

The General Anesthetic Isoflurane Depresses Synaptic Vesicle Exocytosis

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ABSTRACT

General anesthetics have marked effects on synaptic transmission, but the mechanisms of their presynaptic actions are unclear. We used quantitative laser-scanning fluorescence microscopy to analyze the effects of the volatile anesthetic isoflurane on synaptic vesicle cycling in cultured neonatal rat hippocampal neurons monitored using either transfection of a pH-sensitive form of green fluorescent protein fused to the luminal domain of VAMP (vesicle-associated membrane protein), (synapto-pHluorin) or vesicle loading with the fluorescent dye FM 1–43. Isoflurane reversibly inhibited action potential-evoked exocytosis over a range of concentrations, with little effect on vesicle pool size. In contrast, exocytosis evoked by depolarization in response to an elevated extracellular concentration of KCl, which is insensitive to the selective Na⁺ channel blocker tetrodotoxin, was relatively insensitive to isoflurane.

Inhibition of exocytosis by isoflurane was resistant to bicuculline, indicating that this presynaptic effect is not caused by the well known GABA_A receptor modulation by volatile anesthetics. Depression of exocytosis was mimicked by a reduction in stimulus frequency, suggesting a reduction in action potential initiation, conduction, or coupling to Ca²⁺ channel activation. There was no evidence for a direct effect on endocytosis. The effects of isoflurane on synaptic transmission are thus caused primarily by inhibition of action potential-evoked synaptic vesicle exocytosis at a site upstream of Ca²⁺ entry and exocytosis, possibly as a result of Na⁺ channel blockade and/or K⁺ channel activation, with the possibility of lesser contributions from Ca²⁺ channel blockade and/or soluble N-ethylmaleimide-sensitive factor attachment protein receptor-mediated vesicle fusion.

Despite intense interest and scrutiny, the detailed mechanisms by which general anesthetics alter neuronal function to produce amnesia, unconsciousness, and immobility are unknown. Clinically relevant concentrations of various general anesthetics preferentially affect synaptic transmission rather than axonal conduction (Larrabee and Posternak, 1952) by agent-specific postsynaptic and/or presynaptic mechanisms (MacIver, 1997). Ligand-gated ion channels are sensitive to clinical concentrations of many volatile and intravenous general anesthetics and seem to underlie their postsynaptic effects (Franks and Lieb, 1994; Krasowski and Harrison 1999; Campagna et al., 2003). Most general anesthetics enhance inhibitory synaptic transmission by potentiation of postsynaptic GABA_A receptors (Tanelian et al., 1993), whereas specific anesthetics such as nitrous oxide, xenon, and ketamine depress excitatory synaptic transmission by blocking NMDA-type glutamate re-

ceptors, with little effect on GABA_A receptors (Lodge and Johnson, 1990; Mennerick et al., 1998; De Sousa et al., 2000; Yamakura and Harris, 2000). In contrast, the synaptic effects of volatile anesthetics are not fully explained by postsynaptic actions on ligand-gated ion channels.

Volatile anesthetics depress excitatory synaptic transmission in the CNS by poorly characterized presynaptic mechanisms, with little apparent contribution of postsynaptic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid- and NMDA-type glutamate receptor antagonism at clinical concentrations, as indicated indirectly by electrophysiological measurements of postsynaptic responses in the hippocampus (Perouansky et al., 1995; MacIver et al., 1996). Depression of glutamate release from isolated rat cerebrocortical nerve terminals evoked by pharmacological stimulation occurs at clinically relevant concentrations of volatile general anesthetics such as halothane and isoflurane (Schlame and Hemmings, 1995). Isoflurane also inhibits the release of GABA from isolated nerve terminals, but with slightly lower potency than for glutamate (Westphalen and Hemmings, 2003).

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ABBREVIATIONS: NMDA, N-methyl-D-aspartate; CNS, central nervous system; VAMP, vesicle-associated membrane protein; SNARE, soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor; FM 1–43, N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide.

The small size of most nerve terminals in the CNS (diameter $<1\ \mu\text{m}$) prohibits direct electrophysiological analysis of anesthetic effects on presynaptic action potentials and ion channel currents. The inability to directly monitor synaptic vesicle release further impedes detailed studies of presynaptic anesthetic mechanisms. Current electrophysiological and neurochemical techniques are inadequate for probing the effects of general anesthetics on the exocytotic machinery. Analysis of a specialized giant brainstem auditory calyceal synapse in rat provided indirect evidence that isoflurane reduces exocytosis by suppressing presynaptic action potential amplitude modeled by reduced presynaptic current injections (Wu et al., 2004). However, the proposed highly nonlinear coupling to exocytosis made it difficult to rule out other possible sites of action. The effects of anesthetics on the endocytotic aspect of synaptic vesicle function are unknown. Genetic screening in *Caenorhabditis elegans* identified mutations in the three components of the core SNARE complex that alter anesthetic sensitivity, implicating SNARE-mediated vesicle cycling as a target for presynaptic anesthetic effects (van Swinderen et al., 1999), although this has not been confirmed in mammals. We used a recently developed optical technique that involves transfection of the pH-dependent fluorescent probe superecliptic pHluorin fused to the luminal domain of the synaptic vesicle SNARE protein VAMP to simultaneously monitor vesicle exocytosis/endocytosis (Sankaranarayanan et al., 2000). This approach allowed us to determine the effects of the volatile anesthetic isoflurane on action potential-evoked synaptic vesicle cycling including both exocytosis and endocytosis in intact CNS neurons for the first time. Isoflurane at clinical concentrations inhibits action potential-evoked synaptic vesicle exocytosis in cultured rat hippocampal neurons without affecting endocytosis. This effect occurs primarily at a site upstream of Ca^{2+} entry in stimulus-secretion coupling and, thus, does not involve significant SNARE-mediated anesthetic actions.

