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ALTERATION OF CALCIUM MOBILIZATION IN ENDOTHELIAL CELLS BY VOLATILE ANESTHETICS

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Abstract—Halothane and isoflurane have different effects on the peripheral vasculature. Halothane decreases blood pressure primarily by decreasing cardiac contractility, whereas isoflurane acts primarily as a peripheral vasodilator. These peripheral vascular actions may result from different effects of the anesthetics on endothelial cell function and the release of endothelium-derived vasoactive mediators. The ability of these agents at clinically relevant concentrations to alter agonist-induced calcium mobilization in single cultured bovine aortic endothelial cells was tested using the fluorescent indicator fura-2. Neither halothane (0.3, 0.5, and 2 mM) or isoflurane (0.5 and 2 mM) altered basal calcium ($[Ca]_i = 49 \pm 5$ nM); however, the calcium transient normally elicited by 10 nM bradykinin (peak $[Ca]_i = 307 \pm 22$ nM) was inhibited significantly by halothane but not isoflurane. Neither anesthetic altered the calcium response to ATP (10 μM). These findings suggest that anesthetics may have specific effects on receptor-mediated endothelial cell functions that could influence hemodynamics.

Clinically relevant concentrations of the volatile anesthetics halothane and isoflurane produce different effects on the peripheral circulation. Halothane decreases blood pressure primarily by decreasing cardiac contractility without much effect on total peripheral vascular resistance [1–3]. Isoflurane, in contrast, decreases blood pressure by acting primarily as a peripheral vasodilator [3, 4]. Recent reports suggested that at least part of the vascular effects of halothane and isoflurane may result from their differential actions on the synthesis or release of vasoactive mediators from vascular endothelium [5–7].

The volatile anesthetics are known to modify both signal transduction mechanisms and cellular calcium homeostasis [8–15] in a variety of cell types, including cardiac myocytes, skeletal muscle, adrenal medullary cells and PC-12 cells (a cell line derived from pheochromocytoma). However, their effects on calcium signalling in endothelial cells are not defined. It is possible that the differential vascular effects of the anesthetics may result, at least in part, from differential effects on signal transduction and intracellular calcium ($[Ca]_i$) mobilization in endothelial cells, because the synthesis and release of a number of endothelium-derived vasoactive mediators, including prostaglandins [16] and endothelium-derived relaxing factor (EDRF)‖ [17–19], are initiated by agonist-stimulated increases in $[Ca]_i$. We tested this hypothesis in single cultured bovine aortic endothelial cells by studying the effects of halothane and isoflurane on basal $[Ca]_i$ and $[Ca]_i$ mobilization in response to agonists.

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‖ Abbreviations: EDRF, endothelium-derived relaxing factor; and BK, bradykinin.

MATERIALS AND METHODS

Endothelial cell culture. Bovine aortic endothelial cells (BAEC, AG 07684, Coriell Institute, Camden, NJ) were maintained in Ham's F-12 medium (Gibco) supplemented with 15% fetal bovine serum plus penicillin/streptomycin. Cells from passages 9–13 were used.

Intracellular free calcium dye loading and calcium measurements. Endothelial cells were grown on glass coverslips (25 mm diameter). Before use, the cells were loaded with the intracellular calcium indicator dye fura-2. The cells were washed with L-15 medium (Gibco), and fura-2/AM (Molecular Probes), from dimethyl sulfoxide stock solution (5 mM) was added to additional L-15 medium, sonicated and added to each well (final concentration 5 μM). Cells on coverslips were incubated with fura-2/AM at 37° for 30–45 min, and then washed twice with a balanced salt solution (BSS) containing 130 mM NaCl, 5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM dextrose, 20 mM Hepes, buffered to pH 7.4.

