The Action of Halothane on Stimulus–Secretion Coupling in Clonal (GH₃) Pituitary Cells

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The effect of halothane on the physiological response to excitatory stimuli was assessed in clonal (GH₃) pituitary cells. Halothane, at concentrations used to produce general anesthesia in animals (0.25–0.76 mM), inhibited thyrotropin-releasing hormone (TRH)-induced prolactin (PRL) secretion. The sustained (extracellular calcium-dependent) phase of PRL secretion was $70 \pm 7\%$ inhibited by the highest concentration of halothane tested (0.76 mM); 50% inhibition was produced by ≈ 0.4 mM halothane. The early (largely inositol trisphosphate-mediated) phase of secretion was less sensitive to halothane; 0.76 mM halothane produced $18 \pm 2\%$ inhibition of the early phase of secretion. Consistent with these observations, halothane inhibited ($IC_{50} \approx 0.45$ mM) the sustained phase of the TRH-induced rise in intracellular calcium ($[Ca^{2+}]_i$) to a greater extent than the initial $[Ca^{2+}]_i$ peak. The sustained phase of the $[Ca^{2+}]_i$ elevation was inhibited by $75 \pm 7\%$ at the highest concentration of halothane tested (0.76 mM), whereas the peak $[Ca^{2+}]_i$ was only inhibited by $14 \pm 5\%$, consistent with the observation that halothane did not inhibit TRH-stimulated inositol hydrolysis in these cells. Halothane (0.5 mM) did not inhibit phorbol ester- or ionomycin-induced PRL secretion, indicating that halothane has inconsequential effects on the secretory apparatus. Halothane (0.5 mM) also inhibited KCl-induced PRL secretion by 50–80% and the corresponding KCl-induced rise in $[Ca^{2+}]_i$ by $68 \pm 6\%$. These data indicate that halothane inhibits secretagogue-stimulated PRL secretion by reducing the elevation of $[Ca^{2+}]_i$ produced by calcium (Ca^{2+}) influx.

The effects of halothane on average resting membrane potential and on the average KCl- and TRH-induced membrane depolarization were measured to determine if alterations in membrane potential might be responsible for the effect of halothane on the secretagogue-induced elevations of $[Ca^{2+}]_i$. In nimodipine-treated cells, it was found that halothane neither altered resting membrane potential nor affected TRH- or KCl-induced depolarization. These findings support the view that halothane can act to inhibit the phys-

iological response to excitatory stimuli by selectively reducing the elevation in $[Ca^{2+}]_i$ produced by cellular depolarization.

It is thought that volatile anesthetics produce CNS depression, at least in part, as a result of decreased excitatory neurotransmission. The effect of volatile anesthetics on excitatory pathways has been shown to result principally from depression of synaptic transmission rather than from inhibition of axonal or neuronal conduction of the electrical impulse. This was first shown in sympathetic ganglia (Larrabee and Posternak, 1952) and has since been demonstrated in several CNS preparations including olfactory cortex (Richards, 1973; Richards et al., 1975) and dentate gyrus (Richards and White, 1975). It has also been shown that some excitatory synapses [e.g., the CA3–CA1 synapse in rat hippocampus (Pearce et al., 1989)] are resistant to the effects of volatile anesthetics, suggesting some specificity in the molecular targets and/or cellular physiological effects of these drugs.

Further examination of the effects of volatile anesthetics on excitatory synaptic function has yielded convincing evidence that they are capable of depressing both presynaptic and postsynaptic events. More specifically, it has been shown that volatile anesthetics decrease neurotransmitter release at group Ia afferent synapses on spinal motoneurons (Zorychta et al., 1975; Kullmann et al., 1989) and decrease electrical responses to exogenously applied excitatory neurotransmitters in olfactory cortex (Richards and Smaje, 1976). Unfortunately, further details of the molecular basis of volatile anesthetic inhibition of excitatory synaptic transmission remain unclear, at least in part because of the complex biochemical and electrophysiological properties of intact CNS preparations.

To determine more precisely the effects of volatile anesthetics on the release of and response to excitatory substances, we studied the action of volatile anesthetics on stimulus–secretion coupling in a transformed pituitary cell line (GH₃ cells). These cells are electrically excitable neuroendocrine cells in which the signal transduction cascade linking agonist stimulation [thyrotropin-releasing hormone (TRH)] to hormone release [prolactin (PRL)] has been extensively studied (for a review, see Gershengorn, 1986). This system has permitted us to explore the action of anesthetics on well-characterized biochemical pathways and physiological events.

In GH₃ cells, the excitatory signal cascade is activated by the binding of agonist (TRH) to its receptor. This results in the activation of a phospholipase C (PLC) via a G-protein-linked process (Hinkle and Kinsella, 1984; Hinkle and Phillips, 1984). The activated PLC cleaves phosphatidylinositol 4,5-bisphos-

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phate (PIP₂) to yield two second messengers, inositol trisphosphate (IP₃; Martin, 1983; Drummond et al., 1984) and diacylglycerol (Rebecchi et al., 1983). IP₃ causes a rapid, transient rise in [Ca²⁺]_i as a result of Ca²⁺ release from intracellular stores (Albert and Tashjian, 1984a; Gershengorn and Thaw, 1985). After the initial peak in [Ca²⁺]_i, the cells depolarize [apparently as a result of potassium channel inhibition (Ozawa, 1981; Dubinsky and Oxford, 1985; Bauer et al., 1990)], and the Ca²⁺ action potential frequency increases (Kidokoro, 1975). This results in influx of Ca²⁺ through voltage-dependent Ca²⁺ channels (VDCCs; Schlegel et al., 1987) and, consequently, a sustained elevation of [Ca²⁺]_i. Ultimately, the biphasic rise in [Ca²⁺]_i produces a corresponding biphasic increase in the rate of PRL secretion (Albert and Tashjian, 1984a,b; Martin and Kowalczyk, 1984a,b).

