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Effects of Halothane and Isoflurane on Bradykinin-evoked Ca^{2+} Influx in Bovine Aortic Endothelial Cells

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Background: **Volatile anesthetics, such as halothane and isoflurane, have been reported to affect the endothelium-mediated relaxation of vascular smooth muscle cells. Because the activity of the constitutive nitric oxide synthase in endothelial cells depends on the availability of intracellular Ca^{2+}, there is a definite possibility that the observed inhibitory effect of volatile anesthetics involves an action on the agonist-evoked internal Ca^{2+} mobilization and/or Ca^{2+} influx in these cells. Therefore, a study was undertaken to determine how halothane and isoflurane affect the Ca^{2+} signalling process in vascular endothelial cells.**

Methods: **The effect of halothane and isoflurane on the Ca^{2+} response to bradykinin of bovine aortic endothelial (BAE) cells was investigated using the fluorescent Ca^{2+} indicator fura-2. Halothane or isoflurane was applied either to resting cells or after bradykinin stimulation. The agonist-evoked Ca^{2+} influx in BAE cells was estimated by measuring either the rate of fura-2 quenching induced by Mn^{2+} or the increase in cytosolic Ca^{2+} concentration initiated after readmission of external Ca^{2+} after a brief exposure of the cells to a Ca^{2+}-free external medium. The effects of halothane on cell potential and intracellular Ca^{2+} concentration were measured in cell-attached patch-clamp experiments in which a calcium-activated K^+ channel and an inward rectifying Ca^{2+}-independent K^+ channel were used as probes to simultaneously monitor the intracellular Ca^{2+} concentration and the cell transmembrane potential. In addition, combined fura-2 and patch-clamp cell-attached recordings were carried out, to correlate the variations in internal Ca^{2+} caused by halothane and the activity of the Ca^{2+}-dependent K^+ channels, which are known in BAE cells to regulate intracellular potential. Finally, a direct action of halothane and isoflurane on the gating properties of the Ca^{2+}-activated K^+ channel present in these cells was investigated in patch-excised inside-out experiments.**

Results: **The results of the current study indicate that the initial Ca^{2+} increase in response to bradykinin stimulation is not affected by halothane, but that pulse applications of halothane (0.4–2 mM) or isoflurane (0.5–1 mM) reversibly reduce the sustained cytosolic Ca^{2+} increase initiated either by bradykinin or by the Ca^{2+} pump inhibitor thapsigargin. In addition, halothane appeared to dose-dependently inhibit the Ca^{2+} influx evoked by bradykinin, and to cause, concomitant to a decrease in cytosolic Ca^{2+} concentration, a depolarization of the cell potential. Halothane failed, however, to affect internal Ca^{2+} concentration in thapsigargin-treated endothelial cells, which were depolarized using a high K^+ external solution. Finally, halothane and isoflurane decreased the open probability of the Ca^{2+}-dependent K^+ channel present in these cells.**

Conclusions: **These observations suggest that the effects of halothane and isoflurane on Ca^{2+} homeostasis in BAE cells reflect, for the most part, a reduction of the thapsigargin- or bradykinin-evoked Ca^{2+} influx, which would be consequent to a cellular depolarization caused by an inhibition of the Ca^{2+}-dependent K^+ channel activity initiated after cell stimulation. (Key words: Anesthetic, volatile: halothane; isoflurane. Endothelium: calcium, potassium channels.)**

VOLATILE anesthetics such as halothane and isoflurane have been reported to have specific and differing cardiovascular effects.[1] These effects are likely to involve several mechanisms acting on the integration of factors that control smooth muscle cell contraction. It is widely accepted that the endothelium constitutes a key regulator of vascular tone by secreting both vasoconstrictors, such as endothelin-1 (and perhaps other endothelins), and vasorelaxing agents, such as the prostacyclin PGI_2 and the major endothelium-derived relaxing factor nitric oxide (NO).[2] The effects of anesthetics on the endothelium-dependent control of vascular tone were examined in several studies. For instance, Blaise *et al.*[3] showed that isoflurane impairs the phenylephrine-induced contractile response of ca-

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nine coronary arteries in an endothelium-dependent manner. It was suggested that isoflurane could, in this case, stimulate the release of NO. However, most studies supported an inhibitory action of volatile anesthetics on the NO-evoked vascular relaxation. Halothane was found, by Muldoon *et al.*,[4] to inhibit the endothelium-dependent vasodilation induced by acetylcholine and bradykinin in isolated contracted rabbit and canine vascular rings. In addition, Uggeri *et al.'s*[5] study on rat thoracic aorta provided evidence that halothane, enflurane, and isoflurane attenuate the NO-dependent relaxation induced by muscarinic receptor stimulation. Finally, Toda *et al.*[6] confirmed that halothane and isoflurane inhibit both the endothelium-dependent relaxation and the cyclic guanosine monophosphate formation elicited by acetylcholine in rat aorta. On the basis of these observations, it was concluded that halothane and other volatile anesthetics affect either the production and release of NO[4,5] or its stability.[7] Most studies failed to show an effect that could be attributable to a direct action on vascular smooth muscle cells that involve guanylate cyclase, although Hart *et al.*[8] reported, in a work on rat aorta, a series of results suggesting that the site of action of halothane was at the vascular smooth muscle level.

Studies have indicated that the production and release of NO after the stimulation of endothelial cells by bradykinin, histamine, adenosine triphosphate, substance P, or thrombin are linked to an increase in intracellular Ca^{2+}.[9–12] In fact, Ca^{2+} is known to activate, with calmodulin and the nicotinamide adenine dinucleotide NADH, an NO synthase that, in turn, metabolizes L-arginine to citrulline and NO.[10,13] In addition, there is strong evidence that the increase in intracellular Ca^{2+} caused by vasodilators such as bradykinin, adenosine triphosphate, and histamine is a biphasic process that reflects, in part, an inositol 1,4,5-triphosphate ($InsP_3$)-mediated release of Ca^{2+} from intracellular stores, coupled to a Ca^{2+} entry from the external medium.[14–16] The molecular mechanism underlying the agonist-evoked Ca^{2+} influx in endothelial cells remains ill defined.

Electrophysiological and unidirectional $^{45}Ca^{2+}$ flux measurements in cultured or freshly dissociated endothelial cells from large blood vessels have indicated an absence of depolarization-activated Ca^{2+} influx *via* voltage-dependent Ca^{2+} channels in these cells.[17] As in lymphocytes and certain other nonexcitable tissues, there is clear evidence that the Ca^{2+} influx in endothelial cells is augmented or stabilized at hyperpolarizing potentials.[16,18,19] It is generally agreed that this augmentation arises from the increased electrical driving force acting on Ca^{2+} ions under these conditions.[17,19] This situation is at variance with the results obtained in excitable cells where Ca^{2+} influx is normally reduced at hyperpolarizing potentials because of the closure of voltage-dependent Ca^{2+} channels. A hyperpolarizing response to cell stimulation may result from several factors, but there is increasing data that support a determinant role of the K^+ selective channels in the case of endothelial cells.[17,19,20] In particular, studies have shown that Ca^{2+}-activated K^+ channels [$K(Ca^{2+})$] constitute important positive feedback elements of the Ca^{2+} signalling process in vascular endothelial cells.[16,18,19] Chemical agents that affect $K(Ca^{2+})$ channel activity are, therefore, likely to modulate the agonist-evoked Ca^{2+} influx in these cells.

Because volatile anesthetics were shown to alter internal Ca^{2+} homeostasis in many cell types,[21,22] including endothelial cells,[23] there is a possibility that the reported inhibition by halothane or isoflurane of the endothelium-dependent relaxation of vascular endothelial cells involves an action of the anesthetics on the $InsP_3$-dependent Ca^{2+} mobilization and/or agonist-evoked Ca^{2+} influx. Fura-2 and patch-clamp experiments, therefore, were undertaken to characterize the effects of halothane and isoflurane on the Ca^{2+} response of bovine aortic endothelial (BAE) cells to the vasodilating agent bradykinin and to determine the involvement of the endothelial cell $K(Ca^{2+})$ channels in this process. Our results indicate that the action of halothane includes an inhibition of the agonist-evoked Ca^{2+} influx, which is partly related to depolarization of the endothelial cell potential that results from an inhibition of the $K(Ca^{2+})$ channels present in these cells.