For fluorescence measurements in single cells, a coverslip was placed in a thermo-regulated holder mounted on the stage of an inverted epifluorescence microscope (Nikon), as described previously [20], and allowed to equilibrate at 35°. Individual cells were isolated within the field of view using an iris diaphragm. The cells were excited at 340 and 380 nm, using an air-turbine operated filter wheel to alternate the excitation wavelength (Biomedical Instrumentation, University of Pennsylvania). The demodulated fluorescence emission at 505 nm was recorded. Drugs were added directly to the buffer surrounding the cells, or directly on the cell of interest using a low pressure perfusion micropipet (10 μm opening) placed near the surface of the cell. Switching on the perfusion system (Picospritzer, General Valve Corp., Fairfield, NJ) applied nanoliter volumes of agonist solution directly over the cell.

Turning off the perfusion allowed the original medium (nominally agonist free) access to the cell surface, thus removing the agonist by dilution. Using this technique, only those cells adjacent to the pipet tip were exposed to significant concentrations of agonist. Perfusion with buffer only did not alter basal $[Ca]_i$. Therefore, multiple cells on a given coverslip could be tested for their responses to an agonist. Intracellular calcium concentrations were calculated according to the method of Grynkiewicz *et al.* [21] using ionomycin and ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) to generate the fluorescence maximums and minimums, respectively.

Exposure of cultured cells to volatile anesthetics. The anesthetic was equilibrated rapidly with aqueous medium by adding a measured quantity of isoflurane (Anaquest, Madison, WI) or halothane (Halocarbon Laboratories, North Augusta, SC) to an aliquot of BSS, vortexing for 60 sec and allowing it to equilibrate. Then an aliquot of the stock solution of anesthetic was diluted rapidly into the BSS bathing the cells to give the desired final concentration [8, 22]. Concentrations were confirmed with gas chromatography and remained within 10% of the target concentration for at least 3 min. The halothane concentrations (0.3 to 2 mM) correspond to gas concentrations of approximately 0.9 to 6.1 vol% at 37° [23]. This range spans from clinically relevant EC_{50} concentrations (0.3 mM), to anesthetic concentrations (0.5 mM), to more toxic concentrations (2 mM). The EC_{50} for anesthesia (MAC, minimum alveolar concentration preventing movement in response to a surgical stimulus in 50% of subjects) is species specific, and ranges between 0.75 to 1.25 vol% for halothane and 1.1 to 1.5 vol% for isoflurane [24]. As a point of reference, respiratory depression and cardiac failure are seen in rats at concentrations above 2.8% halothane or 4% isoflurane [25].

A typical experiment consisted of obtaining control responses to agonist from at least three widely separated cells on each coverslip. The average amplitude of these calcium transients was then compared to the agonist-induced response in the presence of the anesthetic. In each case, the response to the anesthetic and agonist was compared to its own agonist control response. Therefore, comparisons between agonist and anesthetic + agonist responses for each coverslip were made using paired *t*-tests.

RESULTS

Figure 1 shows results of experiments in which bradykinin (BK) or ATP was perfused over individual endothelial cells for 30 sec. Both agents induced transient increases in $[Ca]_i$ and successive drug applications produced similar $[Ca]_i$ transients in the same cell.

Addition of solutions containing halothane (0.3 or 0.5 mM) or isoflurane (0.5 mM) did not alter basal $[Ca]_i$. However, the $[Ca]_i$ transient normally elicited by BK was attenuated significantly in those cells exposed to halothane for 3 min (Fig. 2, Table 1). This attenuation of the response to BK was not due to the presence of thymol (a preservative in commercially available halothane at 0.01%, or 0.7 mM), because exposure of the cells to thymol alone had no effect on BK-induced calcium transients (data not shown). Treatment of the cells with isoflurane for 3 min did not alter the response to BK (see Fig. 2 and Table 1).