We report here the effects of the volatile anesthetic halothane on secretagogue-induced changes in PRL secretion, membrane potential, and [Ca²⁺]_i.

The results of some of these experiments have been reported in preliminary form (Stern and Evers, 1990).

Materials and Methods

Drugs. Halothane was purchased from Ayerst (New York, NY). All other drugs used were obtained from Sigma (St. Louis, MO) unless otherwise noted.

Cell culture. GH₃ cells were obtained from the American Type Tissue Culture Collection (Rockville, MD). Passage numbers 21–40 were used for experiments. Cells were grown in monolayer cultures at 37°C in a humidified, 5% CO₂ atmosphere. Growth medium consisted of Ham's F-10, 15% horse serum, and 2.5% fetal bovine serum. For experiments in which membrane potential and [Ca²⁺]_i were measured, cells were grown in suspension for 24 hr prior to experimentation.

Fluorescence measurements. Membrane potential and [Ca²⁺]_i were measured using a Photon Technology International Inc. (South Brunswick, NJ) Deltascan spectrophotometer with the fluorescent indicators bis(1,3-dibutylthiobarbituric acid)-trimethine oxonol (bisoxonol) and fura. Dyes were obtained from Molecular Probes (Eugene, OR) and were prepared for use as concentrated stocks in dimethyl sulfoxide. Experiments were performed at 37°C (unless otherwise noted) in saline consisting of (in mM) NaCl, 140; KCl, 5.4; HEPES, 10; CaCl₂, 1.8; and MgCl₂, 2 (pH, 7.5; physiological saline), in stirred, quartz cuvettes. For each experiment, 1–2 × 10⁶ cells were used. All drugs used had no effect on the intrinsic fluorescence of the dyes. Drugs were directly pipetted into the cuvette as 1–60-μl volumes of concentrated stocks to achieve the desired end concentrations. When necessary, compensations were made for the dilutional effects of drug addition on fluorescence.

Calcium measurements. GH₃ cells were loaded with fura-2 AM using a modification of the method reported by Poenie et al. (1986). The cells were incubated at room temperature (RT) for 30 min in 1 ml of physiological saline containing fura-2 AM (2.5 μM), 0.03% Pluronic F-127 (Molecular Probes), and 2% fetal bovine serum. The cells were then washed and resuspended in 2 ml of physiological saline containing probenecid (2.5 mM) to prevent leakage of fura from the cells (Di Virgilio et al., 1988). The presence of probenecid did not qualitatively alter the Ca²⁺ response to agonists or the effect of halothane. Experiments were initiated after allowing 10 min for temperature and baseline fluorescence stabilization. Fluorescence measurements using fura are reported as the ratio of the fluorescence emission at 500 nm [in arbitrary units (a.u.)] generated by rapidly alternating (100-Hz) excitations of 340- and 390-nm light (5-nm bandwidths for excitation and emission). For the purpose of comparison, fluorescence ratios were normalized to values of maximum and minimum fluorescence ratios generated by permeabilizing the cells with Triton X-100 (0.1%) and, subsequently, adding MnCl₂ (2.5 mM) to quench the fura signal.

Membrane potential measurements. Membrane potential measurements were made using the dye bisoxonol [$\lambda_{\text{excitation}} (\lambda_{\text{ex}}) = 395 \text{ nm}$; $\lambda_{\text{emission}} (\lambda_{\text{em}}) = 420 \text{ nm}$; 5-nm bandwidths], according to methods described by Brauner et al. (1984). This dye partitions between the cells and the saline on the basis of the potential across the cell membrane. Because bisoxonol has a net negative charge and the cell-associated dye has a higher quantum efficiency, cellular depolarization results in increased fluorescence,

and hyperpolarization results in decreased fluorescence. It should also be noted that (1) bisoxonol reports the time-averaged membrane potential of many cells and can only accurately resolve changes in membrane potential that occur over tens of seconds and (2) the relationship between bisoxonol fluorescence and membrane potential has previously been shown to be linear over the voltage range studied (Brauner et al., 1984).

After washing and resuspending the cells in physiological saline, cells were incubated with 2 μM bisoxonol for 10 min to allow for temperature equilibration and stabilization of the fluorescence signal. For purposes of comparison, the cells were depolarized to 0 mV by adding ≈100 nM gramicidin (a nonselective, monovalent cation ionophore) at the end of experiments. Fluorescence traces were then background subtracted and normalized to the fluorescence range defined by resting fluorescence and the fluorescence at 0 mV. A quantitative estimate of the relationship between fluorescence and voltage was made by measuring resting membrane potential (V_r) using electrophysiological techniques (J. Herrington, unpublished observations). V_r was $-45.7 \pm 2.4 \text{ mV}$ ($n = 3$); thus, the voltage range defined by the calibration procedure described above is ≈46 mV, and we estimate that changes in membrane potential ≥1.5 mV could be resolved using this technique. The limits of resolution were defined by a signal greater than two times the background noise.

