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Volatile Anesthetics Affect Calcium Mobilization in Bovine Endothelial Cells

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Background: **The site where volatile anesthetics inhibit endothelium-dependent, nitric oxide-mediated vasodilation is unclear. To determine whether anesthetics could limit endothelium-dependent nitric oxide production by inhibiting receptor-mediated increases in cytosolic Ca^{2+}, experiments were performed to see if the inhalational anesthetics halothane, isoflurane, and enflurane affect intracellular Ca^{2+} ($[Ca^{2+}]_i$) transients induced by the agonists bradykinin and adenosine triphosphate in cultured bovine aortic endothelial cells.**

Methods: **Bovine aortic endothelial cells, which had been loaded with the fluorescent Ca^{2+} indicator Fura-2, were added to medium preequilibrated with volatile anesthetic (1.25% and 2.5% for isoflurane, 1.755 and 3.5% for enflurane, and 0.75% and 1.5% for halothane). In Ca^{2+}-containing medium, intracellular Ca^{2+} transients were elicited in response to bradykinin (10 nM and 1 μM) or adenosine triphosphate (1 μM and 100 μM).**

Results: **Both bradykinin and adenosine triphosphate triggered a rapid rise to peak $[Ca^{2+}]_i$ followed by a gradual decline to a plateau above the resting level. Although basal $[Ca^{2+}]_i$ was unaltered by the anesthetics, both halothane and enflurane, in a dose-dependent manner, depressed the peak and plateau of the $[Ca^{2+}]_i$ transient elicited by 10 nM bradykinin, whereas isoflurane had no effect. When $[Ca^{2+}]_i$ transients were elicited by 1 μM bradykinin, halothane (1% and 5%) did not alter peak and plateau levels. Halothane and enflurane also decreased $[Ca^{2+}]_i$ transients evoked by 1 μM and 100 μM adenosine triphosphate, whereas isoflurane also had no effect in this setting.**

Conclusions: **Halothane and enflurane, but not isoflurane, inhibit bradykinin- and adenosine triphosphate-stimulated Ca^{2+} transients in endothelial cells. Limitations of Ca^{2+} availability to activate constitutive endothelial nitric oxide synthase could allow for part, but not all, of the inhibition of endothelium-dependent nitric oxide-mediated vasodilation by inhalational anesthetics. (Key words: Adenosine triphosphate. Anesthetics, volatile: enflurane; halothane; isoflurane. Bradykinin. Calcium, intracellular. Endothelial cells. Fura-2.)**

VASCULAR endothelium is actively involved in regulating blood flow and blood pressure under basal conditions and during adaptive responses to various physiologic and pathologic conditions, including the classical barrier function, macromolecular transport, growth, phagocytosis, free-radical production, thrombosis, vasospasm, and hypoxic dysfunction. Most of these functions are associated with endothelial $[Ca^{2+}]_i$.[1] The endothelium modulates vascular tone by releasing vasodilating substances in response to various physiologic and pharmacologic stimuli. Several agonists, including bradykinin, adenosine triphosphate (ATP), substance P, histamine, thrombin, acetylcholine, and serotonin, can increase $[Ca^{2+}]_i$ in endothelial cells by receptor-mediated mechanisms.[2,3] The endothelial cell agonists bradykinin and ATP stimulate phospholipase C metabolism, resulting in the dose-dependent production of inositol trisphosphate (IP_3), which is thought to initiate mobilization of intracellular calcium stores.[4] These endothelial cell agonists also produce a sustained increase in $[Ca^{2+}]_i$ that is due to the influx from extracellular pools.[5,6]

Endothelium-derived relaxing factor/nitric oxide (EDRF/NO), first discovered in the vascular endothelium,[7] is now recognized as the primary signal transduction mechanism for activating soluble guanylyl cyclase. It is a potent vasodilating agent released by endothelium in response to agonist stimulation and acts as a major modulator of basal vascular tone. In the vasculature, EDRF/NO is produced from L-arginine by the endothelial isoform of nitric oxide synthase (eNOS), an enzyme activated by increased intracellular Ca^{2+} *via* Ca^{2+} calmodulin. Once produced, NO diffuses to the vascular smooth muscle, where it activates soluble guanylyl cyclase-enhancing cyclic guanosine monophosphate pro-

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duction, which subsequently leads to vascular relaxation. Researchers in several laboratories have shown that volatile anesthetics inhibit endothelium-dependent NO-mediated vasodilation.[8-11] Recent studies suggest that only stimulated, but not basal EDRF/NO production is inhibited by volatile anesthetics.[12] We have provided evidence that this inhibitory effect occurred before the steps of NO synthase[13] and guanylyl cyclase[8,14,15] activation. In addition, volatile anesthetics did not alter NOS activity or directly interfere with activation of guanylyl cyclase by either NO itself or NO donors. Inhalational anesthetics have been shown to have profound and specific effects on Ca^{2+} homeostasis in various cell types, including endothelial, neuronal, and cardiac.[16-19] An effect of inhalational anesthetics on Ca^{2+} availability is a likely site of anesthetic interaction with NO generation. Although the inhibition of calcium ionophore A23187-stimulated endothelial relaxation by anesthetics suggests that anesthetic inhibition of receptor-activated Ca^{2+} release cannot account for all available aspects of inhibition of the NO pathway, some effect on receptor activation could not be dismissed.[8]

To determine whether anesthetic inhibition of receptor-mediated increases of cytosolic Ca^{2+} could contribute to the inhibitory action of these volatile anesthetics on NO production, we investigated the dose-dependent effect of halothane, enflurane, and isoflurane on bradykinin- and ATP-stimulated calcium transients in cultured bovine aortic endothelial cells.

Materials and Methods

Cell Culture

Bovine aortae were obtained from a slaughterhouse and the aortic endothelial cells were prepared for culture by lightly scraping the intimal surface of the aortae. The cells adhering to the blade were rinsed into a 35-mm tissue culture dish and grown in Waymouth's medium supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). A pure culture of endothelial cells was obtained by fluorescent-activated cell sorting using Dil-acyl-low-density lipoprotein as the fluorescent marker. Identity of the endothelial cells was further confirmed by immunostaining for Factor VIII antigen and for endothelial cell actin, by demonstrating a single mRNA for actin in endothelium using Northern blot analysis and by observing a characteristic cobblestone appearance. Confluent cells were split in a ratio 1:10 and subcultured up to 12 passages. The following experiments were performed with cells in passages from 9 to 12 using four separate subcultured cell lines.

