Volatile Anesthetic-induced Efflux of Calcium from IP₃-gated Stores in Clonal (GH₃) Pituitary Cells

M. Delavar Hossain, M.B.B.S., Ph.D.,* Alex S. Evers, M.D.†

Background: Many hormones and neurotransmitters produce their effects by stimulating the generation of inositol 1,4,5-trisphosphate (IP₃), a chemical second messenger that releases Ca²⁺ 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 GH₃ cells (a clonal cell line) as a model system in which to characterize the effects of volatile anesthetics on IP₃-induced mobilization of Ca²⁺ from intracellular stores.

Methods: Intracellular Ca²⁺ concentration ([Ca²⁺]) was continuously monitored in suspensions of GH₃ cells at 37°C using the fluorescent Ca²⁺ indicator Fura-2. Thyrotropin releasing hormone (TRH) was used to discharge IP₃-sensitive intracellular Ca²⁺ stores. The effects of halothane, isoflurane, and octanol on TRH-induced Ca²⁺ mobilization were assessed as a function of time and anesthetic concentration. To distinguish between anesthetic effects on Ca²⁺ uptake and Ca²⁺ release, experiments were performed using thapsigargin (a Ca²⁺-ATPase inhibitor) to inhibit Ca²⁺ uptake into IP₃-sensitive stores.

Results: Halothane increased resting [Ca²⁺] and caused a time- and concentration-dependent inhibition of TRH-induced increases in [Ca²⁺]. (IC₅₀ = 0.6 mm). Thapsigargin, in concentrations that completely inhibit Ca²⁺ uptake by IP₃-sensitive stores, also caused a time-dependent reduction in the [Ca²⁺] response to TRH; the time constant of this decay describes the rate of spontaneous leak of Ca²⁺ from IP₃-sensitive stores (τ = 98 ± 9 s). In the presence of thapsigargin, halothane produced concentration-dependent increases in the rate of leak from IP₃-sensitive stores (γ = 74 ± 12 and 46 ± 6 s at 0.3 and 1.0 mm halothane, respectively). Isoflurane and octanol also produced concentration-dependent inhibition of the [Ca²⁺], response to TRH.

Conclusions: Halothane causes a concentration-dependent leak of Ca²⁺ from IP₃-sensitive stores, leading to depletion of the stores and inhibition of IP₃-induced increases in [Ca²⁺]. 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 triphosphate. Thyrotropin releasing hormone. Fluorescent dyes: Fura-2. Pituitary cells: GH₃ 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.1,2 These drugs also can affect slower forms of intercellular signaling, including those mediated by hormones and neuromodulators acting at G-protein coupled receptors.3 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,4 enhancement5 or inhibition of chemical second messenger generation,6 and inhibition of protein phosphorylation.7

Mobilization of intracellular calcium from stores gated by the second messenger inositol 1,4,5-trisphosphate (IP₃) 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 [Ca²⁺], and to inhibit the rise in [Ca²⁺], elicited by arginine vasopressin and platelet-derived growth factor; both arginine vasopressin and platelet-derived growth factor are agonists that stimulate IP₃ generation and subse-
quent release of Ca$^{2+}$ from IP$_3$-sensitive stores. Halothane-induced increases in [Ca$^{2+}$]$_i$ also have been observed in A-10 and BC3H1 cells. These data are consistent with an effect of halothane on IP$_3$ generation, IP$_3$ action, or IP$_3$-gated calcium stores. Second, an analogy can be drawn between intracellular calcium stores gated by ryanodine receptors and those gated by IP$_3$ 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$^{2+}$ through ryanodine-sensitive channels leading to depletion of the calcium stores.$^{9,10}$ The ryanodine receptor and the IP$_3$ channel are homologous proteins$^{11-13}$ that might be effected similarly by volatile anesthetics. This study was performed to characterize the effects of volatile anesthetics on IP$_3$-gated intracellular stores of calcium.