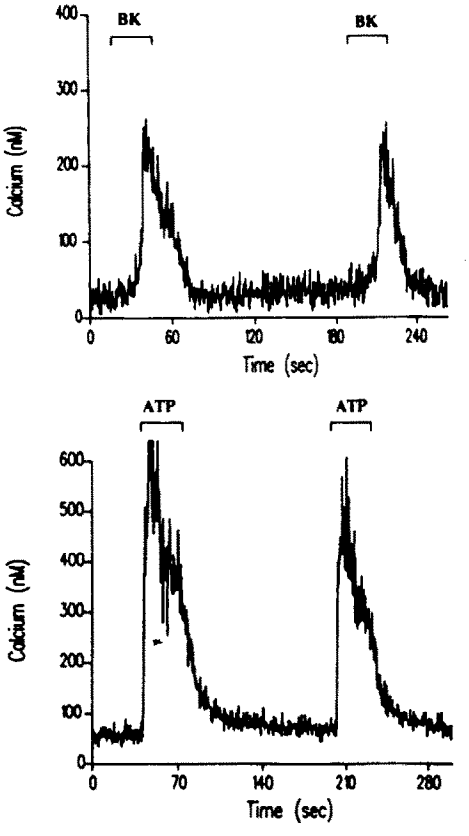

Fig. 1. Representative responses of single endothelial cells to repeated stimulation by either BK (upper) or ATP (lower).

To test whether or not the effects of anesthetics on agonist-induced calcium responses were concentration dependent, we examined the responses of the cells to agonists in the presence of increased concentrations of anesthetic. As shown in Table 1, the response to BK was attenuated significantly in the presence of 0.3, 0.5, and 2 mM halothane, but not in the presence of 0.5 or 2 mM isoflurane (the higher concentrations are equivalent to 6.1% halothane or 8% isoflurane). Although the magnitude of responses to ATP varied in individual cells from different preparations (see Figs. 1 and 3), neither halothane nor isoflurance prevented the subsequent response to ATP, when compared to control responses from the same coverslip (Fig. 3). Addition of 2 mM halothane or isoflurane to the cells sometimes resulted in a transient increase in basal $[Ca]_i$ (for example, see Fig. 3, lower panel). However, the magnitude of increase induced by the