Materials and Methods

Cell Culture and Experimental Conditions. Hippocampal CA3-CA1 regions were dissected from 1- to 3-day-old Sprague-Dawley rats, dissociated, and plated onto polyornithine-coated glass coverslips as described previously (Ryan, 1999). Cultures were maintained at 37°C in 95% air/5% CO_2 in a humidified incubator, and culture medium was replaced every week. Superecliptic synapto-pHluorin was transfected into 6- to 8-day old cultures through calcium phosphate-mediated gene transfer as described previously (Sankaranarayanan et al., 2000). Superecliptic synapto-pHluorin is a modified version of the original ecliptic pHluorin with enhanced optical properties but an identical pK_a (Sankaranarayanan et al., 2000). Cells were used 14 to 21 days after plating (8–14 days after transfection). The coverslips were mounted in a rapid switching, laminar-flow perfusion and stimulation chamber (volume $\sim 75\ \mu\text{l}$) on the stage of a custom-built laser-scanning confocal microscope. Cells were continuously perfused at a rate of 1 to 1.5 ml/min at room temperature ($\sim 25^\circ\text{C}$) in perfusion buffer containing 119 mM NaCl, 2.5 mM KCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 25 mM HEPES (buffered to pH 7.4), 30 mM glucose, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (Sigma/RBI, Natick, MA) and 50 μM DL-2-amino-5-phosphonovaleric acid (Sigma/RBI). Action potentials were evoked by passing 1-ms current pulses, yielding fields of $\sim 10\ \text{V/cm}$, typically at 10 Hz for 20 s (200 action potentials), through the chamber via platinum-iridium electrodes, which induces action potential firing with high fidelity ($>90\%$). With stimulation of vesicle fusion, the pH-sensitive synapto-

pHluorin, which is quenched by the low luminal pH, is exposed to the extracellular solution generating an increase in fluorescence with exocytosis and a subsequent reequenching of fluorescence with endocytosis. For dye studies, nontransfected cells were loaded with FM 1-43 (Molecular Probes, Eugene, OR) at a final concentration of 15 μM in perfusion buffer. The entire releasable pool of vesicles was labeled by stimulating for 40 to 45 s at 30 Hz (a stimulus shown to turn over the entire releasable pool of vesicles at both 25°C and 35°C) in the presence of the dye, followed by an additional 60 s of dye exposure to ensure labeling of all recycling membrane during endocytosis (Ryan and Smith, 1995). After 10 min of rinsing in dye-free solution, the unloading phase consisted of episodes of fluorescence image acquisition continuously during action potential firing.

Optical Measurements, Microscopy, and Analysis. Laser-scanning fluorescence images were acquired using a custom-built laser-scanning microscope through a 40×1.3 numerical aperture Zeiss Fluor objective (Zeiss, Oberkochen, Germany). Specimens were illuminated with ~ 1.5 to 10 μW of the 488-nm line of an argon ion laser that was rapidly shuttered during all non-data-acquiring periods using acousto-optic or electro-optic modulation. Time course of fluorescence responses of synapto-pHluorin and FM 1-43 were measured from time-lapse images. Synapto-pHluorin fluorescence emission was collected using a 498- to 538-nm band pass filter. FM 1-43 emission was collected with a 500-nm long pass filter. Quantitative measurements of fluorescence intensity at individual boutons were obtained by averaging 4×4 area pixels ($0.4 \times 0.4\ \mu\text{m}$) corresponding to the center of individual puncta selected by hand. The same quantitative measurements were applied to the axonal surface, except that the $0.4 \times 0.4\text{-}\mu\text{m}$ boxes spanned axonal areas in between individual boutons. Data are presented as the mean $\Delta F/F$ for control and drug treatments at each time point, and were analyzed by paired t test.

For FM dye experiments on nontransfected cells, each experiment corresponds to measurements from an individual coverslip, which includes boutons from multiple neurons. For each coverslip, a control experiment measured exocytosis without anesthetic, which was followed by reloading with FM dye, equilibration with a single concentration of isoflurane, and measurement of exocytosis in the presence of isoflurane. Reversibility of the isoflurane effect was confirmed by washout of isoflurane, reloading with FM dye, and measurement of exocytosis without anesthetic. For the synapto-pHluorin studies, a low transfection frequency ($\sim 1\%$) allows monitoring of a single neuron per high-power field. Thus, each experiment refers to measurements made on a single neuron from a single culture, even if it involved multiple rounds of stimulation. This method permitted testing of multiple conditions per experiment, because return to baseline control response could be assessed without maximal stimulation for unloading and reloading of vesicles between each condition as in the FM dye experiments.

Preparation and Analysis of Isoflurane Solutions. Perfusion buffer was saturated with isoflurane (Abbott Diagnostics, Abbott Park, IL) by stirring an excess (10 ml/l buffer) for $>12\ \text{h}$ at room temperature ($21\text{--}23^\circ\text{C}$) in an airtight glass bottle. The saturated solution (10–12 mM) was diluted with perfusion buffer into closed glass perfusion reservoirs to ~ 1.4 times the desired chamber concentration. In some experiments, bicuculline methiodide (Sigma-Aldrich, St. Louis MO) was added to both control and isoflurane containing perfusion buffer. The diluted isoflurane solutions were introduced to the perfusion chamber through polyethylene tubing from the closed glass reservoirs. Because of the volatility of isoflurane and attendant losses ($\sim 30\%$), the final isoflurane concentrations exiting the perfusion chamber were determined by gas chromatography after extraction of the isoflurane into heptane (Ratnakumari and Hemmings, 1998). This resulted in some scatter in the actual concentrations of isoflurane present in each experiment. Except for the concentration-effect determination, a concentration of $\sim 1\ \text{mM}$ (~ 2.8 times the EC_{50} for general anesthesia in rat of 0.35 mM; Taheri et al., 1991), which was near the EC_{50} for

inhibition of exocytosis, was chosen for routine analysis. Concentration-effect data were fitted by least-squares analysis to estimate IC_{50} and Hill slope (Prism v. 4.0; GraphPad Software Inc., San Diego, CA). Data are reported as mean \pm S.D.

Results

Isoflurane Inhibits Synapto-pHluorin Responses to Action Potential Firing. Transfection with a pH-sensitive green fluorescent protein variant fused to the luminal domain of the synaptic vesicle-associated membrane protein VAMP (synapto-pHluorin) was used to optically measure synaptic vesicle exocytosis and endocytosis in cultured hippocampal neurons (Sankaranarayanan and Ryan, 2000). This probe can be used to measure the recycling properties of VAMP at hippocampal axon terminals in real time (Sankaranarayanan and Ryan, 2000) because synapto-pHluorin fluorescence increases ~ 20 -fold during transition from the acidic vesicle lumen to the neutral pH in the extracellular solution (Sankaranarayanan and Ryan, 2000; Sankaranarayanan et al., 2000). Because vesicle reacidification is rapid (Sankaranarayanan and Ryan, 2000; Gandhi and Stevens, 2003), changes in fluorescence during action potential firing reflect the net accumulation of VAMP on the presynaptic surface, and changes during the poststimulus period report the kinetics of endocytosis of VAMP.