PRL measurements. In experiments in which PRL was measured, cells were grown in 35-mm six-well plates. To enhance PRL synthesis, cells were supplemented with epidermal growth factor (10 nM) and estradiol (1 nM) 2 d prior to experimentation (Scammel et al., 1986). After a 1-hr preincubation in assay media (Ham's F-12, 0.2% BSA, 15 mM HEPES, pH 7.5), cells were washed, and drugs in 1 ml of fresh assay media were added directly to the six-well plates. Aliquots of the supernatant were then obtained for PRL measurements at specific times. To calculate rates of PRL secretion, it was necessary to subtract the amount of PRL secreted in the previous time period from the cumulative amount of PRL secreted and to divide this amount of PRL by the duration of the collection period. All experiments were performed at 37°C. PRL measurements were carried out using a radioimmunoassay with reagents generously supplied by Dr. Parlow of the National Institute for the Study of Diabetes, Digestive, and Kidney Diseases. Rabbit anti-rat PRL antibody was used at 1:10,000 dilution, and goat anti-rabbit antibody (Cedarlane Laboratories, Hornby, Ontario) was used as the second antibody (1:40 dilution) for immunoprecipitation. PRL measurements were normalized for the protein content of each well using the Pierce BCA protein assay reagents (Pierce, Rockford, IL). PRL secretion was variable in different batches of cells; basal rates varied by as much as fivefold, and TRH-stimulated rates varied by as much as 10-fold. However, the responses to agonists and halothane were qualitatively similar in all experiments despite the variability in absolute amounts of PRL secretion.

Inositol phosphates. GH₃ cells were grown in 35-mm, poly-L-lysine coated, six-well tissue culture plates. For experiments, growth medium was changed to inositol-free Ham's F-12 medium containing 2.5 μCi/ml of ³H-inositol (10–20 Ci/mmol; Amersham, Arlington Heights, IL), 2.5% dialyzed fetal bovine serum, and 15% dialyzed horse serum (Martin, 1986). The labeling medium was also supplemented with triiodothyronine (30 pM), transferrin (10 μg/ml), parathyroid hormone (1 ng/ml), fibroblast growth factor (1 ng/ml), somatomedin C (1 ng/ml), and insulin (10 μg/ml). The cells were labeled for 72 hr to achieve equilibrium labeling of cellular phosphoinositides (Martin, 1986). On the day of experimentation, the labeling medium was removed, and the cells were sequentially washed with inositol-free Ham's F-12 and physiological saline. Physiological saline (0.5 ml) was then added to each well. The cells were then preincubated either with or without halothane for 5 min. Incubations were started by the addition of 1 ml of saline, containing 15 mM LiCl (to give a final concentration of 10 mM LiCl), and the indicated concentrations of TRH and halothane. Incubations were terminated by the addition of 1.5 ml of iced 15% trichloroacetic acid (TCA). The cells were kept on ice for 30 min, and then scraped from the plate using a rubber policeman. The plates were washed twice with 0.5 ml of water, and the TCA-insoluble material was pelleted by centrifugation. TCA was removed from the supernatants by extraction with water-saturated diethyl ether. The aqueous phase from these extractions [containing all of the inositol phosphates (IP_x)] was diluted with 10 mM Tris buffer and pH corrected to 8.5. These samples were then applied to 0.5-ml Dowex AG1-X8 columns. The columns were washed with 30 ml Tris buffer to elute ³H-inositol, and the combined inositol phosphates (IP₁, IP₂, and IP₃) were eluted with 6 ml 0.3 M Na₂SO₄ (Heathers et al., 1989). Aliquots of the eluate (0.5 ml) were then added to Scintiverse II

cocktail and analyzed by scintillation spectrometry. The TCA-insoluble pellets from each incubation were solubilized overnight in a mixture of 10% Triton X-100, 0.07 M SDS, and 0.15 N NaOH for subsequent protein assay. Protein was determined in each sample (described above) to normalize the cpm of inositol phosphates per sample to cellular protein.

Volatile anesthetic administration and measurement. Halothane was delivered to experimental preparations by adding aliquots of halothane-saturated saline (17 mM, at 20°C) to give the desired end concentrations of halothane in saline. The concentration of anesthetic was subsequently maintained by blowing air containing halothane gas over the cuvette or six-well plate. Cells were exposed to halothane for at least 1 min prior to any subsequent addition of agonist. 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 assessed by previously described gas chromatographic methods (Stern et al., 1989). Volatile anesthetic concentrations are reported in millimolar concentrations: concentrations of 0.13, 0.25, 0.50, and 0.76 mM of halothane in saline at 37°C correspond to concentrations of 0.5, 1.0, 2.0, and 3.0 vol% halothane in gas and span the range of clinically effective concentrations of anesthetic in the rat. Halothane produces general anesthesia in rats with an EC_{50} of 1.24 vol% (Evers et al., 1986). This corresponds to a concentration of 0.31 mM in saline.

