Volatile Anesthetic-induced Efflux of Calcium from IP$_3$-gated Stores in Clonal (GH$_3$) Pituitary Cells

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Background: Many hormones and neurotransmitters produce their effects by stimulating the generation of inositol 1,4,5-trisphosphate (IP$_3$), a chemical second messenger that releases Ca$^{2+}$ from intracellular stores. Interruption of this pathway is a potential mechanism through which volatile anesthetics might inhibit chemically mediated communication between cells. This study used GH$_3$ cells (a clonal cell line) as a model system in which to characterize the effects of volatile anesthetics on IP$_3$-induced mobilization of Ca$^{2+}$ from intracellular stores.

Methods: Intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) was continuously monitored in suspensions of GH$_3$ cells at 37°C using the fluorescent Ca$^{2+}$ indicator Fura-2. Thyrotropin releasing hormone (TRH) was used to discharge IP$_3$-sensitive intracellular Ca$^{2+}$ stores. The effects of halothane, isoflurane, and octanol on TRH-induced Ca$^{2+}$ mobilization were assessed as a function of time and anesthetic concentration. To distinguish between anesthetic effects on Ca$^{2+}$ uptake and Ca$^{2+}$ release, experiments were performed using thapsigargin (a Ca$^{2+}$-ATPase inhibitor) to inhibit Ca$^{2+}$ uptake into IP$_3$-sensitive stores.

Results: Halothane increased resting [Ca$^{2+}$] and caused a time- and concentration-dependent inhibition of TRH-induced increases in [Ca$^{2+}$] (IC$_{50}$ = 0.6 mM). Thapsigargin, in concentrations that completely inhibit Ca$^{2+}$ uptake by IP$_3$-sensitive stores, also caused a time-dependent reduction in the [Ca$^{2+}$] response to TRH; the time constant of this decay describes the rate of spontaneous leak of Ca$^{2+}$ from IP$_3$-sensitive stores ($\tau = 98 \pm 9$ s). In the presence of thapsigargin, halothane produced concentration-dependent increases in the rate of leak from IP$_3$-sensitive stores ($\tau = 74 \pm 12$ and 46 $\pm$ 6 s at 0.5 and 1.0 mM halothane, respectively). Isoflurane and octanol also produced concentration-dependent inhibition of the [Ca$^{2+}$] response to TRH.

Conclusions: Halothane causes a concentration-dependent leak of Ca$^{2+}$ from IP$_3$-sensitive stores, leading to depletion of the stores and inhibition of IP$_3$-induced increases in [Ca$^{2+}$]. This effect occurs at clinically relevant concentrations of halothane (as well as isoflurane and octanol) and may be an important mechanism underlying some of the physiologic effects of volatile anesthetics. (Key words: Anesthetics, general; halothane; isoflurane; octanol. Calcium, intracellular. Endoplasmic reticulum. Inositol trisphosphate. Thyrotropin releasing hormone. Fluorescent dyes: Fura-2. Pituitary cells; GH$_3$ cells.)

VOLATILE anesthetics affect excitable tissues, at least in part, by inhibiting intercellular communication. Consistent with this view, volatile anesthetics have been shown to interfere with fast synaptic transmission at some synapses in the central nervous system. These drugs also can affect slower forms of intercellular signaling, including those mediated by hormones and neuromodulators acting at G-protein coupled receptors. Various mechanisms have been proposed through which anesthetics might interfere with receptor-effector coupling in these systems, including inhibition of receptor coupling to G-proteins, enhancement or inhibition of chemical second messenger generation, and inhibition of protein phosphorylation.

Mobilization of intracellular calcium from stores gated by the second messenger inositol 1,4,5-trisphosphate (IP$_3$) is a central mechanism in the action of many hormones and neuromodulators, and there is some evidence suggesting it as a potential locus for anesthetic effects. First, in the A7r5 cell line, halothane has been shown to cause a modest increase in [Ca$^{2+}$], and to inhibit the rise in [Ca$^{2+}$], elicited by arginine vasopressin and platelet-derived growth factor; both arginine vasopressin and platelet-derived growth factor are agonists that stimulate IP$_3$ generation and subse-
quent release of Ca\textsuperscript{2+} from IP\textsubscript{3}-sensitive stores. Halothane-induced increases in [Ca\textsuperscript{2+}]\textsubscript{i} also have been observed in A-10 and BC3H1 cells.\textsuperscript{8} These data are consistent with an effect of halothane on IP\textsubscript{3} generation, IP\textsubscript{3} action, or IP\textsubscript{3}-gated calcium stores. Second, an analogy can be drawn between intracellular calcium stores gated by ryanodine receptors and those gated by IP\textsubscript{3} receptors. Ryanodine-sensitive calcium stores play a central role in excitation-contraction (EC) coupling in cardiac muscle. Current thinking suggests that a major mechanism by which volatile anesthetics inhibit EC coupling in heart is by causing leak of Ca\textsuperscript{2+} through ryanodine-sensitive calcium channels leading to depletion of the calcium stores.\textsuperscript{9,10} The ryanodine receptor and the IP\textsubscript{3} channel are homologous proteins\textsuperscript{11-15} that might be effected similarly by volatile anesthetics. This study was performed to characterize the effects of volatile anesthetics on IP\textsubscript{3}-gated intracellular stores of calcium.