The time course of fluorescence changes during a train of 200 action potentials averaged over a population of individual synaptic boutons from a single neuron transfected with synapto-pHluorin is shown in Fig. 1. Recurrent excitation is avoided by blocking postsynaptic glutamate receptors with DL-2-amino-5-phosphonovaleric acid and CNQX. A rapid increase in fluorescence caused by exocytosis and externalization of synapto-pHluorin reached a peak that decayed after the stimulus because of endocytosis and vesicle reacidification after action potential stimulation. We used this assay to determine the impact of the volatile anesthetic isoflurane on

presynaptic function in intact cultured neurons. When superfused with perfusion buffer containing 0.85 mM isoflurane (2.4 times the EC_{50} for general anesthesia), peak synapto-pHluorin responses to action potential firing were suppressed by $\sim 40\%$ compared with control (Fig. 1). The inhibition of synapto-pHluorin responses by isoflurane was fully reversible; the fluorescence response returned to control after superfusion of the neurons with perfusion buffer in the absence of isoflurane for 10 min. Given the prominent potentiating effect of most intravenous and volatile general anesthetics on GABA_A receptors (Franks and Lieb, 1994), it is important to rule out an indirect depressive effect on excitability mediated by this mechanism as the reason for depression of exocytosis. Addition to the perfusion buffer of 10 μ M bicuculline methiodide, a specific antagonist of GABA_A receptors (Bai et al., 2001), did not prevent the depression of action potential-evoked exocytosis by isoflurane (data not shown).

Synapto-pHluorin measurements are well suited for examining concentration-dependent effects of pharmacological agents, because repeated assays can be carried out on the same cell at multiple drug concentrations. The effect of isoflurane on action potential-evoked synapto-pHluorin responses averaged from multiple boutons derived from a single transfected neuron was concentration-dependent (Fig. 2A). Similar measurements from additional neurons were used to assemble a concentration-effect curve for inhibition of synaptic vesicle cycling by isoflurane (Fig. 2B). Significant inhibition of synapto-pHluorin responses occurred at the clinically relevant concentration of 0.43 mM isoflurane and above: $11 \pm 5\%$ inhibition was observed at 0.43 mM (1.2 times EC_{50} for general anesthesia; $p < 0.05$, $n = 63$ boutons). The average effect of three experiments near 2 times the EC_{50} ($\sim ED_{95}$) for general anesthesia was $27 \pm 3\%$ inhibition by 0.75 ± 0.02 mM isoflurane ($p < 0.05$). Complete blockade of exocytosis was obtained at the highest isoflurane concentrations tested (1.9 mM). The data were fit to a sigmoidal concentration-effect curve with $IC_{50} = 1.0$ mM and Hill slope = 2.95 ($r^2 = 0.89$).

Both Exocytosis and Endocytosis Are Slowed by Isoflurane. The synapto-pHluorin signal reports a net balance of exocytosis and endocytosis (Sankaranarayanan and Ryan, 2001; Fernandez-Alfonso and Ryan, 2004). A net positive change in fluorescence during the stimulus period indicates that the rate of exocytosis exceeded the rate of endocytosis (Fernandez-Alfonso and Ryan, 2004). Thus, the reduction in peak fluorescence amplitude by isoflurane could result from suppression of exocytosis and endocytosis, suppression of exocytosis alone, or acceleration of endocytosis. After stimulation, synapto-pHluorin fluorescence recovers after zero-order kinetics; i.e., the initial rate of internalization is independent of the amount of synapto-pHluorin to be internalized (Sankaranarayanan and Ryan, 2000), and endocytosis proceeds at a constant rate both during and immediately after the stimulus period (Sankaranarayanan and Ryan, 2001). An appropriate measure of the speed of endocytosis is the initial rate of synapto-pHluorin fluorescence change after the stimulus period. We therefore analyzed the initial decay rate of synapto-pHluorin fluorescence for the first three time points poststimulus in the absence and presence of isoflurane. Isoflurane (1.04 ± 0.5 mM) suppressed the synapto-pHluorin peak response by $54 \pm 2.7\%$ and slowed the post-

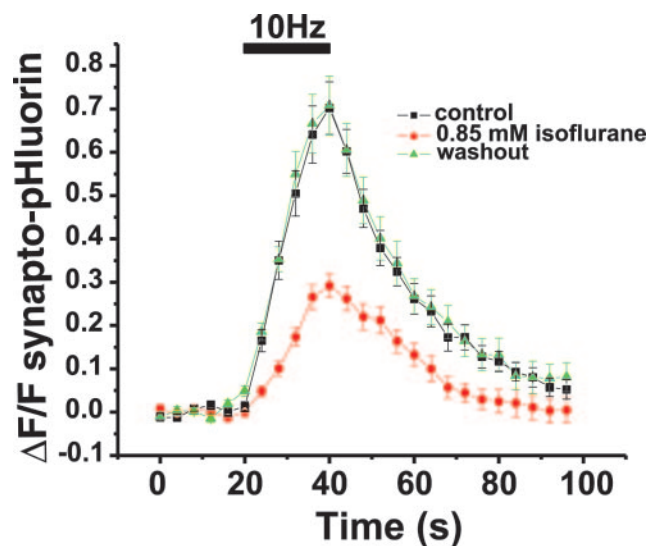


Fig. 1. Action potential-mediated exocytosis at hippocampal nerve terminals is inhibited reversibly by isoflurane. Synapto-pHluorin responses at hippocampal presynaptic terminals (for an ensemble of ~ 30 boutons from a single cell) were monitored during repetitive action potential stimulation. Isoflurane (0.85 mM; $2.4\times$ minimum alveolar concentration) reversibly depressed the response by $\sim 40\%$ compared with bracketing control assays ($p < 0.05$).

