0022-3565/2542-0552$02.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 254, No. 2
Printed in U.S.A.

Halothane Increases Cytosolic Ca^{2+} and Inhibits Na^+/H^+ Exchange in L6 Muscle Cells[1]

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Accepted for publication May 4, 1990

ABSTRACT

The effects of the general anesthetic halothane on the concentration of cytosolic free calcium ($[Ca^{2+}]_i$) and cytosolic pH (pH_i), were investigated in L_6 rat skeletal muscle cells. Basal $[Ca^{2+}]_i$ was 169 ± 8 nM, measured with the fluorescent Ca^{2+}-indicator 1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2′-amino-5-methylphenoxy)ethane-N,N,N′,N′-tetra-acetate. Halothane (5.7 mM) increased $[Ca^{2+}]_i$ to 255 ± 15 nM in the presence of extracellular Ca^{2+}, and from 137 ± 6 nM to 179 ± 9 nM in Ca^{2+} absence. This increase was dose-dependent. The anesthetic released about 50% of the releasable Ca^{2+} from intracellular stores. The resting pH_i of L_6 cells was 7.24 ± 0.04, measured with the fluorescent pH indicator bis-carboxyethylcarboxyfluorescein. Halothane did not affect resting pH_i, but inhibited cytoplasmic alkalinization by hypertonicity or cytoplasmic acidification: (1) The hypertonicity-induced alkalinization *via* activation of Na^+/H^+ exchange (to 7.50 ± 0.08, initial rate 0.10 ± 0.02 pH U/min) was inhibited with 5.7 mM halothane by 67%. (2) Acid-loaded cells (pH_i 6.43 ± 0.01 in cells) recovered towards neutrality *via* activation of Na^+/H^+ exchange (rate 0.47 pH U/min), and halothane inhibited the rate of pH_i recovery by 50%. The halothane-mediated inhibition of alkalinizations after hypertonic exposure or acid-loading was also observed in bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetra-acetate-loaded cells in Ca^{2+}-free medium. Therefore, halothane increases $[Ca^{2+}]_i$ and in parallel inhibits Na^+/H^+ exchange, compromising the ability of muscle cells to recover from imposed acidification.

The mechanism of action of general anesthetics is unknown. One hypothesis proposes that a contributing cause to the depression of neuronal excitability by hydrocarbonic anesthetics such as halothane is a hyperpolarization of neurones, secondary to a rise in potassium conductance of the plasma membrane (Franks and Lieb, 1988). The anesthetics are thought to activate a Ca^{2+}-sensitive K^+ channel by raising the concentration of free $[Ca^{2+}]_i$ (Krnjevic, 1986). Consistent with this hypothesis, we have recently reported that halothane increases $[Ca^{2+}]_i$ in lymphocytes of both normal and MH-susceptible patients (Klip *et al.*, 1987; Klip *et al.*, 1990). This increase in $[Ca^{2+}]_i$ in circulating cells may reflect similar effects of the anesthetic in neuronal and muscle cells. However, there is presently no information on the effect of halothane on $[Ca^{2+}]_i$ in muscle cells.

The effect of general anesthetics of the fluorinated hydrocarbon family on skeletal muscle cells is of primary importance, since skeletal muscle is the tissue involved in the MH crisis that occurs in response to such anesthetics in susceptible individuals and animals (Gronert, 1980; Nelson and Flewellen, 1983; O'Brien *et al.*, 1990). The crises are characterized by muscle hypercatabolism causing hyperthermia, and by concomitant muscle rigidity in 75% of all cases. Although there is no information on the effect of halothane on $[Ca^{2+}]_i$ in normal muscle cells, $[Ca^{2+}]_i$ has been found to be elevated in muscle fibers of MH-susceptible humans (Lopez *et al.*, 1985) relative to controls, and in MH-susceptible pigs before (Lopez *et al.*, 1986) and after (Lopez *et al.*, 1988) a MH crisis. In addition to the hypercatabolism and hyperthermia, the pH of muscle of MH-susceptible pigs is abnormally low immediately after a crisis, as demonstrated by measurements of muscle lactic acid content *in vivo* (Gronert *et al.*, 1986) and immediately after slaughter (Chambers and Hall, 1987). This drop in muscle pH after a MH crisis has been ascribed to the preceding hypercatabolism resulting in overproduction of lactic acid and other metabolic H^+ (Gronert *et al.*, 1986), yet other defects in the mechanisms maintaining pH homeostasis are also possible. The effects of halothane on intracellular pH in normal muscle have not been reported.

In the present study we investigated the effects of halothane

Received for publication November 7, 1989.
[1] This work was supported by a grant from the Muscular Dystrophy Association of Canada.
[2] Recipient of a Medical Research Council Scientist Award.

ABBREVIATIONS: $[Ca^{2+}]_i$, cytoplasmic concentration of ionized Ca^{2+}; pH_i, cytoplasmic pH; MH, malignant hyperthermia; EGTA, [ethylene bis(oxyethylene nitrilo)]tetra-acetate; Indo-1, 1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2′-amino-5′-methylphenoxy)ethane-N,N,N′,N′-tetra-acetate; AM, acetoxymethyl ester; BAPTA, bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetra acetate; HEPES-RPMI, N-2-hydroxyethyl-piperazine-N′-2-ethane-sulfonic acid buffered RPMI 1640 cultured medium; TPA, tetradecanoyl phorbol acetate; BCECF, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein; MMNA, *N*-methyl-*N*-[(2-methylpropyl)amino]amiloride.

on $[Ca^{2+}]_i$ and on pH_i in skeletal muscle cells of the L_6 line. The L_6 cells were selected for this study since they express many characteristics of adult muscle, including the ability to synthesize muscle actin, myosin and acetylcholine receptors, to fuse into myotubes and to develop action potentials and contractile responses (Shainberg *et al.*, 1971; Kidokoro, 1975). In contrast to adult muscles, which are composed of diverse fiber types, the cell line is of homogeneous cell origin. We have previously studied the mechanisms that maintain $[Ca^{2+}]_i$ homeostasis (Klip *et al.*, 1984, 1986b), as well as the regulation of pH_i by growth factors (Klip *et al.*, 1986c) in L_6 muscle cells. Here we demonstrate that halothane increases $[Ca^{2+}]_i$ by a mechanism involving release of Ca^{2+} from intracellular stores, and further we report that the anesthetic concomitantly inhibits ion exchange through the Na^+/H^+ antiport, impairing the ability of the cells to recover from an imposed acidification.

