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Inhibition by Volatile Anesthetics of Endogenous Glutamate Release from Synaptosomes by a Presynaptic Mechanism

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Background: **Synaptic transmission is more sensitive than axonal conduction to the effects of general anesthetics. Previous studies of the synaptic effects of general anesthetics have focused on postsynaptic sites of action. We now provide direct biochemical evidence for a presynaptic effect of volatile anesthetics on neurotransmitter release.**

Methods: **Rat cerebrocortical synaptosomes (isolated presynaptic nerve terminals) were used to determine the effects of general anesthetics on the release of endogenous L-glutamate, the major fast excitatory neurotransmitter. Basal and evoked (by 4-aminopyridine, veratridine, increased KCl, or ionomycin) glutamate release were measured by continuous enzyme-coupled fluorometry.**

Results: **Clinical concentrations of volatile halogenated anesthetics, but not of pentobarbital, inhibited 4-aminopyridine–evoked Ca^{2+}-dependent glutamate release. Halothane also inhibited veratridine-evoked glutamate release but not basal, KCl-evoked, or ionomycin-evoked glutamate release. Halothane inhibited both the 4-aminopyridine–evoked and the KCl-evoked increase in free intrasynaptosomal [Ca^{2+}].**

Conclusions: **Inhibition of glutamate release from presynaptic nerve terminals is a potential mechanism of volatile anesthetic action. Comparison of the sensitivity of glutamate release evoked by secretogogues that act at various steps in the neurotransmitter release process suggests that halothane does not affect Ca^{2+}–secretion coupling or vesicle exocytosis but inhibits glutamate release at a step proximal to Ca^{2+} influx, perhaps by blocking presynaptic Na^+ channels. Synaptosomal glutamate release evoked by 4-aminopyridine should provide a useful system for further characterization of the presynaptic effects of anesthetics. (Key words: Anesthesia: theories. Anesthetics, intravenous: pentobarbital. Anesthetics, volatile: enflurane; halothane; isoflurane. Ions: calcium. Ions, channels: calcium; sodium. Neurotransmitters: exocytosis; glutamate; release.)**

THE mechanisms by which general anesthetics act have not been firmly established. Electrophysiologic studies indicate that synaptic transmission is more sensitive to the effects of general anesthetics than is axonal conduction.[1] Most studies of the synaptic effects of general anesthetics have focused on postsynaptic sites of action, which are more accessible to analysis. Considerable evidence indicates that all known general anesthetics affect postsynaptic responses to neurotransmitters through interactions with ligand-gated ion channels.[2] The presynaptic effects of general anesthetics are not well characterized, however.

The synaptosome preparation is a subcellular fraction containing pinched-off nerve terminals that retain the ability to take up, store, and release various neurotransmitters.[3] Synaptosomes are a useful model for the analysis of presynaptic drug effects because, in contrast to brain slices or neurons *in situ*, they are free of functional glial and neuronal cell body elements and therefore lack the potential for intercellular interactions. We used synaptosomes isolated from rat cerebral cortex[4] to study the effects of general anesthetics on the release of L-glutamate by continuous enzyme-linked fluorometry.[5]

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. It is stored in and released from small synaptic vesicles present in cerebrocortical synaptosomes. In the absence of action potentials, glutamate release from synaptosomes can be evoked by a variety of pharmacologic agents including 4-aminopyridine (4-AP), veratridine, KCl, or ionomycin.[3] 4-AP destabilizes the resting plasma membrane potential by blocking the outward K_A^+ current, a change that leads to sequential activation of voltage-

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dependent Na^+ channels, plasma membrane depolarization, Ca^{2+} entry, and neurotransmitter release. Glutamate release evoked by 4-AP is thought to mimic physiologic presynaptic release mechanisms because 4-AP causes repetitive spontaneous action potentials that result in tetrodotoxin-sensitive release of glutamate.[6] Phorbol esters potentiate 4-AP–evoked glutamate release, possibly by protein kinase C–mediated phosphorylation and inhibition of the delayed rectifier K^+ channel.[7] Veratridine activates voltage-dependent Na^+ channels directly by increasing their open probability at resting potential, and thereby leads to plasma membrane depolarization Ca^{2+} entry (through voltage-dependent Ca^{2+} channels, veratridine-bound Na^+ channels or by reversal of the Na^+/Ca^{2+} exchanger) and neurotransmitter release. Increase in external KCl depolarizes the plasma membrane directly by shifting the K^+ equilibrium potential above the threshold potential for voltage-dependent Ca^{2+} channel activation, a change that leads to Ca^{2+} entry and neurotransmitter release, while Na^+ channels are inactivated. Ionomycin acts as a Ca^{2+} ionophore by inserting into the plasma membrane and allows direct Ca^{2+} entry and neurotransmitter release independent of endogenous ion channel–mediated mechanisms.

