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Inhibition of Presynaptic Sodium Channels by Halothane

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Background: **Recent electrophysiologic studies indicate that clinical concentrations of volatile general anesthetic agents inhibit central nervous system sodium (Na^+) channels. In this study, the biochemical effects of halothane on Na^+ channel function were determined using rat brain synaptosomes (pinched-off nerve terminals) to assess the role of presynaptic Na^+ channels in anesthetic effects.**

Methods: **Synaptosomes from adult rat cerebral cortex were used to determine the effects of halothane on veratridine-evoked Na^+ channel-dependent Na^+ influx (using $^{22}Na^+$), changes in intrasynaptosomal [Na^+] (using ion-specific spectrofluorometry), and neurotoxin interactions with specific receptor sites of the Na^+ channel (by radioligand binding). The potential physiologic and functional significance of these effects was determined by measuring the effects of halothane on veratridine-evoked Na^+ channel-dependent glutamate release (using enzyme-coupled spectrofluorometry).**

Results: **Halothane inhibited veratridine-evoked $^{22}Na^+$ influx (IC_{50} = 1.1 mM) and changes in intrasynaptosomal [Na^+] (concentration for 50% inhibition [IC_{50}] = 0.97 mM), and it specifically antagonized [^{3}H]batrachotoxinin-A 20-α-benzoate binding to receptor site two of the Na^+ channel (IC_{50} = 0.53 mM). Scatchard and kinetic analysis revealed an allosteric competitive mechanism for inhibition of toxin binding. Halothane inhibited veratridine-evoked glutamate release from synaptosomes with comparable potency (IC_{50} = 0.67 mM).**

Conclusions: **Halothane significantly inhibited Na^+ channel-mediated Na^+ influx, increases in intrasynaptosomal [Na^+] and glutamate release, and competed with neurotoxin binding to site two of the Na^+ channel in synaptosomes at concentrations within its clinical range (minimum alveolar concentration, 1–2). These findings support a role for presynaptic Na^+ channels as a molecular target for general anesthetic effects. (Key words: Batrachotoxinin-A 20-α-benzoate; brevetoxin-B; exocytosis; glutamate; volatile anesthetics.)**

NEUROTRANSMISSION consists of action potential propagation along axons and chemical transmission across synapses, both of which involve voltage-dependent sodium channels (Na^+ channels).[1,2] Previous studies in mammalian and nonmammalian tissues have demonstrated inhibition of neuronal Na^+ channels by volatile anesthetic agents, including reduced axonal conduction,[3,4] increased firing threshold,[5,6] and altered Na^+ channel gating and conductance.[7,8] Recent evidence supports inhibition of central nervous system (CNS) Na^+ channels at clinical concentrations in cells transfected with rat brain type IIA Na^+ channels.[9] Voltage- and use-dependent suppression of Na^+ channel currents was found at physiologic resting membrane potentials, which provides direct evidence that Na^+ channels are a sensitive molecular target for volatile anesthetic action.

Volatile anesthetic agents also inhibit release of neurotransmitters in the CNS.[3,10-14] Possible mechanisms include effects on presynaptic terminal depolarization; Ca^{2+} influx; and synthesis, storage, exocytosis, and inactivation of neurotransmitters. Invading action potentials depolarize the presynaptic plasma membrane by activation of Na^+ channels, which leads to Ca^{2+} entry through activation of voltage-dependent Ca^{2+} channels, followed by Ca^{2+}-dependent exocytotic release of neurotransmitters.[2] Inhibition of neuronal Ca^{2+} channels by volatile anesthetic agents has been demonstrated by effects on intracellular [Ca^{2+}],[15,16] radioligand binding,[17,18] and Ca^{2+} currents.[19] Data from our laboratory, however, suggest that a step proximal to Ca^{2+} influx, *i.e.,* Na^+ influx through Na^+ channels, is more sensitive than Ca^{2+} influx through Ca^{2+} channels to the presynaptic actions of volatile anesthetic agents,[12] although this is controversial.[11] Peripheral neuronal Na^+ channels also appear to be more sensitive than Ca^{2+} channels to the action of isoflurane.[20]

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In the current study, we analyzed the effects of the volatile anesthetic agent halothane on presynaptic CNS Na^+ channels by measuring its effects on veratridine-evoked $^{22}Na^+$ influx and increases in intrasynaptosomal $[Na^+]$ ($[Na^+]_i$) and on the binding of radiolabeled neurotoxins to Na^+ channels in rat cerebrocortical synaptosomes. We also examined the functional effects of halothane on presynaptic Na^+ channels by measuring veratridine-evoked release of glutamate, the major excitatory neurotransmitter in the CNS, from the same preparation. Synaptosomes, a subcellular fraction that consists of pinched-off nerve terminals, provide a useful system for analyzing the biochemical pharmacologic characteristics of presynaptic Na^+ channels.[21-24] Our results indicate an interaction between halothane and CNS Na^+ channels and that presynaptic Na^+ channels may mediate some of the inhibitory effects of volatile anesthetic agents on excitatory synaptic transmission.

Materials and Methods

Materials

Reagents were obtained from the following sources: [11-^{3}H]saxitoxin (28 Ci · mmol^{-1}) from Amersham (Arlington Heights, IL); [^{3}H]batrachotoxinin-A 20-α-benzoate (BTX-B; 34 Ci · mmol^{-1}) and $^{22}NaCl$ (1 mCi · ml^{-1}) from DuPont-New England Nuclear (Boston, MA); [42-^{3}H] brevetoxin-3 (14.25 Ci · mmol^{-1}) and *Ptychodiscus brevis* toxin-3 (PbTx-3) from Chiral Corp. (Miami, FL); Percoll density gradient medium from Pharmacia/LKB (Uppsala, Sweden); halothane (thymol-free) from Halocarbon Products (North Augusta, SC); tetrodotoxin, veratridine, scorpion venom (*Leiurus quinquestriatus*), L-glutamate dehydrogenase (*Proteus* sp), and dimethylsulfoxide from Sigma Chemical Co. (St. Louis, MO); and the cell permeant acetoxymethyl ester precursor form of Na^+-binding benzofuran isophthalate (SBFI-AM) and Pluronic F-127 from Molecular Probes, Inc. (Eugene, OR). All other chemicals were of reagent grade.

Dimethylsulfoxide at a final concentration of 0.05% (vol/vol) was used as a vehicle in binding and flux studies to minimize reaction volumes. Control experiments showed that the vehicle alone had no effect on the variables measured (data not shown).