In the current study, GH\textsubscript{3} cells, a transformed rat pituitary cell line, were used to examine the effects of volatile anesthetics on IP\textsubscript{3}-sensitive calcium stores. These clonal cells are thought not to have ryanodine-sensitive intracellular calcium stores but contain IP\textsubscript{3}-releasable stores of calcium that have been studied extensively using fluorescent indicator techniques.\textsuperscript{16,17} In GH\textsubscript{3} cells, a variety of hormones, including thyrotropin releasing hormone (TRH), bind to cell surface receptors that are linked to the G-proteins G\textsubscript{a} and G\textsubscript{b}.\textsuperscript{16} Binding of TRH to its receptor leads to G-protein activation, which in turn activates a phospholipase C-b.\textsuperscript{17} The phospholipase C-b cleaves the membrane lipid phosphatidylinositol bisphosphate yielding two second messengers, IP\textsubscript{3} and diacylglycerol.\textsuperscript{18,19} IP\textsubscript{3} binds to a specific intracellular receptor (a ligand-gated calcium channel), which causes rapid release of calcium from intracellular stores. Diacylglycerol activates protein kinase C, which, in concert with elevated [Ca\textsuperscript{2+}]\textsubscript{i}, leads to secretion of the stored peptide hormones prolactin and growth hormone. TRH also depolarizes GH\textsubscript{3} cells, causing activation of voltage-gated calcium channels and increased frequency of calcium action potentials.\textsuperscript{20,21}

In previous work with GH\textsubscript{3} cells, we showed that clinically relevant concentrations of halothane have minimal effects on TRH-stimulated activation of phospholipase C-b and the attendant generation of inositol phosphates. In keeping with this, halothane did not alter IP\textsubscript{3}-mediated increases in [Ca\textsuperscript{2+}] when applied immediately before TRH stimulation.\textsuperscript{22} However, halothane markedly and specifically inhibited activation of voltage-gated L-type calcium channels.\textsuperscript{23} We now report that halothane, isoflurane, and octanol cause time- and concentration-dependent depletion of Ca\textsuperscript{2+} from IP\textsubscript{3}-sensitive stores. This depletion is the result of an increased rate of efflux from these stores.

The results of some of these experiments have been reported in preliminary form.\textsuperscript{24}

**Materials and Methods**

**Drugs**

Halothane was obtained from Ayerst Laboratories Inc. (New York, NY), and isoflurane was obtained from Anaquest (Madison, WI). Thapsigargin was purchased from LC Services Corporation (Woburn, MA). Fura-2 AM (Cell permeant) was purchased from Molecular Probes, Inc. (Eugene, OR). All other drugs used were obtained from Sigma (St. Louis, MO) unless otherwise noted.

**Cell Culture**

GH\textsubscript{3} cells were obtained from the American Type Tissue Collection (Rockville, MD). Passage numbers 18–38 were used for experiments. Cells were grown at 37°C in a humidified, 5% CO\textsubscript{2} atmosphere. Growth medium consisted of Ham's F-10, 15% horse serum, and 2.5% fetal bovine serum. Cells were grown in suspension for 24 h before experimentation.

**Measurement of [Ca\textsuperscript{2+}]**

GH\textsubscript{3} cells were loaded with Fura-2 AM as previously described.\textsuperscript{22} Cells loaded with Fura-2 were washed and resuspended in physiologic saline consisting of (mm): NaCl 140, KCl 5.4, HEPES 10, CaCl\textsubscript{2} 3, MgCl\textsubscript{2} 2, Dextrose 10, and probenecid 2.5, with a pH of 7.4. Probenecid was included to prevent leakage of Fura-2 from cells\textsuperscript{23} and did not qualitatively alter the [Ca\textsuperscript{2+}] response to any of the drugs used in this study. The cells (1–2 × 10\textsuperscript{6} cells in a volume of 2.0 ml) were placed in a stirred, temperature-controlled (37°C) 4.0-ml cuvette in a Photon Technology International (South Brunswick, NJ) Deltascan spectrofluorimeter. Experiments were initiated after allowing 10 min for temperature and baseline fluorescence stabilization. None of the drugs used in this study had any effect on the intrinsic fluorescence of Fura-2; notably, halothane did not induce or enhance photobleaching of Fura-2, as was observed in one previous report.\textsuperscript{26} Drugs were delivered into the cuvettes as 1–60-μl volumes of con-
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centrated stocks to achieve the desired concentrations. Although the largest volume drug additions produced dilutional effects on fluorescence, this did not affect the ratiometric determination of [Ca<sup>2+</sup>].