We have taken advantage of these pharmacologic probes to analyze the presynaptic effects of volatile anesthetics on endogenous glutamate release from rat brain synaptosomes.

Materials and Methods

Preparation of Rat Brain Synaptosomes

After institutional approval, synaptosomes were prepared by a modification[8] of the method of Dunkley *et al.*[4] Adult male (150 g) Sprague-Dawley rats were obtunded with CO_2 and killed by decapitation. Brains were removed and immediately chilled in ice-cold sucrose solution (0.32 M sucrose, 1 mM ethylenediamine tetraacetic acid, *p*H 7.5). Cerebral cortices from two brains were removed and homogenized in 20 ml sucrose solution using a motor-driven polytetrafluoroethylene–glass homogenizer (Potter-Elvehjem) at 900 rpm for ten up-and-down strokes. The homogenate was centrifuged at 1,000*g* for 10 min, and the pellet was rehomogenized in 20 ml sucrose solution and centrifuged at 1,000*g* for 10 min. The supernatant fractions from the two centrifugations were combined and centrifuged at 15,000*g* for 25 min. The resulting pellet was washed and resuspended in 8 ml sucrose solution (P_2 fraction). Aliquots (2 ml) of the P_2 fraction were loaded onto discontinuous gradients consisting of three 2.5-ml layers of filtered Percoll density gradient medium (23%, 10%, and 3%) in sucrose solution plus 0.25 mM dithiothreitol. The gradients were centrifuged at 25,000*g* for 6 min. The synaptosomal fraction was collected from the 23/10% Percoll interface and diluted about fivefold in buffered saline–glucose (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, *p*H 7.4), which was equilibrated with 95% O_2/5% CO_2. The synaptosomes were centrifuged at 23,000*g* for 10 min and resuspended in 10 ml buffered saline–glucose. The protein concentration of the synaptosomal preparation was determined by the method of Bradford[9] using bovine serum albumin as a standard. The preparation was divided into aliquots consisting of 1 mg protein and centrifuged at 10,000*g* for 7 min. The synaptosomes were stored as pellets on ice for as long as 4 h before use.

Measurement of Glutamate Release from Synaptosomes

Endogenous glutamate release was measured by a modification of the continuous fluorometric assay described by Nicholls *et al.*[5] In this system, released glutamate is oxidized by L-glutamate dehydrogenase, which is coupled to the reduction of the oxidized form of nicotinamide adenine dinucleotide phosphate ($NADP^+$) to NADPH. NADPH gives rise to a strong fluorescence signal at 510 nm when excited at 340 nm. This method allows the detection of glutamate at submicromolar concentrations. An inherent delay is introduced by the time required for the reaction to occur, so that the traces do not reflect the actual glutamate concentration.[10,11] Corrections for this delay were not incorporated into the assay and consequently the initial rates of glutamate release associated with depolarization may not be accurately reflected.

The synaptosome pellets (corresponding to 1 mg protein) were resuspended in 3 ml incubation medium (122 mM NaCl, 3.1 mM KCl, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 20 mM N-tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid, 10 mM D-glucose, 16 μM bovine serum albumin (essentially free of fatty acid), 1 mM $NADP^+$, 100 U L-glutamate dehydrogenase, 1.3 mM $CaCl_2$, *p*H 7.4 with NaOH). The sample was placed in a cuvette in a fluorescence spectrofluorometer (SLM Aminco 8000, SLM Instruments, Inc., Urbana, IL)

equipped with a magnetic stirrer and a temperature-regulated (37°C) cuvette holder. The stirred samples were equilibrated at 37°C for 4 min, and then data acquisition was started. The excitation wavelength was 340 nm, and the monitored emission wavelength was 510 nm.

After recording basal glutamate release (which was Ca^{2+}-independent and is the result of nonvesicular leakage[3]), a secretogogue was added. The kinetics of glutamate release evoked by 4-AP or KCl are biphasic, with a rapid phase complete within 2 s and a more extensive slow phase.[10] The rate of glutamate release from 0–60 s, which was essentially linear with respect to time, was measured and corrected for the stable basal release rate measured before secretogogue addition. The fluorescence signal was calibrated by the addition of 10 nmol L-glutamate to a reaction mixture without synaptosomes or to the reaction mixture at the end of an experiment once release had reached a stable rate. The latter method required considerably more time to perform and therefore was not used routinely, to minimize the time from preparation of synaptosomes to completion of the release assay. The addition to the assay of 0.1% (volume in volume) dimethyl sulfoxide, which was used as a drug vehicle, had no effect on basal glutamate release or on glutamate release evoked by 4-AP, veratridine, KCl, or ionomycin (data not shown).