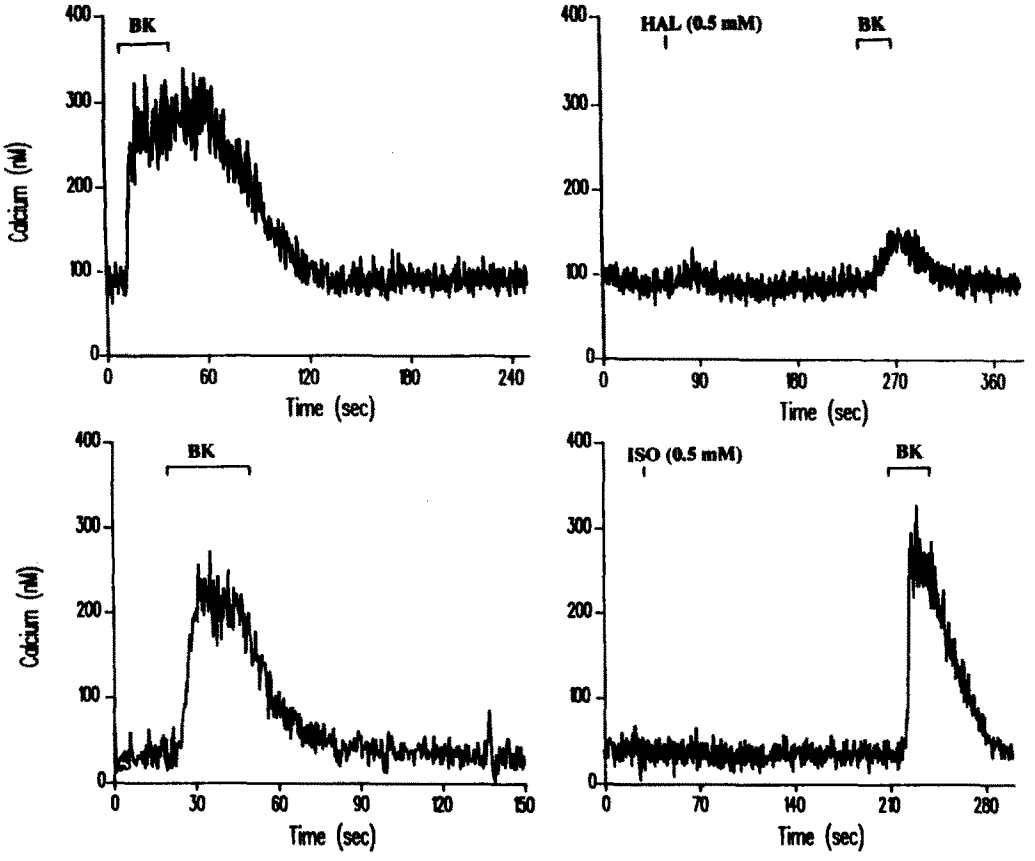

Fig. 2. Representative responses of single endothelial cells to BK (10 nM): Response of a control cell to BK (left) and a different cell on the same coverslip in the presence of either halothane (0.5 mM, upper panel) or isoflurane (0.5 mM, lower panel) for 3 min before BK administration.

Table 1. Effects of halothane and isoflurane on bradykinin-stimulated calcium mobilization

Treatment	Peak $[Ca]_i$ (nM)	N
Basal	49 ± 5	36
Bradykinin (10 nM)	307 ± 22	36
Bradykinin (10 nM) + halothane (0.3 mM)	296 ± 32*	17
Bradykinin (10 nM) + halothane (0.5 mM)	139 ± 35*	17
Bradykinin (10 nM) + halothane (2 mM)	113 ± 15*	4
Bradykinin (10 nM) + isoflurane (0.5 mM)	316 ± 34	13
Bradykinin (10 nM) + isoflurane (2 mM)	344 ± 145	4

Data are expressed as means ± SEM. The data are pooled for presentation purposes; however, statistical comparisons between control responses to bradykinin and the response to bradykinin after anesthetic treatment were made using the paired *t*-test (each cell preparation was used as its own control).

* Significant difference from bradykinin alone ($P < 0.05$).

anesthetics did not reach statistical significance. In addition, these increases were induced by anesthetic concentrations that are considered toxic.

DISCUSSION

Our findings demonstrate that halothane, but not isoflurane, can interfere with the signal transduction pathway stimulated by bradykinin in vascular endothelial cells. Calcium signalling in endothelial cells is associated with both the release of vasoactive compounds, such as prostaglandins and other eicosanoids, and the control of EDRF synthesis, as we [17, 19] and others [16, 18, 26, 27] have shown.

