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Volatile Anesthetics Increase Intracellular Calcium in Cerebrocortical and Hippocampal Neurons

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Background: **An increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in neurons has been proposed as an important effect of volatile anesthetics, because they alter signaling pathways that influence neurotransmission. However, the existing data for anesthetic-induced increases in $[Ca^{2+}]_i$ conflict.**

Methods: **Changes in $[Ca^{2+}]_i$ were measured using fura-2 fluorescence spectroscopy in rat cortical brain slices at 90, 185, 370, and 705 μM isoflurane. To define the causes of an increase in $[Ca^{2+}]_i$, slices were studied in Ca^{2+}-free medium, in the presence of Ca^{2+}-channel blockers, and in the presence of the Ca^{2+}-release inhibitor azumolene. The authors compared the effect of the volatile anesthetic with that of the nonanesthetic compound 1,2-dichlorohexafluorocyclobutane. Single-dose experiments in CA1 neurons in hippocampal slices with halothane (360 μM) and in acutely dissociated CA1 neurons with halothane (360 μM) and isoflurane (445 μM) also were performed.**

Results: **Isoflurane at 0.5, 1, and 2 minimum alveolar concentrations increased basal $[Ca^{2+}]_i$ in cortical slices in a dose-dependent manner ($P < 0.05$). This increase was not altered by Ca^{2+}-channel blockers or Ca^{2+}-free medium but was reduced 85% by azumolene. The nonanesthetic 1,2-dichlorohexafluorocyclobutane did not increase $[Ca^{2+}]_i$. In dissociated CA1 neurons, isoflurane reversibly increased basal $[Ca^{2+}]_i$ by 15 nM ($P < 0.05$). Halothane increased $[Ca^{2+}]_i$ in dissociated CA1 neurons and CA1 neurons in hippocampal slices by approximately 30 nM ($P < 0.05$).**

Conclusions: **(1) Isoflurane and halothane reversibly increase $[Ca^{2+}]_i$ in isolated neurons and in neurons within brain slices. (2) The increase in $[Ca^{2+}]_i$ is caused primarily by release from intracellular stores. (3) Increases in $[Ca^{2+}]_i$ occur with anesthetics but not with the nonanesthetic 1,2-dichlorohexafluorocyclobutane. (Key words: Brain slices; cytosolic free calcium; halothane; isoflurane; nonanesthetics.)**

VOLATILE anesthetics appear to produce their effects by changing the activity of ion channels involved in neurotransmission. A direct interaction of the anesthetic molecule with an ion channel protein at a lipophilic interface is the most commonly proposed mechanism.[1,2] Alternatively, anesthetics may have an indirect effect, activating or inhibiting signaling pathways that regulate ion channel function.

One signaling molecule with potentially substantial effects on various ion channels is intracellular calcium ($[Ca^{2+}]_i$). Previous studies have shown that increased $[Ca^{2+}]_i$ may activate potassium channels,[3] potentiate γ-aminobutyric acid currents,[4] and depress both calcium channel[5,6] and N-methyl-D-aspartate receptor (NMDA) currents[7] *via* Ca^{2+}-dependent mechanisms. Elevations in $[Ca^{2+}]_i$, therefore, could explain the augmentation of inhibitory processes and the inhibition of excitatory pathways that characterize the state of anesthesia at various foci. However, the evidence regarding the anesthetic effect on $[Ca^{2+}]_i$ is controversial. We are aware of only two other studies that have shown an increase in $[Ca^{2+}]_i$ in neurons produced by volatile anesthetics. Bickler *et al.*[8] found in cortical neurons that increases in $[Ca^{2+}]_i$ induced by approximately 1 minimum alveolar concentration (MAC) isoflurane were larger at higher temperatures. Recently, Franks *et al.*[9] reported that halothane, isoflurane, and xenon significantly increased $[Ca^{2+}]_i$ in mouse cortical neurons. In contrast, in other experiments with cultured neurons, investigators concluded that anesthetics did not increase basal $[Ca^{2+}]_i$,[10,11] and studies in mouse brain synaptosomes showed that volatile anesthetics increased $[Ca^{2+}]_i$ only at very high concentrations in the millimolar range.[12] Based on these negative reports, Franks and Lieb[1] dismissed the possibility that volatile anesthetics increase $[Ca^{2+}]_i$ in neurons.

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The goal of the current study was to determine again whether volatile anesthetics increase $[Ca^{2+}]_i$ in central nervous system neurons. Therefore, we measured the effects of isoflurane, halothane, and the nonanesthetic 1,2-dichlorohexafluorocyclobutane (2N) on $[Ca^{2+}]_i$ in cortical brain slices, CA1 neurons in intact hippocampal brain slices, and acutely dissociated CA1 neurons with the fluorescent Ca^{2+} indicator fura-2. Using both models allowed us to determine whether observed elevations in $[Ca^{2+}]_i$ were caused by increases in $[Ca^{2+}]_i$ in neurons or simply by changes occurring in nonneuronal cells in the slices. We also determined whether increases in $[Ca^{2+}]_i$ resulted from the influx of Ca^{2+} from the extracellular fluid or from Ca^{2+} release from intracellular stores. Although little is known about the role of the Ca^{2+}-release channels in the central nervous system, a recent study described for the first time the distribution of the ryanodine receptor isoforms in the human brain.[13] The widespread expression of all isoforms (*ryr 1, ryr 2,* and *ryr 3*) in human hippocampus suggests a role of this gene family in Ca^{2+} signaling, Ca^{2+} homeostasis, and possibly also in fundamental processes such as synaptic plasticity. Finally, we determined whether the effects of a nonanesthetic compound (1,2-dichlorohexafluorocyclobutane) were similar to those of volatile anesthetics.