Materials and Methods

Materials and solutions. Culture medium RPMI 1640 (10×) bicarbonate free, α-minimal essential medium, fetal bovine serum, antibiotic/antimycotic solution (100×) and sterile trypsin solution were from Grand Island Biological Company. Indo 1-free acid and Indo 1/AM and BCECF/AM were from Molecular Probes. Ionomycin, nigericin, bovine serum albumin and [N,N,N′,N′-tetrakis-(2-pyridylmethyl)-ethylenediamine] were from Calbiochem. The Ca^{2+}-chelator precursor BAPTA/AM was from Calbiochem. Halothane was from Ayerst. TPA and monensin were from Sigma. MMNA was kindly provided by Dr. E. J. Cragoe Jr. of Merck, Sharp & Dohme Research Laboratories (West Point, PA).

Na solution contained, in mM: 140 NaCl, 3 KCl, 10 D-glucose, 20 HEPES, 1 $MgCl_2$, pH 7.3. Where indicated, this solution contained 1.5 mM $CaCl_2$, whereas calcium-free Na solution had no added calcium salts and contained 0.5 mM EGTA. K solution and choline solution have the same composition of Na solution except that NaCl is iso-osmotically substituted by KCl or choline chloride, respectively. HEPES-RPMI is bicarbonate-free RPMI containing 20 mM HEPES, pH 7.3.

Cell cultures. L_6 skeletal muscle cells were grown essentially as described previously (Klip *et al.*, 1986b). Briefly cell monolayers were grown to 70 to 80% confluency in 75-cm^2 Falcon tissue culture flasks. The cells were incubated in α-minimal essential medium supplemented with 2% fetal bovine serum and 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B at 37°C in a 5% CO_2 atmosphere. The medium was changed every 48 hr.

Suspension of L_6 cells. Growth medium was decanted from each flask and cells (1.5×10^6) were exposed to 4 ml 0.25% trypsin for 2 to 5 min. The trypsin was decanted, and 10 ml of growth medium were added. Shaking the flasks briskly removed the cells, which were then pelleted by centrifugation. Cells were resuspended in 10% fetal bovine serum-supplemented HEPES-RPMI and allowed to recover at 37°C for 30 min. Cells were agitated periodically during the incubation period. This procedure has been previously shown to yield viable, transport-competent (Klip *et al.*, 1984) and metabolically stable (Klip *et al.*, 1986b) cell suspensions.

Determination of $[Ca^{2+}]_i$. Indo-1 was used to measure cytoplasmic ionized calcium essentially as described earlier for 3T3-L_1 cells (Klip and Ramlal, 1987) and lymphocytes (Klip *et al.*, 1990). The cells from one flask (about 1.5×10^6 cells) were pelleted in 1.5-ml Eppendorf tubes in a Hettich Mikroliter centrifuge for 30 sec at 6000 rpm, resuspended in 1.0 ml HEPES-RPMI containing 2 μM Indo 1/AM and incubated at 37°C for 30 min. The tubes were wrapped in aluminum foil to protect them from light. Cells were resuspended periodically during this incubation. Extracellular Indo1/AM was removed by two centrifugation steps in HEPES-RPMI and the cells were resuspended in 250 μl of the same medium. Indo 1-loaded cells were maintained in the dark prior to fluorescence determinations. Twenty μl of cell suspension were dispersed into 1 ml of filtered Na solution containing 5 μM N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine, a heavy metal chelator. Fluorescence was recorded in a 650-40 Perkin-Elmer spectrofluorometer at 331 nm excitation (3-nm slit width) and 410 nm of emission (10-nm slit width). All determinations were performed at room temperature and thorough mixing was achieved by magnetic stirring. Where indicated, 4 μl of a 1:10 dilution of halothane in dimethyl sulfoxide were added through a Hamilton syringe. After halothane additions, the fluorescence readings changed as described in the text and remained constant for up to 3 min. Routinely, 1 to 2 min after addition of halothane the fluorescence calibration was initiated by permeabilizing the cells to calcium with the ionophore ionomycin (10 μM) to yield the maximum fluorescence, F_{max}, followed by displacement of calcium from its complex with Indo 1 by addition of $MnCl_2$ (3 mM) to yield F_{Mn}. Cytoplasmic ionized calcium was calculated from the equation:

$$[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$$

where $F_{min} = 1/12(F_{max} - F_{Mn}) + F_{Mn}$ and K_d is the dissociation constant of the indicator:calcium complex (Grynkiewicz *et al.*, 1985). The K_d value (175 nM) was determined in our laboratory using Indo 1-free acid and Ca^{2+}-buffers in K solution in the absence of cells (Klip and Ramlal, 1987; Klip *et al.*, 1990).

Determination of intracellular Ca^{2+} stores. The procedure used to measure intracellular Ca^{2+} stores was as previously reported for human lymphocytes (Klip *et al.*, 1986a). Briefly, total releasable Ca^{2+} is defined as the Ca^{2+} released by 10 μM ionomycin into the cytoplasm in cells suspended in Ca^{2+}-free medium containing 0.5 mM EGTA. The effect of halothane on releasable Ca^{2+} was calculated from the ratio of the maximum effect of ionomycin 90 sec after and before addition of halothane.