Data analysis. Statistical comparisons were made using two-tailed Student's *t* tests. Statistically significant differences were defined by $p < 0.05$. Experimental data are reported as the mean $\pm$ the SEM of three experiments unless otherwise indicated. TRH dose-response curves were fit by the first-order equation $f(x) = V_{max}/(1 + (EC_{50}/[TRH]))$ using the Levenberg-Marquardt RMSSQ subroutine of the IMSL statistical library (Bellaire, TX) for minimization of residuals. Values for EC_{50} and V_{max} are reported $\pm 90\%$ confidence limits.

Results

The effect of halothane on TRH-induced PRL secretion

To determine if halothane had physiologically significant effects on the integrated cellular response to TRH, we first examined the effect of halothane on basal and TRH-induced PRL secretion. Halothane, up to 0.76 mM, had small and inconsistent effects on basal PRL secretion. In contrast, the effects of halothane on TRH-induced PRL secretion were appreciable and consistently observed.

TRH application results in a biphasic secretory response (Fig. 1A); there is an initial large increase in the rate of PRL secretion, followed by a sustained, albeit lower, elevation of the PRL secretory rate. Halothane (0.5 mM) did not produce significant changes in either the V_{max} (10 ± 2 and 8.9 ± 1.6 ng PRL/min·mg protein for control and halothane, respectively) or the EC_{50} (3.9 ± 2.8 and 1.9 ± 1.7 nM TRH for control and halothane, respectively) of the early phase of TRH-induced PRL secretion (Fig. 1B). Halothane (0.5 mM) did reduce the V_{max} (5.5 ± 0.7 and 1.1 ± 0.7 ng PRL/min·mg protein for control and halothane, respectively), but not the EC_{50} (1.4 ± 0.9 and 2.3 ± 2.3 nM TRH for control and halothane, respectively) of the late phase of TRH-induced PRL secretion (Fig. 1C). Thus, one (or more) of the physiological processes governing the sustained phase of TRH-induced PRL secretion (i.e., protein kinase C activation, cellular depolarization, Ca^{2+} influx) was particularly sensitive to inhibition by halothane.

The concentration-response curve for the effect of halothane on TRH-induced (100 nM) PRL secretion (Fig. 1D) shows that halothane consistently inhibits the late phase of TRH-induced PRL secretion to a greater extent than the early phase. Halothane, at the highest concentration tested, depressed PRL secretion in the late phase by $70 \pm 7\%$ versus $18 \pm 2\%$ in the early phase. The IC_{50} (defined here as the concentration of halothane that reduces the rise in PRL secretion by 50%) for halothane-induced inhibition of the late phase was ≈ 0.40 mM. Ad-

ditional experiments showed that the effects of halothane on TRH-induced PRL secretion were 95% reversible when cells were tested with TRH after a 3-min preincubation in halothane (0.50 mM) and a subsequent 2-min wash and equilibration period.

The effect of halothane on TRH-stimulated inositol phosphate accumulation

To determine whether halothane inhibited TRH-stimulated PRL secretion via an effect on TRH-stimulated inositol hydrolysis, the following experiments were performed. GH₃ cells were labeled to equilibrium with 3H -inositol and, subsequently, stimulated with TRH in the presence of 10 mM LiCl. The time course of accumulation of 3H -IP_x in response to 3 nM TRH is shown in Figure 2A. Note that IP_x levels are indicative of PLC activity and inositol hydrolysis (because experiments are performed in the presence of the IP₁ phosphatase inhibitor LiCl) and do not directly reflect intracellular IP₃ concentrations. TRH-induced IP_x accumulation was linear over the 10-min time period of the incubations and was unaffected by 0.5 mM halothane. Halothane had only small effects on IP_x accumulation (10 min) elicited by any concentration of TRH (Fig. 2B); in the presence and absence, respectively, of halothane, the V_{max} was 113 ± 2 and 126 ± 6 cpm/10 min·mg protein, and the EC_{50} was 3.4 ± 0.3 and 3.3 ± 1.0 nM TRH. These data indicate that the function of TRH receptors, hormone-stimulated PLC, and the G-protein-linking receptor to PLC are relatively well preserved in the presence of halothane. Thus, inhibition of TRH-stimulated PRL secretion by halothane is not the result of decreased generation of inositol-derived second messengers.

The effect of halothane on the TRH-induced $[Ca^{2+}]_i$ signal

The effects of halothane on the TRH-induced elevation of $[Ca^{2+}]_i$ were measured to determine if alterations in $[Ca^{2+}]_i$ might be responsible for the effect of halothane on TRH-induced PRL secretion. In fura-loaded cells, there was little or no basal Ca^{2+} action potential activity, as evidenced by the fact that resting $[Ca^{2+}]_i$ was unaffected by the Ca^{2+} channel blockers LaCl₃ (50 μ M) or nimodipine (125 nM). Halothane (0.13–0.76 mM) also had no effect on resting $[Ca^{2+}]_i$ levels. In contrast, halothane had marked effects on the TRH-induced elevation in $[Ca^{2+}]_i$.