Fluorescence measurements were made by observing the fluorescence emission at 500 nm generated by exposing the cells to rapidly alternating (100-Hz) excitations of 340- and 390-nm light (5-nm bandwidth for excitation and emission). To obtain maximum and minimum fluorescence, the cells first were permeabilized with Triton X-100 (0.1%), and EGTA (ethylene-glycol-bis-[β-aminoethyl ether]N,N,N′,N′-tetraacetic acid, 20 mM) was added subsequently to give the fluorescence value for zero calcium. [Ca<sup>2+</sup>]<sub>i</sub> was calculated from the fluorescence measurements using the equations described by Gryniewicz et al. All values of [Ca<sup>2+</sup>]<sub>i</sub> are reported as intracellular concentrations (nm). TRH-induced changes in [Ca<sup>2+</sup>]<sub>i</sub> were immediately before TRH addition from the peak value of [Ca<sup>2+</sup>]<sub>i</sub> elicited by TRH. KCl-induced changes in [Ca<sup>2+</sup>]<sub>i</sub> were calculated by subtracting [Ca<sup>2+</sup>]<sub>i</sub> immediately before KCl addition from the stable plateau value of [Ca<sup>2+</sup>]<sub>i</sub> elicited by KCl depolarization.

**Volatile Anesthetic Administration and Measurement**

Halothane was delivered to the cuvettes by adding aliquots of halothane-saturated saline to give the desired final concentrations of halothane in buffer. The concentration of anesthetic was maintained subsequently by blowing air containing halothane gas over the cuvette. Control cells were treated in an identical fashion except that halothane was not present in the added saline or gas. Volatile anesthetic concentrations in gas and saline were measured by gas chromatography.

Volatile anesthetic concentrations are reported in millimolar concentrations, where concentrations of 0.125, 0.25, 0.5, 1.0, 1.5, and 3.0 mm halothane in saline at 37°C correspond to concentrations of 0.5, 1.0, 2.0, 4.0, 6.0, and 12.0 vol% halothane in gas. Halothane produces general anesthesia in rats with an EC<sub>50</sub> of 1.24 vol%, which corresponds to a concentration of 0.31 mm in saline.

**Data Analysis**

Experimental data are reported as the mean ± SEM of at least three experiments unless otherwise indicated. Concentration-response curves were fit to the equation \( y = \text{Min} + (\text{Max} - \text{Min})/[1 + (x/x_{50})^{-\eta}] \), where \( x_{50} \) is the concentration producing half-maximal response and \( \eta \) is the Hill coefficient. Values for IC<sub>50</sub> and EC<sub>50</sub> were obtained from this curve fitting and are reported as ±95% confidence limits. Time-dependent data sets were fit to exponential functions (with or without residuals), and the time constants derived from this fitting are reported as ±95% confidence limits. Curve fitting was performed using an iterative nonlinear least squares procedure for minimization of residuals (FFIT data analysis software, Biosoft, Ferguson, MD).

**Results**

**Isolation of IP<sub>3</sub>-mediated Calcium Mobilization in GH<sub>3</sub> Cells**

Application of TRH to GH<sub>3</sub> cells elicits a transient IP<sub>3</sub>-mediated increase in [Ca<sup>2+</sup>]<sub>i</sub>, followed by a sustained elevation of [Ca<sup>2+</sup>]<sub>i</sub>, resulting from Ca<sup>2+</sup> influx via plasma membrane Ca<sup>2+</sup> channels. The sustained elevation of [Ca<sup>2+</sup>]<sub>i</sub> is completely inhibited by the inorganic Ca<sup>2+</sup> channel antagonist LaCl<sub>3</sub> (5 μM), allowing isolation of the IP<sub>3</sub>-dependent increase in [Ca<sup>2+</sup>]<sub>i</sub> (fig. 1A). It is characteristic of the IP<sub>3</sub> response that, despite the continued presence of agonist (TRH), released Ca<sup>2+</sup> is cleared from the cytosol and [Ca<sup>2+</sup>]<sub>i</sub> returns to near baseline values within 30 s (fig. 1A).

The sustained elevation of [Ca<sup>2+</sup>]<sub>i</sub> occurring after TRH administration results from two pharmacologically distinct components. TRH is known to depolarize GH<sub>3</sub> cells leading to influx of calcium through voltage-gated channels. The component of the [Ca<sup>2+</sup>]<sub>i</sub> response to TRH resulting from Ca<sup>2+</sup> influx through voltage-gated channels is sensitive to both nimbidine (240 nm) and halothane (1 mm; figs. 1B and 1C). An additional component to the sustained phase of the [Ca<sup>2+</sup>]<sub>i</sub> response to TRH is blocked by La<sup>3+</sup> (5 μM) but is insensitive to either halothane or nimbidine (figs. 1B and 1C). This La<sup>3+</sup>-sensitive component may be analogous to the "store-dependent Ca<sup>2+</sup> influx" observed in PC-12 cells. To eliminate the contribution of depolarization-induced calcium influx to TRH-stimulated increases in [Ca<sup>2+</sup>]<sub>i</sub>, nimodipine (240 nm) was included in all experiments examining TRH effect. This concentration of nimodipine completely blocks the voltage-dependent component of Ca<sup>2+</sup> influx, as evidenced by the fact that it completely blocks KCl-induced increases in [Ca<sup>2+</sup>]<sub>i</sub> (fig. 1D). To ensure that Ca<sup>2+</sup> influx did not contribute to any of the observed effects, specifically indicated experiments also were conducted in the...
Fig. 1. The effects of Ca\textsuperscript{2+} channel antagonists on thyrotropin releasing hormone (TRH)- and KCl-induced changes in intracellular Ca\textsuperscript{2+} concentration in GH\textsubscript{3} cells. (A) Continuous application of TRH (100 nM) results in a rapid, transient increase in [Ca\textsuperscript{2+}], followed by a sustained increase in [Ca\textsuperscript{2+}]. The sustained increase in [Ca\textsuperscript{2+}] is eliminated by addition of 5 μM LaCl\textsubscript{3} (left) and prevented by pretreatment with 5 μM LaCl\textsubscript{3} (right). (B and C) The sustained increase in [Ca\textsuperscript{2+}], produced by TRH administration, has two pharmacologically distinct components: one component is sensitive to nimodipine (240 mM), halothane (1 mM), and La\textsuperscript{3+} (5 μM), whereas the other component is sensitive only to La\textsuperscript{3+}. (D) The increase in [Ca\textsuperscript{2+}], produced by KCl (40 mM) is completely inhibited by nimodipine (240 mM).