Methods

Preparation of Brain Slices and Dissociated Neurons

Neocortical brain slices were prepared from 13- to 23-day-old Sprague-Dawley rats according to methods that were approved by the Committee on Animal Research, University of California, San Francisco. After decapitation during 2% halothane anesthesia, brain hemispheres were dissected rapidly and slices were prepared in ice-cold artificial cerebrospinal fluid (aCSF). The aCSF used to prepare and maintain the slices consisted of 116 mM NaCl, 25 mM $NaHCO_3$, 5.4 mM KCl, 1.8 $CaCl_2$ mM, 0.9 mM $MgCl_2$, 0.9 mM NaH_2PO_4, and 10 mM glucose, *p*H 7.40–7.45, oxygenated with 95% oxygen and 5% carbon dioxide. Transverse cortical slices were prepared (300 μm thick) using a vibratome (Campden Instruments, Cambridge, UK), and hippocampal slices were prepared using a tissue chopper (Stoelting Co., Wood Dale, IL). Typically, 8–12 slices were obtained from each animal, and only a few slices within one study group were obtained from the same animal. Slices were stored at room temperature for 45 min to recover from the trauma of slicing and then transferred to vials of oxygenated aCSF containing 2 μM fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) to permit measurement of $[Ca^{2+}]_i$. Fura-2 is primarily a cytosolic dye used to mark cytosolic changes in calcium. It was dissolved in 20% F-127 pluronic acid (Molecular Probes) in anhydrous dimethyl sulfoxide. After 1 h, slices were transferred to fresh oxygenated aCSF containing 2 μM fura-2 for an additional 30 min to facilitate dye loading.[14] Fluorescence signals from fura-loaded slices were five to seven times greater than background fluorescence.

Dissociated neurons were prepared by enzymatic digestion of dissected CA1 regions of hippocampal slices of 1- to 14-day-old rats in 0.1% trypsin and 0.05% pronase in Ca^{2+}-free buffer for 30 min at 37°C.[15] After gentle tituration with a fire-polished pipette, neurons were applied to glass coverslips and loaded with 1–3 μM fura-2 for 15 min.

Measurements of $[Ca^{2+}]_i$

Neocortical brain slices were studied in a Hitachi F-2000 fluorometer (Tokyo, Japan), as described previously.[8,16] Slices were fixed to a nylon mesh holder and placed in a capped quartz fluorometer cuvette containing oxygenated aCSF and a stir bar. $[Ca^{2+}]_i$ was determined by dual-excitation fluorescence spectroscopy at 37°C. Slices were excited alternately at 340 and 380 nm, and the emitted light intensity at 510 nm was recorded every 0.5 s. $[Ca^{2+}]_i$ was estimated using the Grynkiewicz equation[17]:

$$[Ca^{2+}]_i = Kd \cdot \left(\frac{F_{2min}}{F_{2max}}\right)\left(\frac{R - R_{min}}{R_{max} - R}\right)$$

where R is the fluorescence emission ratio (340:380 nm), K_d is the temperature-corrected dissociation constant of fura-2 (K_d = 224 nM),[18] F_{2min}/F_{2max} is the ratio between the 380-nm fluorescence intensities in the absence of Ca^{2+} and in the presence of saturating $[Ca^{2+}]_i$, and R_{min} and R_{max} are the fluorescence ratios in the absence of Ca^{2+} and in the presence of saturating $[Ca^{2+}]_i$, respectively. Tissue autofluorescence (fluorescence in the absence of fura-2) was always subtracted before computing the ratios. F_{2min}, F_{2max}, R_{min}, and R_{max} were determined as follows: Fluorescence emission at saturating $[Ca^{2+}]$ was measured in slices after treatment with 10 μM ionomycin (a calcium ionophore) and 1 mM ouabain. Fluorescence emission at 0 $[Ca^{2+}]$ was measured by replacing the aCSF with calcium-free aCSF containing 5 mM EGTA.