Materials and Methods

Cell Culture

The details of the BAE cell culture procedure and characterization have been described elsewhere.[16] The cells were tested with endothelial cell-labelling reagents or factor VIII antibodies (Daco, Santa Barbara, CA) and responded positively.[24] Bovine aortic endothelial cells were cultured in Dulbecco's modified Eagle Medium (Gibco, Gaithersburg, MD) supplemented with 10% newborn calf serum, 3.7 g/l $NaHCO_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin in hu-

midified air, with 5% CO_2 atmosphere at 37°C. Cells from serial passage 21–26 were reseeded on microscope cover slips, to accommodate the superfusion chamber used for fluorescence and patch clamp measurements.

Solutions and Drugs

Confluent cells were superfused continuously with an Earle's solution that had the standard composition, as follows (in mM): 121.0 NaCl; 5.4 KCl; 1.8 $CaCl_2$; 0.8 $MgSO_4$; 6.0 $NaHCO_3$, 1.0 NaH_2PO_4; 5.5 glucose buffered at pH 7.3 with 25.0 Hepes, and 10.0 NaOH. K^+-Earle's solutions were prepared by equimolar substitution of sodium chloride and NaOH by KCl and potassium hydroxide. Ca^{2+}-free (0 Ca^{2+}) solutions were prepared by omitting $CaCl_2$ and adding 1 mM ethyleneglycol-bis-[B-aminoethylether]N,N,N′,N′ (tetraacetic acid [EGTA]) to standard Earle's or K^+-Earle's solutions.

The low-Ca^{2+} solution used to calibrate the fura-2 signal consisted of an Earle's medium with no $CaCl_2$, to which was added 5.0 mM EGTA, 15.0 mM NH_4Cl, and 5 μM ionomycin. The high-Ca^{2+} solution used for calibration was an Earle's solution, with 5 μM ionomycin buffered at pH 8.0–8.5. Patch pipettes were filled with a solution that contained (in mM): 200 KCl, 0.5 $MgSO_4$, 0.91 $CaCl_2$, and 1 EGTA, for a free Ca^{2+} concentration of 1 μM.

For whole cell recordings, the free Ca^{2+} concentration was reduced to 0.2 μM. The pH was buffered at 7.3, KOH with 25 mM Hepes and 10 mM. Bradykinin, ionomycin, and fura-2 were purchased from Sigma (St. Louis, MO); thapsigargin was obtained from L.C. Services (Woburn, MA). Halothane and isoflurane (Ayerst, Montréal, Québec) containing solutions were prepared in 50-ml gas-tight syringes (Hamilton 1050, Reno, NV), at concentrations ranging from 0.4 mM to 2 mM (2–10 μl halothane in 50 ml solution) or 0.5 mM to 1 mM (3.8–7.9 μl isoflurane in 50 ml solution), respectively. As discussed by Franks and Lieb,[25] a concentration of 1 mM halothane corresponds at room temperature (25°C) to a gaseous partial pressure of 1.7 volume percent (1.7%), whereas 1 mM isoflurane is equal to a partial pressure of 2.0 volume percent (2.0%). At this temperature, the EC_{50} of halothane for general anesthesia ranges from 0.17 mM to 0.24 mM and from 0.23 mM to 0.25 mM for isoflurane. The syringes were mounted on perfusion pumps (Harvard 11, South Natick, MA) set at a perfusion rate of 2–4 ml/min. The effective halothane or isoflurane concentration in the perfusion chamber was estimated as described previously by gas chromatography.[7]

Fura-2 Measurements

Confluent monolayers of BAE cells were loaded with fura-2 by incubation in an Earle's solution that contained 3 μM fura-2 acetoxymethyl ester (fura-2 AM). The incubation time ranged from 30 min to 40 min at room temperature. The fluorescence from 15–20 cells was measured with a Nikon inverted microscope (Tokyo, Japan) attached to a dual-excitation spectrofluorometer (Spex Fluorolog II, Edison, NJ) with excitation wavelengths set at 350 nm and 380 nm, respectively. A dichroic mirror (Nikon FT 400) was placed in the excitation pathway and the emission monitored at 500 nm with a standard bandpass filter (Andover Corporation 500FS40, Salem, NH). The cytosolic Ca^{2+} concentration was calculated from the ratio of the fluorescence measured at 350 nm and 380 nm, respectively, and from the ratio of fluorescence at 380 nm in low-Ca^{2+} relative to that in high-Ca^{2+} conditions, as described previously.[26] For experiments in which Mn^{2+} was used as a quenching agent, one excitation wavelength was set at 358 nm, to monitor fura-2 fluorescence independently of the intracellular Ca^{2+} concentration. The rate of quenching of the fura-2 signal was calculated as follows:

$$QUEN[Mn^{2+}](cps/s) = RLF[Mn^{2+}](cps/s) - RLF \times (cps/s)$$

where cps is the proton count per second, RLF[Mn^{2+}] is the rate at which the fura-2 fluorescence intensity decreased calculated over the first 30 s after the addition of Mn^{2+} (period during which the decrease of fluorescence remains linear), and RLF the equivalent rate calculated during a 60-s period before the addition of Mn^{2+} to the external medium (this measure reflects the leak of fura-2 out of the cells). All the experiments were performed at room temperature (23–25°C).

Patch-clamp Measurements

Patch pipettes were pulled from Pyrex capillaries (Corning 7040, Corning, NY), using a David Kopf (Twinga, CA) programmable pipette puller (Model 750) and used uncoated. The resistance of the patch electrode ranged from 4 to 10 MΩ. Current traces were recorded on frequency modulator wideband tapes (HP 3964, San Diego, CA) at a bandwidth of 1.25 kHz and subsequently transferred to a PC hard disk for offline analysis. Unless otherwise specified, the signal was sampled at 1.5 kHz and filtered at 500 Hz, with two

low-pass four-pole Bessel filters (VVS 300B, Frequency Devices, Haverhill, MA) connected in series. Baseline drift was corrected through a multiple linear interpolation procedure. The open channel probability was computed from current amplitude histograms on the basis of a binomial distribution as described elsewhere.[27] The current amplitude histograms were computed from current segments of 60 s minimum duration. Results are expressed as means ± SEM.

Results

Figure 1 shows the effect of halothane on the cytosolic Ca^{2+} concentration after bradykinin receptor stimulation. Panel A illustrates the Ca^{2+} response evoked by bradykinin in control conditions (n = 14) and in the presence of 2 mM halothane (n = 5). The initial Ca^{2+} rise related to the release of Ca^{2+} from intracellular stores appeared unaffected by halothane at concentrations ranging from 0.2 mM to 2 mM, with a peak Ca^{2+} value estimated at 700 ± 300 nM (n = 5) in control and 700 ± 250 nM (n = 5) in halothane conditions. Identical results were obtained using cells preincubated for 5 min in halothane (2 mM; data not shown). In cells exposed to halothane, however, there was a clear increase in the rate at which the Ca^{2+} concentration returned to a stable resting value. Computation of $t_{1/2}$, the time needed for the Ca^{2+} concentration to reach a value equal to half the initial Ca^{2+} peak amplitude, yielded values of 148 ± 45 s (n = 5) in control and 81 ± 24 s (n = 5) in 2 mM halothane conditions, respectively.

An example of the Ca^{2+} response initiated after the withdrawal of 0.8 mM external halothane is presented in panel B. This experimental maneuver typically produced a transient Ca^{2+} increase, which decayed to a Ca^{2+} concentration value higher than the Ca^{2+} level prevailing before halothane withdrawal. Panel C shows the effects of brief, 50-s halothane applications (2 mM; n = 12) on the Ca^{2+} response triggered by bradykinin. Under these conditions, halothane appeared to significantly decrease the internal Ca^{2+} concentration, especially during the plateau phase of the Ca^{2+} response.

In 2 (16%) of 12 of the experiments performed in 2 mM halothane, the concentration of Ca^{2+} decreased rapidly, after an initial Ca^{2+} increase, and halothane was ineffective in further reducing the Ca^{2+} concentration. In the remaining experiments (10 of 12), 2 mM halothane caused an average reduction of the Ca^{2+} concentration of 145 ± 35 nM (n = 10), for a relative 70% decrease of the Ca^{2+} level maintained during the plateau phase. In addition, each withdrawal of halothane was followed by a transient Ca^{2+} increase, confirming the Ca^{2+} response illustrated in figure 1B. However, because halothane was reapplied during the onset of the Ca^{2+} rise, the Ca^{2+} transients in this case decayed to a value close to the resting Ca^{2+} concentration before bradykinin stimulation.