In the current study, GH$_3$ cells, a transformed rat pituitary cell line, were used to examine the effects of volatile anesthetics on IP$_3$-sensitive calcium stores. These clonal cells are thought not to have ryanodine-sensitive intracellular calcium stores but contain IP$_3$-releasable stores of calcium that have been studied extensively using fluorescent indicator techniques.$^{14,15}$ In GH$_3$ cells, a variety of hormones, including thyrotropin releasing hormone (TRH), bind to cell surface receptors that are linked to the G-proteins $G_4$ and $G_6$.$^{16}$ Binding of TRH to its receptor leads to G-protein activation, which in turn activates a phospholipase C-$\beta$. The phospholipase C-$\beta$ cleaves the membrane lipid phosphatidylinositol bisphosphate yielding two second messengers, IP$_3$ and diacylglycerol.$^{18,19}$ IP$_3$ 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$^{2+}$]$_i$, leads to secretion of the stored peptide hormones prolactin and growth hormone. TRH also depolarizes GH$_3$ cells, causing activation of voltage-gated calcium channels and increased frequency of calcium action potentials.$^{20,21}$

In previous work with GH$_3$ cells, we showed that clinically relevant concentrations of halothane have minimal effects on TRH-stimulated activation of phospholipase C-$\beta$ and the attendant generation of inositol phosphates. In keeping with this, halothane did not alter IP$_3$-mediated increases in [Ca$^{2+}$]$_i$ when applied immediately before TRH stimulation.$^{22}$ However, halothane markedly and specifically inhibited activation of voltage-gated L-type calcium channels.$^{23}$ We now report that halothane, isoflurane, and octanol cause time- and concentration-dependent depletion of Ca$^{2+}$ from IP$_3$-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.$^{24}$

**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$_3$ 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$_2$ 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$^{2+}$]$_i$**

GH$_3$ cells were loaded with Fura-2 AM as previously described.$^{25}$ Cells loaded with Fura-2 were washed and resuspended in physiologic saline consisting of (mm): NaCl 140, KCl 5.4, HEPES 10, CaCl$_2$ 3, MgCl$_2$ 2, Dextrose 10, and probenecid 2.5, with a pH of 7.4. Probenecid was included to prevent leakage of Fura-2 from cells$^{25}$ and did not qualitatively alter the [Ca$^{2+}$]$_i$ response to any of the drugs used in this study. The cells (1-2 x 10$^6$ 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.$^{26}$ Drugs were delivered into the cuvettes as 1–60-μl volumes of con-
HALOTHANE INHIBITS IP₃ RESPONSES

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 [Ca²⁺].

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 bandwidths 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. [Ca²⁺] was calculated from the fluorescence measurements using the equations described by Gryniewicz et al. All values of [Ca²⁺] are reported as intracellular concentrations (nm). TRH-induced changes in [Ca²⁺] were calculated by subtracting the baseline value of [Ca²⁺] immediately before TRH addition from the peak value of [Ca²⁺], elicited by TRH. KCl-induced changes in [Ca²⁺] were calculated by subtracting [Ca²⁺] immediately before KCl addition from the stable plateau value of [Ca²⁺], 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 μM halothane in gas. Halothane produces general anesthesia in rats with an EC₅₀ 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 \( y = \text{Min} + \frac{(\text{Max} - \text{Min})}{(1 + (x/x_{50})^{-n})} \), where \( x_{50} \) is the concentration producing half-maximal response and \( n \) is the Hill coefficient. Values for IC₅₀ and EC₅₀ 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 (PIT data analysis software, Biosoft, Ferguson, MD).

**Results**

**Isolation of IP₃-mediated Calcium Mobilization in GH₃ Cells**

Application of TRH to GH₃ cells elicits a transient IP₃-mediated increase in [Ca²⁺], followed by a sustained elevation of [Ca²⁺], resulting from Ca²⁺ influx via plasma membrane Ca²⁺ channels. The sustained elevation of [Ca²⁺], is completely inhibited by the inorganic Ca²⁺ channel antagonist LaCl₃ (1 μM), allowing isolation of the IP₃-dependent increase in [Ca²⁺] (fig. 1A). It is characteristic of the IP₃ response that, despite the continued presence of agonist (TRH), released Ca²⁺ is cleared from the cytosol and [Ca²⁺], returns to near baseline values within 30 s (fig. 1A).