$[Ca^{2+}]_i$ in CA1 neurons in hippocampal slices and in acutely dissociated CA1 neurons was measured using a PTI D104 fluorometer–inverted microscope system (PTI, South Brunswick, NJ) as described in detail before.[19] Hippocampal slices or coverslips with plated neurons were placed in a 200-μl temperature-controlled (37°C) recording chamber on the stage of an inverted Nikon Diaphot 200 microscope (Nikon Inc. Instrument Group, Melville, NY). The CA1 region of the hippocampal slices was viewed using a microscope. The sealed recording chamber was perfused continuously with aCSF bubbled with 95% oxygen and 5% carbon dioxide at a rate of 4 ml/min. Isoflurane and halothane were added by switching the perfusate from standard aCSF to aCSF bubbled with anesthetic vapor (using a standard calibrated vaporizer) for at least 30 min. Measurements of $[Ca^{2+}]_i$ were acquired from regions of interest in the hippocampal slices (CA1 neurons in a 50 × 100 μM region) or from individual dissociated neurons by adjusting slit apertures in the photomultiplier tube section of the fluorometer–microscope system. Dissociated neurons were identified morphologically by their pyramidal cell body and prominent processes before they were studied, and the slit aperture was focused on the cell body. Calibration experiments and calculation of $[Ca^{2+}]_i$ were performed as described before.

Administration and Determination of Anesthetics

After recording baseline fluorescence for 50–100 s, aCSF saturated with isoflurane or 2N was injected by syringe into the cuvette of the Hitachi F-2000 fluorometer. The actual concentration of isoflurane or 2N in the cuvette was measured by gas chromatography. Trial experiments with isoflurane and 2N were conducted to determine the actual agent concentration and the amount of agent lost from the experimental apparatus. Therefore, different volumes of agent-saturated aCSF were injected into a cuvette containing aCSF to a total volume of 2 ml. Samples of the solutions were taken from the cuvette at the beginning and at the end of the experiments. Samples were equilibrated in air in glass syringes in a constant-temperature water bath. Gas in these syringes was analyzed using a gas chromatograph (Gow Mac, Bridgewater, NJ) calibrated according to the appropriate standards. Measurements at the beginning and end of the experiment showed a loss of approximately 15% for isoflurane and 25–30% for 2N during the experiment. Based on these determinations, the volume of agent-saturated aCSF was adjusted to give target anesthetic concentrations (based on MAC values for rats given by Franks and Lieb[20]) at the middle time point of the studies. 1,2-dichlorohexafluorocyclobutane was purchased from Lancaster Synthesis (Windham, NH).

Losses from the fluorometer–inverted microscope system also were determined in trial experiments. The anesthetic concentrations in the perfusate in the recording chamber were approximately 0.8 of the concentrations expected, when aCSF was equilibrated with a given setting on the vaporizer. The actual concentrations of halothane and isoflurane in the recording chamber also were determined by gas chromatography, as described before.

Experimental Design

Isoflurane-induced changes in $[Ca^{2+}]_i$ in cortical brain slices were studied using the Hitachi F-2000 fluorometer under five experimental conditions: (1) at 90, 185, 370, and 705 μM isoflurane (equivalent to approximately 0.25, 0.5, 1, and 2 MAC, respectively) and standard aCSF (containing 1.8 mM Ca^{2+}); (2) at 0.5, 1, and 2 MAC isoflurane and standard aCSF containing agents to specifically block the N-type and P/Q-type Ca^{2+} channels and Na^+ channels (0.5 μM ω-conotoxin GVIA [Research Biochemicals, Natick, MA], 0.1 μM ω-agatoxin IVa [Pfizer, Groton, NJ], and 0.5 μM tetrodotoxin [Sigma, St. Louis, MO], respectively). These blockers prevent more than 90% of changes in $[Ca^{2+}]_i$ in response to a depolarizing stimulus in this preparation[21]; (3) at 0.5, 1, and 2 MAC isoflurane in Ca^{2+}-free aCSF ($Ca^{2+} < 1$ μM; Corning ion selective electrode #476041; Corning Incorporated Science Products Division, Corning, NY) to determine whether Ca^{2+} influx from extracellular medium contributed to the increase in $[Ca^{2+}]_i$ produced by isoflurane; (4) at 2 MAC isoflurane with standard aCSF after 30 min of incubation with 100 μM azumolene (1-[[[5-(4-bromophenyl)-2-oxazolyl]methylene]amino]-2,4-imidazolidinedione), a dantrolene analog with less fluorescence at 340 and 380 nm and no fluorescence at the emission wavelength of 510 nm (HP 8452 Diode Array Spectrophotometer, Hewlett-Packard, Palo Alto, CA) (this treatment was chosen to block possible isoflurane-mediated release of Ca^{2+} from intracellular stores[22]); and (5) exposure to 30 and 60 μM 2N, corresponding to approximately 1.5 and 3 times the MAC value for this compound predicted by the Meyer-Overton hypothesis based on lipid solubility.[23]

CA1 neurons in intact hippocampal slices were studied at 360 μM halothane (approximately 1.2 MAC), and cell bodies of dissociated neurons were studied at 360 μM halothane (approximately 1.2 MAC) and at 445 μM isoflu-

rane (approximately 1.3 MAC) in the microscope fluorometer with an attached perfusion system that allowed the anesthetic to be washed out from the slices or neurons.

Statistics

Results are reported as the mean ± SD. The n values correspond to the number of brain slices studied. Within each study group, data were analyzed using the paired Student *t* test for comparisons between baseline $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ after exposure to anesthetic. To test for a dose-dependent effect of the increase of $[Ca^{2+}]_i$, linear regression analysis was used (JMP; SAS Institute, Cary, NC). In addition, analysis of variance was used for comparison of the baseline $[Ca^{2+}]_i$ with all treatment groups. $P < 0.05$ was considered significant.