Fig. 2. The effects of thyrotropin releasing hormone (TRH), halothane, and thapsigargin on intracellular Ca\textsuperscript{2+} concentration in GH\textsubscript{3} cells. (A) Continuous application of TRH results in a rapid but transient rise of [Ca\textsuperscript{2+}]. (B and C) Halothane and thapsigargin both increase resting [Ca\textsuperscript{2+}] and inhibit the subsequent TRH-induced rise of [Ca\textsuperscript{2+}]. (D) Administration of halothane (0.5, 1.0, 1.5, and 3.0 mM) increases resting [Ca\textsuperscript{2+}] in GH\textsubscript{3} cells.

Control experiments also were performed to ensure that TRH-mediated mobilization of intracellular Ca\textsuperscript{2+} was not complicated by (Ca\textsuperscript{2+}-induced) calcium release from ryanodine-sensitive stores. These experiments showed that caffeine (10 mM), ryanodine (10 μM), ruthenium red (100 μM), or caffeine and ryanodine in combination affected neither resting [Ca\textsuperscript{2+}] nor the TRH-induced increase in [Ca\textsuperscript{2+}].

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Fig. 3. Time- and concentration-dependent effects of halothane on thyrotropin releasing hormone (TRH)- and KCl-stimulated increases in [Ca$^{2+}$], in GH$_3$ cells. (A) GH$_3$ cells were challenged with 100 nM TRH at various times after the application of 1 mM halothane. Experiments were conducted in the presence of either 240 nM nimodipine (□) or 5 μM LaCl$_3$ (○). Neither LaCl$_3$ (●) nor nimodipine (□) produced any effect on the TRH response in the absence of halothane. Each time point represents the mean ± SEM of the peak rise in [Ca$^{2+}$], induced by TRH as measured in three separate experiments. Cells also were challenged with 40 mM KCl at various times after the application of halothane (△). Each time point in these experiments represents the mean ± SEM of the rise in [Ca$^{2+}$], observed 160 s after KCl administration as measured in three separate experiments. The effect of halothane on the KCl-induced rise of [Ca$^{2+}$], was maximal in less than 30 s, whereas its effect on the TRH-induced rise of [Ca$^{2+}$] was time-dependent. (B) Cells were challenged with TRH (100 nM) at various times after the application of halothane at concentrations of 0.5 (●), 1.0 (▲), 1.5 (●), and 3.0 mM (□). The figure plots peak [Ca$^{2+}$] responses to TRH elicited at various times after halothane administration. Data are expressed as a percentage of the response elicited in the absence of halothane. The data sets (n = 3 for each data point) are fit to a monoexponential function with a residual. (C) Concentration-dependence of halothane's effect on TRH-induced changes in [Ca$^{2+}$], TRH response was elicited 600 s after the administration of halothane. The curve is fit to the Hill equation with an IC$_{50}$ of 0.6 ± 0.03 mM and a Hill coefficient of 1.5 (n = 3 for each data point).