The sustained elevation of [Ca²⁺] occurring after TRH administration results from two pharmacologically distinct components. TRH is known to depolarize GH₃ cells leading to influx of calcium through voltage-gated channels. The component of the [Ca²⁺] response to TRH resulting from Ca²⁺ influx through voltage-gated channels is sensitive to both nimodipine (240 nm) and halothane (1 mM; figs. 1B and 1C). An additional component to the sustained phase of the [Ca²⁺] response to TRH is blocked by La³⁺ (5 μM) but is insensitive to either halothane or nimodipine (figs. 1B and 1C). This La³⁺-sensitive component may be analogous to the "store-dependent Ca²⁺ influx" observed in PC-12 cells. To eliminate the contribution of depolarization-induced calcium influx to TRH-stimulated increases in [Ca²⁺], nimodipine (240 nm) was included in all experiments examining TRH effect. This concentration of nimodipine completely blocks the voltage-dependent component of Ca²⁺ influx, as evidenced by the fact that it completely blocks KCl-induced increases in [Ca²⁺] (fig. 1D). To ensure that Ca²⁺ influx did not contribute to any of the observed effects, specifically indicated experiments also were conducted in the

Anesthesiology, V 80, No 6, Jun 1994
Fig. 1. The effects of Ca\(^{2+}\) channel antagonists on thyrotropin releasing hormone (TRH)- and KCl-induced changes in intracellular Ca\(^{2+}\) concentration in GH\(_3\) cells. (A) Continuous application of TRH (100 nM) results in a rapid, transient increase in [Ca\(^{2+}\)], followed by a sustained increase in [Ca\(^{2+}\)]. The sustained increase in [Ca\(^{2+}\)] is eliminated by addition of 5 \(\mu\)M LaCl\(_3\) (left) and prevented by pretreatment with 5 \(\mu\)M LaCl\(_3\) (right). (B and C) The sustained increase in [Ca\(^{2+}\)], produced by TRH administration, has two pharmacologically distinct components: one component is sensitive to nimodipine (240 nM), halothane (1 mM), and La\(^{3+}\) (5 \(\mu\)M), whereas the other component is sensitive only to La\(^{3+}\). (D) The increase in [Ca\(^{2+}\)], produced by KCl (40 mM) is completely inhibited by nimodipine (240 nM).

Control experiments also were performed to ensure that TRH-mediated mobilization of intracellular Ca\(^{2+}\) was not complicated by (Ca\(^{2+}\)-induced) calcium release from ryanodine-sensitive stores. These experiments showed that caffeine (10 mM), ryanodine (10 \(\mu\)M), ruthenium red (100 \(\mu\)M), or caffeine and ryanodine in combination affected neither resting [Ca\(^{2+}\)] nor the TRH-induced increase in [Ca\(^{2+}\)]. (elicted 1 and 10 min

Fig. 2. The effects of thyrotropin releasing hormone (TRH), halothane, and thapsigargin on intracellular Ca\(^{2+}\) concentration in GH\(_3\) cells. (A) Continuous application of TRH results in a rapid but transient rise of [Ca\(^{2+}\)]. (B and C) Halothane and thapsigargin both increase resting [Ca\(^{2+}\)], and inhibit the subsequent TRH-induced rise of [Ca\(^{2+}\)]. (D) Administration of halothane (0.5, 1.0, 1.5, and 3.0 mM) increases resting [Ca\(^{2+}\)] in GH\(_3\) cells.
HALOTHANE INHIBITS IP₃ RESPONSES