A similar Ca^{2+} response was observed using isoflurane at concentrations of 0.5 mM and 1 mM, suggesting that both anesthetics affect Ca^{2+} signalling in endothelial

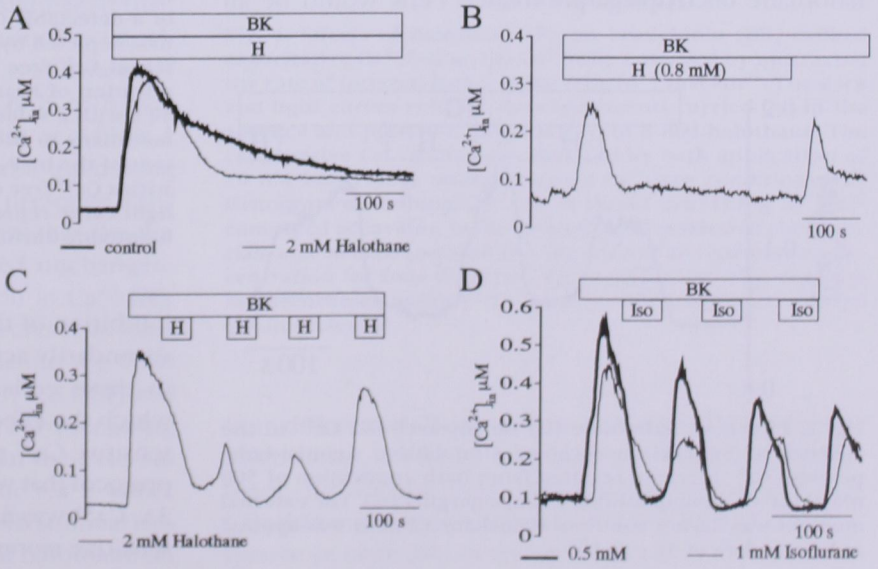

Fig. 1. Effects of halothane (H) and isoflurane (Iso) on the Ca^{2+} response evoked by 10 nM bradykinin (BK) in BAE cells. (*A*) Ca^{2+} response measured with the fluorescent Ca^{2+} indicator fura-2 in control conditions (dark curve) and in the presence of 2 mM halothane (light curve). The bathing medium was Earle's solution (1.8 mM Ca^{2+}) and the fluorescence signal represents the contribution of 10 to 20 cells. (*B*) Transient Ca^{2+} increase generated after the withdrawal of external halothane (0.8 mM). The external medium was Earle's solution (1.8 mM Ca^{2+}). (*C*) Effects of repetitive 50-s applications of 2 mM halothane on the Ca^{2+} response initiated after bradykinin receptor stimulation. (*D*) Effects of repetitive 50-s applications of 0.5 mM or 1 mM isoflurane on the Ca^{2+} response initiated after bradykinin receptor stimulation. Experimental conditions as described in *A*. Experiments were carried out at room temperature.

cells through a common mechanism (panel D). The average reduction in Ca^{2+} concentration was estimated at 247 ± 130 nM (n = 8) and 250 ± 100 nM (n = 9) at 1 mM and 0.5 mM isoflurane, respectively. As observed with halothane, isoflurane appeared ineffective in reducing the initial Ca^{2+} rise after bradykinin receptor stimulation (n = 10; data not shown). These observations indicate that the effects of halothane and isoflurane influence the external Ca^{2+}-dependent phase of the Ca^{2+} increase evoked by bradykinin, rather than the initial Ca^{2+} increase, which is due to the mobilization of Ca^{2+} from intracellular Ca^{2+} stores.

A series of experiments was performed in which the endoplasmic Ca^{2+} pump inhibitor thapsigargin was used to initiate a release of Ca^{2+} from internal pools and generate a capacitative Ca^{2+} influx, independent of $InsP_3$ production.[28,29] Figure 2 shows the effects of 2 mM halothane when applied for time intervals of 50 s to 100 s after 500 nM thapsigargin stimulation (n = 5). A reduction of 50 ± 25 nM (n = 5) of the Ca^{2+} increase caused by thapsigargin was observed under these conditions, indicating that halothane could still affect the cytosolic Ca^{2+} concentration despite the absence of $InsP_3$ production. As in figures 1C and 1D, the withdrawal of halothane resulted in transient Ca^{2+} rises, with peaks that reached, in this case, concentration values superior to the Ca^{2+} concentration measured after thapsigargin application. The fura-2 measurements presented in figure 2 do not support an action of halothane based exclusively on inhibition of the $InsP_3$ production machinery.

One possible explanation for the observed effects of halothane on thapsigargin-treated cells would be an

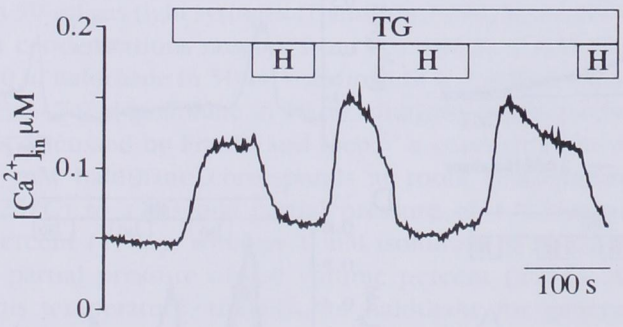

Fig. 2. Effects of halothane (H) on intracellular Ca^{2+} in the absence of bradykinin receptor stimulation. Agonist-independent Ca^{2+} increase resulted from bath application of 500 nM of the Ca^{2+} pump inhibitor thapsigargin (TG). The external medium was Earle's solution. Halothane (2 mM) was applied as brief pulses of 50 s to 100 s.

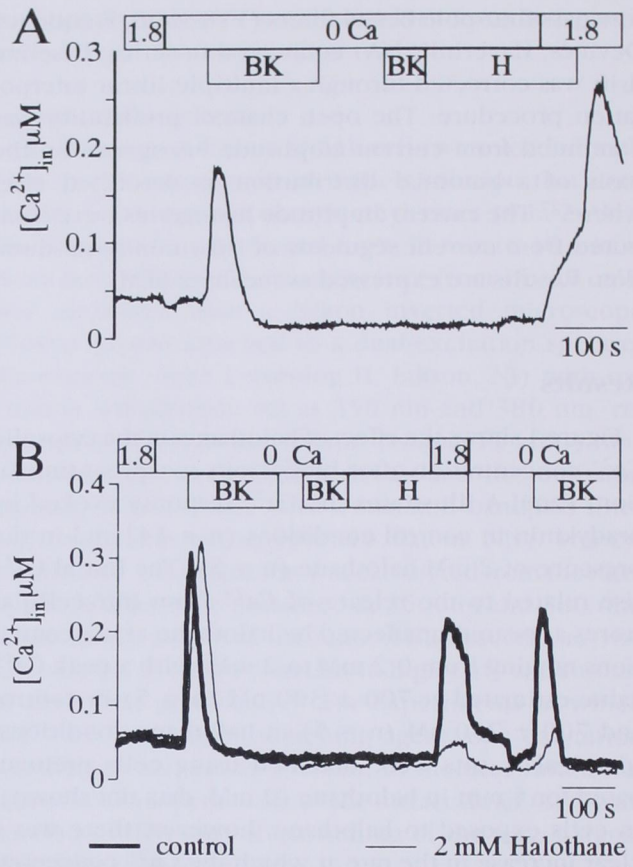

Fig. 3. Effects of halothane (H) on the cytosolic Ca^{2+} concentration under $InsP_3$-sensitive Ca^{2+} stores reloading conditions. (*A*) Effect of halothane on the capacitative Ca^{2+} increase initiated by addition of 1.8 mM Ca^{2+} to the bathing solution after depletion of internal Ca^{2+} pools through bradykinin (BK) receptor stimulation (10 nM) under Ca^{2+}-free conditions. Halothane (2 mM) was applied before the readmission of Ca^{2+} in the external medium and removed 50 s after the occurrence of a detectable Ca^{2+} rise. (*B*) $InsP_3$-sensitive Ca^{2+} pools were first depleted by bath application of 10 nM bradykinin in external Ca^{2+}-free conditions and tested by a second brief application of bradykinin. The cells were then superfused for 50 s with a solution containing 1.8 mM Ca^{2+} with or without halothane to allow reloading of the Ca^{2+} pools. The Ca^{2+} content of the $InsP_3$-sensitive Ca^{2+} pools was tested by perfusing with a Ca^{2+}-free external solution containing bradykinin. The light curve represents the experiment performed with 2 mM halothane during the reloading procedure.