Results

$[Ca^{2+}]_i$ in Neocortical Brain Slices

Figures 1A and B show tracings of the ratio of the two excitation wavelengths (340:380 nm) and $[Ca^{2+}]_i$, respectively, before and during exposure of a cortical brain slice to 705 μM isoflurane (approximately 2 MAC). $[Ca^{2+}]_i$ in untreated brain slices during control conditions (standard aCSF) was 146 ± 41 nM (n = 67; fig. 2A), which is similar to previous studies.[8] An increase in $[Ca^{2+}]_i$ after exposure to 2 MAC isoflurane was observed in all slices (fig. 1C). Exposure to 0.5, 1, and 2 MAC isoflurane increased $[Ca^{2+}]_i$ by 8 ± 11 nM, 23 ± 19 nM, and 22 ± 15 nM (mean ± SD), respectively (fig. 2A). These changes in $[Ca^{2+}]_i$ were dose dependent ($P <$ 0.05) and represent an increase from baseline $[Ca^{2+}]$i of 5–15%. No increase in $[Ca^{2+}]$i was observed with 0.25 MAC isoflurane (149 ± 41 nM *vs.* 149 ± 53 nM).

Relative to untreated slices, baseline $[Ca^{2+}]_i$ in cerebrocortical slices did not differ after pretreatment of slices with 0.5 μM ω-conotoxin GVIA, 0.1 μM ω-agatoxin IVa, and 0.5 μM tetrodotoxin. Exposure to 0.5, 1, and 2 MAC isoflurane increased $[Ca^{2+}]_i$ by 28 ± 16, 32 ± 20, and 41 ± 33 nM, respectively, similar to that in untreated slices (fig. 2B).

In calcium-free extracellular solution, baseline $[Ca^{2+}]_i$ did not differ from that in slices in 1.8 mM Ca^{2+} ($P >$ 0.05 by analysis of variance), and 1 and 2 MAC isoflurane increased $[Ca^{2+}]_i$ by 9 ± 8 nM and 31 ± 29 nM, respectively ($P < 0.05$; fig. 2C).

In azumolene-pretreated slices, the increase in $[Ca^{2+}]_i$ at 2 MAC isoflurane was significantly smaller than that in untreated slices (5 ± 13 nM *vs.* 22 ± 15 nM, respectively; $P < 0.05$; fig. 3).

We also exposed cortical brain slices to the nonanesthetic compound 2N. At concentrations as great as three times the predicted MAC, this compound had no effect on $[Ca^{2+}]_i$ (151 ± 51 *vs.* 156 ± 52 nM; n = 16; $P > 0.05$). Because of the very low saline–gas partition coefficient

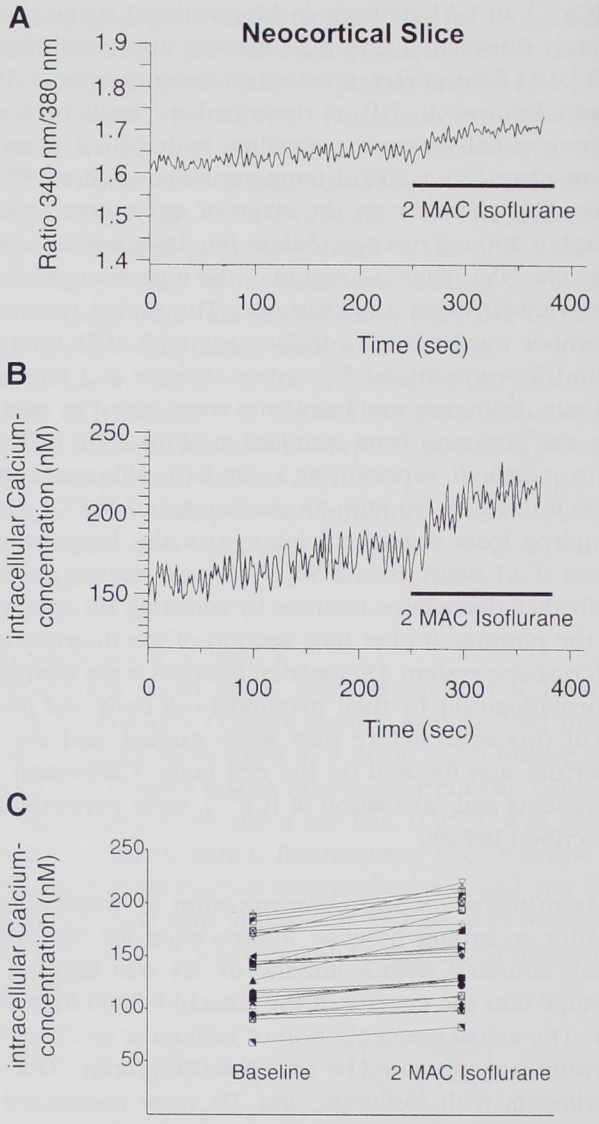

Fig. 1. Increases in the intracellular calcium concentration ($[Ca^{2+}]_i$) in rat neocortical brain slices exposed to 2 minimum alveolar concentration (MAC) isoflurane. (*A*) An original raw tracing of the ratio of the two excitation wavelengths, 340 and 380 nm, before and during exposure of a brain slice to an equivalent of 2 MAC isoflurane. (*B*) The time course of the change in $[Ca^{2+}]_i$ in the same slice after conversion of fluorescence intensity to calculated Ca^{2+} concentration using equation 1 in Methods. (*C*) Individual increases in $[Ca^{2+}]_i$ in different brain slices exposed to 2 MAC isoflurane (n = 25).