Fig. 3. Time- and concentration-dependent effects of halothane on thyrotropin releasing hormone (TRH)- and KCl-stimulated increases in [Ca²⁺] in GH₂ cells. (A) GH₂ 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 (C) or 5 μM LaCl₃ (D). Neither LaCl₃ (A) nor nimodipine (D) produced any effect on the TRH response in the absence of halothane. Each time point represents the mean ± SEM of the peak rise in [Ca²⁺] 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 (C). Each time point in these experiments represents the mean ± SEM of the rise in [Ca²⁺] observed 160 s after KCl administration as measured in three separate experiments. The effect of halothane on the KCl-induced rise of [Ca²⁺] was maximal in less than 30 s, whereas its effect on the TRH-induced rise of [Ca²⁺] was time-dependent. (B) Cells were challenged with TRH (100 nM) at various times after the application of halothane at concentrations of 0.5 (B), 1.0 (γ), 1.5 (γ), and 3.0 mM (E). The figure plots peak [Ca²⁺] 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 [Ca²⁺]. TRH response was elicited 600 s after the administration of halothane. The curve is fit to the Hill equation with an IC₅₀ 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 [Ca²⁺] are reversible. TRH-induced changes in [Ca²⁺] 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 [Ca\(^{2+}\)]. (A) TRH-induced changes in peak [Ca\(^{2+}\)] are plotted as a function of time after the administration of thapsigargin. Each curve represents a different thapsigargin concentration: 0.3 \(\bullet\), 1.0 \(\bigtriangleup\), 3.0 \(\blacksquare\), 10 \(\bigcirc\), 30 \(\triangledown\), and 100 nm \(\square\). 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 [Ca\(^{2+}\)] response elicited 600 s after thapsigargin administration) to TRH and the time constant describing the decay of the peak [Ca\(^{2+}\)] response to TRH. Calculated time constants for each thapsigargin concentration are 305 \(\pm\) 8, 275 \(\pm\) 8, 111 \(\pm\) 8, 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 [Ca\(^{2+}\)]. 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 IC\(_50\) of 273 \(\pm\) 10 pm and a Hill coefficient of 1.97. (C) Time constants of the monoexponential decays of the peak [Ca\(^{2+}\)] 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 Ca\(^{2+}\) from IP\(_3\)-gated stores. Halothane at 0.5 \(\square\) and 1.0 nm \(\triangledown\) causes a concentration-dependent increase in the rate of spontaneous leak of Ca\(^{2+}\) from IP\(_3\)-gated stores. Time constants are reported as \(\pm\)95% confidence limits.

after drug administration) in GH\(_3\) cells. This provides strong evidence for the absence of ryanodine-sensitive calcium stores in the GH\(_3\) cells used in these studies.

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

Administration of halothane to GH\(_3\) cells increased resting [Ca\(^{2+}\)]. 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 [Ca\(^{2+}\)] 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 [Ca\(^{2+}\)] (not shown). This indicates that halothane increases resting [Ca\(^{2+}\)] by releasing Ca\(^{2+}\) from intracellular stores.

Halothane (administered 75 s before TRH challenge) also produced inhibition of the TRH-induced increase in [Ca\(^{2+}\)] (fig. 2B). This contrasts with our previously published data showing that administration of halothane (0–0.75 mm) to GH\(_3\) cells 60 s before TRH challenge has minimal effects on the ensuing change in [Ca\(^{2+}\)]. To resolve this apparent discrepancy, the time
HALOTHANE INHIBITS IP$_3$ RESPONSES

course of halothane inhibition of agonist-stimulated increases in [Ca$^{2+}$]$_i$ was examined. GH$_3$ cells were treated with 1 mm halothane at various times (0–600 s) before challenge with either 100 nm TRH or 40 nm KCl (fig. 3A). (n.b., Experiments using KCl as an agonist were conducted in the absence of Ca$^{2+}$ channel antagonists.) The peak [Ca$^{2+}$]$_i$ response to TRH decayed as a uniaxial 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 nimodipine or LaCl$_3$. In contrast, halothane inhibition of KCl-induced increases in [Ca$^{2+}$]$_i$ 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 [Ca$^{2+}$]$_i$ elicited by halothane (fig. 2D) also shows that halothane is rapidly accessing intracellular compartments. This eliminates the possibility that halothane blocks either the IP$_3$ receptor or its associated channel, because such blockade should have a rapid (<30 s) time course.

To further characterize halothane’s effect on IP$_3$-gated Ca$^{2+}$ stores, we examined the effects of various concentrations of halothane (0–3.0 mm) on the time-dependent decay of the peak [Ca$^{2+}$]$_i$ response to TRH (fig. 3B). Halothane produced a concentration-dependent decrease in the steady-state response to TRH (defined as the peak [Ca$^{2+}$]$_i$ response elicited by TRH 600 s after halothane administration). Inhibition of the [Ca$^{2+}$]$_i$ response to TRH occurred within a clinically relevant range of halothane concentrations. The steady-state response to TRH was inhibited by halothane with an IC$_{50}$ 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 [Ca$^{2+}$]$_i$ 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 IP$_3$-sensitive calcium stores can refill over the 20-min recovery period.