Determination of pH_i. Loading with BCECF/AM and pH_i determinations were carried out using the fluorescent pH indicator BCECF (Rink *et al.*, 1982) essentially as described earlier for L_6 muscle cells (Klip *et al.*, 1986c) and C6 glial cells (Jakubovicz and Klip, 1989). Briefly, after recovery from trypsin treatment, the cells were pelleted and resuspended in 1.0 ml HEPES-RPMI with 1 μg/ml BCECF/AM (from a 2 mg/ml stock in dimethyl sulfoxide) and incubated in the dark at 37°C for 20 to 30 min. All solutions used in fluorescence determinations were kept at 37°C. Extracellular BCECF/AM was removed by two centrifugation steps in HEPES-RPMI, and resuspended in 300 to 400 μl of the same medium. The cells were maintained in the dark prior to fluorescence determinations. Twenty μl of cell suspension (1.0×10^5 cells) were dispersed into 1.0 ml of Na solution at pH 7.3 containing 1.0 mM $CaCl_2$ unless otherwise stated. Where indicated, Na was iso-osmotically replaced by K^+ or $choline^+$ (K solution and choline solution, respectively). Additions of halothane (5.7 mM final concentration) were made with a Hamilton syringe from a 1:10 stock solution in dimethyl sulfoxide. Fluorescence was determined at 37°C at 495 nm of excitation and 525 nm of emission with slit widths of 5 and 10 nm, respectively. Since the fluorescence readings were stable for several minutes, it is assumed that no major loss of halothane occurred in that time. At the end of the experiment, calibration of fluorescence *vs.* pH was performed in parallel samples of cells suspended in K solution using the ionophore nigericin (2 μg/ml) (Klip *et al.*, 1986c; Grinstein *et al.*, 1989a). Under these conditions pH_i approximates pH_o. The extracellular pH was then varied with 1- to 5-μl additions of 1.0 M Tris base or 2.0 M HEPES acid and pH was determined with a glass electrode inserted directly into the cuvette. Intracellular fluorescence was determined and plotted as a function of extracellular pH. Initial rates of alkalinization were calculated from the linear slope of traces, typically during the first 10 sec of the recording.

Acid loading and pH_i recovery. Essentially the procedure of Grinstein *et al.* (1989a) was used to bring pH_i to the desired value. Briefly, BCECF-loaded cells were suspended in 1.0 ml of K solution set at the desired acidic pH. Nigericin (2 μg/ml) was added and mixed thoroughly. After 4 min, fatty acid-free bovine serum albumin (7.5 mg/

ml) was added to scavenge the ionophore. The cells were pelleted by centrifugation, resuspended in 300 μl of choline solution and kept in the dark for up to 60 min prior to fluorescence determinations. To activate Na^+/H^+ exchange, 20 μl of the cell suspension (1.0×10^6 cells) were added to 1.0 ml Na solution and fluorescence was measured. Where indicated, various agents were added to the 1.0 ml of Na solution prior to addition of cells. Initial rates of alkalinization were calculated from the initial traces, typically during the first 10 sec of the recording.

Statistical analysis. Traces are of representative experiments; all values given in the tables are the mean ± S.E. of the number of experiments indicated. Student's *t* test of paired or nonpaired data was applied where indicated.

Results

In the present studies, halothane concentrations in the millimolar range were selected, since: (1) millimolar concentrations of halothane are found in the brain of anesthetized rats (Barany *et al.*, 1985); (2) the concentration of halothane in solutions equilibrated with 1 to 3% halothane used in contractility tests is equivalent to millimolar values; and (3) in this range of concentrations, halothane elicits changes in $[Ca^{2+}]_i$ in blood cells from normal and malignant hyperthermia patients and pigs (Klip *et al.*, 1990).

Effect of halothane on $[Ca^{2+}]_i$ in L_6 cells. Table 1 shows the effect of halothane (5.7 mM) on $[Ca^{2+}]_i$ in L_6 muscle cells. The average resting $[Ca^{2+}]_i$ of these cells was 169 ± 8 nM, and the average increase caused by halothane was 80 ± 8 nM. These values were stable for up to 5 min after addition of halothane. When lower concentrations of halothane were tested (*i.e.*, 1.0, 2.0, 2.9, 3.9 and 4.9 mM), the increases in $[Ca^{2+}]_i$ were 31%, 44%, 54%, and 84%, respectively, of the value observed with 5.7 mM. Thus, the response to the anesthetic is dose-dependent. The effect with 1.0 mM halothane was clearly detectable, but lower effects (*i.e.*, changes in $[Ca^{2+}]_i$ of less than 25 nM) would be in the limit of statistical reproducibility by the technique employed. For most other studies, a concentration of 5.7 mM halothane was used unless indicated otherwise.

Table 1 (*middle column of data*) shows the effect of halothane on cells suspended in Ca^{2+}-free (EGTA-containing) Na solution. This solution is calculated to have <10 nM free Ca^{2+}. In the absence of halothane, the average resting $[Ca^{2+}]_i$ was 137 ± 6 nM, significantly lower than that observed in the presence of extracellular Ca^{2+} ($P < .005$). Halothane increased the peak $[Ca^{2+}]_i$ by an average of 42 ± 6 nM. This elevation was transient, resting levels being recovered after 2 min.

Effect of $[Ca^{2+}]_i$ on intracellular Ca^{2+} stores. The effect of halothane on Ca^{2+} from intracellular stores can be estimated by measuring the Ca^{2+} released by the anesthetic and comparing it with that released by the ionophore ionomycin. In these experiments the extracellular medium was devoid of Ca^{2+} and contained 0.5 mM EGTA. In the experiment illustrated in figure 1*A*, halothane elicited a $[Ca^{2+}]_i$ increase from 152 nM to 211 nM. Figure 1*B* shows that addition of ionomycin (10 μM) to the cell suspension produced a transient increase in $[Ca^{2+}]_i$ from 135 nM to 445 nM. This is considered to be due to the release of Ca^{2+} from intracellular stores into the cytoplasm (Rink *et al.*, 1983; Klip and Ramlal, 1987), and is referred to as maximum releasable Ca^{2+} (Klip *et al.*, 1986a). Figure 1*C* shows that addition of ionomycin after halothane gave a smaller transient $[Ca^{2+}]_i$ increase when compared with the ionomycin-induced Ca^{2+} release in the absence of halothane. This suggests that halothane released Ca^{2+} from the intracellular Ca^{2+} stores, thereby reducing the amount of Ca^{2+} available to be released by ionomycin. In seven independent cell preparations, each assayed two to three times, 49 ± 4% of the total releasable Ca^{2+} was left in the intracellular stores after exposure to halothane. This value was calculated from the ratio of peak fluorescence caused by ionomycin after and before halothane exposure, in experiments similar to that illustrated in figure 1.