Measurement of Intrasynaptosomal [Ca^{2+}]

Synaptosomes (corresponding to 10 mg protein) were resuspended in 5 ml buffered saline–glucose containing 5 μM fura-2 acetoxymethyl ester and incubated at 37°C for 40 min. The mixture was divided into 1-ml aliquots, which were centrifuged at 10,000*g* for 7 min. The pellets were washed with dye-free saline–glucose and were stored on ice until use. For free intrasynaptosomal [Ca^{2+}] measurements, synaptosomal pellets were resuspended in 3 ml incubation medium and incubated in a stirred quartz cuvette at 37°C in the absence or presence of halothane for 5 min followed by the addition of 1 mM 4-AP. Synaptosomal [Ca^{2+}] was calculated from the ratio of the fluorescence intensity of fura-2 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 intrasynaptosomal [Ca^{2+}] using software (SLM Instruments, Inc.) based on the method of Grynkiewicz *et al.*[12] The maximal ratio (saturating [Ca^{2+}]) was obtained by lysing the synaptosomes with 6.2 mM Triton X-100, and the minimal ratio (0 [Ca^{2+}]) was obtained by complexing free Ca^{2+} with 7.7 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid.

Volatile Anesthetic Addition and Quantification

Volatile anesthetics were added to cuvettes by adding aliquots of saturated water solutions. Immediately after the addition of anesthetic, cuvettes were sealed with a polytetrafluorethylene stopper and wrapped with Parafilm. Subsequent additions were made through a hole in the stopper that just accommodated the needle of a 10-μl Hamilton syringe. Actual anesthetic concentrations in the cuvette were determined by gas chromatography.[13] After equilibration, a 0.5-ml aliquot of the synaptosome solution was withdrawn from the cuvette with a gas-tight syringe and extracted with 1 ml n-heptane. The n-heptane extract (7 μl) was injected into a gas chromatograph (GC-8A, Shimadzu Corp., Kyoto, Japan) equipped with a thermal conductivity detector. Separation was achieved on a 1.8 m/6 mm ID glass column packed with Porapak Q (Supelco, Bellefonte, PA). The column temperature was 210°C, the injector temperature was 230°C and carrier gas (He) flow was 40 $ml \cdot min^{-1}$.

Materials

$NADP^+$, NADPH, L-glutamate, L-glutamate dehydrogenase (*Proteus* sp), bovine serum albumin (essentially fatty acid free), Na (±)-pentobarbital, 4-AP, ionomycin, tetrodotoxin, veratridine, fura-2 acetoxymethyl ester, and dimethyl sulfoxide were obtained from Sigma (St. Louis, MO). Percoll density gradient medium (15–30-nm diameter silica particles coated with nondialyzable polyvinylpyrrolidone) was obtained from Pharmacia (Uppsala, Sweden) and was filtered through a 0.45-μm filter before use. β-Phorbol 12,13-dibutyrate (PDBu) was from LC Laboratories (Woburn, MA). Enflurane and isoflurane were obtained from Anaquest (Madison, WI) and halothane (thymol-free) from Halocarbon Products (North Augusta, SC). Ro 31-8220 was kindly provided by Roche Products (Hertfordshire, UK).

Data Analysis

Statistical differences between mean control and experimental values were determined by Student's unpaired *t* test (for single test agents) or by analysis of variance with the Newman-Keuls multiple-range test (for multiple test agents) (PHARM/PCS Pharmacologic

Calculation System, version 4.2, Springer-Verlag, New York, NY).[14] Concentration–effect data were analyzed using a graded dose–response program that carries out linear regression analysis on data between 20% and 80% of the maximal response (PHARM/PCS Pharmacologic Calculation System, version 4.2).[14]

Results

Effect of Anesthetics on Glutamate Release Evoked by 4-Aminopyridine

Glutamate release was monitored by continuous fluorometry in the presence of added $NADP^+$ and L-glutamate dehydrogenase.[5] The following control experiments demonstrated the suitability of this system for studying the effects of anesthetics on neurotransmitter release (data not shown). (1) The fluorescence yield of the assay was linearly proportional to the amount of glutamate or the amount of NADPH added to the incubation mixture. (2) The release of glutamate from synaptosomes stimulated by KCl-induced depolarization was proportional to the amount of synaptosomal protein present in the assay to 2 mg. (3) Halothane (1 mM) did not affect the activity of the coupling enzyme