The Effects of Thapsigargin on Resting [Ca$^{2+}$] and on TRH-induced Ca$^{2+}$ Mobilization

The magnitude of the peak [Ca$^{2+}$]$_i$ response to TRH is a reflection of the amount of Ca$^{2+}$ in the IP$_3$-releasable stores. Halothane could decrease the amount of IP$_3$-releasable Ca$^{2+}$ by either increasing the rate of leak from the stores or inhibiting the rate at which Ca$^{2+}$ is pumped (by the Ca$^{2+}$-ATPase) into the stores. To distinguish between these two mechanisms, we used thapsigargin, a potent inhibitor of the Ca$^{2+}$-ATPase in the endoplasmic reticulum that has been shown to completely inhibit the uptake of calcium into IP$_3$-sensitive stores. Thapsigargin can rapidly increase resting [Ca$^{2+}$]$_i$ and deplete IP$_3$-sensitive intracellular Ca$^{2+}$ stores in a variety of cells. In GH$_3$ cells, thapsigargin caused a slow and rather small (<50 nm) increase in resting [Ca$^{2+}$]$_i$ (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 [Ca$^{2+}$]$_i$ response to TRH (figs. 2C and 5A). The steady-state inhibition (defined as the peak [Ca$^{2+}$]$_i$ response elicited by TRH 600 s after thapsigargin administration) of the TRH response depended on thapsigargin concentration (fig. 5B), with an IC$_{50}$ of 273 ± 10 pm. The thapsigargin-induced decay of the peak [Ca$^{2+}$]$_i$ response to TRH was unexponential, and the time constant of the decay decreased as a function of thapsigargin concentration (fig. 5C), with an IC$_{50}$ 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 Ca$^{2+}$-ATPase is completely inhibited. This value of 98 s is therefore the time constant of the spontaneous leak of Ca$^{2+}$ from IP$_3$-sensitive stores.

The Effects of Halothane on the Rate of Ca$^{2+}$ Leak from IP$_3$-Sensitive Stores

To determine whether halothane affects the rate of leak from IP$_3$-sensitive stores, we examined the time-dependent effects of halothane on the [Ca$^{2+}$]$_i$ response to TRH during complete inhibition of the Ca$^{2+}$-ATPase by 30 nm thapsigargin. Halothane concentrations of 0.5 and 1.0 mm decreased the time constant of Ca$^{2+}$ leak
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.

**Effects of Other Anesthetics on IP\(_3\)-sensitive Ca\(^{2+}\) Stores**

The effects of octanol and isoflurane on the [Ca\(^{2+}\)] response to TRH also were examined. These experiments were performed by administering the anesthetic and challenging with TRH after 10 min. Both isoflurane and octanol produced a concentration-dependent inhibition of the peak [Ca\(^{2+}\)] response to TRH at concentrations similar to those required to produce anesthesia (fig. 7).

---

M. D. HOSSAIN AND A. S. EVERS

*Anesthesiology, V 80, No 6, Jun 1994*
Discussion

In this paper, GH3 cells were used as a model system to study the effects of halothane on receptor-stimulated mobilization of Ca^{2+} from IP3-sensitive, intracellular stores. TRH was used as an agonist to stimulate the generation of IP3, and IP3-mediated changes in [Ca^{2+}]_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 [Ca^{2+}]_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, which eliminates the TRH receptor, its associated G-protein, and phospholipase C-beta 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 Ca^{2+} available to be released by IP3.

The amount of Ca^{2+} in IP3-releasable stores is controlled both by the spontaneous rate of leak of Ca^{2+} from the stores and by the rate of reuptake of Ca^{2+} into the stores by the Ca^{2+}-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

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

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 \( \times 10^{-2} \) s^{-1} (fig. 5C). Halothane produced a concentration-dependent increase in \( k_l \) with values of 1.4 \( \times 10^{-2} \) s^{-1} at 0.5 mM and 2.2 \( \times 10^{-2} \) 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 Ca^{2+} 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 Ca^{2+} leak from intracellular stores.