Effect of halothane on pH_i. During a MH crisis, the sharp rise in muscle catabolic activity demands accelerated H^+ extrusion in order to maintain cytoplasmic neutrality. One of the major cellular mechanisms of H^+ extrusion is Na^+/H^+ exchange. We therefore investigated the effect of halothane on this process. Na^+/H^+ exchange is virtually quiescent at neutral pH_i, but, at least in circulating cells, it is activated by hypertonicity, phorbol esters and cytoplasmic acidification (Grinstein *et al.*, 1983). Table 2 shows that the resting pH_i of L_6 cells suspended in Na solution of pH 7.3 containing 1.5 mM extracellular Ca^{2+} was 7.26 ± 0.05. Addition of halothane either had no effect or caused a slight intracellular acidification. This is compatible with inhibition of a low-activity Na^+/H^+ exchange. When the exchange was activated by hypertonic (0.24 M) NaCl, the pH_i was increased from pH 7.26 ± 0.05 to 7.50 ± 0.08, with an initial rate of 0.095 ± 0.02 pH U/min. This alkalinization was likely due to the extrusion of H^+ in exchange for Na^+, since it was not observed in cells suspended in Na^+-free solution (not shown). When the cells were challenged by hypertonicity in the presence of 5.7 mM halothane, the initial rate of the alkalinization was only 0.037 ± 0.01 pH U/min, *i.e*, it was inhibited by 66%.

Effect of phorbol esters on pH_i. Phorbol esters are also recognized as stimuli that increase the activity of Na^+/H^+ exchange and thus lead to cytoplasmic alkalinization of several cells (Moolenaar *et al.*, 1984; Klip *et al.*, 1988). Figure 2*A* shows that L_6 muscle cells also respond to TPA with a rapid cytoplasmic alkalinization. In the experiment illustrated, TPA elevated the pH_i from 7.08 to 7.25 with an initial rate of 0.035 pH U/min. In the presence of halothane, the TPA-induced alkalinization was almost completely blocked. Virtually iden-

TABLE 1
Cytoplasmic ionized calcium (nM)

	$+Ca^{2+}$		$-Ca^{2+}$		P Not Paired
Halothane, 5.7 mM					
−	169 ± 8 (n = 23)		137 ± 6 (n = 18)		<.005 ($+Ca^{2+}$, −H *vs.* $-Ca^{2+}$, −H)
+	255 ± 15 (n = 19)	P, not paired <.001 (− *vs.* + halothane)	179 ± 9 (n = 18)	P, not paired <.001 (− *vs.* + halothane)	<.001 ($+Ca^{2+}$, +H *vs.* $-Ca^{2+}$, +H)
$\underline{\Delta}$	80 ± 8 (n = 19)	P paired <.001 (− *vs.* + halothane)	42 ± 6 (n = 18)	P paired <.001 (− *vs.* + halothane)	<.001 ($\underline{\Delta}$ $+Ca^{2+}$ *vs.* $\underline{\Delta}$ $-Ca^{2+}$)

Results are the mean ± S.E. of the effect of 5.7 mM halothane in the presence and absence of extracellular Ca^{2+}. The values for $\underline{\Delta}$ $[Ca^{2+}]_i$ are the mean ± S.E. of all individual Δ $[Ca^{2+}]_i$ values (*i.e.*, the values of Δ for each experiment were calculated, and the results indicate the mean ± S.E. of these $\underline{\Delta}$ values); n is the number of independent experiments; within each experiment the number of determinations were two to eight for resting Ca^{2+} and one to three with halothane.

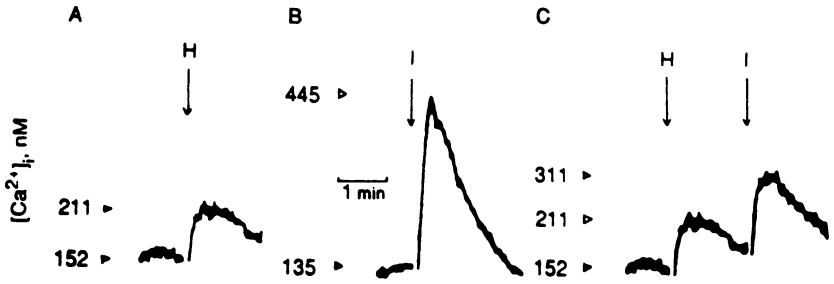

Fig. 1. Effect of ionomycin and halothane on the releasable Ca^{2+} from intracellular stores. Indo 1-loaded cells (1.0×10^5) were suspended in Ca^{2+}-free solution containing 0.5 mM EGTA. Ionomycin (I) 10 μM and halothane (H) 5.7 mM were added where indicated. The fluorescence was calibrated as described in "Materials and Methods," by subsequent additions of $CaCl_2$ and $MnCl_2$ (traces not shown). A, Ca^{2+} released by halothane from intracellular stores; B, total Ca^{2+} releasable by ionomycin from intracellular stores; C, release of Ca^{2+} by halothane and remaining Ca^{2+} released by ionomycin. The results of 18 similar experiments are summarized in table 1.