inhibition of the capacitative Ca^{2+} influx known to be secondarily activated by emptying internal Ca^{2+} stores in these cells.[28–31] Experiments were performed in which the effect of halothane was studied under $InsP_3$-sensitive Ca^{2+} pool reloading conditions. The perfusion protocol that was used in this case is illustrated in figure 3A. Ca^{2+} was first released from the intracellular $InsP_3$-sensitive stores by a double application of 10 nM bra-

dykinin in Earle's Ca^{2+}-free solution. After the second application of bradykinin, the bath solution was replaced by a Ca^{2+}-free Earle's medium with 2mM halothane. The agonist-evoked Ca^{2+} influx was then assayed by superfusing the cells for 50 s with a standard Earle's solution (1.8 mM $CaCl_2$), with and then without 2mM halothane. The fura-2 recording presented in figure 3A indicates a clear decrease of the resulting Ca^{2+} rise under halothane conditions. Such a behavior was observed in three additional experiments based on the same perfusion protocol. These results, therefore, would support a model in which halothane acts through an inhibition of the capacitative Ca^{2+} entry in these cells. This conclusion is also in agreement with the fura-2 experiment illustrated in figure 3B. The perfusion protocol used in this case is similar to the one presented in figure 3A, except that Ca^{2+} reloading was carried out either in the presence or in the absence of halothane. The amount of Ca^{2+} sequestered in the $InsP_3$-sensitive Ca^{2+} pools during the reloading period was assayed by a third application of bradykinin in Ca^{2+}-free conditions. These experiments indicated that the release of Ca^{2+} after reloading in the absence of halothane was always larger than that measured when the reloading was carried out in the presence of halothane in the bathing medium. This observation provides evidence that the decrease in cytosolic Ca^{2+} initiated by halothane cannot be attributed to the stimulation of a Ca^{2+} sequestration process.

Evidence of an inhibitory action of halothane on the agonist-evoked Ca^{2+} influx in BAE cells was also obtained from fura-2 experiments in which Mn^{2+} was used as a quenching agent. Previous studies established that, as internal Ca^{2+} stores release Ca^{2+}, entry of extracellular Ca^{2+} and Mn^{2+} in endothelial cells is enhanced.[32] The bradykinin-evoked Ca^{2+} influx in BAE cells was thereby estimated by measuring the rate at which 1 mM Mn^{2+} in the external medium induced quenching of the intracellular fura-2 signal in the presence and/or absence of halothane. Figure 4A shows that the fluorescence intensity at 358 nm remained unchanged, as expected, after bradykinin stimulation in Ca^{2+}-free external conditions. However, a clear decrease in fura-2 fluorescence was initiated by superfusing the cells with a solution that contained both Ca^{2+} (1.8 mM) and Mn^{2+} (1 mM). In addition, the rate of fura-2 quenching was lower in the presence of halothane in the external medium, indicating an inhibition of the Ca^{2+} influx pathway under these conditions. Figure 4B summarizes the results obtained at 0.8 mM and 2 mM halothane on four different cell preparations. A significant level of inhibition (42–64%) was observed with 2 mM halothane for all the cell preparations considered, but halothane at a concentration of 0.8 mM appeared effective in only two of the cell preparations with inhi-

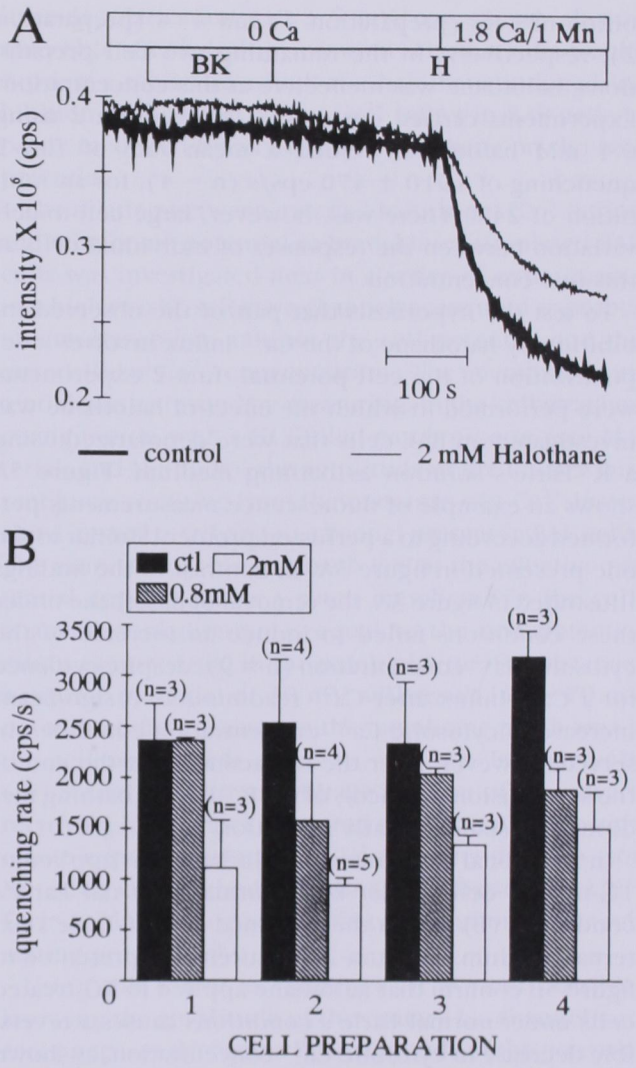

Fig. 4. Effects of halothane (H) on bradykinin (BK)-evoked capacitative Ca^{2+} influx. (*A*) Ca^{2+} influx assayed by measuring the rate of internal fura-2 quenching by 1 mM Mn^{2+}. The dark and light curves refer to the experiments carried out in the absence and presence, respectively, of 2 mM halothane. The capacitative Ca^{2+} influx was initiated by bath application of 10 nM bradykinin under external Ca^{2+}-free conditions. (*B*) Histogram describing the rate of fura-2 quenching by Mn^{2+} computed according to the procedure described in the Materials and Methods section as a function of the halothane concentration for four different cell preparations. The numbers in parentheses indicate the number of experiments carried out in each case.

bition of 41% (preparation 4) and 47% (preparation 2), respectively. In the remaining two cell preparations, halothane was ineffective at this concentration. Experiments carried out on cell preparation 2 using 0.4 mM halothane yielded a mean rate of fura-2 quenching of 1910 ± 470 cps/s (n = 4), for an inhibition of 24%. There was, however, large cell-to-cell variation between the responses of individual cells at this low concentration.

To test the hypothesis that part of the observed inhibition by halothane of the Ca^{2+} influx involves a depolarization of the cell potential, fura-2 experiments were performed in which the effect of halothane was investigated on BAE cells that were depolarized using a K^+-Earle's solution as bathing medium. Figure 5A shows an example of fluorescence measurements performed according to a perfusion protocol similar to the one presented in figure 3A. In contrast to the findings illustrated in figure 3A, the removal of halothane under these conditions failed to induce an increase of the cytosolic Ca^{2+} concentration (n = 9), despite evidence for a Ca^{2+} influx after Ca^{2+} readmission. A significant increase in cytosolic Ca^{2+} concentration could be observed, however, after the replacement, at the end of the superfusion protocol, of the K^+-Earle's bathing medium by a standard Earle's solution (E).