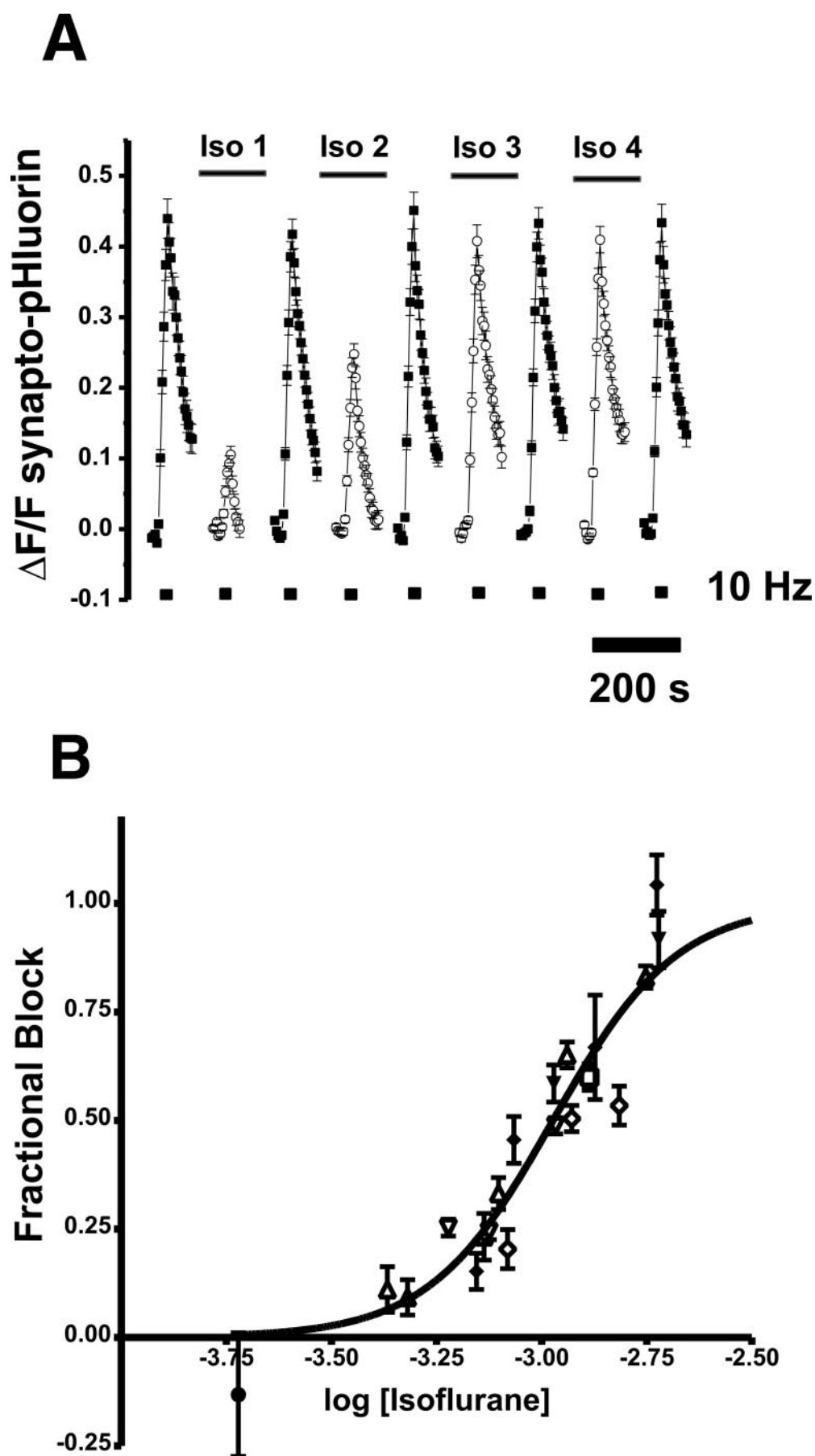


Fig. 2. Isoflurane inhibits action potential-stimulated exocytosis at clinical concentrations. **A**, representative experiment in which synapto-pHluorin responses to 200 action potential stimuli were monitored at the same boutons from a single neuron under control conditions (initial curves) or after equilibration with different concentrations of isoflurane (Iso1 = 1.77 mM, Iso2 = 0.79 mM, Iso3 = 0.47 mM, Iso4 = 0.4 mM). **B**, compiled data for fractional block of exocytosis by various concentrations of isoflurane determined using synapto-pHluorin measurements. Each symbol type represents data from a single cell (30–40 boutons) experiment ($n = 7$ cells total), each representing a different culture and transfection. All concentrations of isoflurane tested at ≥ 0.43 mM significantly inhibited exocytosis versus the respective control values (Student's paired t test, $p < 0.05$).

stimulus endocytosis rate by $52 \pm 8\%$ ($p < 0.05$ for each by paired t test, $n = 4$; Fig. 3).

Because the synapto-pHluorin signal represents the net difference between exocytosis and endocytosis, isoflurane must inhibit exocytosis as well. We therefore examined the effect of isoflurane on the kinetics of FM 1-43 destaining, which should give a faithful measure of its impact on action potential-evoked exocytosis. Synaptic vesicles were labeled with FM 1-43 using repetitive electrical stimulation at 20 Hz to elicit rounds of exocytosis/endocytosis. After washing, the dye released by exocytosis upon subsequent stimulation in the absence or presence of isoflurane was quantified by time-lapse fluorescence imaging of labeled boutons. After a control dye loading/unloading experiment, the same terminals were reloaded with dye and superfused with buffer containing isoflurane during a second round of dye unloading (Fig. 4). Isoflurane at 0.84 mM strongly inhibited the rate of synaptic vesicle exocytosis without affecting the extent of exocytosis; the decay constant τ increased by $31 \pm 7\%$ (from 15.3 ± 0.46 to 21.7 ± 2.4 s, $p < 0.03$ by Student's t test, $n = 4$). This inhibition rate corresponds to a decrease in synaptic efficiency (expressed as percentage of the releasable pool released) from 0.65%/action potential to 0.46%/action potential. The effect of isoflurane was reversible: reloading the same terminals with dye followed by a third round of unloading in the absence of isoflurane showed complete recovery to control values (data not shown).