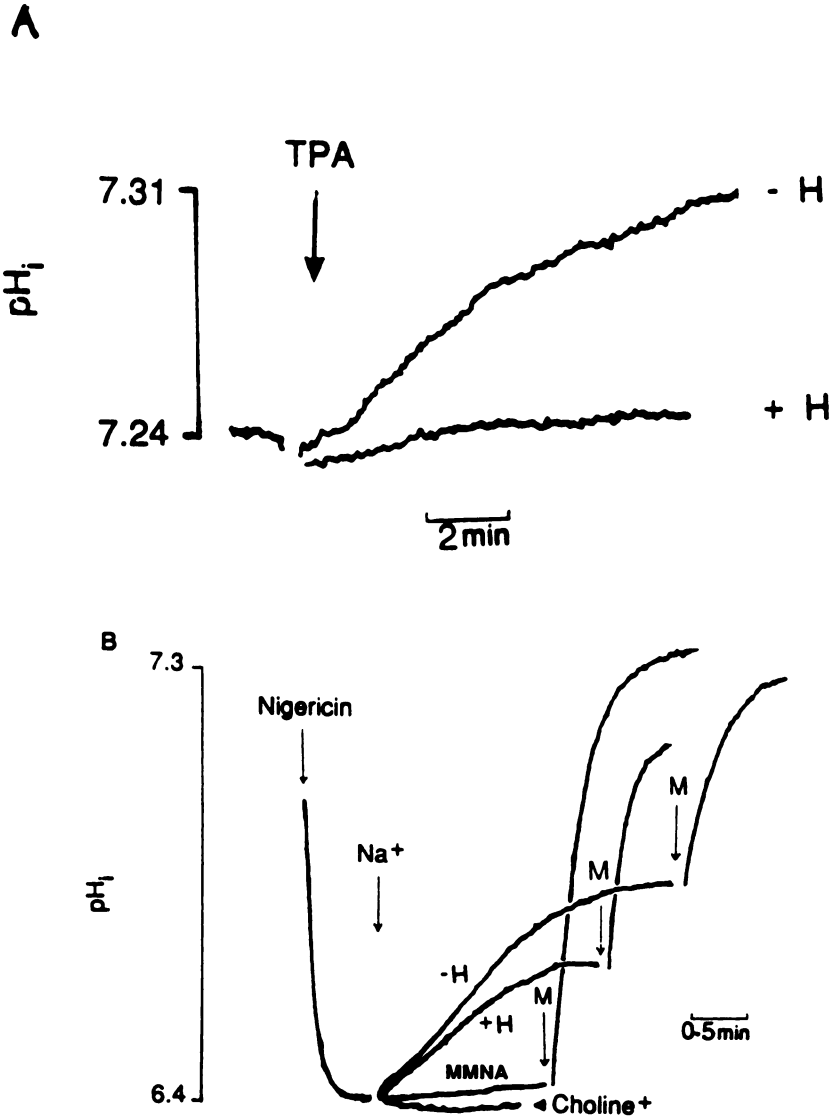

Fig. 2. A, Effect of halothane on phorbol ester-induced alkalinization. Where indicated, TPA (2×10^{-6} M) was added to cells suspended in Na solution in the absence (−H) or presence (+H) of halothane. Similar results were obtained in five independent experiments with TPA (see text). B, Effect of halothane on acid loading-induced alkalinization. BCECF-loaded cells were suspended in Na solution, pH 6.4, and nigericin (2 μg/ml) was added to initiate acid loading. Albumin (7.5 mg/ml) was added to scavenge the nigericin and the cells were centrifuged, resuspended and maintained in choline solution. An aliquot of cells (1.0×10^5) was suspended in Na solution 1 (Na^+) devoid of (−H) or containing (+H) 5.7 mM halothane. Where indicated, halothane (H) or the amiloride analog MMNA were added to the Na solution prior to cell addition. The ionophore monensin was added where indicated. An aliquot of cells was also suspended in choline solution to confirm that the cells remained acidic in the absence of Na^+.

tical results were obtained in five similar experiments, where the phorbol ester caused an increase in pH_i of 0.086 ± 0.0215 U ($P < .025$), and this effect was abolished in the presence of halothane.

Effect of halothane on the activation of Na^+/H^+ exchange by acid loading. Na^+/H^+ exchange is activated not only by hypertonicity or phorbol esters, but also by intracellular acidification (Grinstein *et al.*, 1983, 1989a). The extrusion of H^+ through this pathway leads to the recovery of neutral pH_i after cytoplasmic acidification caused either by increased catabolism or by experimental manipulation. The ionophore nigericin is a useful tool to introduce H^+ to the cytoplasm (Grinstein *et al.*, 1989a). In cells suspended in Na^+-free solution, nigericin equilibrates the extracellular pH with pH_i due to its selectivity for H^+ and K^+ ions (the ionophore drives the cells to the equilibrium dictated by $[H^+]_o/[H^+]_i = [K^+]_o/[K^+]_i$. Thus,

suspending cells in K solution of pH 6.4 lead to cytoplasmic acidification to pH_i near 6.4. Table 2 shows the results of nine independent experiments with acid-loaded cells. The average initial pH_i following acid loading with nigericin in K solution was 6.43 ± 0.01. Introduction of cells into Na solution led to recovery of pH_i to a new steady state of pH_i = 7.00 ± 0.07, with an average initial rate of 0.57 ± 0.05 pH U/min. Halothane decreased the initial rate of pH_i recovery and the final pH_i in a dose-dependent fashion. In these experiments, two concentrations of halothane were tested. At a concentration of 2.85 mM, the anesthetic decreased the rate of pH_i recovery by 36%, and at a concentration of 5.7 mM by 51%. The new steady-state pH_i attained with 5.7 mM halothane was 6.80 ± 0.02.