Previous studies of anesthetic effects on intracellular Ca^{2+} stores have focused largely on anesthetic-induced depletion of [Ca^{2+}] 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 Ca^{2+}-ATPase. These results are consistent with our observations for IP3-sensitive calcium stores and indicate that inhibition of Ca^{2+}-ATPase is not responsible for depletion of intracellular Ca^{2+} stores. Previous studies also indicate that volatile anesthetics stimulate [Ca^{2+}] from SR. In heavy SR preparations, volatile anesthetics promote a calcium leak that is inhibited by ruthenium red, implicating the ryanodine receptor (Ca^{2+}-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 \( \times 10^{-2} \) s^{-1}, values comparable to the value we measured in GH3 cells.

One previous study examined the effects of halothane on IP3-mediated Ca^{2+} 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+}] 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 Ca^{2+} 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 Ca^{2+} leak from IP3-sensitive stores. Assuming that halothane acts by augmenting the spontaneous leak process...

Anesthesiology, V 80, No 6, Jun 1994.
this would suggest the IP₃ receptor as an anesthetic target protein. This is a plausible hypothesis, because the IP₃ 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 Ca²⁺ clearance from the cytosol. TRH stimulation results in a rapid rise of [Ca²⁺], 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 Ca²⁺-ATPase. This indicates that cytosolic Ca²⁺ clearance is not the result of Ca²⁺ reuptake by the endoplasmic reticulum. The plasma membrane Ca²⁺-ATPase and Na-Ca²⁺ exchanger are likely to mediate cytosolic Ca²⁺ clearance. Halothane increased the time constant of the decay of [Ca²⁺], suggesting that it inhibits one of these two processes. The time constant of the [Ca²⁺] decay was sensitive to halothane with a maximal effect observed at 0.5 mM halothane. These observations are consistent with the data of Kosk-Kosicka et al., who showed that the erythrocyte plasma membrane Ca²⁺-ATPase is completely inhibited by clinical concentrations of halothane.⁴¹

The collective data from our current and past studies in GH₃ cells indicate that clinical concentrations of halothane affect three processes involved in regulation of [Ca²⁺]: influx of Ca²⁺ through voltage-gated L-type Ca²⁺ channels, leak of Ca²⁺ from IP₃-gated intracellular stores, and clearance of cytosolic Ca²⁺. Depending on the importance of each of these processes and the interplay of these various effects, halothane may have significantly different effects on Ca²⁺ regulation in different cell types. For example, halothane causes a large transient increase in [Ca²⁺] in several cell types⁻⁸,²⁶,⁴² but not in GH₃ cells. Based on the data from GH₃ cells, it appears unlikely that halothane-induced leak of Ca²⁺ 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 [Ca²⁺] 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 IP₃-releasable Ca²⁺ stores may have important physiologic and clinical implications unrelated to the mechanism of anesthesia. Depletion of intracellular Ca²⁺ stores should attenuate the actions of a variety of hormones and neurotransmitters that use the IP₃ pathway to produce their effects,⁴⁴ potentially contributing to many volatile anesthetic side effects including bronchodilatation, vasodilation, and unresponsiveness to vasoconstrictive agents. Inhibition of IP₃ 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 GH₃ cells,²² whereas it causes significant reduction of TRH-induced Ca²⁺ mobilization. (n.b., The early phase of TRH-induced prolactin secretion is temporally associated with the IP₃-induced rise in [Ca²⁺].) This suggests that the early phase of TRH-induced prolactin secretion predominantly is due to protein kinase C activation⁴⁵; 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 Ca²⁺ from IP₃-sensitive stores. This results in depletion of the IP₃-gated stores with resultant inhibition of IP₃-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

Anesthesiology, V 80, No 6, Jun 1994
HALOTHANE INHIBITS IP₃ RESPONSES


10. Su JY, Kerrick WGL: Effects of halothane on caffeine-induced tension transients in functionally skinned myocardial fibers from rabbits. Pflugers Arch 380:29–34, 1979


36. Frazer MS, Lynch C: Halothane and isoflurane effects on Ca²⁺ fluxes of isolated myocardial sarcoplasmic reticulum. Anesthesiology 77:316–323, 1992


38. Louis CF, Zaalman, Rognhaf T, Michelon JR: The effects of volatile anaesthetics on calcium regulation by malignant hyperthermia-susceptible sarcoplasmic reticulum. Anesthesiology 77:114–125, 1992