Determination of Cytosolic-free Calcium Concentrations in Isolated Endothelial Cells

Monolayer cultures of endothelial cells were grown in 75-cm^2 flasks until 2 or 3 days after confluence. The flasks were rinsed with 10 ml Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, followed by dispersion of the endothelial cells by incubation with 0.05% trypsin and 0.53 mM ethylenediamine tetraacetic acid for 2 min at 37°C. The trypsin was inactivated by adding the cell suspension to a 0.1-mg/ml trypsin inhibitor in a 50-ml centrifuge tube. The flask was rinsed twice with 9 ml HEPES buffer (NaCl, 140 mM; KCl, 5 mM, $MgSO_4$, 1 mM; $CaCl_2$, 1.5 mM; glucose, 10 mM; HEPES, 15 mM; bovine serum albumin, 0.1%) and the rinses were added to the centrifuge tube. Endothelial cells were then formed into pellets by centrifugation at 150*g* for 2 min, followed by a wash with 10 ml HEPES buffer and a subsequent centrifugation. HEPES buffer was used to resuspend the cells at a concentration of 1.5×10^7 cells/ml and the cells were maintained at 37°C in room air. After removal of an aliquot of cells to serve as an autofluorescence control, the remaining cells were incubated with 5 μM Fura-2/AM in HEPES buffer for 20 min at 37°C. The cells were protected from light during this time and gently stirred every 5 min during incubation. The cell suspension was diluted tenfold and incubated at 37°C for 10 min more. Cells were formed into pellets by low-speed centrifugation (at 150*g* for 2 min) and resuspended to a concentration of 1.5×10^6 cells/ml. Fura-2-loaded cells were stored at 5°C until they were needed. Two-milliliter aliquots of cells were used in each experiment. To ensure removal of any extracellular Fura-2, the cell suspensions were gently centrifuged and the supernatants withdrawn, followed by resuspension of the cells in warm (37°C) HEPES buffer. The cells were subjected to centrifugation one final time, resuspended in warm buffer, and placed in cuvettes. Cells were allowed to equilibrate for 5 min at 37°C before each experiment. From preliminary experiments, the intracellular concentration of Fura-2 under these conditions was estimated to be 125 μM, and the dye remained confined to the cytosolic compartment.[20]

Fluorescence measurements were made using a Perkin-Elmer LS50 fluorometer with excitation wavelengths alternating between 345 nm and 380 nm every 500 ms, and emission fluorescence was recorded at 500

nm. Excitation and emission slit widths were generally set at 2 and 16 nm, respectively. The emission intensities at excitation wavelengths of 345 nm (F_{345}) and 380 nm (F_{380}) were corrected automatically for autofluorescence and the fluorescence ratio ($R = F_{345}/F_{380}$) was calculated. The maximum fluorescence ratio, R_{max}, and the minimum fluorescence ratio, R_{min}, were determined by sequentially adding 75 μM digitonin and 20 mM TRIS/5 mM ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid. The $[Ca^{2+}]_i$ was calculated by

$$[Ca^{2+}]_i = K_d\left(\frac{R - R_{min}}{R_{max} - R}\right)\left(\frac{F_{380E}}{F_{380D}}\right)$$

where the K_d for Fura-2 is 224 nm at 37°C, and F_{380D} and F_{380E} are the emission intensities at an excitation wavelength of 380 nm after adding 75 μM digitonin and 20 mM TRIS/5 mM ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid, respectively.[21]

Anesthetic Application and Agonist Stimulation

The incubation medium was preequilibrated in a closed container at 37°C for at least 30 min by bubbling with anesthetic gas mixture in air, which was delivered from a calibrated vaporizer. Fura-2–loaded endothelial cells were gently added to anesthetic-preequilibrated medium in a quartz cuvette. The endothelial cells were incubated for 10 min more while the anesthetic gas–air mixture was maintained over the solution to ensure a constant anesthetic level before measuring $[Ca^{2+}]_i$. Endothelial cells used in control experiments were added to medium treated in an identical manner, but in the absence of anesthetic. Aliquots of media were drawn up into gas-tight syringes to determine anesthetic concentrations using gas chromatography. This analysis confirmed the concentrations delivered from the calibrated vaporizers.

The cells were stimulated by directly adding bradykinin (10 nM and 1 μM, final concentrations) or ATP (1 and 100 μM, final concentrations) into the cell suspension, and $[Ca^{2+}]_i$ was determined as described previously. Each concentration of anesthetic and agonist was repeated at least four separate times, with experiments (control and anesthetic-exposed cells) performed on aliquots obtained from the same pool of endothelial cells. The control and anesthetic-exposed experiments were performed in a randomized manner. The peak transient was defined as the difference between the peak and the basal levels. The plateau was defined as the difference between the plateau and the basal levels. The measurement for the plateau was taken at the end of the experiment. In limited preliminary experiments, the reversibility of the effect of volatile anesthetics on calcium transients was determined by exposing the endothelial cell suspension to the volatile anesthetic and then washing out the anesthetic by further gassing the cell suspension with air alone. Stimulation of the cells with agonist revealed no difference in the calcium transient when compared with cells that had not been exposed to volatile anesthetic.

Statistical Analysis

Results are expressed as mean ± SEM. Comparisons between the groups were made using Fisher's protected least-significant difference test (Statview 4.0). An effect was considered statistically significant at $P < 0.05$.