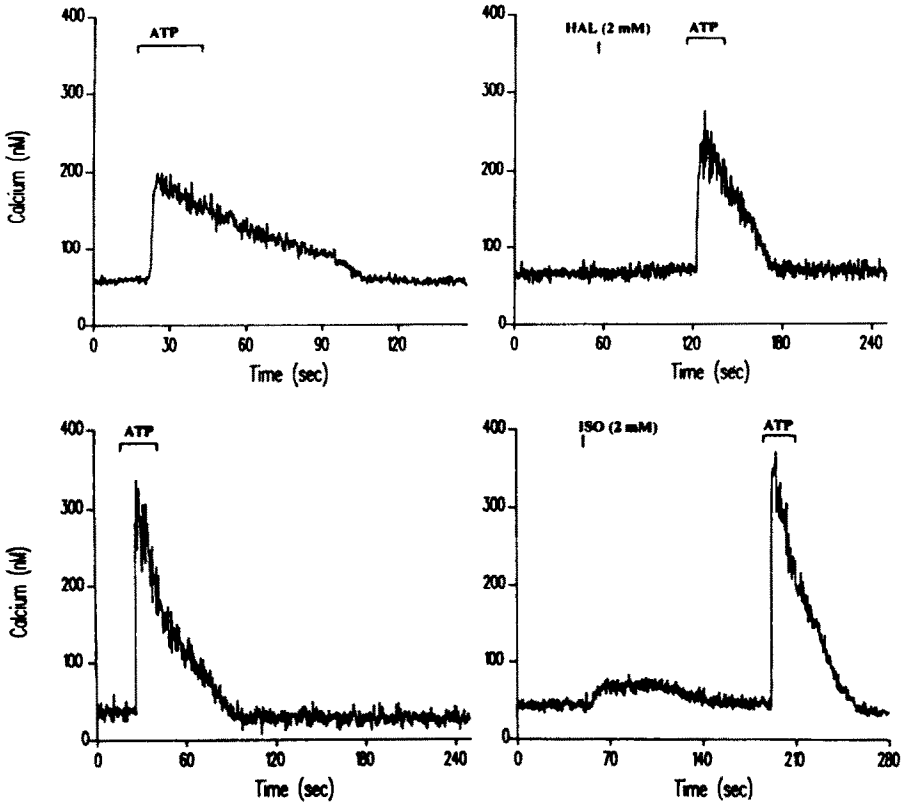

Fig. 3. Representative responses of endothelial cells to ATP (10 μM): Response of control cell to ATP (left) and a different cell on the same coverslip in the presence of anesthetic. The upper panel depicts responses to ATP in the presence of halothane (2 mM); the lower panel depicts responses in the presence of isoflurane (2 mM).

The ability of halothane to attenuate responses to BK but not ATP may result from the action of halothane on receptor–agonist interactions, or specific differences in receptor–G-protein–effector coupling that mediate the responses to agonists. In our studies, the calcium release mechanism was probably not altered by halothane because both ATP and BK activate phospholipase C and stimulate inositol phosphate turnover and subsequent $[Ca]_i$ mobilization in endothelial cells [16, 17, 28].

Recent studies have shown that halothane can decrease functional coupling between G-proteins and both muscarinic and α_2-adrenergic receptors in rat brain [29, 30]. In addition, Narayanan *et al.* [31] found that halothane could disrupt muscarinic inhibition of adenylyl cyclase (G_i-mediated) while having no effect on β-adrenergic stimulation of adenylyl cyclase (G_s-mediated). Similarly, Puig *et al.* [32] showed that the inhibitory action of halothane on electrically stimulated contractions of guinea pig myenteric plexus was prevented when G_i was inactivated by pretreatment with pertussis toxin. In endothelial cells, BK-stimulated phosphoinositide metabolism is not coupled to a pertussis toxin sensitive G-protein [33]; nevertheless, it is possible that the inhibitory effect of halothane that we observed could have been due to an effect on a different G-protein coupled to the BK receptor.

The volatile anesthetics are known to modify signal transduction and cellular calcium homeostasis in a variety of cells types. Analogous to our findings with BK, Freeman and Li [15] found that halothane reduces the amplitude of isoproterenol-stimulated calcium transients in ventricular myocytes, and suggested that halothane alters intracellular calcium stores. In A7r5 cells (derived from rat vascular smooth muscle), Sill *et al.* [34] demonstrated that halothane increases basal $[Ca]_i$ acutely and inhibits calcium signalling in response to arginine vasopressin and platelet-derived growth factor. They also showed that halothane inhibits the arginine vasopressin-induced increase in total inositol phosphates without affecting basal levels. In PC-12 cells [13], and in isolated bovine adrenal medullary cells [10], volatile anesthetics were found to depress stimulus–secretion coupling and the depolarization-induced rise in cytoplasmic calcium. In isolated cardiac myocytes, halothane transiently increases intracellular free calcium concentrations [12] and decreases electrically simulated Ca^+ transients [13]. Although others have reported that halothane alters calcium uptake and release from internal stores [12, 14, 35], we believe this mechanism to be an unlikely possibility in endothelial cells because the response to ATP was unaffected by either anesthetic.

In summary, the differential actions of halothane

and isoflurane on endothelial cell signal transduction may be one explanation for how these anesthetics can have such different effects on the peripheral circulation [2, 3] and differentially alter endothelium-dependent processes *in vitro* and *in vivo* [5, 6, 36]. The precise endothelial mechanism that is affected by halothane remains to be determined. Our findings indicate that halothane specifically inhibits BK-stimulated increases in $[Ca]_i$ in vascular endothelial cells whereas isoflurane does not. Neither anesthetic altered the response to ATP. The ability of halothane to attenuate calcium mobilization in endothelial cells in response to specific agonists may result from alterations in receptor–G-protein coupling [30, 31, 34] which regulates the production of endothelium-derived vasoactive mediators [6, 7]. Differential effects of anesthetic agents in altering mediator production may at least partially explain the differential vascular effects of the anesthetics.