In additional experiments, halothane was applied to TG-treated cells under both normal external Earle's conditions (E) and in the presence of a K^+-Earle's external medium. The fura-2 measurements presented in figure 5B confirm that halothane applied to TG-treated cells under normal Earle's conditions causes a reversible decrease in cytosolic Ca^{2+} concentration, as shown previously in figure 2. A significant decrease in internal Ca^{2+} concentration was also initiated after the superfusion of the TG-treated cells with K^+-Earle's medium. Under these conditions, 2 mM halothane failed to cause a decrease in cytosolic Ca^{2+} concentration, indicating that the action of halothane on intracellular Ca^{2+} can be significantly impaired by maintaining the cells in a depolarized state. The results in figure 5B are representative of six other experiments carried out under the same conditions. In one experiment, however, a small decrease in cytosolic Ca^{2+} was observed after the superfusion of the cells with an external solution that contained 2 mM halothane. The amplitude of the Ca^{2+} decrease was significantly smaller than that measured under normal Earle's conditions, and the withdrawal of halothane did not result in a transient Ca^{2+} rise (data not shown).

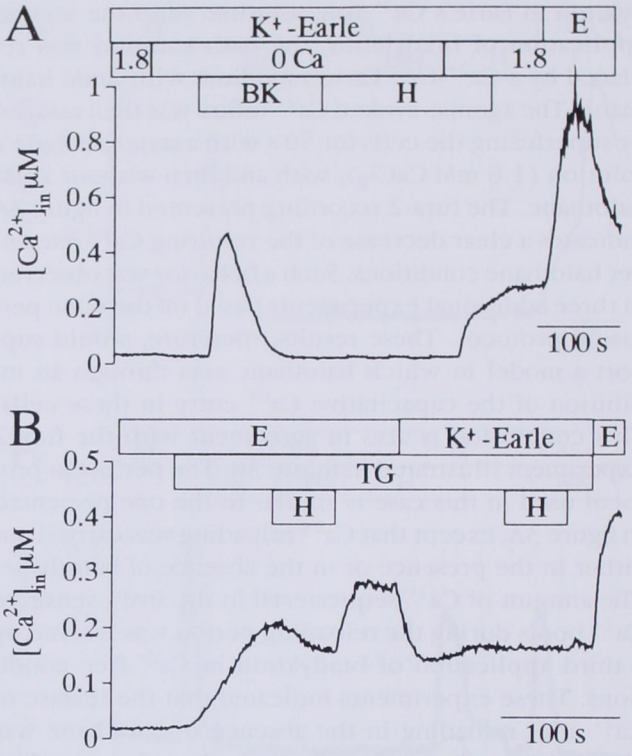

Fig. 5. Effects of halothane (H) on the capacitative Ca^{2+} influx in BAE cells after cell depolarization. (*A*) Effect of 2 mM halothane on depolarized cells after pool depletion *via* 10nM bradykinin receptor stimulation. The cells were depolarized using K^+-Earle's solutions. Perfusion protocol as in figure 3A. In contrast to the results presented in figure 3A, the removal of halothane failed to initiate an additional Ca^{2+} increase under K^+-Earle's conditions. The resulting Ca^{2+} increase observed when cells were repolarized by perfusing with a standard Earle's solution (E) confirmed the voltage dependence of the capacitative Ca^{2+} influx in these cells. (*B*) Effect of 2 mM halothane on depolarized cells treated with 500 nM of the Ca^{2+} pump inhibitor thapsigargin. Bath application of halothane was first carried out under standard Earle's conditions (E). Halothane was then applied on depolarized cells bathed in a K^+-Earle's external solution. Halothane was ineffective in reducing the Ca^{2+} level under these conditions.

To determine the effect of halothane on membrane potential, whole cell experiments were performed on mechanically dissociated BAE cells in zero current clamp conditions, using 200 mM KCl + 0.2 µM Ca^{2+} filled patch electrodes (see Materials and Methods). An example of the resulting voltage changes is presented in figure 6A. As expected, the superfusion with an external solution containing 10 nM bradykinin caused an initial hyperpolarization of the cell potential, the magnitude of which ranged from −45 mV to −55 mV (mean −49 ± 4 mV; n = 4). An external application of halothane under these conditions induced a significant cel-

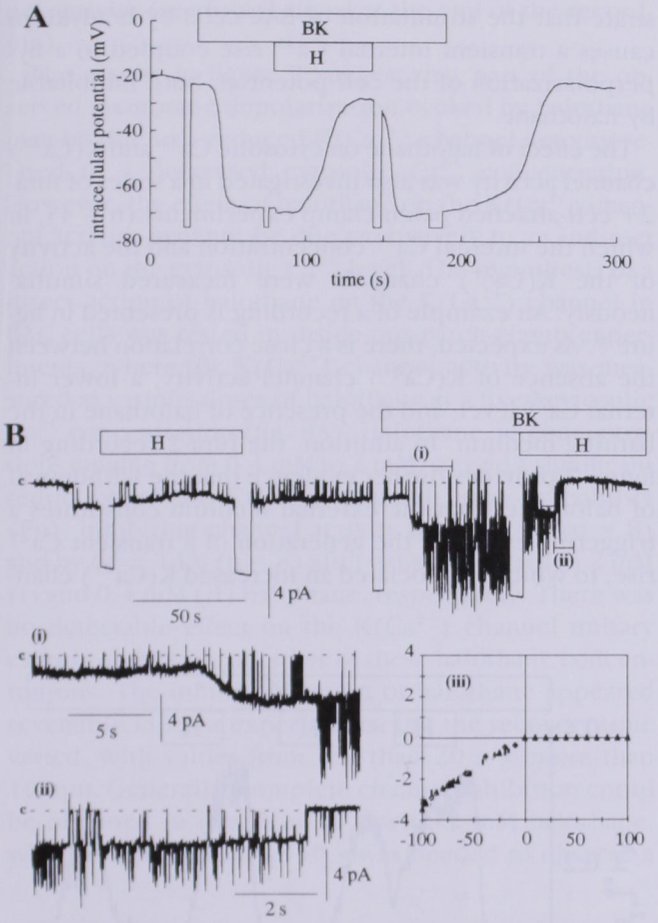

Fig. 6. Effects of halothane (H) on the intracellular potential of bradykinin (BK)-stimulated cells. (*A*) Whole cell experiment carried out in zero current conditions using a patch electrode filled with a 200 mM KCl + 0.2 μM free Ca^{2+} solution. The bathing medium was an Earle's solution. Under control conditions, the cell potential was −21 mV. Superfusion with bradykinin caused a 47-mV hyperpolarization that could be reversed by the addition of 2 mM halothane. (*B*) Single channel activity recorded in the cell-attached configuration. Cells were superfused continuously with a normal Earle's medium. The pipette was filled with a 200 mM KCl + 1 μM free Ca^{2+} solution, and a potential of 30 mV was applied throughout. Expanded portions of the original recording are illustrated in (*i*) and (*ii*). The initial portion of the recording shows a single channel activity typical of the inward rectifying I_{K1} channel. After bradykinin stimulation, the amplitude of I_{K1} increased, indicating a 63-mV hyperpolarization of the cell potential [see enlargement (*i*)]. This increase was correlated with the activation of a $K(Ca^{2+})$ channel of 40 pS conductance. The addition of halothane led to a reduction of the I_{K1} current jump amplitude compatible with a depolarization of more than 47 mV of the cell potential, and to a marked inhibition of the $K(Ca^{2+})$ channel activity [see enlargement (*ii*)]. The current/voltage relation of the inward rectifying I_{K1} channel is presented in (*iii*). The current/voltage curve was measured in cell-attached, patch-clamp experiments with KCl-containing pipettes on BAE cells bathed in a K^+-Earle's solution, to abolish the contribution of the cell resting potential.

lular depolarization (39 ± 5 mV, n = 4), which was reversible after halothane removal. These results would support a model in which the halothane-induced inhibition of the capacitative Ca^{2+} influx in BAE cells is partly mediated by an action of halothane on the cell potential.