Results

Under control conditions, both bradykinin and ATP, as shown in figure 1, triggered a rapid rise to peak $[Ca^{2+}]_i$ followed by a gradual decline to a plateau above the resting level, as described previously.[3] Overall, the peak and plateau of the $[Ca^{2+}]_i$ transient elicited by 10 nM bradykinin were depressed by both halothane and enflurane in a dose-dependent manner. However, each of the anesthetics failed to affect the basal $[Ca^{2+}]_i$ level, which ranged from 70 to 110 nM,

Both agonists induced transient increases in $[Ca^{2+}]_i$, and successive applications of the same agonist produced similar $[Ca^{2+}]_i$ transients in the cell suspension. It was notable that when the two agonists were given in succession to the same cell suspension, altering the order of addition produced contrasting responses. As shown in figure 2, preceding the addition of ATP (100 μM) with an addition of bradykinin (10 nM) resulted in the production of $[Ca^{2+}]_i$ transients after each agonist application, whereas preceding the addition of bradykinin with ATP application produced a $[Ca^{2+}]_i$ transient only after the ATP application.

The concentration dependence of volatile anesthetics on agonist-induced changes in $[Ca^{2+}]_i$ was subsequently investigated (figs. 3 to 5). As indicated in table 1, halothane (0.75% and 1.5%) decreased the peak of the bradykinin-induced $[Ca^{2+}]_i$ transient by 22 ± 8% and 44 ± 5% from the control level. The $[Ca^{2+}]_i$ plateau was reduced by 38 ± 8% and 38 ± 5% for the 0.75% and 1.5%

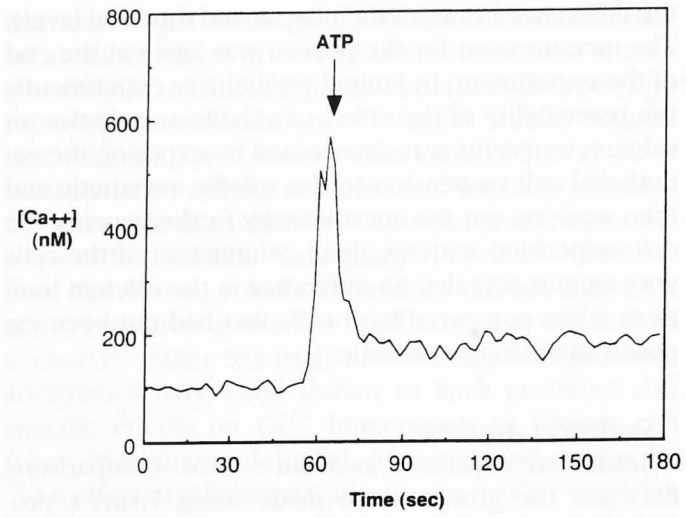
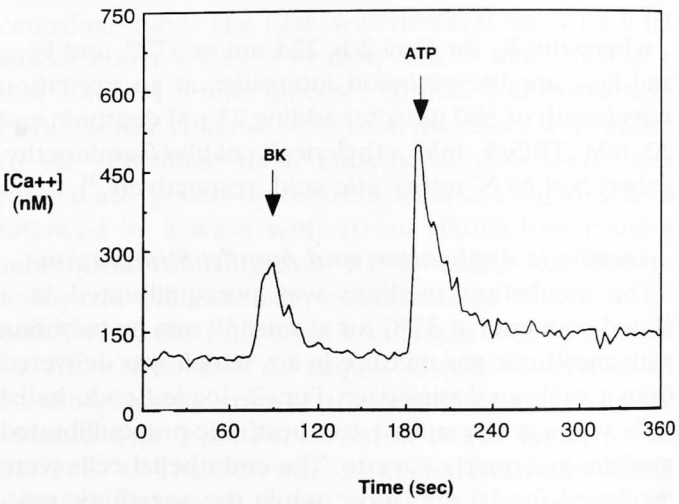


Fig. 1. Induction of $[Ca^{2+}]_i$ transients by ATP (10^{-4} M) and bradykinin (10^{-8} M) as measured in Fura-2–loaded cultured bovine aortic endothelial cells. The figures are representative tracings of single experiments.

concentrations of halothane, respectively. Enflurane at a concentration of 1.75% had no effect on the bradykinin-induced $[Ca^{2+}]_i$ transient peak, but it resulted in a 20 ± 6% reduction in the bradykinin-induced $[Ca^{2+}]_i$ plateau. At the higher concentration of 3.5% enflurane, the bradykinin-induced $[Ca^{2+}]_i$ transient peak and plateau were reduced by 35 ± 6% and 19 ± 5%, respectively. In contrast, isoflurane at either concentration (1.25% and 2.5%) had no effect on the bradykinin-induced $[Ca^{2+}]_i$ transient peak and plateau phases. Examination of the $[Ca^{2+}]_i$ transients elicited by bradykinin at a much higher concentration, 1 μM, revealed that halothane at 1% and 5% had no effect on the peak and plateau levels.

$[Ca^{2+}]_i$ transients evoked by ATP were less sensitive to modulation by halothane and enflurane than were those evoked by bradykinin, as shown in table 2. Halothane (1.5%) decreased the peak of the $[Ca^{2+}]_i$ transient induced by 1 μM ATP by only 17 ± 3% from its control level, along with a reduction in the $[Ca^{2+}]_i$ plateau by 30 ± 14%. Enflurane (3.5%) decreased the peak of the $[Ca^{2+}]_i$ transient induced by 1 μM ATP by only 19 ± 4% from its control level, with no change in the $[Ca^{2+}]_i$ plateau.