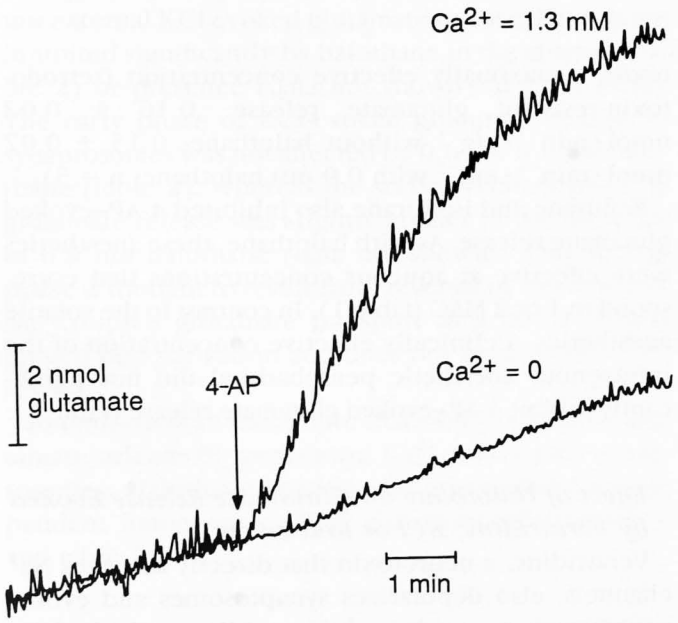

Fig. 1. 4-Aminopyridine (4-AP)–evoked release of glutamate from rat brain synaptosomes in the absence or presence of added external Ca^{2+}. Synaptosomes were preincubated in the presence of 1 μM β-phorbol 12,13-dibutyrate in the absence or presence of 1.3 mM $CaCl_2$. Glutamate release evoked by the addition of 1 mM 4-AP was monitored spectrofluorometrically.

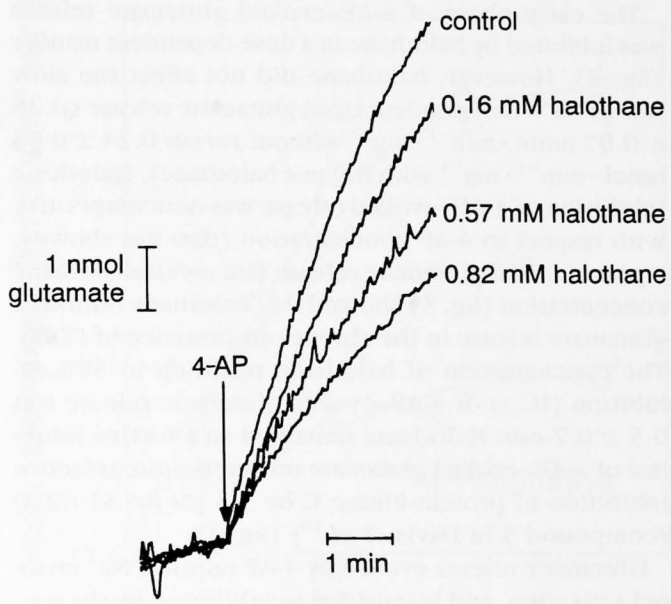

Fig. 2. Effect of halothane on the rate of glutamate release evoked by 4-aminopyridine (4-AP). Synaptosomes were preincubated in the presence of 1 μM β-phorbol 12,13-dibutyrate and various concentrations of halothane. Glutamate release evoked by the addition of 1 mM 4-AP was monitored spectrofluorometrically.

L-glutamate dehydrogenase and did not interfere with the fluorescence yield of NADPH in our assay conditions; a previous investigation demonstrating inhibition of L-glutamate dehydrogenase by halothane[15] used suprapharmacologic concentrations (20 vol% or ≈6 mM).