Normally, the TRH-induced rise in $[Ca^{2+}]_i$ consists of an initial IP₃-mediated, transient elevation of $[Ca^{2+}]_i$, followed by a sustained elevation of $[Ca^{2+}]_i$ above resting levels (Fig. 3A). Ca^{2+} channels play an important role in the sustained elevation of $[Ca^{2+}]_i$, as evidenced by the fact that 125 nM nimodipine [a concentration shown to inhibit fully L-type Ca^{2+} currents in GH₃C₁ cells (Cohen and McCarthy, 1987)] inhibits the sustained phase of $[Ca^{2+}]_i$ elevation by $70 \pm 4\%$ (data not shown). Halothane at all concentrations tested had little effect on the initial $[Ca^{2+}]_i$ peak. In contrast, the secondary phase of $[Ca^{2+}]_i$ elevation was inhibited in a dose-dependent fashion ($IC_{50} \approx 0.45$ mM, defined here as the concentration of halothane which reduces the rise in $[Ca^{2+}]_i$ by 50%) and by up to $75 \pm 7\%$ at the highest concentration of halothane tested (Fig. 3A,B). The inhibitory effect of halothane on the TRH-induced elevation of $[Ca^{2+}]_i$ was fully reversible; when cells were tested with TRH after a 3-min preincubation in halothane (0.50 mM) and a subsequent 5-min wash and equilibration period, the secondary phase was not significantly different from control responses (data not shown).

To assess the time course of the action of halothane on the plateau phase of the Ca^{2+} signal, halothane (0.50 mM) was added

Figure 1. The effects of halothane on basal and TRH-induced PRL secretion in GH₃ cells. *A*, The time course of TRH-induced PRL secretion elicited by 100 nM TRH. *B* and *C*, Dose–response curves for TRH-induced PRL secretion in the presence (▲) and absence (■) of 0.5 mM halothane. *B* shows the average PRL secretory rate in the first 5-min period following TRH addition (peak phase), and *C* shows the average rate of PRL secretion in the subsequent 10-min period (plateau phase). *D*, The concentration dependence of the effect of halothane on the two phases of the TRH-induced PRL response. In these experiments, the peak phase (▲) represents the average rate of PRL secretion in the first 2 min following 100 nM TRH application, and the plateau phase (■) represents the average PRL secretory rate over the subsequent 13 min. Note that basal rates of PRL secretion in the presence and absence of halothane were subtracted from all values in *D*. Data points and error bars represent the mean of three experiments and the SEM in *B–D*; in *A*, data points represent the mean of two experiments. Data points in *B* and *C* were fit by curves generated by the first-order equation $f(x) = V_{\max}/(1 + (EC_{50}/[TRH]))$. *B*: $V_{\max}$ is 10 ± 2 and 8.9 ± 1.6 ng PRL/min·mg protein for control and halothane, respectively; EC_{50} is 3.9 ± 2.8 and 1.9 ± 1.7 nM TRH for control and halothane, respectively. *C*: $V_{\max}$ is 5.5 ± 0.7 and 1.1 ± 0.7 ng PRL/min·mg protein for control and halothane, respectively; EC_{50} is 1.4 ± 0.9 and 2.3 ± 2.3 nM TRH for control and halothane, respectively. Statistically significant differences ($p < 0.05$) between the effect of halothane on the early and late phases are indicated by asterisks in *D*. All experiments were performed at 37°C, in the continuous presence of the indicated drugs.

≈5 min after TRH stimulation instead of prior to the addition of TRH (Fig. 3C). This resulted in a rapid (within 2 sec), sustained drop in $[Ca^{2+}]_i$ ($71 \pm 4\%$ inhibition) to approximately the same level observed in cells preincubated with halothane. It is possible that the onset time for the effect of halothane is even faster than 2 sec; however, it is not possible to obtain finer temporal resolution with the cuvette system employed in these experiments.

The effect of halothane on membrane potential

In response to TRH, GH₃ cells are also known to undergo a biphasic change in membrane potential. There is an initial hyperpolarization due to activation of Ca^{2+} -dependent outward conductances during the IP₃-induced $[Ca^{2+}]_i$ elevation (Ozawa, 1981; Dubinsky and Oxford, 1985). Unfortunately, cellular hyperpolarization cannot be reliably observed in our experiments (Fig. 4A) because of the slow response of the dye to changes in

membrane potential and the relatively fast nature of this event (≈20 sec). Hyperpolarization is followed by a sustained cellular depolarization that is thought to result from a decrease in potassium conductance (Ozawa, 1981; Dubinsky and Oxford, 1985; Bauer et al., 1990). Cellular depolarization may result in increased Ca^{2+} action potential frequency and in part account for the elevation of $[Ca^{2+}]_i$ during the sustained phase of the TRH response. Thus, the average membrane depolarization produced by TRH may represent not only depolarization due to closure of K^+ channels, but also the averaged effect of increased action potential frequency.

Halothane could act to block the sustained rise in $[Ca^{2+}]_i$ by hyperpolarizing the resting cell, by inhibiting the TRH-induced depolarization, or by a more direct action on Ca^{2+} influx. In order to distinguish among these possibilities, the effect of halothane (0.5 mM) was measured on V_i and the TRH-induced change in membrane potential. The addition of halothane to unstimulated cells produced a dilutional depression in fluorescence of

$8.9 \pm 0.2\%$ of the range defined by V_r and $V = 0$. This was equivalent to the decrease in fluorescence produced by the same volume of non-halothane-containing saline ($9.1 \pm 0.3\%$), indicating that halothane has little effect on resting membrane potential.