At the higher dose of 100 μM ATP, the effects of halothane and isoflurane were similar to those seen



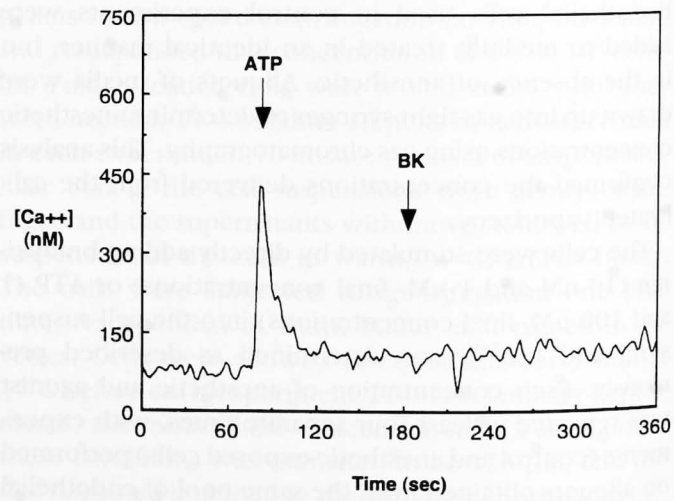
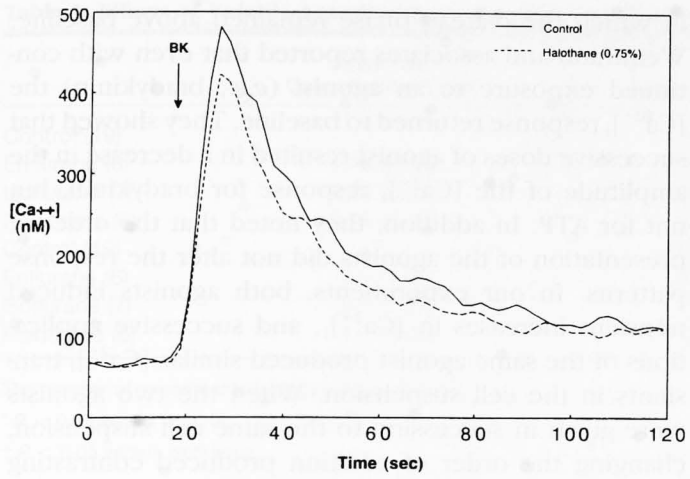


Fig. 2. Effect of the order of the successive addition of ATP (10^{-4} M) and bradykinin (10^{-8} M) on $[Ca^{2+}]_i$ transients as measured in Fura-2–loaded cultured bovine aortic endothelial cells. The figures are representative tracings of single experiments.



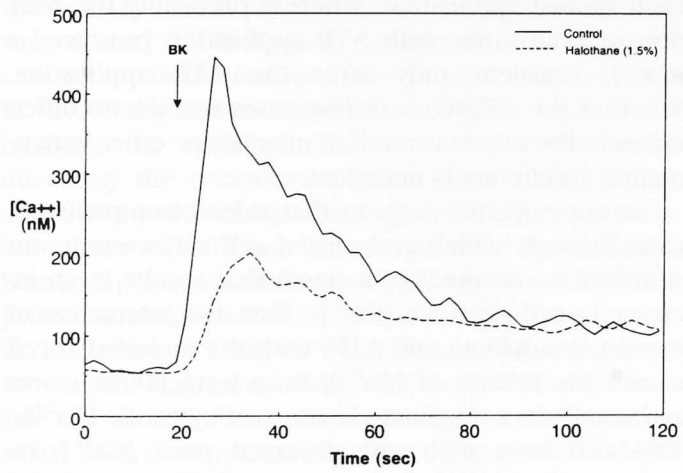


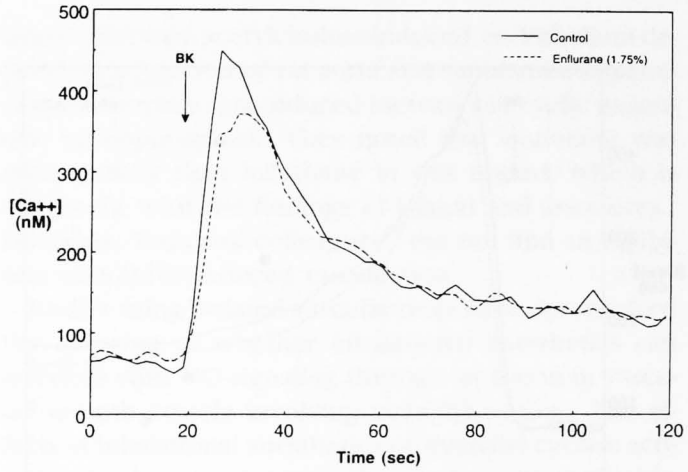
Fig. 3. Effect of halothane (0.75% and 1.5%) on the bradykinin-induced (10^{-8} M) $[Ca^{2+}]_i$ transients as measured in Fura-2–loaded cultured bovine aortic endothelial cells. The figures are representative tracings of single experiments.

with the lower concentration. Halothane (1.5%) decreased the peak of the $[Ca^{2+}]_i$ transient by 14 ± 3%, with a reduction in the $[Ca^{2+}]_i$ plateau by 25 ± 4%. Similarly, enflurane (3.5%) decreased the peak of the $[Ca^{2+}]_i$ transient induced by 100 μM ATP by 20 ± 3% from its control level, along with a reduction in the $[Ca^{2+}]_i$ plateau by 31 ± 2%. Again, isoflurane had no effect on the ATP-evoked (1 μM and 100 μM) $[Ca^{2+}]_i$ transient.

Discussion

Although enflurane, halothane, and isoflurane did not change the fluorometrically determined level of endothelial cell Ca^{2+}, enflurane and halothane depressed bradykinin- and ATP-induced Ca^{2+} transients. Each of the anesthetics failed to affect the basal $[Ca^{2+}]_i$ levels. After bradykinin (10 nM) stimulation, we observed that halothane decreased the peak and plateau $[Ca^{2+}]_i$ transients in a dose-dependent manner, whereas enflurane only affected the peak and plateau $[Ca^{2+}]_i$ transients at the higher (3.5%) concentration. Isoflurane, in contrast, did not affect either the peak or plateau $[Ca^{2+}]_i$ transient after bradykinin stimulation, even at the higher concentration.