Fig. 4. The effects of halothane on thyrotropin releasing hormone (TRH)-induced changes in peak [Ca$^{2+}$], are reversible. TRH-induced changes in [Ca$^{2+}$], were measured in the absence of halothane (Control), in cells treated with 1 mM halothane for 10 min (Halothane), and in cells treated with 1 mM halothane for 10 min followed by a 20-min period for volatilization of halothane (Recovery). Each bar represents the mean ± SEM of three separate experiments.
Fig. 5. Time- and concentration-dependent effect of thapsigargin on the thyrotropin releasing hormone (TRH)-induced rise in \([\text{Ca}^{2+}]_i\). (A) TRH-Induced changes in peak \([\text{Ca}^{2+}]_i\) are plotted as a function of time after the administration of thapsigargin. Each curve represents a different thapsigargin concentration: 0.3 \(\bullet\), 1.0 \(\triangle\), 3.0 \(\Delta\), 10 \(\bullet\), 30 \(\bigcirc\), and 100 \(\square\) nM. Each time point represents the mean \pm SEM of three separate experiments. Each curve is fit to a monoexponential function with a residual. Thapsigargin causes concentration-dependent decreases in both the steady-state response (defined as the peak \([\text{Ca}^{2+}]_i\), response elicited 600 s after thapsigargin administration) to TRH and the time constant describing the decay of the peak \([\text{Ca}^{2+}]_i\) response to TRH. Calculated time constants for each thapsigargin concentration are 305 \(\pm\) 8, 275 \(\pm\) 8, 111 \(\pm\) 6, 95 \(\pm\) 5, 92 \(\pm\) 9, and 93 \(\pm\) 5 s for 0.3, 1.0, 3.0, 10.0, 30.0, and 100.0 nM, respectively. (B) Concentration-dependence of thapsigargin's effect on TRH-induced changes in peak \([\text{Ca}^{2+}]_i\). TRH response was elicited 600 s after the administration of thapsigargin (n = 3 for each data point). The curve is fit to the Hill equation with an IC50 of 273 \(\pm\) 10 pm and a Hill coefficient of 1.97. (C) Time constants of the monoexponential decays of the peak \([\text{Ca}^{2+}]_i\) response to TRH are plotted as a function of thapsigargin concentration. The effect of thapsigargin on the decay of the TRH response saturates at thapsigargin concentrations above 3 nM. The limiting time constant is 98 \(\pm\) 9 s. This corresponds to the time constant of spontaneous leak of \text{Ca}^{2+} \text{ from IP}_{3}-gated stores. Halothane at 0.5 (\(\bigcirc\)) and 1.0 mm (\(\bigtriangledown\)) causes a concentration-dependent increase in the rate of spontaneous leak of \text{Ca}^{2+} \text{ from IP}_{3}-gated stores. Time constants are reported as \(\pm 95\%\) confidence limits.

after drug administration) in GH3 cells. This provides strong evidence for the absence ofryanodine-sensitive calcium stores in the GH3 cells used in these studies.

The Effects of Halothane on Resting \([\text{Ca}^{2+}]_i\), and on TRH-Induced Calcium Mobilization

Administration of halothane to GH3 cells increased resting \([\text{Ca}^{2+}]_i\). The magnitude of this increase was small at clinically relevant concentrations but was clearly concentration-dependent (fig. 2D). Figure 2B shows a particularly dramatic example of a rapid increase in \([\text{Ca}^{2+}]_i\), elicited by 3 mM halothane. When intracellular stores were emptied by pretreatment with thapsigargin (30 nM for 10 min), halothane (3 mM) no longer caused an increase in resting \([\text{Ca}^{2+}]_i\) (not shown). This indicates that halothane increases resting \([\text{Ca}^{2+}]_i\) by releasing \text{Ca}^{2+} \text{ from intracellular stores.}

Halothane (administered 75 s before TRH challenge) also produced inhibition of the TRH-induced increase in \([\text{Ca}^{2+}]_i\) (fig. 2B). This contrasts with our previously published data showing that administration of halothane (0–0.75 mM) to GH3 cells 60 s before TRH challenge has minimal effects on the ensuing change in \([\text{Ca}^{2+}]_i\). To resolve this apparent discrepancy, the time
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course of halothane inhibition of agonist-stimulated increases in [Ca²⁺], was examined. GH₃ cells were treated with 1 mM halothane at various times (0–600 s) before challenge with either 100 nM TRH or 40 mM KCl (fig. 3A). (n.b., Experiments using KCl as an agonist were conducted in the absence of Ca²⁺ channel antagonists.) The peak [Ca²⁺] response to TRH decayed as a unexponential function of time after halothane administration, reaching a new steady-state level (≈40% of control) within 300–600 s. The magnitude and time course of halothane’s effect on the TRH response were the same in the presence of either nifedipine or LaCl₃. In contrast, halothane inhibition of KCl-induced increases in [Ca²⁺] was immediate (<30 s) and time-independent (over 30–600 s). The rapid inhibition of the KCl response demonstrates that halothane is rapidly equilibrating with the cells. This rapid equilibration indicates that the slow time course of halothane inhibition of the TRH response is not a mixing artifact. The rapid increase in resting [Ca²⁺] elicited by halothane (fig. 2D) also shows that halothane is rapidly accessing intracellular compartments. This eliminates the possibility that halothane blocks either the IP₃ receptor or its associated channel, because such blockade should have a rapid (<30 s) time course.

To further characterize halothane’s effect on IP₃-gated Ca²⁺ stores, we examined the effects of various concentrations of halothane (0–3.0 mM) on the time-dependent decay of the peak [Ca²⁺] response to TRH (fig. 3B). Halothane produced a concentration-dependent decrease in the steady-state response to TRH (defined as the peak [Ca²⁺] response elicited by TRH 600 s after halothane administration). Inhibition of the [Ca²⁺] response to TRH occurred within a clinically relevant range of halothane concentrations. The steady-state response to TRH was inhibited by halothane with an IC₅₀ of 0.6 ± 0.03 mM (fig. 3C).