Preparation of Synaptosomes

Synaptosomes from rat cerebral cortex were prepared using a modification of the procedure of Dunkley *et al.*[25] Adult male (150-175 g) Sprague-Dawley rats were anesthetized with 80% CO_2/20% O_2, were killed by decapitation, and their brains were immediately removed and rinsed in ice-cold 0.32 M sucrose. Cortical gray matter was dissected and homogenized in ten volumes of 0.32 M sucrose using a motor-driven Teflon glass homogenizer at 900 rpm for 10 up-and-down strokes. The homogenate was centrifuged at 1,000 × *g* for 2 min. The supernatant was collected and centrifuged at 15,000 × *g* for 12 min. The resulting pellet was resuspended in 8 ml of 0.32 M sucrose. Aliquots (2 ml) of this fraction were loaded onto discontinuous gradients consisting of three 2.5-ml layers of filtered (0.45 μm) Percoll density gradient medium (23%, 10%, and 3%) in 0.32 M sucrose plus 0.25 mM dithiothreitol and 1 mM ethylenediaminetetraacetic acid, *p*H 7.4. The gradients were centrifuged at 25,000 × *g* for 6.5 min. The synaptosome fraction was collected from the 23%/10% Percoll interface and diluted approximately fivefold in low Na^+ buffer for $^{22}Na^+$ influx studies, Na^+-free buffer for $[Na^+]_i$ and for neurotoxin binding studies, or high Na^+ buffer for glutamate release assays (buffer compositions given subsequently); all buffers were equilibrated with 95% O_2/5% CO_2. The synaptosomes were centrifuged at 23,000 × *g* for 10 min and resuspended in the appropriate buffer. Protein concentrations were determined by the method of Bradford[26] using bovine serum albumin as a standard.

Measurement of $^{22}Na^+$ Influx

$^{22}Na^+$ influx was measured by a modification of the method of Tamkun and Catterall.[21] Synaptosomes (600-700 μg protein in 150 μl low Na^+ buffer, consisting of 130 mM choline chloride, 5.4 mM KCl, 5 mM NaCl, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, and 50 mM HEPES-Tris, *p*H 7.4) were preincubated at 37°C for 5 min in the absence or presence of halothane (added as a diluted solution in dimethylsulfoxide). After preincubation, 60 μM veratridine with or without 80 μg/ml scorpion venom was added, and the samples were incubated for 10 min at 37°C. Uptake was initiated by the addition of 1.3 μCi of carrier-free $^{22}NaCl$ in 50 μl low Na^+ buffer and was terminated after 5 s by the addition of 3 ml of ice-cold washing buffer (163 mM choline chloride, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, and 5 mM HEPES-Tris, *p*H 7.4) and rapid vacuum filtration through GF/C glass fiber filters (Whatman, Kent, UK). Filters were washed twice with 3 ml washing buffer, and filter radioactivity was determined by liquid scintillation spectrometry using Bio-Safe NA scintillation cocktail (Research Products International Corp., Mount Prospect, IL). Nonspecific

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(Na^+ channel-independent) $^{22}Na^+$ uptake was determined in the presence of 1 μM tetrodotoxin, a specific Na^+ channel blocker.[22]

Measurement of Free Intrasynaptosomal [Na^+]

Na^+ concentration was determined by ion-specific spectrofluorometry using a spectrofluorometer (Perkin Elmer LS-50B; Beaconsfield, UK) with continuous computer-assisted data acquisition.[24] SBFI-AM was used as the fluorescent indicator. Synaptosomes (5 mg protein) were suspended in 1 ml of a Na^+-free buffer (120 mM choline chloride, 5 mM KCl, 0.8 mM $MgSO_4$, 5 mM D-glucose, and 50 mM HEPES-Tris, *p*H 7.4) containing 10 μM SBFI-AM and 0.01% (vol/vol) Pluronic F-127 (a nonionic detergent that facilitates indicator uptake) and incubated for 2 h at room temperature. At the end of the loading period, synaptosomes were centrifuged at 5,000 × *g*, resuspended in indicator-free buffer, and centrifuged again at 5,000 × *g* to remove excess indicator. The synaptosomes were suspended in Na^+-free buffer and incubated for an additional 30 min to allow indicator hydrolysis. After incubation, aliquots of synaptosomes (0.5 mg protein) were centrifuged, and the pellets were stored on ice until use. For free $[Na^+]_i$ determination, synaptosome pellets were resuspended in 1.5 ml of 120 mM Na^+ buffer (same as Na^+-free buffer, except NaCl was replaced choline chloride) and incubated in a stirred quartz cuvette at 37°C in the absence or presence of halothane (added as aliquots of saturated buffer solution) for 5 min, followed by the addition of 60 μM veratridine to activate Na^+ channels. Synaptosomal $[Na^+]_i$ was calculated by the fluorescence ratio method at an emission wavelength of 510 nm, with excitation wavelengths of 340 and 380 nm (switched every 2 s). The signal ratio was converted into free $[Na^+]_i$ based on the method of Grynkiewicz *et al.*[27] Calibration of the 340:380 nm excitation ratio in terms of free $[Na^+]_i$ was performed for each synaptosome preparation. For calibration, SBFI-loaded synaptosomes were added to solutions of known extracellular $[Na^+]$ made by appropriate mixtures of high-$[Na^+]$ and high-potassium ($[K^+]$) solutions in the presence of 40 μM monensin, 2 μM gramicidin, and 100 μM ouabain. The high-$[Na^+]$ solution contained 120 mM NaCl, 2 mM EGTA, and 10 mM HEPES-Tris, *p*H 7.4. The high-$[K^+]$ solution was identical except that K^+ replaced Na^+. In control experiments, no quenching of SBFI fluorescence by veratridine or halothane was observed in the presence of monensin, a Na^+ ionophore (data not shown).

Equilibrium Binding Assays

All reactions were performed at 37°C in Teflon-sealed glass vials to minimize loss of halothane.

[^{3}H]Batrachotoxinin-A 20-α-Benzoate Binding. [^{3}H]BTX-B binding was determined as described by Postma and Catterall[28] using a Na^+-free buffer (135 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, and 50 mM HEPES-Tris, *p*H 7.4) plus 10 nM [^{3}H]BTX-B, 1 μM tetrodotoxin, 80 μg/ml scorpion venom, and 1 mg/ml bovine serum albumin. Tetrodotoxin inhibits membrane depolarization due to Na^+ flux through Na^+ channels activated by BTX-B and scorpion venom.[29] Binding reactions were initiated by rapid mixing of synaptosomes (200 μg protein in 100 μl) with 150 μl of the reaction mixture just described in the absence or presence of halothane (added as a diluted solution in dimethylsulfoxide) and were terminated after 60 min at 37°C by the addition of 3 ml of ice-cold washing buffer. Synaptosomes were collected on GF/C glass fiber filters (Brandel, Gaithersburg, MD) by vacuum filtration and washed three times with 3 ml washing buffer. Bound [^{3}H]BTX-B was determined by liquid scintillation spectrometry. Nonspecific binding (10–20% of total binding) was determined in the presence of 0.3 mM veratridine, which binds at the same site as BTX-B.[29]

[^{3}H]Brevetoxin-3 Binding. [^{3}H]Brevetoxin-3 binding was determined as described by Edwards *et al.*,[30] with minor modifications. Synaptosomes (100 μg protein in 100 μl) were suspended in a Na^+-free buffer (135 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, 50 mM HEPES-Tris, *p*H 7.4) plus 25 nM [^{3}H]brevetoxin-3 and 0.01% (vol/vol) Pluronic F-127, a nonionic detergent required to solubilize the high concentrations of unlabeled brevetoxin-3 used to determine nonspecific binding,[31] in the absence or presence of halothane (added as a diluted solution in dimethylsulfoxide). After rapid mixing, synaptosomes were incubated at 4°C for 1 h, after which the reaction was stopped by the addition of 3 ml ice-cold washing buffer. The synaptosomes were collected on GF/C glass fiber filters under vacuum and washed twice with 3 ml washing buffer. Bound [^{3}H]brevetoxin-3 was determined by liquid scintillation spectrometry. Nonspecific binding (10–15% of total binding) was measured in the presence of 10 μM unlabeled brevetoxin-3.