The $[Ca^{2+}]_i$ transients evoked by ATP were less sensitive to modulation by halothane and enflurane. At both



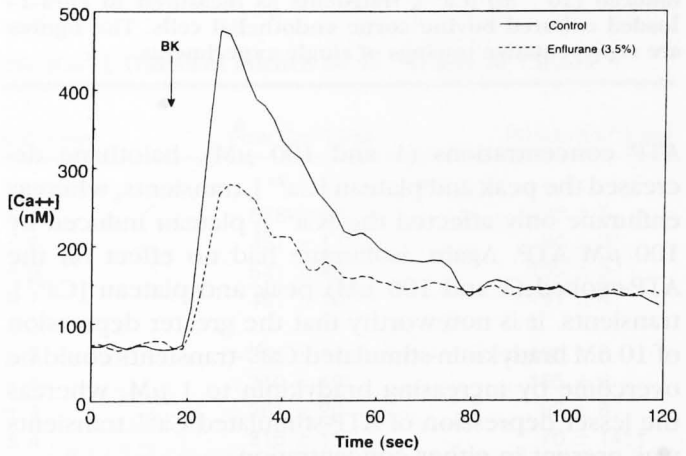
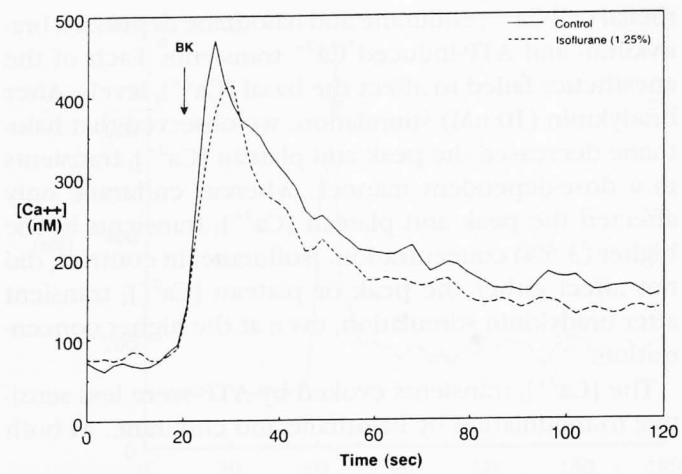


Fig. 4. Effect of enflurane (1.75% and 3.5%) on the bradykinin-induced (10^{-8M} M) $[Ca^{2+}]_i$ transients as measured in Fura-2–loaded cultured bovine aortic endothelial cells. The figures are representative tracings of single experiments.



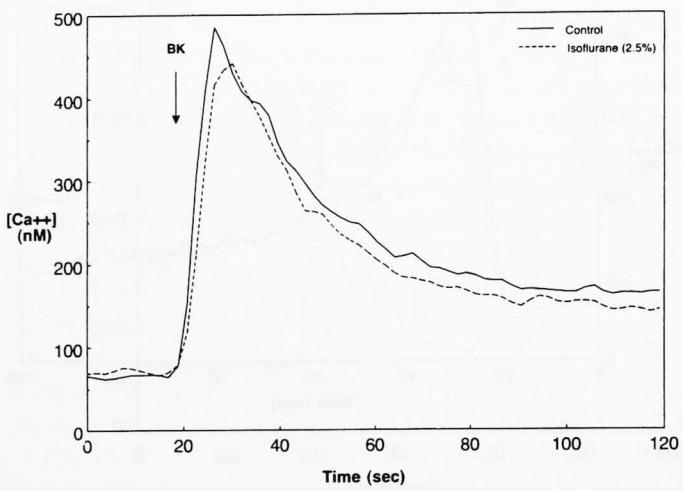


Fig. 5. Effect of isoflurane (1.25% and 3.5%) on the bradykinin-induced (10^{-8} M) $[Ca^{2+}]_i$ transients as measured in Fura-2–loaded cultured bovine aortic endothelial cells. The figures are representative tracings of single experiments.

ATP concentrations (1 and 100 μM), halothane decreased the peak and plateau $[Ca^{2+}]_i$ transients, whereas enflurane only affected the $[Ca^{2+}]_i$ plateau induced by 100 μM ATP. Again, isoflurane had no effect on the ATP-evoked (1 and 100 μM) peak and plateau $[Ca^{2+}]_i$ transients. It is noteworthy that the greater depression of 10 nM bradykinin-stimulated Ca^{2+}-transients could be overcome by increasing bradykinin to 1 μM, whereas the lesser depression of ATP-stimulated Ca^{2+} transients was present in either concentration.

Changing the order of agonist addition resulted in the $[Ca^{2+}]_i$ responses, which differed from those of Weintraub and associates.[22] In contrast to our experiments, in which the plateau phase remained above baseline, Weintraub and associates reported that even with continued exposure to an agonist (*e.g.*, bradykinin) the $[Ca^{2+}]_i$ response returned to baseline. They showed that successive doses of agonist resulted in a decrease in the amplitude of the $[Ca^{2+}]_i$ response for bradykinin, but not for ATP. In addition, they noted that the order of presentation of the agonists did not alter the response patterns. In our experiments, both agonists induced transient increases in $[Ca^{2+}]_i$, and successive applications of the same agonist produced similar $[Ca^{2+}]_i$ transients in the cell suspension. When the two agonists were given in succession to the same cell suspension, changing the order of addition produced contrasting responses. We noted that preceding the addition of ATP (100 μM) with an addition of bradykinin (10 nM) resulted in the production of $[Ca^{2+}]_i$ transients after each agonist application, whereas preceding the addition of bradykinin with ATP application produced a $[Ca^{2+}]_i$ transient only after the ATP application. Whether the difference in responses was due to differences in the concentration of agonists or other experimental conditions is uncertain.

Current evidence suggests that at least two pathways exist through which endothelial cell surface receptor stimulation can mediate a signal that results in an increase in cell cytosolic $[Ca^{2+}]_i$. First, the interaction of agonist (bradykinin and ATP) with the endothelial cell causes the release of $[Ca^{2+}]_i$ from intracellular stores and results in a transient elevation of cytosolic $[Ca^{2+}]_i$. This correlates with our observed peak $[Ca^{2+}]_i$ responses. This initial increase in $[Ca^{2+}]_i$ in endothelial cells is very rapid and occurs in the absence of external Ca^{2+}, indicating that Ca^{2+} is released from intracellular storage sites.[23] Second, the agonist stimulates Ca^{2+} influx across the surface membrane of the endothelial cell, which is responsible for the plateau phase of the calcium transient.[4,6,24,25] This influx results in a sustained elevation of cytosolic $[Ca^{2+}]_i$ above the resting level, an elevation that could be abolished when the cells were superfused with Ca^{2+}-free buffer.[26]

This agonist-stimulated Ca^{2+} influx responsible for the plateau phase may occur by Ca^{2+} depletion-activated cation channels (I_{CRAC}), as seen in human leukemic T lymphocytes[27] or activation of a channel *via* an internal messenger other than Ca^{2+}, such as IP_4.[28,29] These two pathways could help explain the maintained increase in cytosolic Ca^{2+} in response to agonist stimulation of endothelial cells.