The relation between external halothane, Ca^{2+} influx, and membrane potential in bradykinin-stimulated BAE cells was investigated next in a series of experiments in which two K^+ selective channels were used as probes to simultaneously monitor the cell potential and the intracellular Ca^{2+} concentration. The membrane potential was estimated by measuring, in the cell-attached configuration with a KCl-filled patch pipette (see Materials and Methods), the amplitude of the unitary current jump resulting from the openings of a Ca^{2+}-insensitive inward rectifying I_{K1} channel present in BAE cells. The rationale of this approach is that the effective potential applied across a patch membrane in the cell-attached configuration is equal to the cell potential minus the potential in the patch pipette. For instance, a pipette potential of 30 mV will result in a −70 mV potential difference across the membrane patch area if the cell potential is equal to −40 mV. In figure 6B, (iii) shows the current/voltage relation of the inward rectifying I_{K1} channel measured in cell-attached, patch-clamp experiments with KCl-containing pipettes (see Materials and Methods) on BAE cells bathed in a K^+-Earle's solution, to abolish the contribution of the cell resting potential.[20]

Using this curve as reference, it is then possible, knowing the amplitude of the unitary I_{K1} channel current jumps, to determine the magnitude of the potential acting on the I_{K1} channel. Because the pipette potential is maintained at a constant known value, it follows that any variation of the I_{K1} channel unitary current jump amplitude is a reflection of a variation in intracellular potential. Cell-attached experiments can, therefore, be used to directly monitor the cell potential during bradykinin stimulation, with no significant disturbance of the cytoplasmic medium. Similarly, the fluctuations in intracellular Ca^{2+} concentration in BAE cells can be estimated by monitoring the changes in activity of the $K(Ca^{2+})$ channels present in the BAE cell plasma membrane. Unlike the $K(Ca^{2+})$ channel of large conductance measured in a variety of excitable and nonexcitable cells, the $K(Ca^{2+})$ channel identified in BAE cells exhibits inward rectification, with a slope conductance of 40 pS and 10 pS for inward and outward currents, respectively. In inside-out patch clamp experiments,

this channel was activated at submicromolar cytosolic Ca^{2+} concentrations, and channel activity appeared voltage insensitive within the voltage range −100 mV to 0 mV.[16] As a consequence, both I_{K1} and $K(Ca^{2+})$ constitute endogeneous probes that can be used to simultaneously record changes in cell potential and internal Ca^{2+} concentration.

Figure 6B shows an example of single channel recording in which both I_{K1} and $K(Ca^{2+})$ are present.[20] Downward current deflections corresponding to spontaneous I_{K1} channel long openings were observed before the first addition of halothane to the external medium. The pipette potential was maintained at 30 mV throughout, and the measured unitary current amplitude corresponded to 1.8 pA. Because a potential difference of 53 mV is known to be required to generate unitary current jumps of 1.8 pA amplitude, it was concluded in this case that the cell resting potential was equal to −23 mV. This value is in good agreement with the whole cell measurements reported by Mehrke *et al.*[33] and the zero current clamp results in figure 6A.

It should also be apparent from this recording that the application of 2 mM halothane, before bradykinin stimulation, did not result in any significant changes in the current fluctuation pattern. A small decrease of the I_{K1} unitary current amplitude from 1.8 pA to 1.5 pA could, nevertheless, be detected, an observation compatible with a 9-mV depolarization of the cell potential. In contrast, a subsequent superfusion with a solution that contained 10 nM bradykinin resulted in an increase of the I_{K1} channel unitary current jump amplitude from 1.6 pA to 3.8 pA, indicating a 63-mV hyperpolarization of the cell potential, to a value close to −86 mV [see enlargement (i)]. In addition, the presence of bradykinin in the external medium caused the appearance of current bursts, characterized by rapid channel openings, superimposed on the I_{K1} slow current fluctuations [see enlargement (i)]. These fast openings were due to the activation of $K(Ca^{2+})$ channels in response to the bradykinin-evoked increase in cytosolic Ca^{2+} concentration. Under these conditions, the addition of halothane (2mM) to the bathing medium led to a marked reduction in $K(Ca^{2+})$ channel activity concomitant to a decrease of the I_{K1} channel unitary current jump amplitude to 2.4 pA [see enlargement (ii)]. These observations were confirmed in four identical experiments, which showed an average reduction in unitary current amplitude of 1.5 ± 0.4 pA (n = 5), signifying an average halothane-induced cell depolarization of 47 ± 10 mV. These results demonstrate that the stimulation of BAE cells by bradykinin causes a transient internal Ca^{2+} rise coupled to a hyperpolarization of the cell potential, both inhibitable by halothane.

The effect of halothane on cytosolic Ca^{2+} and $K(Ca^{2+})$ channel activity was also investigated in a series of fura-2+ cell-attached patch-clamp experiments (n = 4), in which the internal Ca^{2+} concentration and the activity of the $K(Ca^{2+})$ channels were measured simultaneously. An example of a recording is presented in figure 7. As expected, there is a close correlation between the absence of $K(Ca^{2+})$ channel activity, a lower internal Ca^{2+} level, and the presence of halothane in the bathing medium. In addition, the fura-2 recording in figure 7 provides further evidence that the withdrawal of halothane from the external medium constitutes a triggering event for the generation of a transient Ca^{2+} rise, to which is associated an increased $K(Ca^{2+})$ chan-

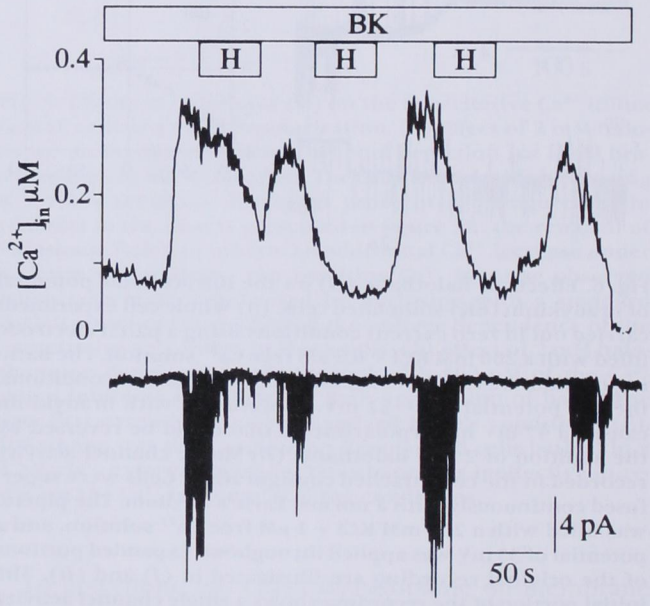

Fig. 7. Correlation between $K(Ca^{2+})$ channel activity and cytosolic Ca^{2+} fluctuations in bradykinin (BK)-stimulated cells exposed to pulse applications of halothane (H). Combined cell-attached + fura-2 experiment carried out on BAE cells stimulated with 10 nM bradykinin. The patch pipette was filled with a 200 mM KCl + 1 μM free Ca^{2+} solution, and the bath contained a normal Earle's medium. The potential in the patch electrode was 30 mV throughout. $K(Ca^{2+})$ channel activity was abolished systematically in the presence of halothane, despite minor variations of the Ca^{2+} level during the first perfusion with halothane. In subsequent halothane pulses, halothane caused a decrease in internal Ca^{2+} concentration that was correlated with a marked reduction in the $K(Ca^{2+})$ channel activity.

nel activity (see fura-2 signal at the end of the recording).

The results in figure 7 suggest that part of the observed membrane depolarization evoked by halothane may be due to a reduced $K(Ca^{2+})$ channel activity related to a decreased cytosolic Ca^{2+} concentration. However, the effect of halothane on the $K(Ca^{2+})$ channel activity may not be due exclusively to an indirect action on the cytosolic Ca^{2+} level. The hypothesis of a direct action of halothane on the $K(Ca^{2+})$ channel in BAE cells was tested in inside-out patch-clamp experiments, where the $K(Ca^{2+})$ channel activity was measured at various doses of halothane at a fixed cytosolic Ca^{2+} concentration (fig. 8A). Halothane, at concentrations ranging from 0.4 mM to 2 mM, caused a significant reduction of the $K(Ca^{2+})$ channel open probability (Po), inhibiting channel activity by 96 ± 3% (n = 8) and by 65 ± 14% (n = 8) after 40-s exposures to 2 mM (i) and 0.4 mM (ii) halothane, respectively. There was no detectable effect on the $K(Ca^{2+})$ channel unitary current amplitude at either of these halothane concentrations. The inhibitory action of halothane appeared reversible in most experiments, but the recovery time varied, with values from less than 20 s to more than 10 min. Generally, complete channel inhibition could be obtained in less than 10 s with 2 mM halothane, whereas a minimum of 40 s was needed to observe a stable reduction in channel activity at 0.4 mM. Channel inhibition could also be observed in the presence of isoflurane (fig. 8B). Reductions of channel activity varying from 35% to 85% (mean 52 ± 16%; n = 6) were measured with 0.5 mM isoflurane (i). Similar values were obtained with 1 mM isoflurane (ii), in which a mean inhibition of 54 ± 15% (n = 4) was observed (fig. 8). These two volatile anesthetics, therefore, constitute potent $K(Ca^{2+})$ channel blockers.