[^{3}H]Saxitoxin Binding. [^{3}H]Saxitoxin binding was determined as described by Catterall *et al.*[32] Synaptosomes (100 μg protein in 100 μl) were added to a reaction mixture (100 μl) consisting of a Na^+-free buffer

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(135 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, and 50 mM HEPES-Tris, *p*H 7.4) plus 3 nM [^{3}H]saxitoxin, in the absence or presence of halothane (added as a diluted solution in dimethylsulfoxide). Samples were rapidly mixed and incubated at 37°C for 30 min. Binding reactions were stopped by the addition of 3 ml of ice-cold washing buffer, and synaptosomes were collected on GF/C glass fiber filters under vacuum and washed twice over 10–15 s. Bound [^{3}H]saxitoxin was determined by liquid scintillation spectrometry. Nonspecific binding (10–15% of total binding) was determined in the presence of 1 μM tetrodotoxin, which binds at the same site as saxitoxin.

Kinetic Binding Assays

The time course of [^{3}H]BTX-B dissociation from the Na^+ channel receptor complex was analyzed by preincubating synaptosomes for 60 min with 10 nM [^{3}H]BTX-B, 80 μg/ml scorpion venom, and 1 μM tetrodotoxin at 37°C, as in the equilibrium binding assays. Dissociation was initiated by adding 0.3 mM veratridine in the absence or presence of halothane (0.74 mM; minimum alveolar concentration [MAC]≈2; added as a diluted solution in dimethylsulfoxide). Reactions were terminated (after 5, 10, 20, or 30 min) by vacuum filtration and washing, followed by determination of bound [^{3}H]BTX-B. The dissociation rate constant (k_{-1}) was calculated using the equation[33] $\ln(B_t/B_o) = -k_{-1} \cdot t$, where B_t is the specific binding of [^{3}H]BTX-B at time t, and B_o is the specific binding of [^{3}H]BTX-B at time zero. A plot of $\ln(B_t/B_o)$ *versus* t, in the absence or presence of halothane, was linear with a slope of k_{-1}.

The rate of association of [^{3}H]BTX-B was measured by incubating synaptosomes with 80 μg/ml scorpion venom for 15 min at 37°C in the absence or presence of halothane (0.74 mM; added as a diluted solution in dimethylsulfoxide) as in the equilibrium binding assays. [^{3}H]BTX-B (10 nM) was then added to the synaptosomes to initiate binding. Parallel assays were performed in the presence of 0.3 mM veratridine to determine nonspecific binding at each time point. Incubations were terminated (after 5, 10, 20, or 30 min) by vacuum filtration and washing followed by determination of bound [^{3}H]BTX-B. The association rate constant (k_{+1}) of [^{3}H]BTX-B binding was calculated using the equation[33] $\ln(B_{eq}/B_{eq} - B_t) = ([L]k_{+1} + k_{-1})t$, where B_{eq} is the specific binding of [^{3}H]BTX-B at equilibrium, B_t is the specific binding of [^{3}H]BTX-B at time t, [L] is the concentration of [^{3}H]BTX-B, and k_{-1} is the dissociation rate constant for [^{3}H]BTX-B from the Na^+ channel receptor complex at the ambient drug concentration. A plot of $\ln(B_{eq}/B_{eq} - B_t)$ *versus* t was linear, with a slope of $[L]k_{+1} + k_{-1}$, from which k_{+1} was calculated. The inhibition constant, K_i, for halothane was calculated from the equation: $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of [^{3}H]BTX-B (10 nM), K_d is the equilibrium dissociation constant for [^{3}H]BTX-B (122 nM), and IC_{50} is the concentration of halothane which produced 50% inhibition of [^{3}H]BTX-B binding. The dissociation constant K_d also was calculated from kinetic data as k_{-1}/k_{+1}.

Measurement of Release of Glutamate from Synaptosomes

Endogenous release of glutamate was measured by the method of Nicholls *et al.*[34] Synaptosomal pellets (0.5 mg protein) were resuspended in 1.5 ml release buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 5 mM $NaHCO_3$, 10 mM D-glucose, and 20 mM HEPES, *p*H 7.4 with NaOH) plus 16 μM bovine serum albumin (essentially free of fatty acid), 1 mM $NADP^+$, 100 U L-glutamate dehydrogenase, and 1.3 mM $CaCl_2$. Stirred samples were equilibrated at 37°C for 4 min in a spectrofluorometer cuvette, and data acquisition was started with an excitation wavelength at 340 nm and an emission wavelength at 510 nm. After recording basal glutamate release, 50–350 μl buffer solution saturated with halothane was added, and the rate of release of glutamate (from 0–60 s) was measured. After recording release for 200 s after the addition of halothane, veratridine (60 μM) was added, and once again the rate of release of glutamate (from 0–60 s) was measured. The fluorescence signal was calibrated by adding 5 nmol L-glutamate to the cuvette at the end of each experiment.

Volatile Anesthetic Quantification

Volatile anesthetic agents were added as aliquots of saturated buffer solutions for measurement of $[Na^+]_i$ and release of glutamate and were diluted in dimethylsulfoxide for measurement of $^{22}Na^+$ influx and neurotoxin binding. Final anesthetic concentrations in each assay mixture were determined by gas chromatography.[35] A fixed amount of the assay mixture was withdrawn from the tube/cuvette with a gas-tight syringe and extracted into n-heptane. The n-heptane extract was injected onto a gas chromatograph (GC-8A; Shimadzu Corp., Kyoto, Japan) equipped with a thermal conductivity detector. Separation was achieved on a 1.8-m-long, 6-mm ID glass column packed with Porapak Q (Supelco, Bellefonte, PA). The column temperature

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was 210°C, the injector temperature was 230°C, and carrier gas flow was 40 ml/min.