The available evidence supports the observation that

Table 1. Effects of Volatile Anesthetics on the Peak and Plateau of the $[Ca^{2+}]_i$ Transient Elicited by 10^{-8} M Bradykinin

	Anesthetic Concentration % (nM)	Baseline $[Ca^{2+}]_i$ (nM)	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
Control (19)		68 ± 3	366 ± 13	73 ± 4
Enflurane (6)	1.75 (0.44)	75 ± 6	336 ± 17	59 ± 5*
Isoflurane (7)	1.25 (0.28)	64 ± 5	332 ± 15	62 ± 3
Halothane (6)	0.75 (0.23)	63 ± 2	287 ± 30*†	45 ± 6*†
Control (19)		65 ± 3	354 ± 12	73 ± 3
Enflurane (6)	3.5 (1.01)	65 ± 4	245 ± 28*†	52 ± 3*†
Isoflurane (7)	2.5 (0.57)	70 ± 6	347 ± 17	73 ± 3
Halothane (6)	1.5 (0.44)	56 ± 5	204 ± 20*†	45 ± 3*†

The number of experiments is shown in parentheses.

* $P < 0.05$ *versus* control.

† $P < 0.05$ *versus* isoflurane.

inhalational anesthetics are potent inhibitors of endothelium-dependent vascular relaxation at clinically relevant doses.[8,10,30] Isoflurane, halothane, and enflurane can inhibit both receptor- and nonreceptor-mediated EDRF/NO-dependent vasodilation.[8] These anesthetics inhibited the endothelium-dependent vasodilation induced by the receptor-mediated agent methacholine and that by the receptor-independent agent calcium ionophore A23187 but had no effect on the endothelium-independent vasodilation induced by sodium nitroprusside. Although this study showed that the anesthetic inhibition of EDRF/NO vasodilation occurred distal to receptor activation of the endothelial cell, it did not rule out an additional effect on receptor mechanisms, as has been demonstrated in other cell types.[8,31-35] Toda and colleagues[30] reported that both isoflurane and halothane inhibited acetylcholine-induced endothelium-dependent relaxation of rat aorta and simultaneously prevented acetylcholine-induced increases in cyclic guanosine monophosphate. They noted that isoflurane was more potent than halothane in this regard, which is consistent with the findings of Uggeri and associates.[8] However, Toda and colleagues[30] did not find an inhibition of A23187-induced vasodilation.

Studies using isolated vascular rings have not resolved the question of whether inhalational anesthetics can interfere with NO signaling through an action in vascular smooth muscle involving guanylyl cyclase. The effects of inhalational anesthetics on guanylyl cyclase activation in the vascular smooth muscle were studied in the context of EDRF-dependent vasodilation. Conflicting data exist on whether NO and NO donor (sodium

Table 2. Effects of Volatile Anesthetics on the Peak and Plateau of the $[Ca^{2+}]_i$ Transient Elicited by 10^{-4} M and 10^{-6} M ATP

	Anesthetic Concentration % (mM)	Baseline $[Ca^{2+}]_i$ (nM)	Peak $[Ca^{2+}]_i$ (nM)	Plateau $[Ca^{2+}]_i$ (nM)
ATP 10^{-4}				
Control (14)		71 ± 5	573 ± 18	122 ± 5
Enflurane (5)	2.5 (0.57)	70 ± 5	457 ± 17*	84 ± 3*
Isoflurane (4)	3.5 (1.01)	68 ± 3	541 ± 49	105 ± 5
Halothane (5)	1.5 (0.44)	72 ± 7	491 ± 16*	91 ± 4*
ATP 10^{-6}				
Control (17)		72 ± 3	381 ± 11	100 ± 3
Enflurane (7)	2.5 (0.57)	69 ± 5	310 ± 15*	105 ± 6
Isoflurane (5)	3.5 (1.01)	76 ± 4	351 ± 20	92 ± 8
Halothane (7)	1.5 (0.44)	70 ± 4	316 ± 11*	70 ± 14*†

The number of experiments is shown in parentheses.

* $P < 0.05$ *versus* control.

† $P < 0.05$ *versus* enflurane.

nitroprusside and nitroglycerin) compound-induced vasodilation was inhibited by inhalational anesthetics.[8,10,11,36] The possibility that inhalational anesthetics affect NO signaling through interaction with guanylyl cyclase was investigated using partially isolated soluble and particulate guanylyl cyclase from the rat brain. Inhalational anesthetics, at wide-ranging concentrations, did not affect either the basal or the agonist-stimulated activity.[14]

Although anesthetic inhibition of muscarinic receptor (or other receptor) activation may be a component of the mechanism by which anesthetics inhibit EDRF/NO production in response to those specific agonists, receptor activation is not likely to be the major site of anesthetic inhibition of NO signaling. We know this from the findings of Uggeri and associates,[9] who found significant anesthetic inhibition of calcium ionophore A23187-stimulated EDRF/NO production that bypasses the receptor effects. However, it could be argued that due to subcellular localization of Ca^{2+}, release of intracellular Ca^{2+} may play a greater role in NO synthase activation than that mediated by ionophore.

We have shown that halothane and enflurane, but not isoflurane, inhibit bradykinin- and ATP-stimulated Ca^{2+} transients in endothelial cells. Limitations of Ca^{2+} availability to activate constitutive endothelial NO synthase thus could account for part, but not all of the inhibition of endothelium-dependent NO-mediated vasodilation by inhalational anesthetics, because isoflurane, which inhibits the NO pathway more potently than halothane or enflurane, had no effect on Ca^{2+} transients induced by NO synthase-activating concentrations of bradykinin and ATP.