Discussion

Our data suggest that part of the effects exerted by halothane and isoflurane on the Ca^{2+} response of endothelial cells after bradykinin stimulation is related to a depolarization of the cell potential resulting from the inhibition of a $K(Ca^{2+})$ channel. Through this mechanism, halothane would decrease the capacitative Ca^{2+} influx linked to the mobilization of internal Ca^{2+} pools by decreasing the electrochemical driving force acting on Ca^{2+} ions.

Effects of Halothane on Ca^{2+} Mobilization

In several studies, it was reported that halothane impairs Ca^{2+} retention in isolated cardiac sarcoplasmic reticulum vesicles by increasing passive Ca^{2+} efflux.[21,34]

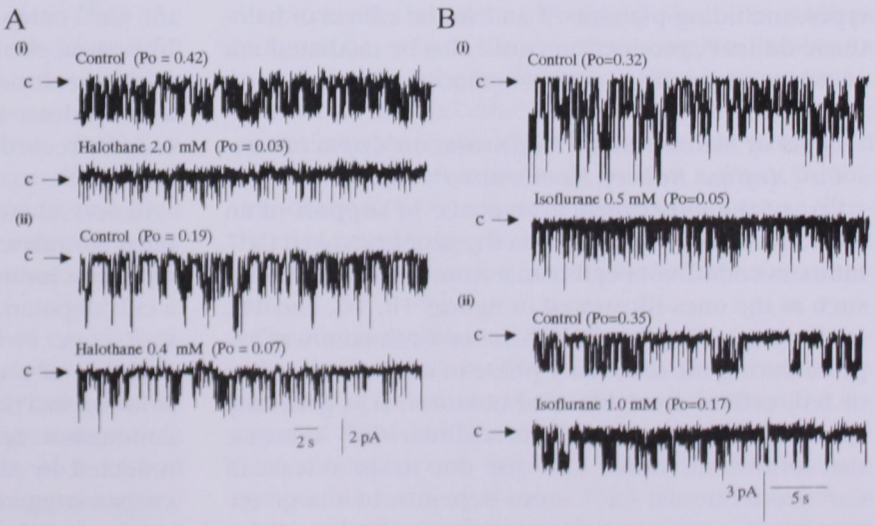

Fig. 8. Direct inhibition by halothane and isoflurane of $K(Ca^{2+})$ channel activity. Single channel activity measured in the inside-out, patch-clamp configuration. The patch pipette was filled with a 200 mM KCl + 1 μM free Ca^{2+} solution, and the bath was superfused with a 200 mM KCl solution containing 0.8 μM free Ca^{2+} plus halothane or isoflurane at various concentrations. Single channel activity was measured in control conditions and 40 s after perfusing with the anesthetics. The applied potential was equal to 60 mV throughout. (*A*) (*i*) Effect of 2 mM halothane. In this example, halothane caused a 94% inhibition of the channel activity. (*ii*) Effect of 0.4 mM halothane. A 66% reduction in channel activity was observed in this case. (*B*) (*i*) Effect of 0.5 mM isoflurane. In this example, isoflurane caused a 83% inhibition of the open probability. (*ii*) Effect of 1 mM isoflurane. A 52% reduction in channel activity was observed in this case. The channel open probability was estimated from current amplitude histogram taken throughout 30-s periods. Arrows (*c*) point to zero current level of channel.

This effect appeared to be linked to the activation by halothane of the sarcoplasmic reticulum Ca^{2+} release channel.[35] However, despite evidence for the existence of functional caffeine-sensitive Ca^{2+} pools in BAE cells,[9,29] our experiments (data not shown) failed to detect any significant rise in cytosolic Ca^{2+} after bath application of halothane on resting cells. Our observations are, therefore, in accordance with those of Loeb *et al.*,[23] who studied the alteration by volatile anesthetics of Ca^{2+} mobilization in BAE cells. In addition, the fura-2 results in figure 1A showed that the initial Ca^{2+} rise in response to bradykinin stimulation is not affected by halothane. This is at variance with the findings published by Loeb *et al.*,[23] who reported that Ca^{2+} transients elicited by brief applications of bradykinin were significantly inhibited by halothane but not isoflurane. It is clear, however, from figure 2 that the production of $InsP_3$ is not essential to the halothane-induced decrease in cytosolic Ca^{2+} concentration. This would be in agreement with the results of Kress *et al.*[36] and Stern *et al.*[37] on neuronal cells, which suggested that halothane at clinical concentrations (<0.8 mM) does not affect the phosphatidyl inositol signalling pathway nor significantly depress the $InsP_3$-induced Ca^{2+} mobilization. Nevertheless, it must be pointed out that, in the absence of direct measurements of the $InsP_3$ and $InsP_4$ levels in cells exposed to halothane, we cannot entirely rule out that this agent exerts part of its inhibitory action *via* the phosphatidyl inositol pathway. In addition, halothane has been reported to affect cyclic adenosine monophosphate production in several cell types, including platelets,[38] and so the effects of halothane on $InsP_3$ production could also be mediated *via* an action on cyclic adenosine monophosphate.

Effect of Halothane or Isoflurane on Capacitative Ca^{2+} Influx: Role of Membrane Potential

Part of the experimental evidence in support of an effect of volatile anesthetics on the agonist-evoked Ca^{2+} influx in endothelial cells came from fura-2 recordings such as the ones illustrated in figures 1B, 1C, and 1D. In these experiments, halothane or isoflurane was applied during the secondary phase of the Ca^{2+} response of BAE cells to bradykinin stimulation. It is generally agreed that the sustained intracellular Ca^{2+} increase that follows the initial Ca^{2+} rise due to the release of Ca^{2+} from internal Ca^{2+} stores depends, to a large extent, on the entry of Ca^{2+} from the external medium. The fact that halothane and isoflurane caused a significant decrease of the cytosolic Ca^{2+} concentration during the external Ca^{2+}-dependent phase of the BAE cell response to bradykinin stimulation provides indirect support for an action of volatile anesthetics on the agonist-evoked Ca^{2+} entry mechanism in these cells.

More importantly, it is clear from the fura-2 measurements presented in figures 1B, 1C, 1D, 2, and 7 that the withdrawal of halothane or isoflurane from the external medium led to transient Ca^{2+} increases whose peak values could exceed the Ca^{2+} level measured before the first exposure of the cell to the anesthetic (fig. 2). Similar transient changes in cytosolic Ca^{2+} concentration were reported for a variety of nonexcitable cells, including BAE cells, upon readmission of external Ca^{2+} after a short incubation in Ca^{2+}-free solution.[39,40] The transient Ca^{2+} increases measured under these conditions were usually interpreted as reflecting an entry of Ca^{2+} *via* a Ca^{2+}-permeable pathway activated by the decreased state of filling of the internal Ca^{2+} stores (capacitative influx).[31]

These results are also in agreement with the observation illustrated in figure 3A, in which the removal of halothane enhanced the Ca^{2+} rise initiated upon readmission of external Ca^{2+} after Ca^{2+} pool depletion. One likely explanation compatible with the results in figures 1B, 1C, 1D, 2, and 7 would be that the withdrawal of halothane or isoflurane removed the volatile anesthetic-induced inhibition of the capacitative Ca^{2+} influx evoked either by bradykinin or TG. The exact mechanism underlying the time course of the transient peaks initiated after Ca^{2+} readmission or anesthetic removal is not yet fully resolved. One possibility would be that the Ca^{2+} entry is inactivated at high and reactivated at low intracellular Ca^{2+}.[40] This would be in line with the observation of Vaca and Kunze,[41] who reported that a Ca^{2+}-release activated Ca^{2+} current in BAE cells could only be recorded in the presence of very low intracellular Ca^{2+}.