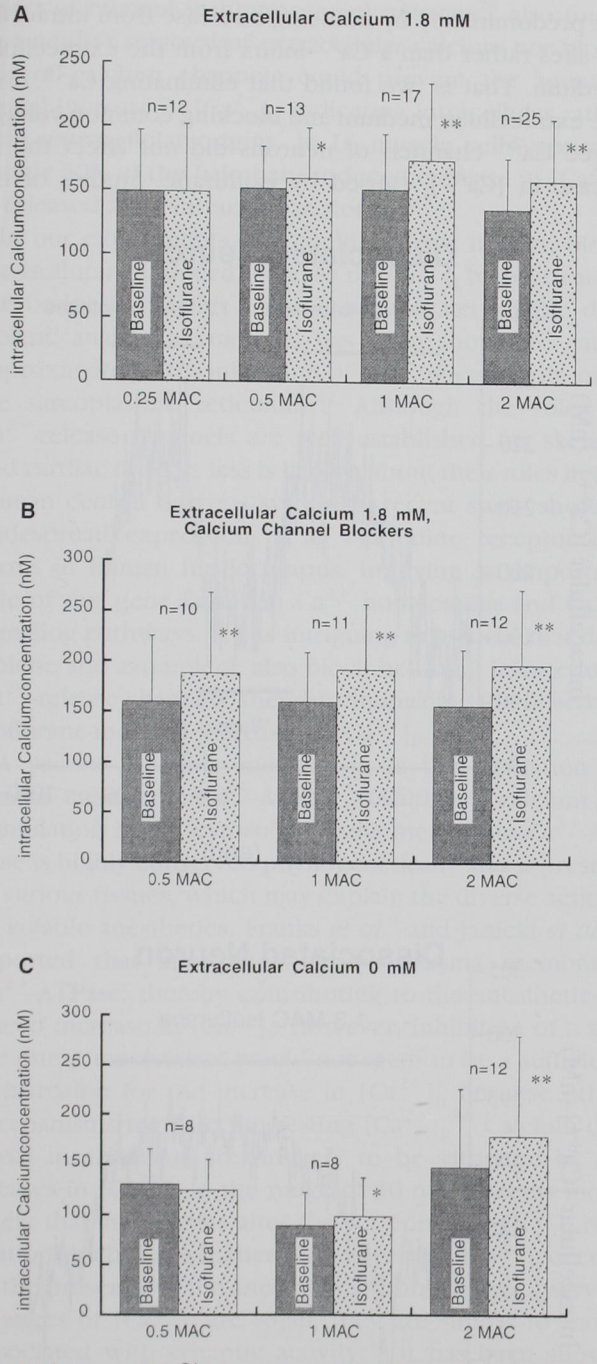

Fig. 2. Changes of $[Ca^{2+}]_i$ in rat neocortical brain slices induced by isoflurane compared with baseline. (*A*) With normal extracellular calcium (1.8 mM) present, 0.5, 1, and 2 minimum alveolar concentration (MAC) isoflurane increased $[Ca^{2+}]_i$ significantly compared with baseline, whereas 0.25 MAC had no effect. (*B*) After pretreatment of the slices with conotoxin GVIA, agatoxin IVa, and tetrodotoxin, 0.5, 1, and 2 MAC isoflurane increased $[Ca^{2+}]_i$ significantly compared with baseline. (*C*) With no extracellular calcium present, 1 and 2 MAC isoflurane increased $[Ca^{2+}]_i$ significantly compared with baseline. Data are expressed as the mean ± SD. *$P < 0.05$, **$P < 0.01$ (by the paired Student's *t* test).

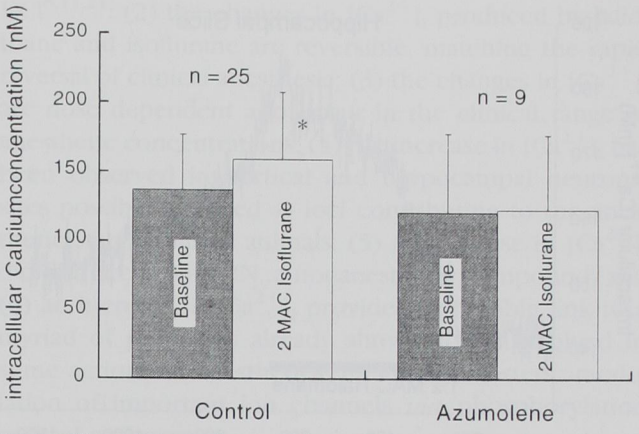

Fig. 3. Effect of azumolene on the isoflurane-induced increase of $[Ca^{2+}]_i$ in rat neocortical brain slices. After pretreatment of the slices with 100 μM azumolene, the increase in $[Ca^{2+}]_i$ was significantly smaller than in untreated slices. Data are expressed as the mean ± SD. *$P < 0.05$.

at 37°C (0.00011),[23] we injected a relatively large volume (800 μl) of 2N-saturated saline into the cuvette to achieve three times the predicted MAC. To control whether brain slices remained adequately oxygenated during this procedure, we measured the oxygen tension in the cuvette using an oxygen electrode (Cameron Instrument Co., Port Aransas, TX). The mean oxygen tension after addition of 800 μl 2N-saturated solution was 236 ± 13 mmHg (n = 4).