In order to test that the recovery of pH_i in Na solution was due to activation of Na^+/H^+ exchange, two characteristics were tested: an obligatory dependence on extracellular Na^+ and inhibition by amiloride analogs (Grinstein and Rothstein, 1986). When acid-loaded cells were introduced into Na^+-free, choline solution pH 7.3, pH_i remained steady at the acidic value of 6.43 (fig. 2*B*). This indicates that in the absence of Na^+ the pH_i does not recover from an imposed acidification. In contrast, and as described in table 2, introduction of cells into Na solution pH 7.4 caused a rapid pH_i recovery (initial rate of 0.43 pH U/min, to a final value of 6.99, fig. 2*B*), and halothane decreased the initial rate of recovery (0.21 pH U/min) and the final pH_i (6.76) (fig. 2*B*). The effect of a specific inhibitor of the Na^+/H^+ antiport was subsequently tested on the pH_i recovery from imposed acidification. Amiloride and its 5-N,N′-disubstituted analogs such as MMNA are potent inhibitors of Na^+/H^+ exchange (Vigne *et al.*, 1984). Figure 2*B* shows that 10 μM MMNA completely blocked pH_i recovery. Hence, it can be concluded that recovery of pH_i from acid-loading is largely mediated by the Na^+/H^+ antiport. All traces were followed by addition of 5 μM monensin, an exogenous ionophore capable of exchanging H^+ for Na^+ in a manner analogous to that of the endogenous Na^+/H^+ antiport. It is clearly observed that in all instances monensin alkalinized the cells substantially. This indicates that the failure of the cells to recover from acidification in the presence of MMNA and the slower response observed in the presence of halothane were not the result of interference with BCECF fluorescence or of collapse of the Na^+ gradient, since an exogenous Na^+/H^+ ionophore caused rapid pH_i recovery. Therefore, it is suggested that halothane, like MMNA, inhibited the activity of the endogenous Na^+/H^+ exchange mechanism.

Effect of halothane on pH_i in BAPTA-loaded cells. Because halothane increased $[Ca^{2+}]_i$ as described in figure 1 and table 1, it was possible that this increase could be causally related to the inhibition of Na^+/H^+ exchange activity described in table 2 and figure 2. We therefore tested whether the anesthetic could inhibit the cytoplasmic alkalinization when cells were loaded with the Ca^{2+} chelator BAPTA and suspended in Ca^{2+}-free Na solution. As expected for the liberation of a limited intracellular Ca^{2+} pool, this chelator effectively prevented the halothane-dependent increase in $[Ca^{2+}]_i$ in cells suspended in Ca^{2+}-free, EGTA-containing Na solution (Klip *et al.*, 1990). In the experiments reported here, the cells were loaded with BAPTA simultaneously to loading with BCECF. Figure 3 shows the results of such experiments. Loading of cells with BAPTA did not appreciably alter the resting pH_i, nor the ability of the cells to respond to hypertonic NaCl solution with a rapid cytoplasmic alkalinization (fig. 3*A*). In the experiment illustrated, the resting pH_i was 7.17, and hypertonic NaCl elevated it with an initial rate of 0.03 pH U/min to a new steady-state value of pH_i = 7.33 after 10 min. Halothane (5.7 mM) completely blocked the alkalinization in BAPTA-loaded cells (fig. 3*A*), as was the case for non-loaded cells (table 3).

The results of pH_i recovery after an imposed acidification were similarly unaffected by cytoplasmic Ca^{2+} chelation (fig. 3*B*). In the experiment illustrated with BAPTA-loaded cells, nigericin treatment brought cells to a pH_i value of 6.56. The lower trace in figure 3*B* shows that, as expected, the cells remain at the acidic pH_i of 6.56 when transferred from nigericin-containing Ca^{2+}-free K solution into nigericin-free Ca^{2+}-free choline solution. Introducing cells into Na solution at pH 7.3 led to pH_i recovery with an initial rate of 0.31 pH U/min, to a final value of 6.74. The initial rate of pH_i recovery in the presence of 5.7 mM halothane was only 0.07 pH U/min in BAPTA-loaded cells, and the final pH_i value was 6.67. This indicates that the anesthetic decreased the initial rate of pH_i recovery by >50% in BAPTA-loaded cells. These results indicate that under conditions that preclude increases in $[Ca^{2+}]_i$, halothane still effectively depressed the activity of the Na^+/H^+ antiport.

Discussion

The intracellular concentration of ionized Ca^{2+} plays a key role in the regulation of enzymatic activities and overall cellular excitability. $[Ca^{2+}]_i$ is maintained by the concerted action of Ca^{2+} influx and extrusion from/to the medium, and release and uptake from/to intracellular stores. Until recently, measurements of $[Ca^{2+}]_i$ were not available, and cellular Ca^{2+} homeostasis was studied mostly by measuring Ca^{2+} fluxes. The results

TABLE 2
Effect of halothane on cytoplasmic alkalinization by hypertonic solution

	Resting		Hypertonic NaCl	
	−H	+H	−H	+H
pH after 4 min	7.26 ± 0.05	7.19 ± 0.04	7.50 ± 0.08	7.39 ± 0.06
n	6	6	7	7
Paired P (−H *vs.* +H)		NS[a]	<.005	
Initial rate[b] of alkalinization, Δ pH U/min (%)[c]			0.095 ± 0.02	0.037 ± 0.01
%			100	34 ± 5

Results in the top half of the table are the mean pH_i ± S.E.
[a] NS, not significant.
[b] Initial rate is the change in pH_i/min in the first 20 sec (linear increase).
[c] % is the percentage ratio of alkalinization rate after:before addition of halothane.

TABLE 3
Effect of halothane on pH_i recovery after acid loading

Initial Acid pH_i	[Halothane] in Na Solution	pH_i Recovery	
		Initial Rate, pH U/min	Final pH_i
6.43 ± 0.01 (9)		0.47 ± 0.05 (9)	7.00 ± 0.07 (9)
	2.85 mM	0.30 ± 0.03 (6)	6.89 ± 0.07 (6)
	5.7 mM	0.23 ± 0.02 (9)	6.80 ± 0.02 (9)

L_6 muscle cells were acidified by nigericin in K solution, followed by removal of the ionophore through successive washing in bovine serum albumin-containing K solution. The initial pH_i was recorded by introducing an aliquot of cells into choline solution. The recovery of pH_i was induced by introducing aliquots of cells into Na solution without or with the indicated concentrations of halothane. The initial rate of pH_i recovery was calculated from the slope of the increase in fluorescence, which was linear over the first 10 sec. The final pH_i was calculated from the steady-state fluorescence value, which was usually attained after 10 min. Fluorescence readings of each experiment were calculated by additions of Tris base and pH recordings as described in "Materials and Methods." Numbers in parentheses are the number of independent experiments. The results are of independent experiments, and are expressed as the mean ± S.E.