Statistical Analysis

Statistical differences between control and experimental values were determined by analysis of variance with Fisher's *post hoc* test. Concentration-effect data were analyzed using a graded dose-response program that performs linear regression analysis on data between 20% and 80% of the maximal response (Pharm/PCS Pharmacologic Calculation System, Version 4.2; Springer Verlag, New York, NY). Confidence limits follow the derived IC_{50} values in the text. K_d values and maximum number of binding sites (B_{max}) for [^{3}H]BTX-B were calculated from Scatchard plots using Enzfit (Elsevier-Biosoft, Cambridge, UK). Kinetic (association and dissociation) parameters were estimated by linear regression using the Pharm/PCS Pharmacologic Calculation System. Curves were fit to data by simple polynomial (figs. 1, 2, 3, 6, and 8) or linear (figs. 4, 5A, 5B, and 9) functions using Origin software (Microcal Software, Inc., Northampton, MA). Each experiment contained two to three replicates for each data point and was performed n times, as specified. Values are expressed as mean ± SD.

Experiments were performed in accordance with the National Institutes of Health guide for the care and use of laboratory animals as approved by the Cornell University Medical College Institutional Animal Care and Use Committee.

Results

Na^+ Influx

Veratridine, an alkaloid neurotoxin that causes persistent activation of Na^+ channels by binding to site two,[22] was used to stimulate $^{22}Na^+$ influx into synaptosomes. Uptake was linear from 2-10 s (data not shown); a 5-s uptake period was used in subsequent experiments. Specific $^{22}Na^+$ uptake stimulated by 60 μM veratridine, which causes maximal stimulation of $^{22}Na^+$ influx into rat cortical synaptosomes,[24] was 73 ± 6 nmol · mg^{-1} · min^{-1} (n = 3). Veratridine-evoked $^{22}Na^+$ uptake was completely inhibited by 1 μM tetrodotoxin, indicating that it is Na^+ channel mediated. Some experiments included *Leirus quinquestriatus* scorpion venom, which contains α-scorpion toxins, small polypeptide neurotoxins that bind to site three to inhibit Na^+ channel inactivation and that interact cooperatively with toxin binding to site two.[29] Scorpion venom augmented veratridine-evoked uptake to 138 ± 9

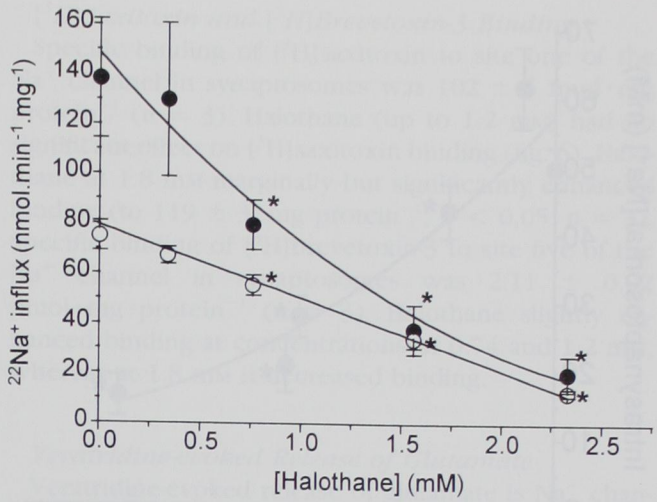

Fig. 1. Inhibition of veratridine-evoked $^{22}Na^+$ influx into synaptosomes by halothane. Na^+ channel-dependent Na^+ uptake was evoked with 60 μM veratridine in the absence (*open circles*) or presence (*filled circles*) of 80 μg/ml scorpion venom. Nonspecific uptake in the presence of 1 μM tetrodotoxin was subtracted to obtain specific uptake (points represent mean ± SD of three different experiments performed in duplicate). *$P < 0.05$ *versus* control (no halothane) by analysis of variance with Fisher's *post hoc* test.

nmol · mg^{-1} · min^{-1} (n = 3). Halothane inhibited veratridine-evoked $^{22}Na^+$ uptake in a concentration-dependent manner in the absence (IC_{50} = 1.1 mM; range, 0.8-1.4 mM) or presence (IC_{50} = 1.1 mM; range, 0.8-1.4 mM) of scorpion venom with comparable efficacy and potency (fig. 1). Basal uptake (in the absence of veratridine) was not affected by halothane (data not shown).

Intrasynaptosomal [Na^+]

Veratridine (60 μM) increased free $[Na^+]_i$ in synaptosomes fourfold, from 17 ± 2 to 67 ± 5 mM, in 120 mM Na^+-containing buffer (n = 4); this effect was completely blocked by 1 μM tetrodotoxin (data not shown). Halothane did not affect resting $[Na^+]_i$ but significantly inhibited veratridine-evoked increase in $[Na^+]_i$ in a concentration-dependent manner (IC_{50} = 0.97 mM; range, 0.6-1.4 mM; n = 3; fig. 2).

[^{3}H]Batrachotoxinin-A 20-α-Benzoate Binding

[^{3}H]BTX-B binding to synaptosomes was measured in the presence of scorpion venom, which enhanced specific [^{3}H]BTX-B binding nearly 16-fold, from 24 ± 3 to 380 ± 55 fmol/mg (n = 6), without affecting nonspecific binding. Scatchard analysis of [^{3}H]BTX-B binding in the absence of halothane revealed binding to a single

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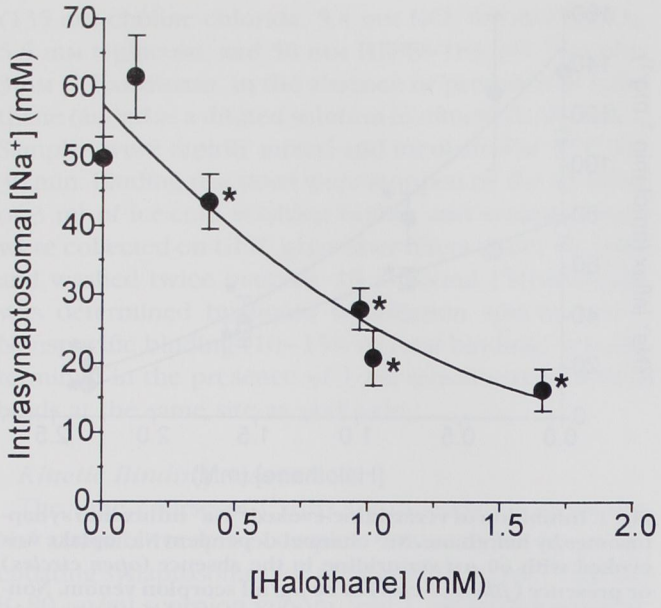

Fig. 2. Inhibition of the veratridine-evoked increase in free intrasynaptosomal $[Na^+]$ ($[Na^+]_i$) by halothane. Synaptosomes were loaded with SBFI-AM, and $[Na^+]_i$ was determined after stimulation with 60 μM veratridine using the fluorescence ratio method. Fluorescence data were converted to $[Na^+]_i$ by calibration with Na^+ standards. Data (mean ± SD of three different experiments performed in duplicate) are presented as the veratridine-evoked increase in $[Na^+]_i$ (the difference between resting and veratridine-evoked $[Na^+]_i$). *$P < 0.05$ *versus* control (no halothane) by analysis of variance with Fisher's *post hoc* test.