The effect of halothane on TRH-induced depolarization was measured in the presence of nimodipine (125 nM) to assess specifically the effect of halothane on the non- Ca^{2+} channel-dependent aspects of TRH-induced changes in membrane potential. Nimodipine (125 nM) itself reduced the net TRH-induced cellular depolarization by $17 \pm 6\%$. The significance of the small effect of nimodipine is somewhat difficult to assess because both Ca^{2+} influx through Ca^{2+} channels and Ca^{2+} -activated potassium channel activity will be diminished. However, the result is at least consistent with previous reports (Ozawa, 1981) that demonstrate that the extent of cellular depolarization by TRH is not affected by blockade of Ca^{2+} channels. Halothane (0.5 mM) did not inhibit the TRH-induced depolarization in the presence of nimodipine (Fig. 4A). These results indicate that halothane does not inhibit the TRH-induced increase in $[\text{Ca}^{2+}]_i$ by altering resting membrane potential or the TRH-induced cellular depolarization.

The effects of halothane on KCl-induced changes in $[\text{Ca}^{2+}]_i$, membrane potential, and PRL secretion

To test further the hypothesis that halothane inhibits PRL secretion by reducing Ca^{2+} influx, the effect of halothane on cells depolarized with KCl was measured. To approximate the average physiological depolarization produced by TRH, cells were depolarized with 10 mM KCl.

The addition of KCl (10 mM) causes a biphasic increase in $[\text{Ca}^{2+}]_i$ (Fig. 5). The cause of the initial $[\text{Ca}^{2+}]_i$ spike may be a consequence of the addition of a highly concentrated KCl solution (10 μl of 2 M KCl), which initially depolarizes a portion of the cells to a much greater degree. Halothane (0.5 mM) inhibited the KCl-induced increase in $[\text{Ca}^{2+}]_i$ by $68 \pm 6\%$ (Fig. 5) when calculated as the percent reduction in the Ca^{2+} signal integrated over the 5-min period after KCl addition.

To facilitate comparison of the effects of halothane on Ca^{2+} currents measured at RT [reported in the accompanying article (Herrington et al., 1991)] with physiological observations made at 37°C , the effect of halothane on $[\text{Ca}^{2+}]_i$ was measured at RT. In contrast to experiments at 37°C , basal $[\text{Ca}^{2+}]_i$ was reduced by 125 nM nimodipine at RT. This indicates that influx of Ca^{2+} through VDCCs contributes to basal $[\text{Ca}^{2+}]_i$ in the unstimulated state at this temperature. Halothane (0.5 mM) also reduced basal $[\text{Ca}^{2+}]_i$ at this temperature by about 50% as much as nimodipine. This complicated quantitation of the effects of halothane pretreatment on the KCl-induced rise in $[\text{Ca}^{2+}]_i$, because baseline $[\text{Ca}^{2+}]_i$ was changed by halothane. To circumvent this problem, the effect of halothane addition on the KCl-induced rise in $[\text{Ca}^{2+}]_i$ was measured 3 min after KCl stimulation and compared to the effect of 125 nM nimodipine. Similar to its actions at 37°C , halothane (0.5 mM) reduced the KCl-induced rise in $[\text{Ca}^{2+}]_i$; the decrease in $[\text{Ca}^{2+}]_i$ was $62 \pm 8\%$ of that produced by nimodipine (data not shown). This result indicates that effects of halothane on elevations of $[\text{Ca}^{2+}]_i$ produced by Ca^{2+} influx are comparable at different temperatures and validates the comparison of these data to the electrophysiological data described in the accompanying article (Herrington et al., 1991).

To investigate the possibility that halothane might act to re-

Figure 2. The effect of halothane on TRH-stimulated IP_3 accumulation in GH₃ cells. *A*, The time course of IP_3 accumulation in response to 3 nM TRH in the presence (▲) and absence (■) of 0.5 mM halothane. *B*, The dose-response curve for TRH-induced IP_3 accumulation over a 10-min period in the presence (▲) and absence (■) of 0.5 mM halothane. All experiments were performed at 37°C , in the continuous presence of the indicated drugs and 10 mM LiCl. Data points and error bars represent the mean and the SEM of the results of three experiments. Data points in *B* were fit by a curve generated using the equation $f(x) = V_{\text{max}}/(1 + (\text{EC}_{50}/[\text{TRH}]))$. V_{max} is 113 ± 2 and 126 ± 6 cpm/10 min·mg protein for halothane and control, respectively; the EC_{50} is 3.4 ± 0.3 and 3.3 ± 1.0 nM TRH for halothane and control, respectively.

duce the KCl-induced $[\text{Ca}^{2+}]_i$ rise by inhibiting Ca^{2+} -induced Ca^{2+} release (CICR), we measured the extent to which this process contributes to $[\text{Ca}^{2+}]_i$ elevations using caffeine. Caffeine (10 mM) did not produce an increase in $[\text{Ca}^{2+}]_i$, nor did it reduce the rise in $[\text{Ca}^{2+}]_i$ produced by KCl. These results provide evidence that (1) there is no CICR in these cells, though it is hard to rule out completely the existence of such a process; (2) CICR probably does not contribute to the Ca^{2+} signal produced by KCl; and (3) the caffeine-sensitive Ca^{2+} pool is not the target of the action of halothane in these cells.