Reversibility of Halothane Inhibition of the TRH Response

To ensure that halothane’s effects on the TRH response were not due to protein denaturation, cell death, or other irreversible phenomena, recovery experiments were performed. Cells were incubated with halothane for 10 min, with or without a subsequent 20-min period for volatilization of halothane, before challenge with TRH (100 nM). As shown in figure 4, 1 mM halothane inhibits the TRH-induced increase in [Ca²⁺] by about 65%. A 20-min period for volatilization of halothane allows the response to recover to more than 90% of control. Ten-, 20-, and 30-min exposures to halothane produce equal inhibition of the TRH response (data not shown). This indicates that the effects of halothane are reversible and that the IP₃-sensitive calcium stores can refill over the 20-min recovery period.

The Effects of Thapsigargin on Resting [Ca²⁺], and on TRH-induced Ca²⁺ Mobilization

The magnitude of the peak [Ca²⁺] response to TRH is a reflection of the amount of Ca²⁺ in the IP₃-releasable stores. Halothane could decrease the amount of IP₃-releasable Ca²⁺ by either increasing the rate of leak from the stores or inhibiting the rate at which Ca²⁺ is pumped (by the Ca²⁺-ATPase) into the stores. To distinguish between these two mechanisms, we used thapsigargin, a potent inhibitor of the Ca²⁺-ATPase in endoplasmic reticulum that has been shown to completely inhibit the uptake of calcium into IP₃-sensitive stores. Thapsigargin can rapidly increase resting [Ca²⁺] and deplete IP₃-sensitive intracellular Ca²⁺ stores in a variety of cells. In GH₃ cells, thapsigargin caused a slow and rather small (<50 nm) increase in resting [Ca²⁺] (fig. 2C), suggesting that the spontaneous leak of calcium from intracellular stores is relatively slow.

Thapsigargin, like halothane, produced a time-dependent inhibition of the peak [Ca²⁺] response to TRH (figs. 2C and 5A). The steady-state inhibition (defined as the peak [Ca²⁺] response elicited by TRH 600 s after thapsigargin administration) of the TRH response depended on thapsigargin concentration (fig. 5B), with an IC₅₀ of 273 ± 10 pm. The thapsigargin-induced decay of the peak [Ca²⁺] response to TRH was unexponential, and the time constant of the decay decreased as a function of thapsigargin concentration (fig. 5C), with an IC₅₀ of 1.3 nm. The time constant of the decay of the response reached a minimum value of 98 ± 9 s at a thapsigargin concentration of 3 nm, indicating that the Ca²⁺-ATPase is completely inhibited. This value of 98 s is therefore the time constant of the spontaneous leak of Ca²⁺ from IP₃-sensitive stores.

The Effects of Halothane on the Rate of Ca²⁺ Leak from IP₃-sensitive Stores

To determine whether halothane affects the rate of leak from IP₃-sensitive stores, we examined the time-dependent effects of halothane on the [Ca²⁺] response to TRH during complete inhibition of the Ca²⁺-ATPase by 30 nm thapsigargin. Halothane concentrations of 0.5 and 1.0 mM decreased the time constant of Ca²⁺ leak
Fig. 6. The effects of halothane and thapsigargin on clearance of Ca\(^{2+}\) from the cytosol of GH\(_3\) cells. After thyrotropin releasing hormone (TRH)-induced mobilization of Ca\(^{2+}\), [Ca\(^{2+}\)] declines exponentially (inset). The time constant of this decay was calculated after the administration of TRH in the absence (Control) and presence of halothane and thapsigargin. The decay of [Ca\(^{2+}\)] is well fit by a monoexponential function in all experiments. The time constant of the decay was 7.0 ± 0.7 s for Control. Thapsigargin at all points after administration and at all concentrations had no effect on the time constant of decay (7.1 ± 0.8 s). Halothane at all time points after administration and at all concentrations tested (0.5–3.0 mM) increased the time constant of the decay to 13.5 ± 0.8 s. All reported time constants are the mean ± SEM from at least 10 experiments. a.u. = arbitrary units.

from a control value of 98 ± 9 s to 74 ± 12 and 46 ± 6 s, respectively. This indicates that halothane, at clinically relevant concentrations, causes a marked concentration-dependent increase in the rate of Ca\(^{2+}\) leak from IP\(_3\)-sensitive stores.

**The Effects of Halothane and Thapsigargin on Clearance of Ca\(^{2+}\) from the Cytosol**

IP\(_3\) rapidly empties Ca\(^{2+}\) stores producing a peak in [Ca\(^{2+}\)]. The subsequent decrease in [Ca\(^{2+}\)], is caused by clearance of Ca\(^{2+}\) from the cytosol. This is illustrated in figure 1A, where TRH elicits a sharp peak in [Ca\(^{2+}\)], followed by an exponential decay toward resting [Ca\(^{2+}\)] levels. Both thapsigargin and halothane reduce the peak response to TRH, but only halothane appears to prolong the decay (figs. 2B and 2C). To quantify this effect, we fit the decay of [Ca\(^{2+}\)], from the peak of the TRH response to a steady baseline. In all cases, these curves were well fit by a unexponential function (fig. 6, inset). Interestingly, thapsigargin at all concentrations and time points after administration had no effect on the decay of [Ca\(^{2+}\)], (fig. 6). This indicates that the endoplasmic reticulum Ca\(^{2+}\)-ATPase has little role in rapid clearance of Ca\(^{2+}\) from the cytosol. Halothane, in contrast, prolonged the time constant of decay of [Ca\(^{2+}\)], nearly twofold (fig. 6). This prolongation occurred at even the earliest times (15 s) after halothane administration, confirming rapid equilibration of halothane with intracellular sites. The effect of halothane on the time constant of [Ca\(^{2+}\)] decay was also independent of concentration (0.5–3.0 mM), suggesting that the effect of halothane on the relevant clearance process is saturated at very low concentrations. These experiments were initially conducted in the presence of nimodipine and subsequently repeated in the presence of LaCl\(_3\) with identical results.
HALOTHANE INHIBITS IP₃ RESPONSES