Hippocampal Slices and Dissociated CA1 Neurons

$[Ca^{2+}]_i$ was measured in the CA1 cell bodies within intact hippocampal slices using the microscope fluorometer. Baseline $[Ca^{2+}]_i$ during perfusion of slices with standard aCSF without additional stimulus was 108 ± 37 nM and increased to 139 ± 48 nM by superfusing the slices with aCSF bubbled with 1.2 MAC halothane (n = 12; $P < 0.05$). This increase was reversible after the anesthetic was washed out (fig. 4).

$[Ca^{2+}]_i$ also was measured in acutely dissociated CA1 neurons from hippocampi of 1- to 14-day-old rats. Neurons were identified by their shape, long cell processes, and phase-bright appearance. Baseline $[Ca^{2+}]_i$ was 186 ± 48 nM (n = 14) in these dissociated neurons. In all neurons, $[Ca^{2+}]_i$ increased with exposure to either 1.2 MAC halothane (29 ± 17 nM; n = 7) or 1.3 MAC isoflurane (15 ± 9 nM; n = 7) ($P < 0.05$). As in the hippocampal slices, the increases of $[Ca^{2+}]_i$ in the dissociated neurons were reversible with washout of the anesthetics (fig. 5).

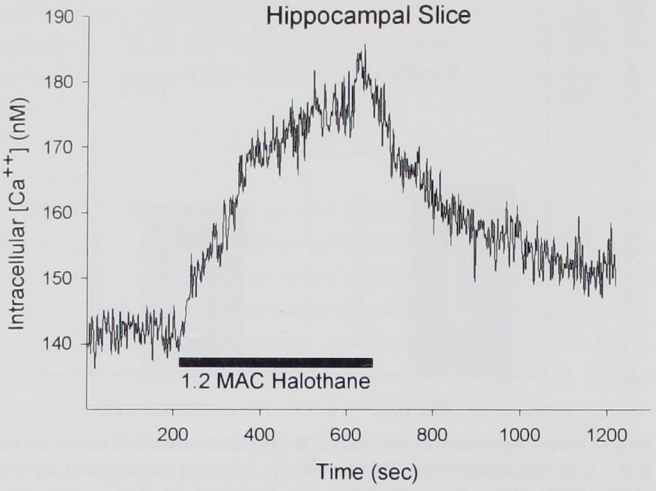

Fig. 4. Effects of halothane on $[Ca^{2+}]_i$ of CA1 neurons in an intact hippocampal slice. An equivalent of approximately 1.2 MAC halothane was present in the perfusate (horizontal bar).

Discussion

Previous reports of the effect of volatile anesthetics on $[Ca^{2+}]_i$ are contradictory. Two studies have shown an anesthetic-induced increase in $[Ca^{2+}]_i$ in neurons,[8,9] whereas other investigators found no effect or one only at high concentrations.[10–12] Several factors might have contributed to a failure to detect an increase in $[Ca^{2+}]_i$. First, cultured neurons may be vulnerable to a loss of sensitivity to anesthetics, possibly by downregulation of volatile anesthetic-sensitive Ca^{2+}-adenosine triphosphatase (ATPase) pumps. Second, studies with negative results[10,11] used relatively high concentrations of fura-2 (approximately 5 μM), which can buffer small calcium changes. Finally, increases in $[Ca^{2+}]_i$ are temperature dependent,[8,9] suggesting that the use of room temperature conditions in both studies may have masked the detection of any change in $[Ca^{2+}]_i$.

Several studies have proposed that increases in $[Ca^{2+}]_i$ may be responsible in part for the actions of volatile anesthetics in neurons. Krnjevic[3] suggested that anesthetics might increase K^+-conductance by increasing $[Ca^{2+}]_i$ in neurons. Nicoll and Madison[24] observed such hyperpolarization for various anesthetic compounds. Mody *et al.*[4] suggested that augmented γ-aminobutyric acid A currents induced by volatile anesthetics might result from increased $[Ca^{2+}]_i$ because BAPTA, a calcium chelator, reduced the augmentation. In other tissues, volatile anesthetics are recognized more widely to increase $[Ca^{2+}]_i$.[25–27]

The mechanism by which anesthetics increase $[Ca^{2+}]_i$ in neurons remains unclear. Our results suggest that they act predominately by inducing a release from intracellular sites rather than a Ca^{2+}-influx from the extracellular medium. That is, we found that eliminating Ca^{2+} from the extracellular medium and blocking common voltage-gated Ca^{2+} channels of neurons did not affect the increase in $[Ca^{2+}]_i$ caused by isoflurane. Studies of the