Our findings are consistent with those of Loeb and coworkers,[37] who investigated the effects of halothane and isoflurane on agonist-induced $[Ca^{2+}]_i$ transient peaks. Although both studies showed a decrease in the $[Ca^{2+}]_i$ transient elicited by bradykinin in the presence of halothane, but not in the presence of isoflurane, Loeb and coworkers reported that neither halothane nor isoflurane, at concentrations as high as 2 mM, altered the $[Ca^{2+}]_i$ response to ATP (10 μM). In addition, they did not observe an increase in agonist-induced $[Ca^{2+}]_i$ plateau levels, possibly due to the transient application of the agonists.

Activation of a small conductance Ca^{2+}-activated K^+ channel, perhaps *via* a G-protein,[38] appears to be critical for maintaining a driving force for Ca^{2+} entry during the plateau phases. In fact, a blocker of Ca^{2+}-dependent K^+ current, tetrabutylammonium chloride (5 mM), completely blunts the plateau phase with little or no effect on the peak Ca^{2+} transient elicited by bradykinin (Miao and Lynch, unpublished data).[39] Our findings indicate that the reduction of bradykinin- and ATP-induced Ca^{2+} plateau phases by halothane and enflurane could be explained by anesthetic effects at any of the putative mechanisms of Ca^{2+} entry or by the function of Ca^{2+}-activated K^+ channels. Another difference between the anesthetics is their ability to deplete intracellular Ca^{2+} stores by activating Ca^{2+} release channels (ryanodine receptors). Such channels, which have been histologically[40] and physiologically[41] demonstrated in endothelial cells, are activated by halothane and possibly enflurane, but not isoflurane,[42,43] which is consistent with the effects we observed.

Our data show that the $[Ca^{2+}]_i$ transients evoked by ATP (1 and 100 μM) were far less sensitive to modulation by halothane and enflurane than those evoked by bradykinin. The ability of halothane and enflurane (at the higher dose) to attenuate responses to bradykinin and, to a lesser extent, ATP may result from the action of the volatile anesthetic on receptor-agonist interactions or on specific differences in receptor G-protein-effector coupling that mediate the responses to agonists. There are clearly differential effects of halothane and enflurane when compared with isoflurane in their ability to inhibit the mobilization of $[Ca^{2+}]_i$ by receptor-operated mechanisms in endothelial cells. The data of Loeb and colleagues[44] showed that halothane, but not isoflurane, inhibited bradykinin-stimulated prostaglandin I_2 (PGI_2) production by vascular endothelial cells, suggesting that halothane-mediated inhibition of bradykinin-stimulated prostacyclin production does not involve a pertussis toxin-sensitive G protein and may result from an interaction of halothane at some other step in the signal transduction pathway, including the inhibition of protein kinase C. Given the importance of intracellular $[Ca^{2+}]_i$ mobilization in activating NO synthase to produce NO, with its physiologically critical role in modulating vascular tone, such anesthetic actions have considerable implications. The property of halothane and enflurane to decrease intracellular $[Ca^{2+}]_i$ mobilization may potentially interfere with physiologic $[Ca^{2+}]_i$ mechanisms mediated by the endothelium in various organs. In contrast, we might anticipate that such regulation would be better maintained by isoflurane. Our findings, consistent with those of Loeb and colleagues,[37,44] show that halothane and enflurane, but not isoflurane, can interfere with the receptor-mediated Ca^{2+} mobilization in vascular endothelial cells. How the

mechanisms by which the differential effects of the volatile anesthetics act on the signal transduction pathway remains to be clarified.

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References

1. Graier WF, Sturek M, Kukovetz WR: Ca^{2+} regulation and endothelial vascular function. Endothelium 1994; 1:223-36

2. Cannell MB, Sage SO: Bradykinin-evoked changes in cytosolic calcium and membrane currents in cultured bovine pulmonary artery endothelial cells. J Physiology 1989; 419:555-68

3. Himmel HM, Whorton AR, Strauss HC: Intracellular calcium, currents, and stimulus-response coupling in endothelial cells. Hypertension 1993; 21:112-27

4. Lambert TL, Kent RS, Whorton AR: Bradykinin stimulation of inositol polyphosphate production in porcine aortic endothelial cells. J Biol Chem 1986; 261:15288-93

5. Freay A, Johns A, Adams DJ, Ryan US, Van Breemen C: Bradykinin and inositol 1,4,5-trisphosphate-stimulated calcium release from intracellular stores in cultured bovine endothelial cells. Pflugers Arch 1989; 414:377-84

6. Hallman TJ, Pearson JD: Exogenous ATP raises cytosolic free calcium in fura-2 loaded piglet aortic endothelial cells. FEBS Lett 1986; 207:95-9

7. Furchgott RF, Zawadzki JV: The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980; 288:373-6

8. Uggeri MJ, Proctor GJ, Johns RA: Halothane, enflurane, and isoflurane attenuate both receptor and non-receptor mediated EDRF production in rat thoracic aorta. ANESTHESIOLOGY 1992; 76:1012-7

9. Toda H, Nakamura K, Hatano Y, Nishiwada M, Kakuyama M, Mori K: Halothane and isoflurane inhibit endothelium-dependent relaxation elicited by acetylcholine. Anesth Analg 1992; 75:198-203

10. Muldoon SM, Hart JL, Bowen KA, Freas W: Attenuation of endothelium-mediated vasodilation by halothane. ANESTHESIOLOGY 1988; 68:31-7

11. Nakamura K, Terasako K, Toda H, Miyawaki I, Kakuyama M, Nishiwada M, Hatano Y, Mori K: Mechanisms of inhibition of endothelium-dependent relaxation by halothane, isoflurane, and sevoflurane. Can J Anaesth 1994; 41:340-6

12. Zuo Z, Tichotsky A, Johns RA: Halothane and isoflurane inhibit vasodilation due to constitutive but not inducible nitric oxide synthase. ANESTHESIOLOGY 1996; 84:1156-65

13. Rengasamy A, Ravichandran LV, Reikersdorfer CG, Johns RA: Inhalational anesthetics do not alter nitric oxide synthase activity. J Pharmacol Exp Ther 1995; 273:599-604