class of high affinity binding sites with a K_d value of 122 ± 8 nM and a B_{max} of 2.43 ± 0.18 pmol · mg protein^{-1} (n = 3; fig. 3), values which are similar to those reported previously.[23,29,36] Halothane inhibited specific [^{3}H]BTX-B binding in a concentration-dependent manner (IC_{50} = 0.53 mM; range, 0.37–0.71 mM; n = 3; fig. 4) without affecting nonspecific binding (data not shown); the calculated K_i value for halothane was 0.49 mM. Halothane (0.74 mM) increased the K_d value for [^{3}H]BTX-B to 478 ± 60 nM ($P < 0.05$) without significantly affecting B_{max} (3.14 ± 0.43 pmol · mg protein^{-1}; $P = 0.14$; n = 3). This effect is most consistent with a competitive mechanism for inhibition of [^{3}H]BTX-B binding. The Hill coefficient for inhibition of [^{3}H]BTX-B binding by halothane was 1.0 (data not shown), suggesting an interaction with a single class of binding sites.

Kinetics of [^{3}H]Batrachotoxinin-A 20-α-Benzoate Binding

The k_{+1} value for [^{3}H]BTX-B binding in the absence of halothane was 0.0032 ± 0.0002 min^{-1} (fig. 5A). Halothane (0.74 mM) decreased the rate of association of [^{3}H]BTX-B to 0.0023 ± 0.0002 min^{-1} ($P < 0.05$; n = 3).

The k_{-1} value for [^{3}H]BTX-B dissociation from the Na^+ channel receptor complex was 0.005 ± 0.001 min^{-1} (fig. 5B). Halothane (0.74 mM) significantly enhanced the dissociation rate to 0.010 ± 0.002 min^{-1} ($P < 0.05$; n = 3). The calculated K_d values ($K_d = k_{-1}/k_{+1}$) for [^{3}H]BTX-B were 1.54 ± 0.23 nM in the absence and 4.35 ± 0.5 nM in the presence of halothane ($P < 0.05$; n = 3). Lower K_d values calculated from the kinetic data compared with those obtained from equilibrium binding also were observed for [^{3}H]BTX-B binding in cardiac myocytes.[33] The halothane-induced increase in K_d calculated from equilibrium binding (3.9-fold) is in good agreement with the increase calculated from the kinetic data (2.8-fold).

An effect of halothane on rebinding of dissociated [^{3}H]-BTX-B is unlikely, because dissociation assays contained a saturating concentration of veratridine (0.3 mM), a direct competitive inhibitor of [^{3}H]-BTX-B binding.[29]

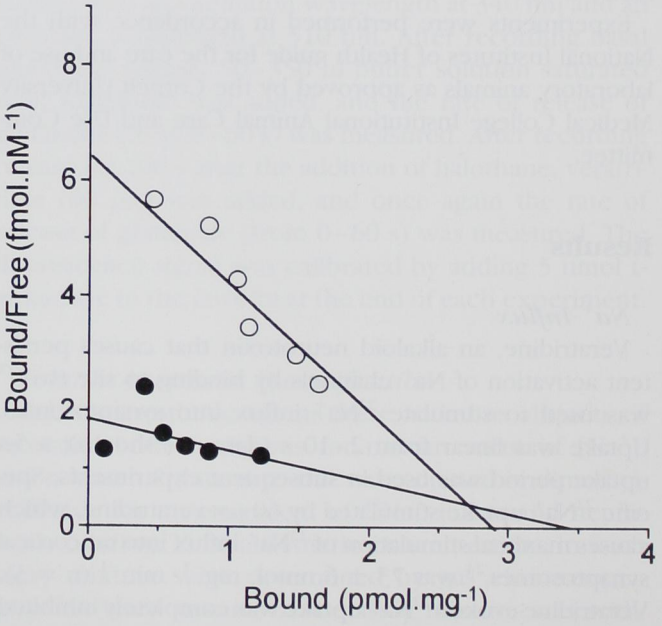

Fig. 3. Scratched analysis of the effect of halothane on [^{3}H]BTX-B binding. Synaptosomes were incubated with various concentrations of [^{3}H]BTX-B in the absence (*open circles*) or presence (*filled circles*) of 0.74 mM halothane. Data shown are from a representative experiment (n = 3) in which duplicate determinations were made. Values for B_{max} were 2.9 and 3.4 pmol · mg protein^{-1} in the absence and presence of halothane, respectively ($P = 0.25$). Values for K_d were 125 and 450 nM in the absence or presence of halothane, respectively ($P < 0.05$).

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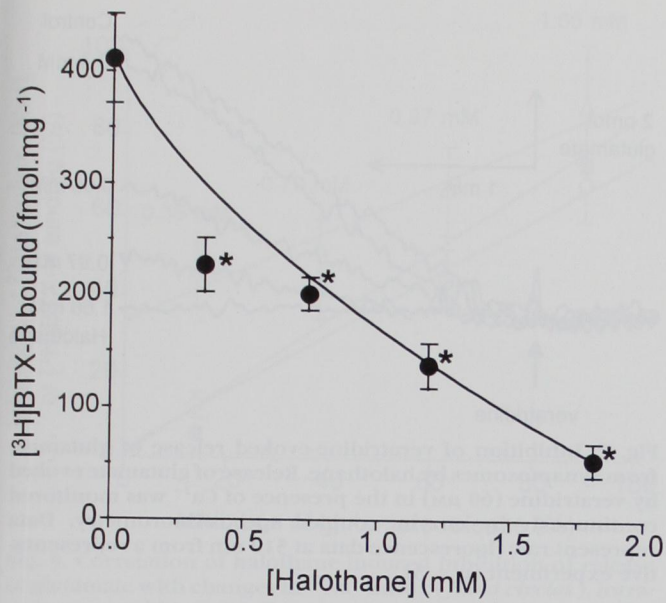

Fig. 4. Inhibition of [^{3}H]BTX-B binding to synaptosomes by halothane. Synaptosomes were incubated with 10 nM [^{3}H]BTX-B for 1 h at 37°C. Data represent mean ± SD (n = 3) with duplicate determinations. *$P < 0.05$ *versus* control (no halothane) by analysis of variance with Fisher's *post hoc* test.

[^{3}H]Saxitoxin and [^{3}H]Brevetoxin-3 Binding

Specific binding of [^{3}H]saxitoxin to site one of the Na^+ channel in synaptosomes was 102 ± 5 fmol·mg protein^{-1} (n = 3). Halothane (up to 1.2 mM) had no significant effect on [^{3}H]saxitoxin binding (fig. 6). Halothane at 1.8 mM marginally but significantly enhanced binding (to 119 ± 3·mg protein^{-1}; $P < 0.05$; n = 3). Specific binding of [^{3}H]brevetoxin-3 to site five of the Na^+ channel in synaptosomes was 2.11 ± 0.02 pmol·mg protein^{-1} (n = 3). Halothane slightly enhanced binding at concentrations of 0.74 and 1.2 mM, whereas at 1.8 mM it decreased binding.