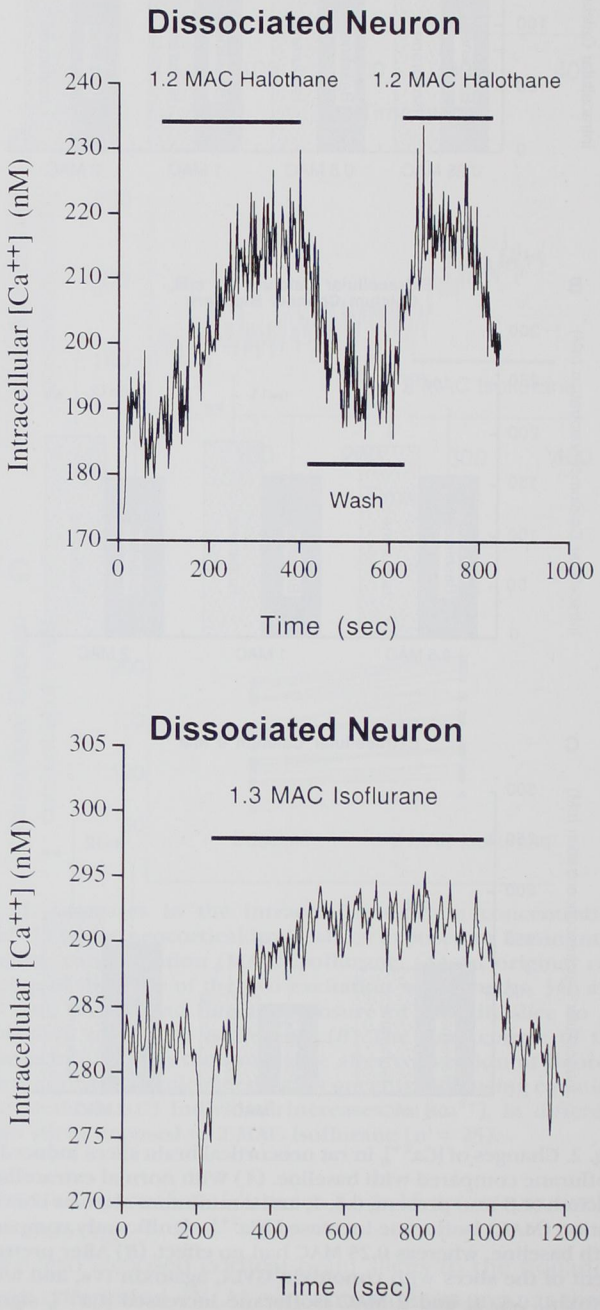

Fig. 5. (*Upper*) $[Ca^{2+}]_i$ changes in a dissociated CA1 neuron during repeated exposure to 1.2 minimum alveolar concentration (MAC) halothane. (*Lower*) $[Ca^{2+}]_i$ changes in a dissociated CA1 neuron during exposure to 1.3 MAC isoflurane.

effect of ethanol in hippocampal neurons[28] also found that neither removal of extracellular calcium nor blockade of calcium channels could prevent the agent-induced increase in $[Ca^{2+}]_i$, indicating intracellular rather than extracellular origins. In L6 muscle cells, approximately 50% of the halothane-induced increase in $[Ca^{2+}]_i$ is released from intracellular stores.[26]

In our experiments, we could, at least in part, block the isoflurane-induced increase of $[Ca^{2+}]_i$ by pretreating the brain slices with azumolene. A water-soluble dantrolene analog, azumolene, has been shown to cause approximately 30% inhibition of Ca^{2+} release from muscle sarcoplasmic reticulum.[22] Although the roles of Ca^{2+}-release channels are well established for skeletal and cardiac muscle, less is known about their roles in the human central nervous system. A recent study showed widespread expression of all ryanodine receptor isoforms in human hippocampus, implying an important role of this gene family in Ca^{2+} homeostasis and Ca^{2+}-signaling pathways.[13] It is intriguing to assume that dantrolene and azumolene also block neuronal intracellular Ca^{2+}-release channels, thereby diminishing the observed isoflurane-induced increase of $[Ca^{2+}]_i$.

A second possible mechanism is the inhibition of plasma membrane Ca^{2+}-ATPase, resulting in calcium accumulation in the cytosol.[9] Plasma membrane Ca^{2+}-ATPase is highly conserved phyllogenetically and is present in various tissues, which may explain the diverse actions of volatile anesthetics. Franks *et al.*[9] and Janicki *et al.*[29] reported that anesthetics inhibit plasma membrane Ca^{2+}-ATPase, thereby contributing to the anesthetic-induced increase in $[Ca^{2+}]_i$. However, inhibition of a single pump mechanism would not seem to be a sufficient explanation for the increase in $[Ca^{2+}]_i$, because other mechanisms regulate the resting $[Ca^{2+}]_i$.[30] Calcium disposal mechanisms are unlikely to be saturated by increases in $[Ca^{2+}]_i$ in the range of 30 nM. It seems more likely that anesthetics alter the set point of $[Ca^{2+}]_i$ regulation, such that a higher level of resting $[Ca^{2+}]_i$ occurs in the presence of the anesthetic. Although the observed changes of $[Ca^{2+}]_i$ are small, they are similar to those associated with synaptic activity.[31] It has been shown that an increase in $[Ca^{2+}]_i$ of 10–30 nM produced a twofold synaptic enhancement.[32]