demonstrated that halothane induces Ca^{2+} release from the sarcoplasmic reticulum (Britt and Kalow, 1970; Strobel and Bianchi, 1971; Beeler and Gable, 1985), and lowers the critical threshold Ca^{2+} concentration required for Ca^{2+}-induced Ca^{2+} release from sarcoplasmic reticulum vesicles (Nelson and Sweo, 1988). These effects on Ca^{2+} fluxes, however, do not prove that general anesthetics alter $[Ca^{2+}]_i$ in intact cells. Importantly, it is this parameter that is the regulatory element in Ca^{2+}-dependent processes. Clearly, direct measurements of $[Ca^{2+}]_i$ are required to confirm an effect of general anesthetics. Previous studies using aequorin to measure $[Ca^{2+}]_i$ were controversial. Halothane decreased aequorin fluorescence, and hence presumably $[Ca^{2+}]_i$, in the cat papillary cardiac muscle (Bosnjak and Kampine, 1986), but increased aequorin fluorescence in squid axon (Baker and Schapira, 1980). Regrettably, in both cases the anesthetic caused nonspecific changes in the fluorescence of aequorin.

The most suggestive indication of a halothane-dependent increase in $[Ca^{2+}]_i$ in skeletal muscle is the reproducible observation of anesthetic-mediated muscle contraction (Bruton *et al.*, 1986; Deuster *et al.*, 1985; Su and Bell, 1986). This effect is blocked by dantrolene (Deuster *et al.*, 1985), a drug known to decrease cytoplasmic $[Ca^{2+}]_i$ in this tissue (Lopez *et al.*, 1987). In recent years, new fluorescent Ca^{2+} indicators were developed that measure $[Ca^{2+}]_i$ more quantitatively (Grynkiewicz *et al.*, 1985). In the present study, using the fluorescent Ca^{2+} indicator Indo-1, we show that anesthetic concentrations of halothane effectively increase $[Ca^{2+}]_i$ in muscle cells in culture (table 1). This response is partly due to Ca^{2+} release from intracellular stores, since it is observed in cells suspended in Ca^{2+}-free medium. Moreover, halothane effectively decreased the site of the releasable Ca^{2+} pool. In Ca^{2+}-free medium, the increase in $[Ca^{2+}]_i$ is smaller than in the presence of extracellular Ca^{2+}, probably reflecting that in the lower prevailing $[Ca^{2+}]_i$ halothane opens fewer Ca^{2+} release channels of intracellular stores, which are under $[Ca^{2+}]_i$ control. Alternatively, external Ca^{2+} ions may participate in the halothane-mediated increase in $[Ca^{2+}]_i$ by replenishing the intracellular Ca^{2+} stores. Thus, halothane may have a dual effect, eliciting Ca^{2+} release from intracellular stores and increasing Ca^{2+} influx from the medium.

When this study was in progress, Daniell and Harris (1989) reported that halothane increases $[Ca^{2+}]_i$ in brain synaptosomes detected by fluorescence spectroscopy of trapped fura-2, and Iaizzo *et al.* (1988) reported that halothane increases $[Ca^{2+}]_i$ in bundles of skeletal muscle fibers from MH-susceptible pigs measured by fura-2 fluorescence microscopy. In contrast to the results with synaptosomes and L_6 muscle cells, however, no increase in $[Ca^{2+}]_i$ was detected in muscle from normal pigs. It is possible that the differences are due to the different cellular preparations and optical methods used.

In contrast to the vast literature on effects of halothane on Ca^{2+} homeostasis, little is known about the effect of the anesthetic on cellular pH maintenance. Gronert *et al.* (1986) reported that halothane increases both circulating lactate levels and the lactate gradient from muscle to blood in MH pig muscle. Interestingly, in these muscles administration of bicarbonate did not relieve muscle acidity unless dantrolene was added, leading to the suggestion that the Ca^{2+}-induced increase in muscle catabolism was responsible for the increased lactate production.

The mechanisms that participate in the maintenance of cellular pH are primarily Cl^-/HCO_3^- exchange (Na^+-dependent and -independent forms) and Na^+/H^+ exchange (Grinstein *et al.*, 1989b). The former plays a relevant role in the recovery of resting pH in the alkaline range, whereas the latter is more significant in the recovery from cell acidification (Grinstein *et al.*, 1989b). In the present study we investigated whether halothane affects the activity of Na^+/H^+ exchange, and in this way alters the ability of cells to cope with cytoplasmic acidification. In order to dissect out the activity of Na^+/H^+ exchange, the experiments described were carried out in nominally HCO_3^- free solutions, where Cl^-/HCO_3^- exchange is virtually eliminated. The results in figures 2 and 3 indicate that the anesthetic markedly inhibited the increase in pH_i caused by two experimental protocols known to measure the activity of the Na^+/H^+

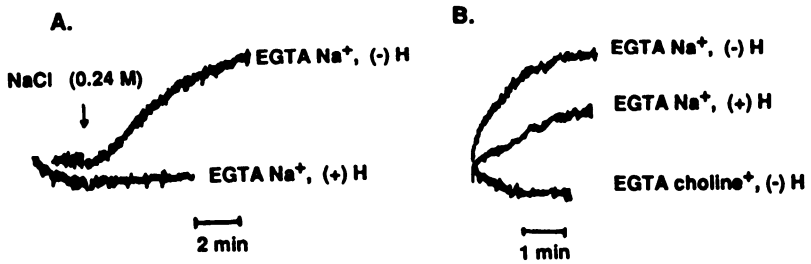

Fig. 3. Effect of halothane on pH_i in BAPTA-loaded cells suspended in Ca^{2+}-free solution. Cells were loaded with BCECF and BAPTA by simultaneously incubating them with 1 μg/ml BCECF/AM and 10 μM BAPTA/AM for 30 min at 37°C, and then washed free of the AM compounds as described in "Materials and Methods." The effects of hypertonic (0.24 M) NaCl solution (A) or of nigericin-induced cytoplasmic acidification (B) were then measured in the absence and presence of halothane as described in table 2 and figure 3, respectively. The results are of one experiment representative of three independent ones.

