapsigargin.[45]) Current results indicate that the mechanism of halothane-induced attenuation of agonist-mediated Ca^{2+} mobilization does not involve facilitated cAMP formation.

In conclusion, results suggest that in vascular smooth muscle, clinically relevant concentrations of isoflurane and halothane variably attenuate contractions evoked by Ca^{2+} mobilizing agonists—by a cellular action beyond the level of surface receptors. Neither isoflurane nor halothane inhibited phorbol ester–induced protein kinase C-dependent contractions. Serotonin- and endothelin-induced $Ca^{2+}{}_i$ mobilization was attenuated by the anesthetics, but by a mechanism that was not dependent upon facilitated cAMP formation.

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