Veratridine-evoked Release of Glutamate

Veratridine-evoked release of glutamate is Na^+ channel-dependent (tetrodotoxin-sensitive)[2] and therefore can be used to assess the functional significance of presynaptic Na^+ channel inhibition by halothane. Veratridine-evoked release of glutamate in the presence of 1.3 mM Ca^{2+} was completely blocked by 1 μM tetrodotoxin (data not shown). Halothane inhibited veratridine-evoked release of glutamate in a concentration-dependent manner (IC_{50} = 0.67 mM; range, 0.57–0.89 mM; n = 3; figs. 7 and 8). Correlations between the percentage

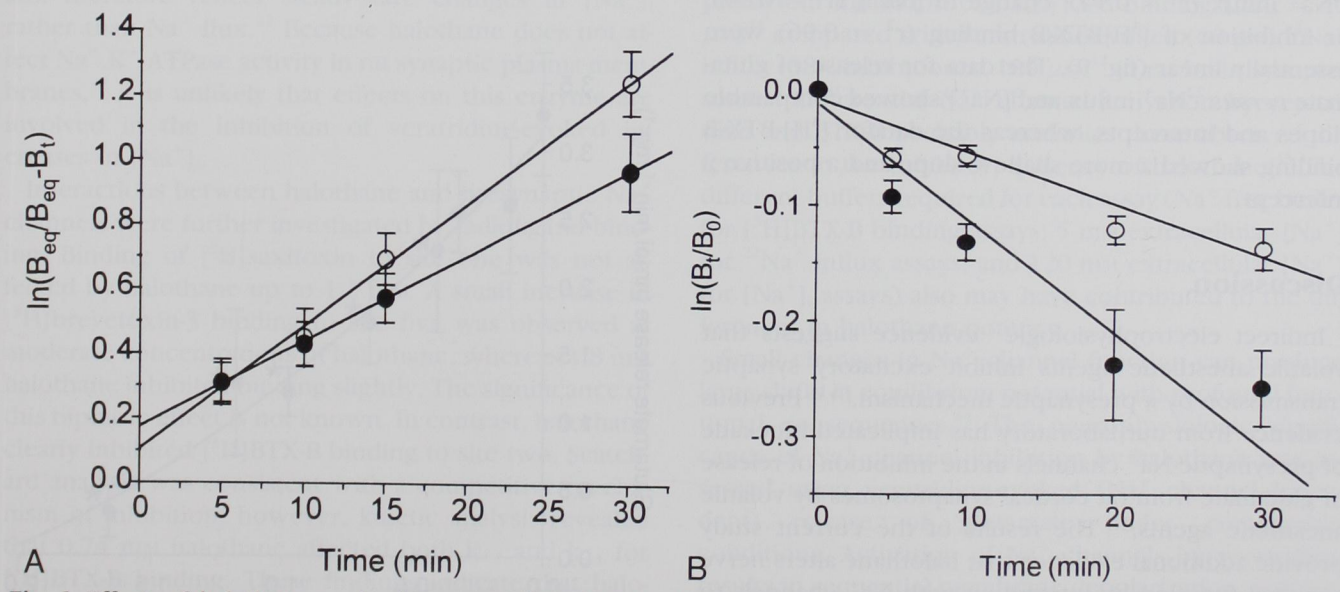

Fig. 5. Effects of halothane on the kinetics of [^{3}H]BTX-B binding. (*A*) Rate of association of [^{3}H]BTX-B. Synaptosomes were incubated with 10 nM [^{3}H]BTX-B in the absence (*open circles*) or presence (*filled circles*) of 0.74 mM halothane for the indicated times at 37°C, and specific [^{3}H]BTX-B binding was determined. Association rate constants (k_{+1}) were calculated from the slopes by linear regression. (*B*) Rate of dissociation of [^{3}H]BTX-B. Synaptosomes were preincubated with 10 nM [^{3}H]BTX-B for 60 min at 37°C. At time zero, dissociation was initiated by the addition of 0.3 mM veratridine in the absence (*open circles*) or presence (*filled circles*) of 0.74 mM halothane, and specific [^{3}H]BTX-B binding was determined. Dissociation rate constants (k_{-1}) were determined from the slopes by linear regression. Data represent mean ± SD (n = 3) with duplicate determinations.

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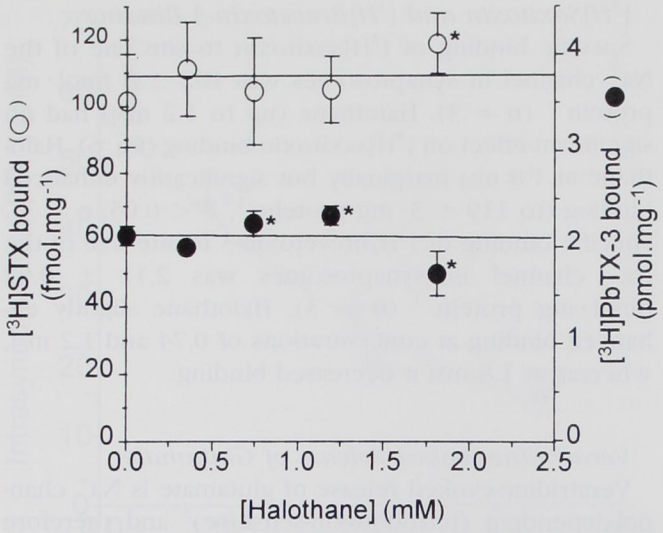

Fig. 6. Effects of halothane on [^{3}H]saxitoxin and [^{3}H]brevetoxin-3 binding to synaptosomes. Synaptosomes were incubated with either 3 nM [^{3}H]saxitoxin (*open circles*) or 25 nM [^{3}H]brevetoxin-3 (*filled circles*) for 30 min at 37°C (saxitoxin) or for 1 h at 4°C (brevetoxin-3). Data represent mean ± SD (n = 3) with duplicate determinations. *$P < 0.05$ *versus* control (no halothane) by analysis of variance with Fisher's *post hoc* test.