Discussion

In this paper, GH₃ cells were used as a model system to study the effects of halothane on receptor-stimulated mobilization of Ca²⁺ from IP₃-sensitive, intracellular stores. TRH was used as an agonist to stimulate the generation of IP₃, and IP₃-mediated changes in [Ca²⁺]ᵢ were monitored using the fluorescent indicator Fura-2. The results show that halothane causes a time- and concentration-dependent inhibition of TRH-induced elevation of [Ca²⁺]ᵢ and that this effect occurs within a clinically relevant range of halothane concentrations.

The results also provide some localization of halothane's action within the TRH signaling pathway. Previous data from our laboratory showed that halothane (0.5 mM) does not affect TRH-stimulated inositol phosphate generation, thus eliminating the TRH receptor, its associated G-protein, and phospholipase C as loci for halothane's action. The current results suggest that halothane neither interferes with IP₃ binding to its receptor nor blocks ion flux through the IP₃ receptor/ion channel. By exclusion, the action of halothane on the TRH response must be mediated by reduction of the Ca²⁺ available to be released by IP₃.

The amount of Ca²⁺ in IP₃-releasable stores is controlled both by the spontaneous rate of leak of Ca²⁺ from the stores and by the rate of reuptake of Ca²⁺ into the stores by the Ca²⁺-ATPase. At steady-state, the rate of leak from the stores must be equal to the rate of reuptake. The rate of leak can be simply described as

\[
\text{Rate of leak} = k_l [Ca^{2+}]_i
\]

where \( k_l \) is the rate constant of the leak process and \([Ca^{2+}]_i\) is the calcium concentration in the intracellular stores. Using thapsigargin to block reuptake, we measured the time constant of leak from intracellular stores and calculated its reciprocal, \( k_l \), to be \(1.0 \times 10^{-2} \text{ s}^{-1}\) (fig. 5C). Halothane produced a concentration-dependent increase in \( k_l \) with values of \(1.4 \times 10^{-2} \text{ s}^{-1}\) at 0.5 mM and \(2.2 \times 10^{-2} \text{ s}^{-1}\) at 1.0 mM.

Are anesthetic-induced increases in \( k_l \) sufficient to account for the effect of halothane on the TRH response? In the absence of a change in the rate of Ca²⁺ reuptake, an increase in \( k_l \) must result in a reciprocal decrease in \([Ca^{2+}]_i\). The amplitude of the peak increase in \([Ca^{2+}]_i\) elicited by TRH challenge 10 min after halothane administration (fig. 3B) provides a reasonable index of steady-state \([Ca^{2+}]_i\), at any given concentration of halothane. Halothane concentrations of 0.5 mM and 1.0 mM halothane reduce the TRH response to \(\approx 70\%\) and \(\approx 40\%\) of the control response, respectively (fig. 3C). These are almost precise reciprocals of the measured changes in \( k_l \). These calculations indicate that halothane's effect on the TRH response can be explained by the increase in the rate of Ca²⁺ leak from intracellular stores.

Previous studies of anesthetic effects on intracellular Ca²⁺ stores have focused largely on anesthetic-induced depletion of \([Ca^{2+}]_i\) from skeletal muscle and cardiac sarcoplasmic reticulum (SR). These studies indicate, with some exception, that clinically relevant concentrations of halothane produce either no effect or mild stimulation of the SR Ca²⁺-ATPase. These results are consistent with our observations for IP₃-sensitive calcium stores and indicate that inhibition of Ca²⁺-ATPase is not responsible for depletion of intracellular Ca²⁺ stores. Previous studies also indicate that volatile anesthetics stimulate \([Ca^{2+}]_i\) leak from SR. In heavy SR preparations, volatile anesthetics promote a calcium leak that is inhibited by ruthenium red, implicating the ryanodine receptor (Ca²⁺-release channel) as an anesthetic target. In both heavy and light SR, a second anesthetic-induced leak is insensitive to ruthenium red, which may be similar to the anesthetic-induced leak that we have observed in IP₃-sensitive stores. In porcine skeletal muscle SR, the rate constant of this ruthenium red-resistant leak ranges from 1 to \(5 \times 10^{-2} \text{ s}^{-1}\), values comparable to the value we measured in GH₃ cells.