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REFERENCES

1. Miller ED, Kistner JR and Epstein RM, Whole body distribution of radioactive microspheres in the rat during anesthesia with halothane, enflurane or ketamine. *Anesthesiology* **52**: 296–302, 1980.
2. Eger EI, Smith NT, Cullen DJ, Cullen BF and Gregory GA, A comparison of the cardiovascular effects of halothane, fluroxene, ether, and cyclopropane in man. A resumè. *Anesthesiology* **34**: 25–41, 1971.
3. Seyde WC and Longnecker DE, Anesthetic influences on regional hemodynamics in normal and hemorrhaged rats. *Anesthesiology* **61**: 686–698, 1984.
4. Stevens WC, Cromwell TH, Halsey MJ, Eger EI, Shakespeare TF and Bahlman SH, The cardiovascular effects of a new inhalation anesthetic, Forane, in human volunteers at constant arterial carbon dioxide tension. *Anesthesiology* **35**: 8–16, 1971.
5. Blaise G, Sill JC, Nugent M, Van Dyke RA and Vanhoutte PM, Isoflurane causes endothelium-dependent inhibition of contractile responses of canine coronary arteries. *Anesthesiology* **67**: 513–517, 1987.
6. Muldoon SM, Hart JL, Bowen KA and Freas W, Attenuation of endothelium-mediated vasodilation by halothane. *Anesthesiology* **68**: 31–37, 1988.
7. Stone DJ and Johns RA, Endothelium-dependent effects of halothane, enflurane, and isoflurane on isolated rat aortic vascular rings. *Anesthesiology* **71**: 126–132, 1989.
8. Kress HG, Eckhardt-Wallasch H, Tas PWL and Koschel K, Volatile anesthetics depress the depolarization-induced cytoplasmic calcium rise in PC 12 cells. *FEBS Lett* **221**: 28–32, 1987.
9. Lynch C and Frazer MJ, Depressant effects of volatile anesthetics upon rat and amphibian ventricular myocardium: Insights into anesthetic mechanisms of action. *Anesthesiology* **70**: 511–522, 1989.
10. Pocock G and Richards CD, The action of volatile anaesthetics on stimulus–secretion coupling in bovine adrenal chromaffin cells. *Br J Pharmacol* **95**: 209–217, 1988.
11. Su JY and Bell JG, Intracellular mechanism of action of isoflurane and halothane on striated muscle of the rabbit. *Anesth Analg* **65**: 457–462, 1986.
12. Wheeler DM, Rice RT, Hansford RG and Lakatta EG, The effect of halothane on the free intracellular calcium concentration of isolated rat heart cells. *Anesthesiology* **69**: 578–583, 1988.
13. Wilde DW, Knight PR, Sheth N and Williams BA, Halothane alters control of intracellular Ca^{2+} mobilization in single rat ventricular myocytes. *Anesthesiology* **75**: 1075–1086, 1991.
14. Bosnjak ZJ, Aggarwal A, Turner LA, Kampine JM and Kampine JP, Differential effects of halothane, enflurane, and isoflurane on Ca^{2+} transients and papillary muscle tension in guinea pigs. *Anesthesiology* **76**: 123–131, 1992.
15. Freeman LC and Li Q, Effects of halothane on delayed afterdepolarization and calcium transients in dog ventricular myocytes exposed to isoproterenol. *Anesthesiology* **74**: 146–155, 1991.
16. Carter TD, Hallam TJ, Cusack NJ and Pearson JD, Regulation of P_{2y}-purinoceptor-mediated prostacyclin release from human endothelial cells by cytoplasmic calcium concentration. *Br J Pharmacol* **95**: 1181–1190, 1988.
17. Loeb AL, Izzo NJ, Johnson RM, Garrison JC and Peach MJ, Endothelium-derived relaxing factor release associated with increased endothelial cell inositol triphosphate and intracellular calcium. *Am J Cardiol* **62**: 36G–40G, 1988.
18. Lückhoff A and Busse R, Increased free calcium in endothelial cells under stimulation with adenine nucleotides. *J Cell Physiol* **126**: 414–420, 1986.
19. Peach MJ, Singer HA, Izzo NJ and Loeb AL, Role of calcium in endothelium-dependent relaxation of arterial smooth muscle. *Am J Cardiol* **59**: 35A–43A, 1987.
20. Hansen CA, Monck JR and Williamson JR, Measurement of intracellular free calcium to investigate receptor-mediated calcium signalling. *Methods Enzymol* **191**: 691–706, 1990.
21. Grynkiewicz G, Poenie M and Tsien RY, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**: 3440–3450, 1985.
22. Bazil CW and Minneman KP, Effects of clinically effective concentrations of halothane on adrenergic and cholinergic synapses in rat brain *in vitro*. *J Pharmacol Exp Ther* **248**: 143–148, 1989.
23. Renzi F and Waud BE, Partition coefficients of volatile anesthetics in Kreb's solution. *Anesthesiology* **47**: 62–63, 1977.
24. Committee on Pain and Distress in Laboratory Animals, Control of pain. In: *Recognition and Alleviation of Pain and Distress in Laboratory Animals*, pp. 53–84. National Research Council, National Academy Press, Washington, DC, 1992.
25. Wolfson B, Hetrick WD, Lake CL and Siker ES, Anesthetic indices—Further data. *Anesthesiology* **48**: 187–190, 1978.
26. Bredt DS and Snyder SH, Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci USA* **87**: 682–685, 1990.
27. Mülsch A, Bassenge E and Busse R, Nitric oxide synthesis in endothelial cytosol: Evidence for a calcium-dependent and a calcium-independent mechanism. *Naunyn Schmiedebergs Arch Pharmacol* **340**: 767–770, 1989.
28. Brock TA, Dennis PA, Griendling KK, Diehl TS and Davies PF, GTPγS loading of endothelial cells stimulates phospholipase C and uncouples ATP receptors. *Am J Physiol* **255**: C667–C673, 1988.
29. Dennison RL Jr, Anthony B, Narayanan TK and Aronstam RS, Effects of halothane on high affinity agonist binding and guanine nucleotide sensitivity of muscarinic acetylcholine receptors from brainstem of rat. *Neuropharmacology* **26**: 1201–1205, 1987.