Synaptosomal glutamate release can be evoked by 4-AP, a K^+ channel blocker that destabilizes membrane potential and causes repetitive spontaneous Na^+ channel-dependent depolarizations that closely mimic electric stimulation.[3,6] The addition of 4-AP to synaptosomes resulted in a marked stimulation of glutamate release that was Ca^{2+}-dependent (fig. 1). The ability of 4-AP to evoke glutamate release was potentiated by the phorbol ester PDBu (1.37 ± 0.11 nmol · min^{-1} · mg^{-1} without *versus* 2.43 ± 0.22 nmol · min^{-1} · mg^{-1} with 1 μM PDBu; $P < 0.01$; n = 3). The rate of formation of NADPH, which is proportional to the amount of glutamate released, is essentially linear over the first 60-s period in these assay conditions. The rate of Ca^{2+}-dependent glutamate release during this interval (early phase) can be quantified and used to screen for pharmacologic effects on this phase of release. After about 2 min, the rate of glutamate release stabilized at a rate somewhat greater than the basal rate; this release is Ca^{2+} independent and is probably attributable to reversal of the Na^+/glutamate transporter.[3]

The early phase of 4-AP–evoked glutamate release was inhibited by halothane in a dose-dependent manner (fig. 2). However, halothane did not affect the slow rate of Ca^{2+}-independent basal glutamate release (0.28 ± 0.07 nmol · min^{-1} · mg^{-1} without *versus* 0.24 ± 0.03 nmol · min^{-1} · mg^{-1} with 0.9 mM halothane). Halothane inhibition of 4-AP–evoked release was noncompetitive with respect to 4-AP concentration (data not shown). A plot of initial glutamate release rate *versus* halothane concentration (fig. 3) showed that halothane inhibited glutamate release in the absence or presence of PDBu. The concentration of halothane resulting in 50% inhibition (IC_{50}) of 4-AP–evoked glutamate release was 0.5 ± 0.2 mM. Halothane remained an effective inhibitor of 4-AP–evoked glutamate release despite selective inhibition of protein kinase C by 3.3 μM Ro 31-8220 (compound 3 in Davis *et al.*[16]) (fig. 4).

Glutamate release evoked by 4-AP requires Na^+ channel activation, and is sensitive to inhibition by the specific Na^+ channel blocker tetrodotoxin.[6] Halothane caused no further inhibition of the residual 4-AP-evoked glutamate release in the presence of 0.3 μM tetrodotoxin, a maximally effective concentration (tetrodotoxin-resistant glutamate release: 0.16 ± 0.02 nmol · min^{-1} · mg^{-1} without halothane; 0.15 ± 0.02 nmol · min^{-1} · mg^{-1} with 0.9 mM halothane; n = 3).

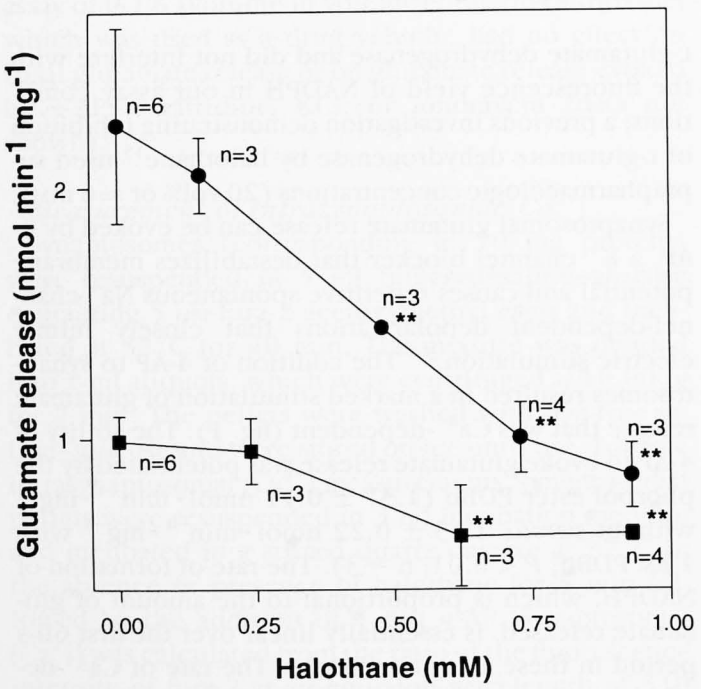

Fig. 3. Dose–response curve for halothane-induced inhibition of glutamate release in the absence or presence of phorbol ester. The effect of various concentrations of halothane on 4-aminopyridine–evoked glutamate release (mean ± SD [SD shown where larger than symbol]; n as indicated) was measured in the absence (squares) or presence (circles) of 1 μM β-phorbol 12,13-dibutyrate. **$P < 0.01$ *versus* control (no halothane) by Student's unpaired two-tailed *t* test.