One previous study examined the effects of halothane on IP₃-mediated Ca²⁺ mobilization. This study in A7r5 cells showed that halothane attenuates the \([Ca^{2+}]_i\) response to arginine vasopressin and platelet-derived growth factor, two agents that stimulate IP₃ production via different signaling pathways. These anesthetic effects were attributed partly to depletion of intracellular \([Ca^{2+}]_i\) stores and partly to inhibition of arginine vasopressin-stimulated phospholipase C activity. This contrasts with our previous results showing no effect of halothane on TRH-stimulated inositol phosphate generation. This apparent discrepancy may be due to selective effects of halothane on certain receptors, G-proteins, or phospholipase C isozymes.

The current results do not define the molecular basis for the spontaneous and halothane-induced leak of Ca²⁺ from IP₃-sensitive stores in GH₃ cells. Studies in permeabilized RBL-1 cells indicate that the IP₃ receptor/channel itself is the major source of spontaneous Ca²⁺ leak from IP₃-sensitive stores. Assuming that halothane acts by augmenting the spontaneous leak process.
this would suggest the IP$_3$ receptor as an anesthetic target protein. This is a plausible hypothesis, because the IP$_3$ receptor shares significant partial sequence homology with the ryanodine receptor,$^9$-11 a demonstrated anesthetic target protein. Alternatively, it is possible that halothane-induced leak and spontaneous leak occur through different pores.

This study also provides some insight into the cellular mechanisms involved in Ca$^{2+}$ clearance from the cytosol. TRH stimulation results in a rapid rise of [Ca$^{2+}$], followed by a monoeponential decay (fig. 6). The time constant of this decay process is unaffected by pretreatment with concentrations of thapsigargin that completely inhibit the endoplasmic reticulum Ca$^{2+}$-ATPase. This indicates that cytosolic Ca$^{2+}$ clearance is not the result of Ca$^{2+}$ reuptake by the endoplasmic reticulum. The plasma membrane Ca$^{2+}$-ATPase and Na-Ca$^{2+}$ exchanger are likely to mediate cytosolic Ca$^{2+}$ clearance. Halothane increased the time constant of the decay of [Ca$^{2+}$], suggesting that it inhibits one of these two processes. The time constant of the [Ca$^{2+}$] decay was sensitive to halothane with a maximal effect observed at 0.5 mM halothane. These observations are consistent with the data of Koss-Kosicka et al., who showed that the erythrocyte plasma membrane Ca$^{2+}$-ATPase is completely inhibited by clinical concentrations of halothane.$^{41}$

The collective data from our current and past studies in GH$_3$ cells indicate that clinical concentrations of halothane affect three processes involved in regulation of [Ca$^{2+}$]: influx of Ca$^{2+}$ through voltage-gated L-type Ca$^{2+}$ channels, leak of Ca$^{2+}$ from IP$_3$-gated intracellular stores, and clearance of cytosolic Ca$^{2+}$. Depending on the importance of each of these processes and the interplay of these various effects, halothane may have significantly different effects on Ca$^{2+}$ regulation in different cell types. For example, halothane causes a large transient increase in [Ca$^{2+}$] in several cell types$^{8,26,42}$ but not in GH$_3$ cells. Based on the data from GH$_3$ cells, it appears unlikely that halothane-induced leak of Ca$^{2+}$ from intracellular stores contributes to central nervous system depression; the slow time course of leak from intracellular stores appears to be inconsistent with the fact that clinical anesthesia can be induced in a matter of seconds. However, it is possible that, if halothane produces a large [Ca$^{2+}$] transient in central nervous system neurons, this could contribute to the anesthetic state by facilitating anesthetic inhibition of GABAA channels.$^{43}$

The ability of anesthetics to deplete IP$_3$-releasable Ca$^{2+}$ stores may have important physiologic and clinical implications unrelated to the mechanism of anesthesia. Depletion of intracellular Ca$^{2+}$ stores should attenuate the actions of a variety of hormones and neurotransmitters that use the IP$_3$ pathway to produce their effects,$^{44}$ potentially contributing to many volatile anesthetic side effects including bronchodilatation, vasodilation, and unresponsiveness to vasoconstrictive agents. Inhibition of IP$_3$ responses also could inhibit neuronal and endocrine secretory processes. In this regard, it is notable that 10-min pretreatment with halothane causes minimal inhibition of the early phase of TRH-induced prolactin secretion in GH$_3$ cells,$^{22}$ whereas it causes significant reduction of TRH-induced Ca$^{2+}$ mobilization. (n.b., The early phase of TRH-induced prolactin secretion is temporally associated with the IP$_3$-induced rise in [Ca$^{2+}$],.) This suggests that the early phase of TRH-induced prolactin secretion predominantly is due to protein kinase C activation$^{45}$; we previously showed that protein kinase C-induced prolactin secretion is unaffected by halothane.$^{22}$

The major finding of this paper is that clinical concentrations of halothane cause a concentration-dependent leak of Ca$^{2+}$ from IP$_3$-sensitive stores. This results in depletion of the IP$_3$-gated stores with resultant inhibition of IP$_3$-mediated processes. Isoflurane and octanol appear to produce similar effects. Elucidation of the molecular basis of the anesthetic-induced leak should prove instructive in understanding molecular mechanisms of anesthesia and anesthetic side effects.

References


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