A Major Presynaptic Target of Isoflurane Is Upstream of Ca^{2+} Entry. Synaptic vesicle exocytosis and endocytosis are both Ca^{2+} -sensitive (Sanakaryanan and Ryan, 2001); thus, potential targets for isoflurane include voltage-gated Ca^{2+} channels and/or the driving force for gated Ca^{2+} entry during stimulation (i.e., action potential-driven membrane depolarization). We tested the possibility that inhibition of exocytosis by isoflurane arises directly from blockade of Ca^{2+} entry by attempting to relieve the blockade by stimulation in the presence of elevated external Ca^{2+} . Figure 5A

shows an example of the synapto-pHluorin responses to 200 action potentials delivered in 2 mM external Ca^{2+} in the absence or presence of 1 mM isoflurane. This measurement was then performed in 3.5 mM external Ca^{2+} in the same cell, comparing controls to a similar isoflurane concentration. Although elevation of external Ca^{2+} increased the size of the response by $24 \pm 0.05\%$ ($p < 0.05$, $n = 4$), the degree of block by isoflurane was identical at 2 mM and 3.5 mM external Ca^{2+} (mean block by 1.0 mM isoflurane was $49 \pm 5\%$ for 2 mM Ca^{2+} ($p < 0.05$) versus $51 \pm 1\%$ block by 1.1 mM isoflurane for 3.5 mM Ca^{2+} ; $p < 0.05$ by Student's t test, $n = 4$).

Evidence from isolated nerve terminals suggests that pharmacologically evoked glutamate release is inhibited by volatile anesthetics acting to block presynaptic voltage-gated Na^+ channels (Ratnakumari and Hemmings, 1996; Ratnakumari et al., 2000; Lingamaneni et al., 2001). Field electrical stimulation of exocytosis in cultured hippocampal neurons depends upon voltage-gated Na^+ channel function, because it can be blocked by tetrodotoxin, a selective antagonist of neuronal voltage-gated Na^+ channels that prevents downstream activation of voltage-gated Ca^{2+} channels for excitation-exocytosis coupling (T. A. Ryan, unpublished data). An alternate, Na^+ channel-independent stimulation of exocytosis is produced by elevated extracellular K^+ stimulation. Elevated extracellular KCl has been used extensively as a pharmacological method of evoking exocytosis in isolated nerve terminals (Nicholls, 1993) and cultured hippocampal neurons (Ryan et al., 1993). Exocytosis evoked by a pulse of 40 mM KCl in cultured hippocampal neurons was insensitive to $1 \mu\text{M}$ tetrodotoxin (T. A. Ryan unpublished data). Thus, in contrast to action potential-evoked exocytosis, activation of tetrodotoxin-sensitive Na^+ channels is not involved in KCl depolarization-evoked release. We therefore compared the sensitivity to isoflurane of exocytosis evoked by a train of action potentials or by a pulse of elevated external KCl. To simplify the comparison, we chose a KCl concentration ([KCl]) that elicited a synapto-pHluorin response of a magnitude similar to the peak response obtained using our stan-

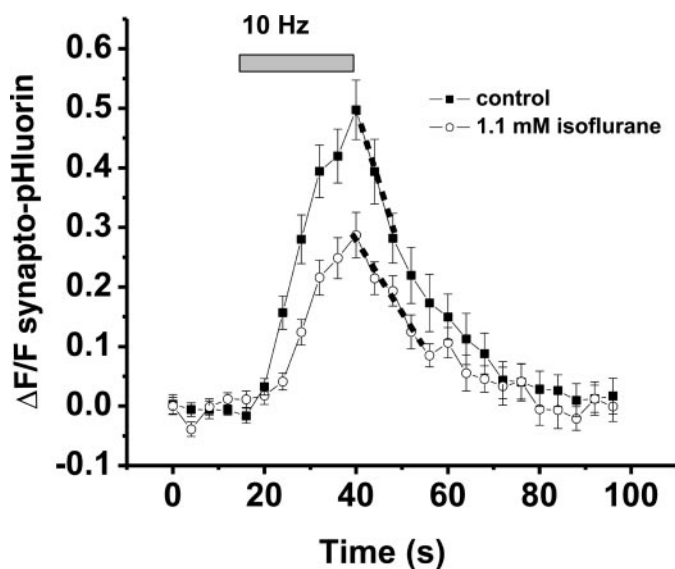


Fig. 3. Isoflurane reduces the peak response and slows recovery of the synapto-pHluorin response after action potential stimulation. In addition to suppressing the peak synapto-pHluorin response, isoflurane also inhibits the poststimulus rate of endocytosis, as determined by the initial slope in the poststimulus period (heavy dashed lines).

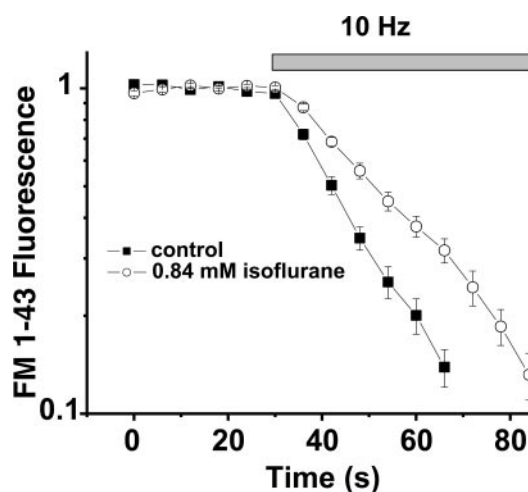


Fig. 4. FM 1-43 destaining kinetics are slowed by isoflurane. Hippocampal synaptic terminals were preloaded with FM 1-43 using action potential stimulation. The rate of dye destaining either in the presence or absence of isoflurane was followed for the same boutons in sequential loading/unloading runs. The depression of FM 1-43 destaining was reversible upon washout of isoflurane (not shown).

dard test condition (200 action potentials at 10 Hz). Although the overall dynamic profile of intracellular Ca^{2+} almost certainly differs for elevated KCl superfusion compared with action potential stimulation, the similar kinetics of sustained secretion indicate that the exocytic machinery must be operating under similar kinetic constraints. Although 1 mM isoflurane inhibited synapto-pHluorin responses driven by action potential stimulation (Fig. 5A), this effect was greatly diminished when exocytosis was driven by elevated [KCl] (Fig. 5B). Side-by-side comparisons on the same neurons indicated that, on average, the inhibitory effect of 1 mM isoflurane was reduced by $\sim 65\%$, from a mean of $56 \pm 3.7\%$ inhibition with action potential stimulation to $19 \pm 4.7\%$ inhibition with elevated [KCl] ($p < 0.05$ by analysis of variance, $n = 6$; Fig. 5B). Because KCl-evoked exocytosis is less sensitive to isoflurane, the *major* presynaptic target for inhibition of exocytosis by isoflurane must lie upstream of Ca^{2+} entry through nerve terminal voltage-gated Ca^{2+} channels coupled to synaptic vesicle exocytosis.