antiporter (see Grinstein *et al.*, 1989a). The first one is the cytoplasmic alkalinization caused by hypertonic solutions and phorbol esters (table 2 and fig. 2*A*). Our results indicate that these responses operate in L_6 muscle cells and that halothane, at anesthetic concentrations, effectively slows down such alkalinization, presumably by preventing the full activation of Na^+/H^+ exchange. The second protocol is the pH_i recovery from an imposed cytoplasmic acidification. The results in figure 2*B* indicate that L_6 muscle cells are capable of fast recovery from nigericin-induced cytoplasmic acidification through activation of the Na^+/H^+ antiport. Halothane interfered markedly with this process, leading to slower pH_i recovery (fig. 2*B*).

The mechanism of inhibition of Na^+/H^+ exchange by halothane is not fully understood at present. Our results indicate that the inhibition of cytoplasmic alkalinization does not result from collapse of the Na^+ or pH gradients, since monensin was effective in raising pH substantially in the presence and absence of halothane (fig. 2*B*). It is therefore likely that the anesthetic interacts directly with the antiport, whose activity is inhibited by cationic guanidinium compounds such as amiloride (Vigne *et al.*, 1984). The fluorinated hydrocarbon halothane bears no structural resemblance to this compound. However, it has been proposed that a hydrophobic site in the antiport molecule also participates in inhibitor binding, since hydrophobic amiloride derivatives are more effective inhibitors and ligands of the Na^+/H^+ antiport (Dixon *et al.*, 1987). It is possible that halothane could interact with this site in the antiport molecule. In this regard, it is compelling to recall the hypothesis that general anesthetics interfere competitively with the function of sensitive membrane proteins by binding to hydrophobic pockets on these molecules (Franks and Lieb, 1982). This hypothesis has been in part demonstrated for the $Na^+/K^+/Cl^-$ cotransport system of glioma cells (Tas *et al.*, 1987).

We further examined whether the halothane-mediated increase in $[Ca^{2+}]_i$ could mediate the inhibition of Na^+/H^+ exchange. Previous studies have shown that in certain cell types such as fibroblasts and lymphocytes, an increase in $[Ca^{2+}]_i$ actually *activates* Na^+/H^+ exchange (Grinstein and Rothstein, 1986; Grinstein and Cohen, 1987). Our results indicate that there is no direct relationship between the ability of halothane to elevate $[Ca^{2+}]_i$ and to inhibit Na^+/H^+ exchange activity. The results in figure 3 indicate that the anesthetic slows down significantly the alkalinization caused by either hypertonic solutions or a sudden cytoplasmic acidification in cells loaded with a Ca^{2+} chelator and suspended in Ca^{2+} free medium. These conditions should prevent the effects of the anesthetic on $[Ca^{2+}]_i$. This suggests that the halothane-dependent increase in $[Ca^{2+}]_i$ does not lead to the inhibition of Na^+/H^+ exchange. The converse situation, *i.e.*, that inhibition of Na^+/H^+ exchange could lead to the increase in $[Ca^{2+}]_i$ is also unlikely, since under resting conditions halothane affected pH_i only minimally, its effect on Na^+/H^+ exchange becoming manifest mainly in the presence of stimuli of the exchange. This suggests that the effects of halothane on $[Ca^{2+}]_i$ and pH_i occur in parallel rather than in series.

The effects of halothane on $[Ca^{2+}]_i$ and pH_i are part of a spectrum of actions on ion permeabilities including those of K^+, Na^+ and Cl^-. Halothane increases K^+ permeability, reduces Na^+ conductance and potentiates Cl^- flux by the following mechanisms: (1) The anesthetic augments a Ca^{2+}-dependent K^+ conductance leading to cell hyperpolarization (Nicoll and Madison, 1982); this may contribute to anesthetic action by preventing depolarization elicited by stimuli that normally trigger nerve or muscle activity (Daniell and Harris, 1989). (2) General anesthetics reduce the maximum Na^+ conductance of squid giant axons (Hayden and Urban, 1983), the activity of glial sodium channels (Tas *et al.*, 1985) and the burst duration of low- and high-amplitude acetylcholine receptor channels of amphibian muscle cells (Lechleiter and Gruener, 1984). Such inhibitory action on Na^+ permeabilities could depress excitability. Interestingly, both peak action potential and repolarization rates are decreased by halothane in MH-susceptible porcine muscle, and these effects are attenuated by dantrolene (Iaizzo *et al.*, 1988) suggesting mediation by the halothane-dependent increase in $[Ca^{2+}]_i$. (3) Halothane exerts a potentiation of the nondesensitized Cl^- current (both peak and sustained) through the γ-aminobutyric acid receptor channel of rat dorsal root ganglion neurons (Nakahiro *et al.*, 1989). The resulting augmentation in activity of inhibitory synapses (where γ-aminobutyric acid is a primary neurotransmitter) could potentially suppress central nervous system activity.

In summary, we have demonstrated that, in muscle cells, halothane increases $[Ca^{2+}]_i$ by allowing Ca^{2+} influx and intracellular Ca^{2+} release into the cytoplasm, and decreases Na^+/H^+ activity, thereby lowering the capacity of cells to cope with increases in cytosolic acid. Other studies have shown that the anesthetic increases a Ca^{2+}-dependent K^+ permeability and hence causes membrane hyperpolarization, decreases Na^+ conductance and acetylcholine channel kinetics leading to decreased electrical activity, and increases inhibitory synaptic activity of γ-aminobutyric acid receptors. This constellation of ionic effects could constitute the basis for anesthetic action.

Acknowledgments

We thank Drs. S. Grinstein and G. B. Mills for careful reading of this manuscript.

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