In several studies, researchers indicated that the agonist-stimulated Ca^{2+} influx in several endothelial cell types, including BAE cells, is decreased in response to a cell depolarization.[18,19] The diminution in cytosolic Ca^{2+} concentration shown in figure 5B after the substitution of a normal Earle's medium by a K^+-Earle's solution in TG-stimulated BAE cells fully supports this conclusion. Schilling[19] reported that the Ca^{2+} influx, reflected by the amplitude of the transient Ca^{2+} increases triggered by readmission of external Ca^{2+} after exposure of bradykinin-stimulated BAE cells to a Ca^{2+}-free external solution, was reduced, but not abolished, in 150 mM external KCl. This would be in agreement

with the results presented in figure 5A, where a significant increase in internal Ca^{2+} could still be detected after the replacement of a Ca^{2+}-free K^+-Earle's medium by a Ca^{2+}-containing K^+-Earle's solution. The internal Ca^{2+} augmentation observed under these conditions is expected to reflect a capacitative Ca^{2+} influx, because control experiments indicated no changes in cytosolic Ca^{2+} concentration after the replacement of a K^+-Earle's solution by a Ca^{2+}-free K^+-Earle's bathing medium in resting cells (data not shown). However, in contrast to the fura-2 recording presented in figure 3A, the removal of halothane in K^+-Earle's conditions did not result in an increased intracellular Ca^{2+} concentration (fig. 5A). Similarly, the withdrawal of halothane from a K^+-Earle's medium illustrated in figure 5B failed to initiate a detectable transient Ca^{2+} rise, as was observed with the same cells when bathed in a normal Earle's solution (see also fig. 2). An absence of halothane-induced Ca^{2+}-decrease and/or cytosolic Ca^{2+} rise after halothane removal was confirmed in five additional experiments carried out on TG-treated cells superfused with a K^+-Earle's medium. These observations provide strong evidence that a depolarization of the cell potential is sufficient to impair the effects of halothane on internal Ca^{2+}, suggesting that most of the inhibitory action of halothane involves a depolarization of the cell potential. Because cells bathed in a K^+-Earle's medium are maintained in a depolarized state, there should be a minimal impact on the cell potential due to an inhibition by halothane of the $K(Ca^{2+})$ conductance, thereby explaining the lack of halothane effects on Ca^{2+} influx, as observed. In addition, it is clear from the fura-2 measurements illustrated in figures 5A and 5B that reperfusion with a normal Earle's solution after exposure to a K^+-Earle's medium causes an important increase of the cytosolic Ca^{2+} concentration. This latter result would be in agreement with the expected voltage dependency of the capacitative Ca^{2+} influx triggered either by TG or bradykinin, in which more negative membrane potential values have been associated with an enhanced Ca^{2+} entry.[19]

The variation in membrane potential of bradykinin-stimulated BAE cells in response to halothane application is illustrated in figure 6. The bradykinin-evoked hyperpolarization shown in fig 6A is in agreement with the potential measurements reported by Mehrke and others in whole-cell patch-clamp experiments carried out on cultured BAE cells.[17,33] More importantly, figure 6A provides direct evidence of a halothane-evoked depolarization with a magnitude comparable to the hyperpolarization triggered by bradykinin. These measurements offer additional support to the conclusions drawn from the cell-attached experiments illustrated in figure 6B, where the amplitude of the current jumps resulting from the openings of the Ca^{2+}-independent IK_1 channel was used to monitor membrane potential changes. It is clear, from these experiments, that part of the halothane action on the Ca^{2+} response of BAE cells to bradykinin involves a depolarization of the cell potential, which is expected, in turn, to contribute to the Ca^{2+} influx inhibition observed in halothane conditions. In addition, figure 6B shows that there is a correlation between the presence of halothane in the external medium, a depolarization of the cell potential, and a reduced activity of the $K(Ca^{2+})$ channels present in these cells. These results suggest that the halothane-induced depolarization reported in figure 6A is related to an inhibition of the $K(Ca^{2+})$ channels under these conditions.

A direct inhibitory action of halothane on the $K(Ca^{2+})$ channel in BAE cells was observed in a series of inside-out patch-clamp experiments (fig. 8). Halothane was reported to decrease the activity of a large variety of potassium selective channels, including several Ca^{2+}-activated potassium channels.[42–46] The halothane concentration needed for half inhibition of the $K(Ca^{2+})$ channel of large conductance in smooth muscle cells[42] and for the intermediate conductance $K(Ca^{2+})$ channel of red blood cells[44] was evaluated at 0.5 mM, a value in agreement with the results presented in figure 8. The inside-out measurements presented in figure 8, therefore, are consistent with the whole cell recordings in figure 6A, which show a near complete inhibition by halothane of the bradykinin-evoked hyperpolarization. However, the effect of halothane and isoflurane on the activity of the $K(Ca^{2+})$ channels in stimulated BAE cells may not exclusively involve a direct inhibition of the $K(Ca^{2+})$ channel gating processes, as illustrated in figure 8, but may also include an indirect effect coming from a decreased intracellular Ca^{2+} concentration.

The fura-2 + cell-attached recordings in figure 7 provide direct evidence for a correlation between the presence of halothane in the external medium, an absence of $K(Ca^{2+})$ channel activity, and a low cytosolic Ca^{2+} concentration. Together, these observations are compatible with a positive feedback mechanism in which volatile anesthetics inhibit the $K(Ca^{2+})$ channels in BAE cells directly, thereby causing a depolarization of the cell potential, a reduced Ca^{2+} influx, and a low-

ering of the internal Ca^{2+} concentration (figs. 1C, 1D, 2, and 7). This latter effect should, in turn, fuel the depolarization process by enhancing the inhibition of the $K(Ca^{2+})$ channel activity. However, despite the importance of this mechanism, we cannot totally rule out a direct interaction of the anesthetics, with the structures responsible for the agonist-evoked Ca^{2+} entry. For instance, isoflurane was found to initiate a clear decrease in Ca^{2+} concentration in bradykinin-stimulated cells (fig. 1D), without totally inhibiting $K(Ca^{2+})$ channel activity (fig. 8). These observations suggest either that a total inhibition of the $K(Ca^{2+})$ channel activity may not be required to initiate a cell depolarization of sufficient magnitude to impair the bradykinin-evoked Ca^{2+} influx, or that part of the effect of halothane and isoflurane on Ca^{2+} influx involves a direct inhibition of the Ca^{2+} entry mechanism. In fact, there is increasing evidence that volatile anesthetics, such as halothane, bind competitively to specific hydrophobic regions of proteins.[47] Because halothane has been reported to interact with a large variety of ionic channels, this anesthetic is likely to affect Ca^{2+} permeable channels, such as the second messenger-operated Ca^{2+} channel described by Lückhoff and Clapham[48] in BAE cells or the Ca^{2+} permeable channel activated by Ca^{2+} store depletion reported by Vaca and Kunze[41] using the same cell preparation. Such inhibitory effects would contribute to further decreasing the capacitative Ca^{2+} influx, leading to a more significant reduction, by halothane or isoflurane, of the cell internal Ca^{2+} concentration. The significance of this mechanism for BAE cells remains to be established, because no additional effect of halothane accountable by a direct interaction with the Ca^{2+} entry machinery was observed in cells maintained in a depolarized state (fig. 5).

Conclusion

The cardiovascular effects of halothane and isoflurane are likely to be manifold. Our results show that halothane and isoflurane decrease the Ca^{2+} availability in endothelial cells mainly through an action on the agonist-evoked Ca^{2+} entry. This phenomenon involved an effect on the cell membrane potential characterized by a cell depolarization consequent to an inhibition by the volatile anesthetics of the $K(Ca^{2+})$ channels present in these cells. The resulting decrease in Ca^{2+} availability is likely to interfere with NO production by modulating the NO synthase activity. However, the overall contribution of this mechanism to the inhibition by halothane and isoflurane of the endothelium-dependent vascular smooth muscle relaxation process needs further investigation. Other mechanisms, such as a direct effect of halothane on NO stability, were shown to play a significant role in this regard.[7] Similarly, Johns *et al.*[49] provided evidence that halothane could affect the activity of the NO synthase independently of cytosolic Ca^{2+} variations. An action of halothane and isoflurane on internal Ca^{2+} homeostasis would, nevertheless, constitute a general phenomenon that would affect not only NO production but also the entire Ca^{2+} signalling process in these cells.

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