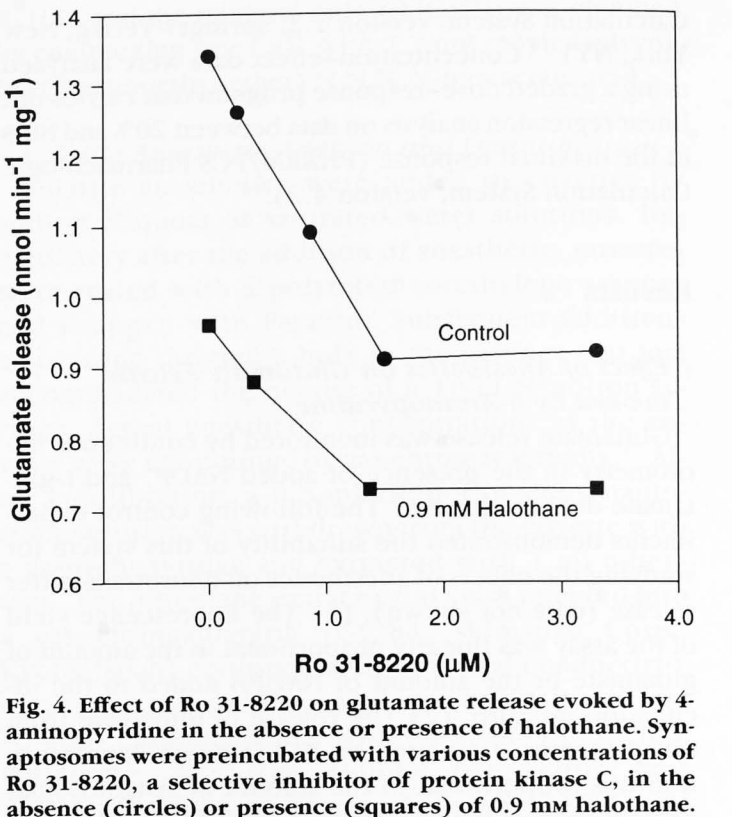

Fig. 4. Effect of Ro 31-8220 on glutamate release evoked by 4-aminopyridine in the absence or presence of halothane. Synaptosomes were preincubated with various concentrations of Ro 31-8220, a selective inhibitor of protein kinase C, in the absence (circles) or presence (squares) of 0.9 mM halothane. Glutamate release was evoked with 1 mM 4-aminopyridine. The data are averages of two independent experiments (ranges less than 10%).

Enflurane and isoflurane also inhibited 4-AP–evoked glutamate release. As with halothane, these anesthetics were effective at aqueous concentrations that correspond to 1 or 2 MAC (table 1). In contrast to the volatile anesthetics, a clinically effective concentration of the intravenous anesthetic pentobarbital did not significantly inhibit 4-AP–evoked glutamate release (table 1).

Effect of Halothane on Glutamate Release Evoked by Veratridine, KCl or Ionomycin

Veratridine, a neurotoxin that directly activates Na^+ channels, also depolarizes synaptosomes and evokes neurotransmitter release.[3] Veratridine-evoked glutamate release, like 4-AP–evoked glutamate release, was significantly inhibited by 0.9 mM halothane (fig. 5), and by lower concentrations of halothane (data not shown).

Table 1. Effect of Various Anesthetics on the Rate of Glutamate Release Evoked by 4-Aminopyridine

Anesthetic (n)	Concentration (mM)	Glutamate Release (nmol · min^{-1} · mg^{-1})*	Inhibition (%)
Control (6)		2.22 ± 0.35	0
Halothane† (4)	0.29 (1 MAC)	1.82 ± 0.27‡	18
	0.58 (2 MAC)	1.26 ± 0.25‡	43
Isoflurane† (4)	0.35 (1 MAC)	1.50 ± 0.39‡	32
	0.70 (2 MAC)	1.27 ± 0.24‡	42
Enflurane† (4)	0.68 (1 MAC)	1.72 ± 0.13‡	23
	1.36 (2 MAC)	1.55 ± 0.18‡	30
Pentobarbital (4)	0.05§	2.06 ± 0.46	7
	0.5	1.65 ± 0.18‡	26

Synaptosomes were preincubated in the presence of 1 μM β phorbol 12,13-dibutyrate and the anesthetic indicated. Glutamate release evoked by 1 mM 4-aminopyridine was measured as described in Materials and Methods.

* Values are mean ± SD.

† Aqueous concentrations, determined by gas chromatography[14] corresponding to MAC values for rats[39] (in parentheses).

§ Estimated free aqueous concentration corresponding to the EC_{50}[2].

‡ $P < 0.01$ *versus* control by ANOVA with Newman–Keuls multiple-range test.