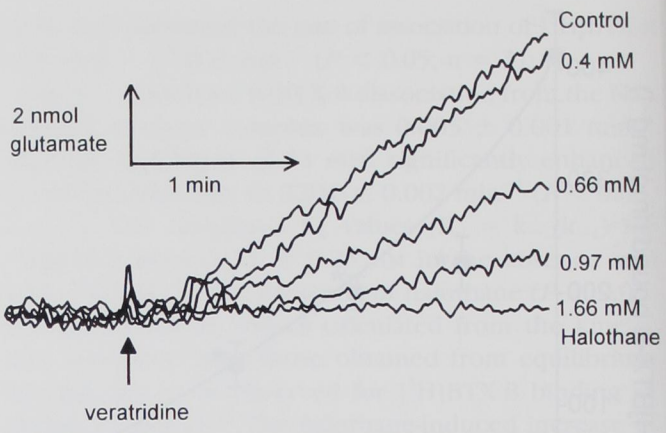

Fig. 7. Inhibition of veratridine-evoked release of glutamate from synaptosomes by halothane. Release of glutamate evoked by veratridine (60 μM) in the presence of Ca^{2+} was monitored continuously by enzyme-coupled spectrofluorometry. Data represent raw fluorescence data at 510 nm from a representative experiment (n = 3).

inhibition by halothane of release of glutamate *versus* $^{22}Na^+$ influx ($r^2 = 0.94$), change in $[Na^+]_i$ ($r^2 = 0.99$), or inhibition of [^{3}H]BTX-B binding ($r^2 = 0.96$) were essentially linear (fig. 9). The data for release of glutamate *versus* $^{22}Na^+$ influx and $[Na^+]$ showed comparable slopes and intercepts, whereas the datafor [^{3}H]-BTX-B binding showed a more shallow slope and a positive y intercept.

Discussion

Indirect electrophysiologic evidence suggests that volatile anesthetic agents inhibit excitatory synaptic transmission by a presynaptic mechanism.[13,14] Previous evidence from our laboratory has implicated blockade of presynaptic Na^+ channels in the inhibition of release of glutamate from rat cortical synaptosomes by volatile anesthetic agents.[12] The results of the current study provide additional evidence that halothane alters nerve terminal function by interacting with Na^+ channels to inhibit release of glutamate.

Halothane inhibited veratridine-evoked $^{22}Na^+$ influx into synaptosomes. Because $^{22}Na^+$ influx is directly proportional to ion channel Na^+ permeability and to the number of open channels,[22] reductions in Na^+ influx reflect reduced presynaptic Na^+ channel opening or permeability. Halothane did not affect $^{22}Na^+$ influx into synaptosomes in the presence of tetrodotoxin, which indicates a lack of effects on other modes of Na^+ entry.

Inhibition by halothane of $^{22}Na^+$ influx through presynaptic Na^+ channels was confirmed by its inhibition

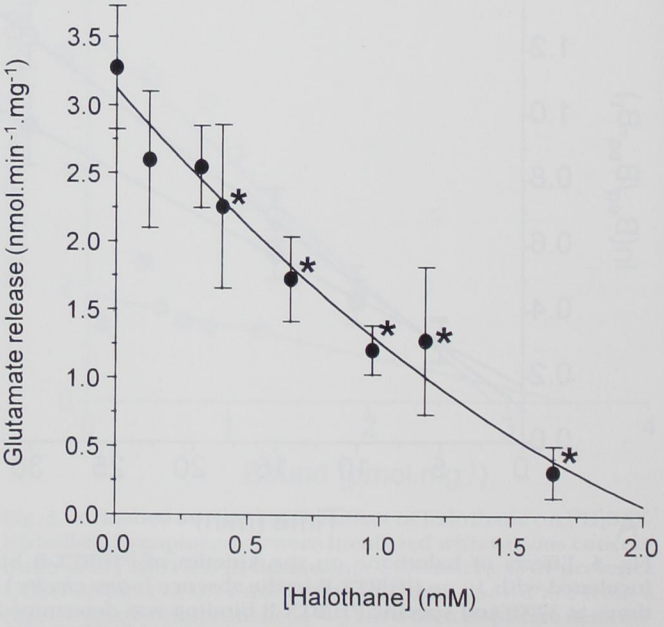

Fig. 8. Concentration–effect curve for halothane on veratridine-evoked release of glutamate from synaptosomes. Data are mean ± SD (n = 3). *$P < 0.05$ *versus* control (no halothane) by analysis of variance with Fisher's *post hoc* test.

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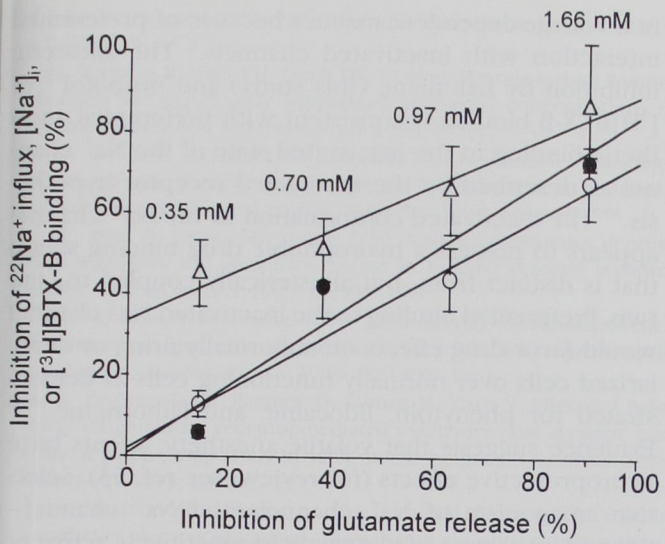

Fig. 9. Correlation of halothane-induced inhibition of release of glutamate with changes in $^{22}Na^+$ influx (*filled circles*), intrasynaptosomal $[Na^+]$ ($[Na^+]_i$; *open circles*), and [^{3}H]BTX-B binding (*open triangles*). Points represent mean ± SD (n = 3) observed in the presence of 0.35, 0.70, 0.97, or 1.66 mM halothane.

of veratridine-evoked changes in $[Na^+]_i$ measured using a Na^+-sensitive fluorescent probe. These changes were monitored for longer periods than in the flux studies and therefore reflect steady-state changes in $[Na^+]_i$ rather than Na^+ flux.[22] Because halothane does not affect Na^+,K^+-ATPase activity in rat synaptic plasma membranes,[37] it is unlikely that effects on this enzyme are involved in the inhibition of veratridine-evoked increases in $[Na^+]_i$.

Interactions between halothane and presynaptic Na^+ channels were further investigated by radioligand binding. Binding of [^{3}H]saxitoxin to site one was not affected by halothane up to 1.2 mM. A small increase in [^{3}H]brevetoxin-3 binding to site five was observed at moderate concentrations of halothane, whereas 1.8 mM halothane inhibited binding slightly. The significance of this biphasic effect is not known. In contrast, halothane clearly inhibited [^{3}H]BTX-B binding to site two. Scatchard analysis was consistent with a competitive mechanism of inhibition; however, kinetic analysis revealed that 0.74 mM halothane affected both k_{+1} and k_{-1} for [^{3}H]BTX-B binding. These findings indicate that halothane inhibits [^{3}H]BTX-B binding by an allosteric mechanism, primarily by an increase in the dissociation rate. The effect of halothane on [^{3}H]BTX-B binding does not appear to be due to an indirect effect on scorpion toxin binding to site three (used to enhance [^{3}H]BTX-B binding), because halothane inhibited $^{22}Na^+$ influx with similar potency in the absence or presence of scorpion venom.