The corresponding 5 min of KCl-induced PRL secretion was also inhibited by 0.5 mM halothane; between 54% (Fig. 6) and 77% inhibition was observed in three separate sets of experiments ($n = 3$ for each set). Parallel experiments using bisoxonol showed that the KCl-induced increase in membrane potential was unaffected by halothane (Fig. 4B). These experiments, similar to experiments in which the effect of halothane on TRH-induced changes in membrane potential was studied, were also performed in the presence of nimodipine (nimodipine reduced the KCl-induced rise in $[\text{Ca}^{2+}]_i$ by $24 \pm 5\%$). Thus, halothane consistently inhibits depolarization-associated PRL secretion by reducing the elevation of $[\text{Ca}^{2+}]_i$ (most likely as a result of reduced Ca^{2+} influx) produced by cellular depolarization.

The effect of halothane on ionomycin- and phorbol ester-stimulated PRL secretion

It remained possible that halothane-induced depression of the PRL secretory apparatus also contributed to the reduction in agonist-stimulated PRL secretion. In order to examine this possibility, the effect of halothane on PRL secretion elicited by phorbol 12-myristate 13-acetate (PMA) and ionomycin was measured. These two agonists trigger PRL secretion independent of Ca^{2+} influx through VDCCs; ionomycin increases PRL

Figure 3. The effects of halothane on basal and TRH-induced changes in $[Ca^{2+}]_i$. **A**, Four representative, superimposed traces of the TRH-induced change in the ratio of intracellular fura fluorescence emission (λ_{em} , 500 nm; λ_{ex} , 340 and 390 nm) as a function of time in the presence of the indicated concentrations of halothane. Note that halothane was introduced into the cuvette 1 min prior to TRH (100 nM) application and had no effect on the resting fluorescence ratio. Traces were calibrated (see *inset*) at the end of experiments by the sequential addition of Triton X-100 (0.01%) and $MnCl_2$ (2.5 mM) to yield the fluorescence ratio maxima and minima, respectively. *Inset*: vertical axis, 1.4 a.u.; horizontal axis, 900 sec. **B**, The concentration dependence of the effect of halothane on the TRH-induced (100 nM TRH) peak (■) and plateau (▲) calcium elevation is reported here in quantitative form. The fraction of peak control response was calculated as the quotient of the maximum TRH-induced change in 340/390 fluorescence emission in halothane-treated and control experiments. The fraction of the plateau control response was calculated as the quotient of the TRH-induced change in 340/390 fluorescence emission measured 5 min after TRH application in halothane-treated and control experiments. *Data points and error bars* represent the mean and the SEM of the results of three experiments. Statistically significant differences ($p < 0.05$) between the effects of halothane on the early and late phases are indicated by stars. **C**, The effect of 0.5 mM halothane added during the sustained phase of the TRH-induced rise in $[Ca^{2+}]_i$.

secretion via its ability to act as a non-voltage-gated Ca^{2+} ionophore, whereas PMA-induced PRL secretion occurs independently of $[Ca^{2+}]_i$ elevation. The latter effect is probably due to the action of protein kinase C on the secretory apparatus (R. C. Stern and A. S. Evers, unpublished observations). Halothane (0.5 mM) did not inhibit ionomycin- or PMA-induced PRL secretion (Fig. 6). This indicates that the inhibition of KCl- and TRH-induced PRL secretion by halothane probably does not result from a direct effect of halothane on the PRL secretory apparatus.

Discussion

In GH₃ cells, halothane inhibits the secretion of PRL elicited by TRH. The effect of halothane on secretion was dose-dependent and occurred at concentrations that produce general an-

esthesia in animals. Furthermore, the reduction in $[Ca^{2+}]_i$ produced by halothane had a rapid onset (within 2 sec) and was fully reversible, consistent with the characteristics of anesthetic action in the CNS. These features of the action of halothane in GH₃ cells suggest that these cells may provide a good model for the study of the molecular basis of the action of volatile anesthetics.

By stepwise analysis of the signal transduction cascade, it was determined that the effect of halothane on TRH-induced PRL secretion was a consequence of a reduction in the depolarization-associated elevation of $[Ca^{2+}]_i$. In fact, the effects of the Ca^{2+} channel blocker nimodipine are quite similar to the effects of halothane on TRH-activated Ca^{2+} signals. The present experiments indirectly support the hypothesis that halothane is acting by directly interfering with Ca^{2+} influx via VDCCs. This hy-

Figure 4. The effect of halothane on TRH- and KCl-induced changes in membrane potential: changes induced by 100 nM TRH (*A*) and 10 mM KCl (*B*) in the bisoxonol fluorescence emission (λ_{ex} , 495 nm; λ_{em} , 520 nm) as a function of time in the presence and absence of 0.5 mM halothane. These experiments were performed in the continuous presence of 125 nM nimodipine, which had no effect on the resting fluorescence. Halothane was introduced into the cuvette $\approx$ 3 min prior to TRH or KCl addition. Note that previous experiments (not shown) demonstrated that halothane also had no effect on resting fluorescence levels. Traces were calibrated at the end of experiments by adding gramicidin, which effectively clamps the membrane potential at 0 mV. These traces are representative of the results of three experiments for each condition.