Depolarization of synaptosomes with 10 mM KCl results in submaximal glutamate release and increase in intracellular free [Ca^{2+}], whereas 30 mM KCl produces essentially maximal glutamate release and increase in [Ca^{2+}].[10] Depolarization of synaptosomes by 10 or 30 mM external KCl evoked glutamate release that was not inhibited significantly by halothane in the absence (table 2) or presence (data not shown) of 1 μM PDBu. The early phase of KCl-evoked glutamate release in synaptosomes was not affected by 0.65 or 0.9 mM halothane (table 2), whereas the second, slower phase of glutamate release was slightly greater in the presence of 0.9 mM halothane (data not shown). This second phase is thought to result from reversal of electrogenic Na^+-coupled glutamate transport as a result of prolonged depolarization and does not involve vesicular release.[3]

Ionomycin is an ionophore that evokes vesicular glutamate release by permitting Ca^{2+} influx into synaptosomes. Halothane significantly stimulated Ca^{2+}-dependent ionomycin-evoked glutamate release (fig. 6 and table 2).

Effect of Halothane on Intrasynaptosomal [Ca^{2+}]

To determine whether the effects of halothane on glutamate release were mediated by changes in [Ca^{2+}], intrasynaptosomal free [Ca^{2+}] was measured in conditions similar to those used to determine glutamate release. Halothane did not affect resting intrasynaptosomal [Ca^{2+}] before or after addition of $CaCl_2$ to the external medium (table 3). Halothane significantly

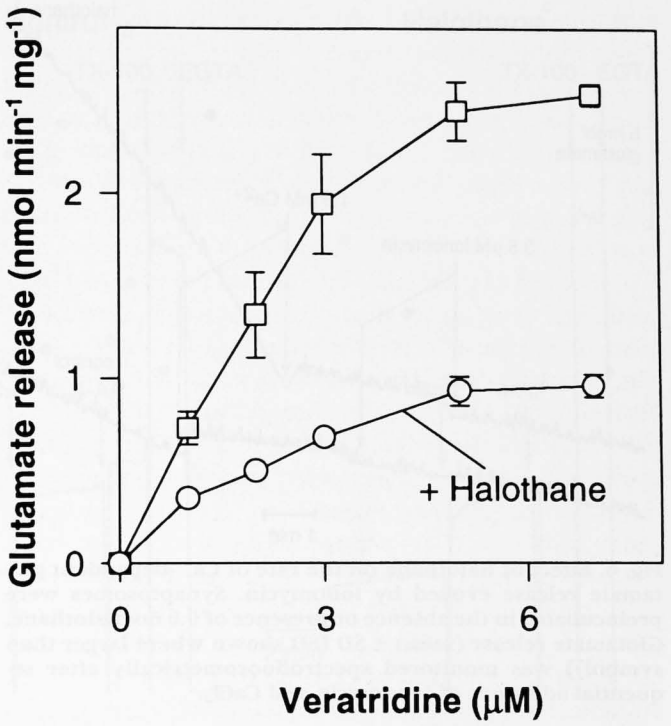

Fig. 5. Effect of halothane on the rate of glutamate release evoked by veratridine. Synaptosomes were preincubated with 1.3 mM $CaCl_2$ in the absence (squares) or presence (circles) of 0.9 mM halothane. Glutamate release (mean ± SD [SD shown where larger than symbol]; n = 3) evoked by various concentrations of veratridine was monitored spectrofluorometrically. Halothane values were significantly less than control values at all concentrations of veratridine ($P < 0.01$ by Student's unpaired two-tailed *t* test).

Table 2. Effect of Halothane on the Rate of Glutamate Release Evoked by KCl or Ionomycin

Stimulus (n)	Glutamate Release (nmol · min^{-1} · mg^{-1}) Control	+ Halothane†
10 mM KCl (4)	2.07 ± 0.59	1.96 ± 0.71
30 mM KCl (4)	3.30 ± 0.88	3.44 ± 0.68
3 μM Ionomycin (3)	1.17 ± 0.37	2.76 ± 0.28‡

Synaptosomes were incubated in the absence or presence of halothane. The release of glutamate evoked by the indicated agents was measured as described in Materials and Methods.

* Values are mean ± SD.

† 0.65 mM.

‡ $P < 0.01$ *versus* control by Student's unpaired two-tailed *t* test.