14. Zuo Z, Johns RA: Halothane, enflurane, and isoflurane do not affect the basal or agonist stimulated activity of partially isolated soluble and particulate guanylyl cyclase of rat brain. ANESTHESIOLOGY 1995; 83:395-404

15. Johns RA, Tichotsky A, Muro M, Spaeth J, Le Cras TD, Rengasamy A: Halothane and isoflurane inhibit endothelium-derived relaxing factor-dependent cyclic guanosine monophosphate accumulation in endothelial cell-vascular smooth muscle co-cultures independent of an effect on guanylyl cyclase activation. ANESTHESIOLOGY 1995; 83:823-34

16. Kress HG, Muller J, Eisert A, Gilge U, Tas PW, Koschel K: Effects of volatile anesthetics on cytoplasmic Ca^{2+} signaling and transmitter release in a neural cell line. ANESTHESIOLOGY 1991; 74:309-19

17. Rusy BF, Komai H: Anesthetic depression of myocardial contractility: A review of possible mechanisms. ANESTHESIOLOGY 1987; 67:745-66

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

19. Loeb AL, Longnecker DL, Williamson JR: Alteration of calcium mobilization in endothelial cells by volatile anesthetics. Biochem Pharmacol 1993; 45:1137-42

20. Tracey WR, Johns RA, Romero G, Peach MJ: Mechanism of phospholipase C-induced release of EDRF from pulmonary artery endothelium. Am J Physiol 1991; 261:C927-35

21. Grynkiewicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985; 260:3440-50

22. Weintraub WH, Negulescu PA, Machen TE: Calcium signaling in endothelia: Cellular heterogeneity and receptor internalization. Am J Physiol 1992; 263:C1029-39

23. Buchan KW, Martin W: Bradykinin induces elevations of cytosolic calcium through mobilisation of intracellular and extracellular pools in bovine aortic endothelial cells. Br J Pharmacol 1991; 102:35-40

24. Colden-Stanfield M, Schilling WP, Ritchie AK, Eskin SG, Navarro LT, Kunze DL: Bradykinin-induced increases in cytosolic calcium and ionic channels in cultured bovine aortic endothelial cells. Circ Res 1987; 61:632-40

25. Morgan-Boyd R, Stewart JM, Vaurek RJ, Hasid A: Effects of bradykinin and angiotensin II on intracellular Ca^{2+} dynamics in endothelial cells. Am J Physiol 1987; 253 (Cell Physiol 22):C588-98

26. Hosoki E, Iijima T: Chloride-sensitive Ca^{2+} entry by histamine and ATP in human aortic endothelial cells. Eur J Pharmacol 1994; 266:213-8

27. Zweifach A, Lewis RS: Rapid inactivation of depletion-activated calcium current (ICRAC) due to local calcium feedback. J Gen Physiol 1995; 105:209-26

28. Luckhoff A, Clapham DE: Inositol 1,3,4,5-tetrakisphosphate activates an endothelial $Ca(^{2+})$-permeable channel. Nature 1992; 355:356-8

29. Parekh AB, Terlau H, Stuhmer W: Depletion of InsP3 stores activates a Ca^{2+} and K+ current by means of a phosphatase and a diffusible messenger. Nature 1993; 364:814-8

30. Toda H, Nakamura K, Hatano Y, Nishiwada M, Kakuyama M, Mori K: Halothane and isoflurane inhibit endothelium-dependent relaxation elicited by acetylcholine. Anesth Analg 1992; 75:198-203

31. Aronstam RS, Anthony BL, Dennison RL: Halothane effects on muscarinic acetylcholine receptor complexes in brainstem. Biochem Pharmacol 1986; 35:667-72

32. Dennison RL, Anthony BL, Narayanan TK, Aronstam RS: Effects of halothane on high affinity agonist binding and guanine nucleotide sensitivity on muscarinic acetylcholine receptors from brainstem of rat. Neuropharmacology 1987; 26:1201-5

33. Brett RS, Dilger JP, Yland KF: Isoflurane causes flickering of the acetylcholine receptor channel: Observations using patch clamp. ANESTHESIOLOGY 1988; 69:157-60

34. Anthony BL, Dennison RL, Aronstam RS: Disruption of muscarinic receptor-G protein coupling is a general property of liquid volatile anesthetics. Neurosci Lett 1989; 99:191–6

35. Lechleiter J, Greuner R: Halothane shortens acetylcholine receptor channel kinetics without affecting conductance. Proc Natl Acad Sci USA 1989; 81:2929–33

36. Hart JL, Jing M, Bina S, Freas W, Van Dyke RA, Muldoon SM: Effects of halothane on EDRF/cGMP-mediated vascular smooth muscle relaxations. ANESTHESIOLOGY 1993; 79:323–31

37. Loeb AL, Longnecker DL, Williamson JR: Alteration of calcium mobilization in endothelial cells by volatile anesthetics. Biochem Pharmacol 1993; 45:1137–42

38. Vaca L, Schilling WP, Kunze DL: G-protein-mediated regulation of a Ca^{2+}-dependent K+ channel in cultured vascular endothelial cells. Pflugers Arch 1992; 422:66–74

39. Groschner K, Graier WF, Kukovetz WR: Activation of a small-conductance Ca^{2+}-dependent K+ channel contributes to bradykinin-induced stimulation of nitric oxide synthesis in pig aortic endothelial cells. Biochem Biophys Acta 1992; 1137:162–70

40. Lesh RE, Marks AR, Somlyo AV, Fleischer S, Somlyo AP: Anti-ryanodine receptor antibody binding sites in vascular and endocardial endothelium. Circ Res 1993; 72:481–8

41. Ziegelstein RC, Spurgeon HA, Pili R, Passaniti A, Cheng L, Corda S, Capogrossi MC: A functional ryanodine-sensitive intracellular Ca^{2+} store is present in vascular endothelial cells. Circ Res 1994; 74:151–6

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

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

44. Loeb AL, O'Brien DK, Longnecker DL: Halothane inhibits bradykinin-stimulated prostacyclin production in endothelial cells. ANESTHESIOLOGY 1994; 81:931–8