Figure 5. The effect of halothane on KCl-induced increases in $[\text{Ca}^{2+}]_i$. Shown are two superimposed traces of the KCl-induced change in the ratio of intracellular fura fluorescence emission (λ_{em} , 500 nm; λ_{ex} , 340 and 390 nm) as a function of time in the presence and absence of 0.5 mM halothane. Note that halothane was introduced into the cuvette 1 min prior to KCl (10 mM) addition and had no effect on the resting fluorescence ratio. Traces were calibrated as shown in the inset of Figure 3*A*. These traces are representative of the results of three experiments for each condition.

pothesis is further supported in the accompanying article (Herrington et al., 1991), in which it is shown that halothane, over a similar concentration range, selectively inhibits VDCCs in GH₃ cells. Taken together, these data strongly support the views that halothane inhibits TRH-induced PRL secretion via its action on VDCCs and that the effect of volatile anesthetics on cell physiology may be localized to precise molecular targets.

At concentrations of halothane that depress depolarization-associated increases in $[\text{Ca}^{2+}]_i$, we showed that many processes essential to basal cell function and the transduction of excitatory stimuli are well preserved. Halothane did not significantly affect the function of the PRL secretory apparatus as supported by the fact that PMA- and ionomycin-induced PRL secretion was unaffected by halothane, nor did halothane interfere with inositol hydrolysis, IP₃-mediated intracellular Ca^{2+} release, or the TRH-induced cellular depolarization. In addition, halothane had little effect on resting membrane potential and resting $[\text{Ca}^{2+}]_i$, suggesting that halothane has little effect on the basal function of the various pumps and channels regulating resting membrane potential and $[\text{Ca}^{2+}]_i$. Thus, halothane does not produce its effects (in GH₃ cells) on the cellular response to excitatory stimuli by nonspecifically inhibiting a number of different cellular processes, but rather by its selective actions on a well-defined functional target.

Comparable investigations of volatile anesthetic action on stimulus-secretion coupling have been made in adrenal chromaffin cells (Yashima et al., 1986; Pocock and Richards, 1988). Similar to our experimental results, these studies show that volatile anesthetics inhibit depolarization-associated Ca^{2+} flux and that this results in decreased nicotinic-ACh receptor (nAChR)-activated catecholamine secretion. Also in agreement with our observations, these studies demonstrate that the secretory apparatus of chromaffin cells is unaffected by volatile anesthetics. However, both groups observe little effect of volatile anesthetics on KCl-induced secretion and the attendant $^{45}\text{Ca}^{2+}$

influx. Therefore, they conclude that the action of volatile anesthetics principally results from inhibition of the nAChR, and not VDCC-mediated Ca^{2+} influx. These observations on the effects of volatile anesthetics on KCl-induced Ca^{2+} influx contrast with the observations we have made in GH₃ cells. The disparity in results may be a consequence of differences in Ca^{2+} regulation in GH₃ cells and adrenal chromaffin cells or a result of the large differences in KCl concentrations used to study Ca^{2+}

Figure 6. The effect of halothane on PRL secretion induced by several secretagogues. PRL secretion in response to KCl, ionomycin, and PMA was measured in the presence (hatched bars) and absence (open bars) of 0.5 mM halothane. PRL secretory rates are reported as the change in the average rate of PRL secretion over the 5-min period following secretagogue application. Data points and error bars represent the mean and SEM of three experiments. Statistically significant difference ($p < 0.05$) between halothane-treated and untreated cells is indicated by the star.

flux. Concentrations of KCl used to depolarize adrenal chromaffin cells (55 or 77 mM) were approximately fivefold the concentrations we used to test the effects of halothane on depolarization-activated increases in $[Ca^{2+}]_i$. It is possible that the sensitivity of Ca^{2+} influx to anesthetics depends on the concentration of KCl used to depolarize the cells.

The present data provide strong evidence that halothane produces its inhibitory effects on agonist-stimulated PRL secretion by selectively inhibiting the function of VDCCs. Thus, the GH_3 cell model suggests that it is plausible that volatile anesthetics may act to inhibit the response to excitatory stimuli by selectively perturbing the function of a single class of proteins. It is also interesting to consider the possibility that the effect of halothane on VDCCs may contribute to the depressant effects of halothane at excitatory synapses, because it is thought that L-type Ca^{2+} channels play an important role in neurotransmitter release in the CNS (Perney et al., 1986; Rane et al., 1987). Consistent with this idea is the observation that halothane inhibits neuronal VDCCs in hippocampal neurons (Krnjevic and Puil, 1988).

While halothane may act to depress synaptic function by its action on VDCCs, it should be recognized that volatile anesthetics have been shown to affect other proteins, which could account for their CNS-depressant actions. For example, volatile anesthetics have been shown to activate resting K^+ and Cl^- conductances (Franks and Lieb, 1988; Yang and Zorumski, 1990), which reduce neuronal excitability, and to facilitate inhibitory synapses by enhancing GABAergic activity (Nicoll, 1972; Mody et al., 1990). At present it is not known which, if any, of the described effects of volatile anesthetics on neuronal function produce the general anesthetic state. It is, however, becoming increasingly clear that only a few of the many plausible sites at which anesthetics may act to depress CNS function are affected by these drugs.

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