If the principal target of isoflurane is action potential depression, the effect of isoflurane on synapto-pHluorin responses should be mimicked by a reduction in the action potential-firing frequency compared with control stimulus conditions (20 s at 10 Hz). In the next series of experiments, we sought to test this hypothesis by comparing the time course of synapto-pHluorin responses during 10-Hz repetitive stimulation in the presence of isoflurane to synapto-pHluorin responses at the same synapses stimulated at lower frequency in the absence of isoflurane. In principle, even the slowing of poststimulus endocytosis could result from reduced firing frequency, because that in turn would lead to lower total Ca^{2+} influx over a given stimulus period. Because

intracellular Ca^{2+} also controls endocytosis, slowing firing rate should slow both exocytosis and endocytosis. The effect of isoflurane (0.94 ± 0.03 mM leading to $42 \pm 1\%$ inhibition; $p < 0.05$, $n = 4$) on the synapto-pHluorin peak response and on postendocytic recovery to a 20-s stimulation at 10 Hz was well matched by a 20-s stimulation at an average rate of 6.8 ± 0.1 Hz ($n = 4$) (Table 1). A representative experiment is shown in Fig. 6. Although the very late phase of endocytosis is somewhat slower in the presence of isoflurane at 10 Hz compared with a control at 7.3 Hz, the initial poststimulus slopes (as defined in Fig. 3) were almost identical (ratio of the poststimulus endocytosis slope in isoflurane to the frequency-shifted control = 0.95 ± 0.1 ; $p > 0.05$ by paired t test, $n = 4$). Thus, isoflurane inhibition of synapto-pHluorin responses is most consistent with an effective block of action potential firing.

Discussion

Our results demonstrate that isoflurane, a widely used polyhalogenated ether anesthetic, has profound inhibitory effects on action potential-evoked synaptic vesicle exocytosis in hippocampal neurons. This study represents the first direct demonstration of presynaptic general anesthetic actions on action potential-evoked synaptic vesicle cycling in intact CNS neurons representative of most central synapses in mammalian brain. These findings indicate that the principal presynaptic target for anesthetic inhibition of synaptic transmission is reduced exocytosis because of action potential disruption. Other potential sites of action downstream of the action potential, including inhibition of Ca^{2+} channels coupled to vesicle exocytosis and direct effects on SNARE-

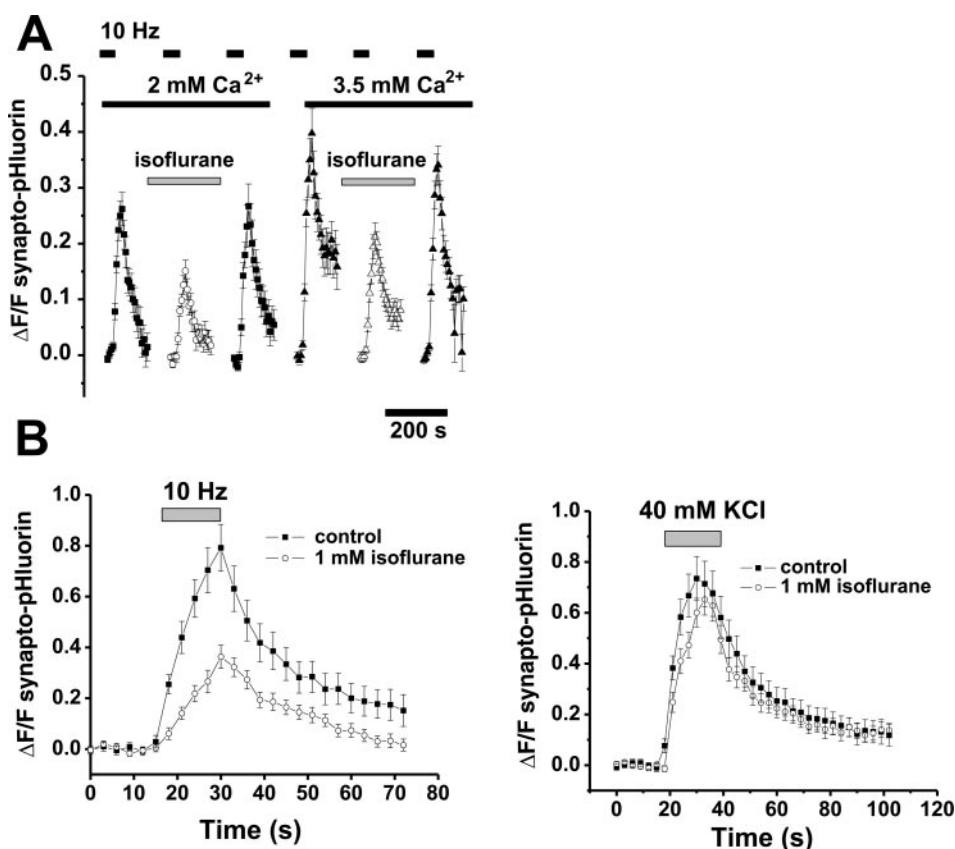


Fig. 5. Isoflurane block of action potential-mediated exocytosis occurs upstream of Ca^{2+} influx. **A**, elevated external Ca^{2+} does not relieve isoflurane block. The degree of isoflurane block was measured for both 2 mM and 3.5 mM external Ca^{2+} . Synapto-pHluorin responses to a 200-action potential stimulus in the presence and absence of 1.1 mM isoflurane (for 2 mM Ca^{2+}) or 1.2 mM isoflurane (for 3.5 mM Ca^{2+}) at the same synaptic terminals are shown. The fractional block for each condition was very similar (51% for 2 mM Ca^{2+} and 53% for 3.5 mM Ca^{2+}). **B**, stimulation by high [KCl] is resistant to isoflurane block. Left, an example of action potential-mediated synapto-pHluorin responses in the presence or absence of 1 mM isoflurane. In this example, 1 mM isoflurane resulted in 56% block of exocytosis. The same cell was then stimulated using a pulse of 40 mM KCl in perfusion buffer (with an equivalent reduction in NaCl) in the presence or absence of 1 mM isoflurane (right). The peak responses were almost identical for action potential-induced and elevated [KCl]-induced exocytosis under control conditions. However, only the action potential-mediated responses are inhibited significantly by isoflurane.

mediated synaptic vesicle exocytosis, do not seem to be major presynaptic targets of isoflurane, but may contribute to the overall effect.