30. Baumgartner MK, Dennison RL, Narayanan TK and Aronstam RS, Halothane disruption of α_2-adrenergic receptor-mediated inhibition of adenylate cyclase and receptor G-protein coupling in rat brain. *Biochem Pharmacol* **39**: 223–225, 1990.
31. Narayanan TK, Confer RA, Dennison RL, Anthony BL and Aronstam RS, Halothane attenuation of muscarinic inhibition of adenylate cyclase in rat heart. *Biochem Pharmacol* **37**: 1219–1223, 1988.
32. Puig MM, Turndorf H and Warner W, Synergistic interaction of morphine and halothane in the guinea pig ileum: Effects of pertussis toxin. *Anesthesiology* **72**: 699–703, 1990.
33. Voyno-Yasenetskaya TA, Panchenko MP, Nupanko EV, Rybin VO and Tkachuk VA, Histamine and bradykinin stimulate the phosphoinositide turnover in human umbilical vein endothelial cells via different G-proteins. *FEBS Lett* **259**: 67–70, 1989.
34. Sill JC, Uhl C, Eskuri S, Van Dyke R and Tarara J, Halothane inhibits agonist-induced inositol phosphate and Ca^{2+} signalling in A7r5 cultured vascular smooth muscle cells. *Mol Pharmacol* **40**: 1006–1013, 1992.
35. Komai H and Rusy BF, Direct effect of halothane and isoflurane on the function of the sarcoplasmic reticulum in intact rabbit atria. *Anesthesiology* **72**: 694–698, 1990.
36. Greenblatt EP, Loeb AL and Longnecker DE, Endothelium-dependent circulatory control: A mechanism for the differing peripheral vascular effects of isoflurane versus halothane. *Anesthesiology* **77**: 1178–1185, 1992.