An increase in $[Ca^{2+}]_i$ caused by volatile anesthetics might be related to anesthesia because (1) the increases in $[Ca^{2+}]_i$ are significant in size, at approximately one tenth of those that occur with maximal stimulation of NMDA receptors or activation of voltage-gated calcium channels with a depolarizing stimulus of 50 mM KCl[8,11,21]; (2) the changes in $[Ca^{2+}]_i$ produced by halothane and isoflurane are reversible, matching the rapid reversal of clinical anesthesia; (3) the changes in $[Ca^{2+}]_i$ are dose dependent and occur in the clinical range of anesthetic concentrations; (4) the increase in $[Ca^{2+}]_i$ has been observed in cortical and hippocampal neurons, sites possibly involved as loci contributing to the anesthetic state in intact animals; (5) an increase in $[Ca^{2+}]_i$ did not occur with 2N, a nonanesthetic compound; and (6) an increase in $[Ca^{2+}]_i$ provides a plausible link to a myriad of processes already shown to be involved in some actions of anesthetics, including allosteric modulation of important ion channels *via* phosphorylation control pathways.

Inhibition of neurotransmission at central synapses is likely to be an important locus of anesthetic action. Anesthetics might interrupt synaptic transmission by acting presynaptically to cause a Ca^{2+}-dependent inhibition of neurotransmitter release. It also has been suggested that halothane potentiated inhibitory postsynaptic currents *via* increased $[Ca^{2+}]_i$, based on the partial prevention of depression by the calcium buffer 1,2bis(2-aminophenoxy)ethane-N,N,N′-tetraacetic acid or the calcium release inhibitor dantrolene.[4] However, other investigators could not confirm such a role for $[Ca^{2+}]_i$ in modulating inhibitory synapses in cultured rat hippocampal neurons.[33]

Elevated $[Ca^{2+}]_i$ has important inhibitory influences in the postsynapse. The activity of the NMDA receptor is decreased by increased $[Ca^{2+}]_i$. Inhibition of this receptor, a proposed target for anesthetic action,[11] is controlled by calcium-bound calmodulin, which activates calcineurin.[34] Rosenmund and Westbrook[7] showed that increased $[Ca^{2+}]_i$ decreases NMDA channel activity by increasing actin depolymerization. With respect to volatile anesthetics, elevated $[Ca^{2+}]_i$ could tip the balance from an active to an inactive NMDA receptor.

Our results differ from those of previous studies that found a decrease of $[Ca^{2+}]_i$ produced by volatile anesthetics and barbiturates.[35,36] However, both these studies examined the effect of anesthetics on a potassium chloride–evoked increase in $[Ca^{2+}]_i$ and not from changes of baseline $[Ca^{2+}]_i$. We propose that a small increase in $[Ca^{2+}]_i$ induced by volatile anesthetics may have complex effects on the regulation of ion channels by means of calcineurine phosphatases, and Ca^{2+}–calmodulin-dependent protein kinase II.[37] An elevated $[Ca^{2+}]_i$ can cause enhanced inhibition (*e.g.*, Ca^{2+}-sensitive potassium channel currents and γ-aminobutyric acid A receptor currents) and decreased excitation (inhibi-

tion of excitatory neurotransmitter release, inhibition of postsynaptic receptors).

Study Limitations

The limitations of measuring $[Ca^{2+}]_i$ in brain slices are well recognized. Most important is the injury layer in each slice, which may respond differently to volatile anesthetics than undamaged tissue does. In addition, the calibration of $[Ca^{2+}]_i$ measurements in slices is difficult because of the challenges imposed by equilibrating intracellular and extracellular pools of Ca^{2+}. We overcame both these difficulties, in part, by also studying morphologically intact dissociated neurons in which Ca^{2+} levels are more easily calibrated. The fact that volatile anesthetics produced increases in $[Ca^{2+}]_i$ by amounts in cortical and hippocampal slices similar to those in dissociated neurons indicates that data in slices are representative of changes in $[Ca^{2+}]_i$. This suggests that the changes in $[Ca^{2+}]_i$ observed in neurons in intact slices were not due to the signal contribution from nonneuronal cells or from neurotransmitter release, because the very low density of cells on the coverslips used for the dissociated neurons and the constant perfusion system would have eliminated any released neurotransmitter.

Conclusions

Our results suggest that (1) volatile anesthetics reversibly increase $[Ca^{2+}]_i$ in central nervous system neurons, (2) the increase of $[Ca^{2+}]_i$ is, in part, the result of a release of Ca^{2+} from intracellular stores, and (3) the increase in $[Ca^{2+}]_i$ is not observed in a compound similar in structure to anesthetics but devoid of anesthetic potency.

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