Genetic studies in *C. elegans* raised the intriguing possibility that volatile anesthetics may exert some of their presynaptic effects by interacting with key elements of the exocytotic protein machinery. Mutants with hypersensitivity or resistance to the immobilizing effects of volatile anesthetics harbor mutations in homologs of syntaxin, SNAP-25 or VAMP, consistent with an anesthetic effect on the vesicle fusion step regulated by these proteins (van Swinderen et al., 1999). Our observations that exocytosis evoked by elevated extracellular K^+ is less sensitive to isoflurane compared with action potential-evoked exocytosis indicate that SNARE-mediated vesicle fusion is not the major presynaptic target for volatile anesthetic depression of exocytosis in the rat CNS. Likewise, Wu et al. (2004) concluded that reduction in excitatory postsynaptic currents at the rat calyx of Held is caused primarily by reduction of presynaptic action potential amplitude, with minor contributions from targets downstream from terminal depolarization. The failure of elevated extracellular Ca^{2+} to rescue isoflurane inhibition of action potential-evoked exocytosis, which should antagonize Ca^{2+} channel-mediated blockade, provides additional evidence that inhibition of action potential-evoked exocytosis does not in-

TABLE 1

Average values of the synapto-pHluorin responses to stimulation as well as the initial rate of post-stimulus synapto-pHluorin decay (endocytosis rate)

Synapto-pHluorin responses were measured over an 8-s period for three different stimulation conditions in each cell (mean \pm S.E.M., $n = 4$ cells). Isoflurane (0.95 ± 0.03 mM) led to a $42 \pm 1.7\%$ reduction in peak response ($p < 0.05$ by paired t test) and a $51 \pm 3.4\%$ reduction in endocytosis rate ($p < 0.05$ by paired t test) at a stimulus frequency of 10 Hz. At a lower stimulus frequency (6.8 Hz), where the peak response matched that for 10-Hz stimulation in isoflurane, endocytosis was identical ($p > 0.05$ by paired t test).

	Peak Response	Endocytosis Rate
	arbitrary fluorescence units	
10 Hz control	0.77 ± 0.17	0.23 ± 0.04
10 Hz isoflurane	0.43 ± 0.09	0.12 ± 0.02
Frequency match (mean 6.8 ± 0.1 Hz)	0.40 ± 0.13	0.13 ± 0.03

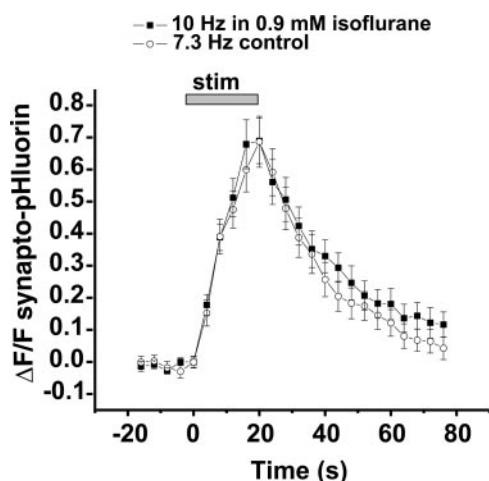


Fig. 6. Isoflurane block of synapto-pHluorin responses is equivalent to lowering firing frequency. Lowering the stimulus frequency mimicked the blocking effect of isoflurane on synapto-pHluorin responses. In the example shown here, the synapto-pHluorin response to a 10-Hz stimulus in the presence of 0.9 mM isoflurane was very similar to the synapto-pHluorin response obtained in the absence of isoflurane with a 7.3-Hz stimulus.

volve Ca^{2+} entry through voltage-gated Ca^{2+} channels. Taken together, our findings that inhibition of exocytosis by isoflurane is equivalent to reducing the rate of action potential firing, that elevated external Ca^{2+} fails to relieve inhibition by isoflurane, and that exocytosis stimulated by elevated extracellular K^+ is approximately one-third as sensitive to isoflurane as action potential-evoked exocytosis implicate action potential depression as the principal presynaptic action of isoflurane. Considerable evidence supports a role for Na^+ channel blockade in the inhibition of synaptic vesicle exocytosis by volatile anesthetics (Ratnakumari and Hemmings, 1998; Lingamaneni et al., 2001; Ouyang et al., 2003; Westphalen et al., 2003; Wu et al., 2004), but the current data do not rule out other potential targets. Putative anesthetic actions upstream of Ca^{2+} entry and vesicle fusion that may contribute to depression of exocytosis include activation of voltage-gated or two-pore domain K^+ channels, some of which are sensitive to volatile anesthetics (Patel et al., 1999). The functions and subcellular localizations of the anesthetic-sensitive background two-pore domain K^+ channels are not known, but a presynaptic localization could stabilize membrane potential and prevent nerve terminal depolarization and exocytosis via enhanced outward K^+ current. Further studies will be required to investigate the intriguing proposal that anesthetic-activated two-pore domain K^+ channels are present in presynaptic terminals and contribute to presynaptic anesthetic effects (Patel et al., 1999).