Allosteric competitive inhibition of [^{3}H]BTX-B binding also has been observed for other clinically useful drugs, including local anesthetic agents, class I anticonvulsants, class I antiarrhythmic agents,[38] and propofol.[23] Most of these compounds have no significant effects on neurotoxin binding to other receptor sites on the Na^+ channel. Taken together, these studies suggest a common general mechanism for Na^+ channel inhibition by several classes of drugs with distinct chemical structures mediated by a common conformational effect on the channel.

The MAC of halothane for surgical anesthesia is 0.76 vol% in humans and 1.24 vol% in rats.[39] Corresponding aqueous halothane concentrations at 37°C were calculated as 0.21 and 0.35 mM for humans and rats, respectively.[40] Halothane significantly inhibited veratridine-evoked increases in $^{22}Na^+$ influx, $[Na^+]_i$, and [^{3}H]BTX-B binding in rat cortical synaptosomes at clinical concentrations. The potency for inhibition of [^{3}H]BTX-B binding (IC_{50} = 0.53 mM; 1.5 MAC) was greater than for inhibition of veratridine-evoked changes in $[Na^+]_i$ (IC_{50} = 0.97 mM; 2.8 MAC) and $^{22}Na^+$ influx (IC_{50} = 1.1 mM; 3.1 MAC). A similar difference was reported for phenytoin inhibition of [^{3}H]BTX-B binding (IC_{50} = 40 μM)[41] compared with veratridine-evoked (60 μM) $^{24}Na^+$ influx (38% inhibition at 100 μM)[42] in rat brain synaptosomes. Differential Na^+ channel activation by veratridine and BTX-B probably underlies these observed differences in the inhibitory potency of halothane.[43] The different buffers required for each assay (Na^+-free buffer for [^{3}H]BTX-B binding assays; 5 mM extracellular $[Na^+]$ for $^{22}Na^+$ influx assays, and 120 mM extracellular $[Na^+]$ for $[Na^+]_i$ assays) also may have contributed to the differences in halothane potency.

Small changes in Na^+ channel function can produce large shifts in equilibrium potential with profound functional consequences.[44] The neurophysiologic significance of Na^+ channel inhibition by halothane was assessed using veratridine-evoked (Na^+ channel-dependent) release of glutamate under comparable conditions. Activation of Na^+ channels by veratridine results in sequential membrane depolarization, voltage-dependent opening of Ca^{2+} channels, and Ca^{2+}-dependent exocytotic release of neurotransmitters.[2] The rise in intracellular $[Na^+]$ also results in reversal of Na^+/Ca^{2+} antiport and reversal of Na^+/glutamate cotransport. Although veratridine evokes both Ca^{2+}-independent (car-

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rier-mediated) and -dependent (exocytotic) glutamate release from synaptosomes, the major component of release is Ca^{2+}-dependent,[24] similar to physiologic release; however, the pathway for Ca^{2+} entry is not clear and may differ from action potential-evoked Ca^{2+}.

Inhibition of veratridine-evoked release of glutamate by halothane (IC_{50} = 0.67 mM; 1.9 MAC) *via* Na^+ channel blockade could involve one or more of the following mechanisms: (1) inhibition of Na^+ channel-dependent membrane depolarization and consequently of Ca^{2+} channel activation; (2) inhibition of Ca^{2+} entry through veratridine-modified Na^+ channels[45]; (3) inhibition of Na^+/glutamate transporter reversal or stimulation of glutamate reuptake; or (4) inhibition of Na^+/Ca^{2+} exchange. Blockade of presynaptic Ca^{2+} channels coupled to release of glutamate or interference with subsequent release mechanisms also could contribute to the effect of halothane.[11] Na^+ channel-independent mechanisms appear quantitatively less important than an effect at Na^+ channels because release of glutamate evoked by KCl, a secretogogue not dependent on Na^+ channel function, was insensitive to halothane under our assay conditions.[12] The sensitivity of veratridine-evoked release of glutamate to halothane was comparable to that of release of glutamate evoked by 4-aminopyridine (IC_{50} = 0.5 mM[12]), which also evokes Na^+ channel-dependent (tetrodotoxin-sensitive) release. Because halothane had no effect on basal or spontaneous release of glutamate, which is due primarily to reversed Na^+/glutamate uptake,[46] a direct effect on the Na^+/glutamate transporter is unlikely. Stimulation of glutamate reuptake is also an unlikely mechanism because halothane (3–4 vol%) did not affect [^{3}H]glutamate uptake into rat cortical synaptosomes significantly.[47,48]

Halothane and other volatile anesthetic agents also affect other targets, including ligand-gated ion channels, which have been proposed as principal sites for anesthetic action.[49] For example, halothane potentiated γaminobutyric acid type A receptor-mediated Cl^- current in rat hippocampal neurons (at 1.0–1.5 MAC)[50]; increased $^{36}Cl^-$ uptake through γaminobutyric acid–gated Cl^- channels (50% effective concentration [EC_{50}] = 2.2 mM)[51]; and inhibited *t*-[^{35}S]butylbicyclophosphorothionate binding to cortical membranes (IC_{50} = 1.68 mM).[51] These actions of halothane occur at concentrations comparable to or higher than those reported here for inhibition of Na^+ channels. Thus, Na^+ channel inhibition must also be considered as a potential target for the effects of halothane on the CNS.

Volatile anesthetic agents suppress CNS Na^+ channels in a voltage-dependent manner because of preferential interaction with inactivated channels.[9] The allosteric inhibition by halothane (this study) and propofol[23] of [^{3}H]BTX-B binding is consistent with preferential anesthetic binding to the inactivated state of the Na^+ channel as described by the modulated receptor hypothesis.[38] The inactivated conformation of the Na^+ channel appears to possess a hydrophobic drug binding site(s) that is distinct from, but allosterically coupled to, site two. Preferential binding to the inactivated Na^+ channel would favor drug effects on abnormally firing or depolarized cells over normally functioning cells as demonstrated for phenytoin, lidocaine, and lamotrigine.[38,52] Evidence suggests that volatile anesthetic agents have neuroprotective effects (for review, see ref. 53). Selective antagonism of Na^+ channels and Na^+ channel–dependent release of glutamate in repetitively active or ischemically depolarized neurons underlies the neuroprotective mechanism of several drugs[54] and also may contribute to the neuroprotective properties of some general anesthetic agents.[55]

Halothane interacts with presynaptic Na^+ channels to inhibit veratridine-evoked Na^+ influx, $[Na^+]_i$ changes, and release of glutamate, actions which may contribute to its anesthetic and neuroprotective properties. Taken together with our previous studies of volatile anesthetic agents[12] and propofol,[23,24] these findings emphasize that presynaptic Na^+ channels may be important targets for general anesthetic inhibition of excitatory neurotransmission in the CNS.

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