The available data cannot distinguish between anesthetic blockade of axonal Na^+ channels that support axonal action potential conduction (Mikulec et al., 1998) from blockade of Na^+ channels in the presynaptic bouton that are required for sufficient nerve terminal depolarization to activate the voltage-gated Ca^{2+} channels coupled to exocytosis (Nicholls, 1993). Evidence from isolated nerve terminals (Schlame and Hemmings, 1995; Ouyang et al., 2003) and the calyx of Held (Wu et al., 2004) suggest that inhibition of Na^+ channels in the presynaptic bouton is sufficient to inhibit transmitter release, although a contribution from depression of axonal action potential conduction may also be involved (Mikulec et al., 1998). The specific neuronal Na^+ channel isoform(s) affected by isoflurane in cultured hippocampal neurons is not known. All major adult CNS Na^+ channel isoforms are inhibited by isoflurane (Shiraishi and Harris, 2004); the sensitivities of neonatal splicing variants are unknown.

Previous studies have suggested anesthetic blockade of voltage-gated Ca^{2+} channels as a mechanism for inhibition of transmitter release. Halothane augments paired-pulse facilitation in hippocampal CA1 neurons, consistent with a reduction in presynaptic Ca^{2+} influx (MacIver et al., 1996; Kirson et al., 1998), and a study in isolated guinea pig nerve terminals suggested inhibition of voltage-gated Ca^{2+} channels in the depression of glutamate release by volatile anesthetics (Miao et al., 1995), although this finding was not reproducible (Lingamaneni et al., 2001). However, pharmacological evidence indicates that the principal target for inhibition of glutamate release from isolated rat, mouse, and guinea pig cortical nerve terminals occurs at a target upstream of the voltage-gated Ca^{2+} channels coupled to exocytosis (Ratnakumari and Hemmings, 1998; Lingamaneni et al., 2001; Westphalen et al., 2003). Electrophysiological studies in isolated rat neurohypophyseal nerve terminals indicate that isoflurane potently inhibits presynaptic voltage-gated

Na⁺ channels (Ouyang et al., 2003) and action potential amplitude with a less potent effect on Ca²⁺ channels (Ouyang et al., 2005), consistent with our results in hippocampal neurons. In the calyx of Held synapse stimulated by intraterminal current injections, isoflurane inhibited exocytosis detected by presynaptic capacitance changes and reduced the amplitude of presynaptic action potentials (Wu et al., 2004). Voltage-gated N- and P/Q-type Ca²⁺ channels (Ca 2.1 and 2.2) have been implicated as the source of presynaptic Ca²⁺ entry in hippocampal synaptic transmission (Wheeler et al., 1994; Wu and Saggau, 1994). Despite findings that somatic and heterologously expressed N- and P/Q-type voltage-gated Ca²⁺ channels can be inhibited by volatile anesthetics (Study, 1994; Kamatchi et al., 1999), the available evidence (reduced sensitivity of KCl-evoked exocytosis, no Ca²⁺ rescue) does not support presynaptic Ca²⁺ channel blockade as the principal target for depression of transmitter release by volatile anesthetics.

This study supports the hypothesis that depression of excitatory synaptic transmission by volatile anesthetics can be accounted for largely by presynaptic mechanisms. In particular, inhibition of exocytosis by the volatile anesthetic isoflurane occurs primarily upstream of Ca²⁺ influx. Our results can be explained by a reduction in nerve terminal excitation caused by impaired action potential conduction within the presynaptic bouton, resulting in impaired synaptic efficiency. In fact, the effects of isoflurane on synaptic vesicle cycling are mimicked by application of the selective Na⁺ channel blocker tetrodotoxin (data not shown) or by reducing the number of action potential stimuli. Pharmacological evidence in isolated nerve terminals indicates that blockade of presynaptic voltage-gated Na⁺ channels is the probable mechanism for this effect, although effects on K⁺ channels cannot be ruled out. Volatile anesthetics inhibit Na⁺ channel-dependent depolarization-evoked glutamate release from isolated rat CNS nerve terminals (Schlame and Hemmings, 1995; Lingamaneni et al., 2001; Westphalen and Hemmings, 2003) and also antagonize depolarization-evoked Na⁺ flux and batrachotoxin-B binding to Na⁺ channels (Ratnakumari and Hemmings, 1998). Although it has not been possible to record Na⁺ currents from typical small CNS nerve terminals, volatile anesthetics block somatic (Ratnakumari et al., 2000) and heterologously expressed (Rehberg et al., 1996; Shiraishi and Harris, 2004) neuronal voltage-gated Na⁺ channels, as well as voltage-gated Na⁺ channels in isolated neurohypophysial nerve terminals (Ouyang et al., 2003, 2005).

Cultured neonatal rat hippocampal neurons are phenotypically heterogeneous; both glutamatergic pyramidal neurons and GABAergic interneurons are represented in the cultures. The synapto-pHluorin method selectively labels a single neuron per field because of the low transfection efficiency and thus provides data from a single neuron of unknown phenotype. The FM 1–43 method labels active boutons of all functioning neurons present in the culture and provides an ensemble average of exocytosis from multiple neuron types. These complementary methods indicate comparable sensitivity to isoflurane, consistent with anesthetic suppression of synaptic vesicle exocytosis in both glutamatergic and GABAergic terminals, which represent the bulk of terminals in this preparation (T. A. Ryan, unpublished observations). We have also shown that isoflurane inhibits release of both glutamate and GABA from isolated rat cortical nerve termi-

nals by an independent dual-label isotopic method (Westphalen and Hemmings, 2003), although GABA release is somewhat less sensitive than glutamate release. This heterogeneity may explain some of the variation observed in sensitivity of exocytosis to isoflurane in individual neurons.

Considerable uncertainty has surrounded the mechanisms of action of general anesthetics for more than a century (Franks and Lieb, 1994; Campagna et al., 2003). Recent progress favors multiple agent-specific targets for anesthetics in the CNS: facilitation of inhibitory synaptic transmission via GABA_A receptor potentiation by most intravenous and volatile anesthetics, depression of excitatory synaptic transmission via blockade of postsynaptic NMDA receptors by gaseous and dissociative anesthetics, and depression of excitatory transmission via presynaptic inhibition of transmitter release by volatile anesthetics. Our findings solidify the latter mechanism by demonstrating profound inhibition of action potential-evoked synaptic vesicle fusion in intact cultured CNS neurons. Our results implicate multiple presynaptic targets for depression of exocytosis by isoflurane: a major target upstream of Ca²⁺ entry, probably involving Na⁺ channel blockade and/or two-pore domain K⁺ channel activation, and a lesser contribution from Ca²⁺ channel blockade and/or an effect on SNARE-mediated fusion.

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