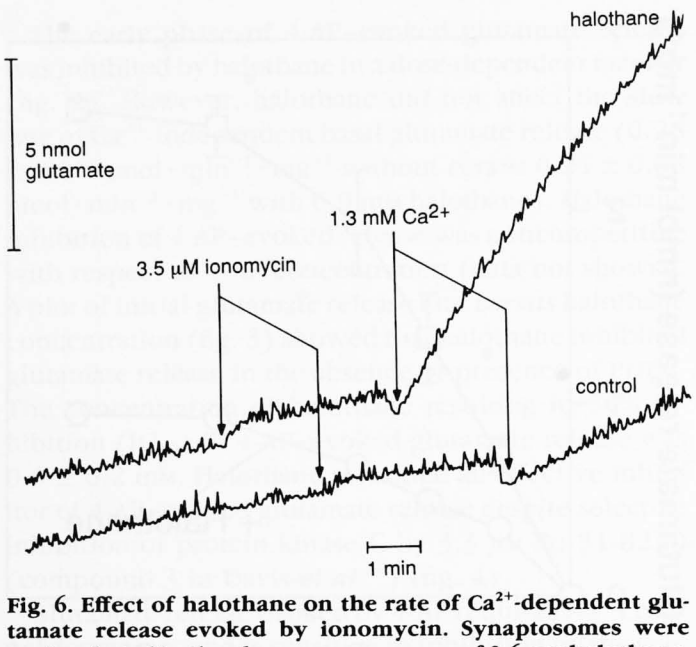

Fig. 6. Effect of halothane on the rate of Ca^{2+}-dependent glutamate release evoked by ionomycin. Synaptosomes were preincubated in the absence or presence of 0.6 mM halothane. Glutamate release (mean ± SD (SD shown where larger than symbol)) was monitored spectrofluorometrically after sequential additions of ionomycin and $CaCl_2$.

depressed the increase in the plateau phase of intrasynaptosomal [Ca^{2+}] induced by 4-AP in the absence (79% inhibition) or presence (73% inhibition) of PDBu (fig. 7 and table 3). A less marked effect of halothane was observed on the KCl-induced increase in intrasynaptosomal [Ca^{2+}] (40% inhibition; table 3). In contrast, halothane potentiated the increase in [Ca^{2+}] induced by ionomycin by 3.5-fold (table 3).

Discussion

This study demonstrates a direct inhibitory effect of volatile halogenated anesthetics at clinically relevant concentrations on the release of the excitatory neurotransmitter glutamate evoked by 4-AP. Inhibition of the release of glutamate, the major excitatory neurotransmitter in the mammalian central nervous system, is consistent with the generalized neuronal depression characteristic of general anesthesia.[1] Our results suggest that depression of glutamate release may not be a universal anesthetic mechanism, however, because the barbiturate pentobarbital, a structurally unrelated anesthetic compound, did not inhibit 4-AP–evoked glutamate release at anesthetic concentrations. Inhibition of glutamate release, in various combinations with postsynaptic glutamate receptor antagonism,[17] γ-aminobutyric acid A receptor potentiation,[18] and Ca^{2+} channel antagonism,[19] may contribute to the molecular mechanisms of volatile anesthetic action, depending on the specific anesthetic agent and synaptic physiologic characteristics.

The basal rate of glutamate release before secretogogue addition was not affected by halothane in this study. A previous study using mouse cortical synaptosomes demonstrated a 14% increase in basal glutamate release by 0.75 mM halothane at 32°C during a 15-min assay.[20] However, the interpretation of these results is complicated by the use of such a long assay interval and by the low assay temperature.

The mechanism of the inhibitory effect of halothane on glutamate release was investigated in more detail. In general, exocytotic neurotransmitter release from synaptic vesicles involves multiple steps after invasion of the presynaptic nerve terminal by an action potential.[21] The plasma membrane depolarizes as a result of voltage-dependent Na^+ channel activation. This elicits Ca^{2+} entry through voltage-dependent Ca^{2+} channels. Presynaptic Ca^{2+} entry is then coupled to the exocytosis of synaptic vesicles. Each of these steps is subject to modulation by cellular regulatory mechanisms, such as protein phosphorylation,[22] and by specific drugs and

Table 3. Effect of Halothane on Free Intrasynaptosomal Ca^{2+} Concentration

Secretagogue (n)	External Ca^{2+} (mM)	Control	+ Halothane*
		Intrasynaptosomal [Ca^{2+}] (nM)†	
Control (6)	0	164 ± 17	162 ± 19
Control (12)	1.3	369 ± 66	362 ± 68
		Secretagogue-induced increase in intrasynaptosomal [Ca^{2+}] (nM)†	
1 mM 4-AP (3)	1.3	94 ± 4	20 ± 9‡
1 mM 4-AP + 1 μM PDBu (3)	1.3	127 ± 18	35 ± 7‡
30 mM KCl (3)	1.3	106 ± 13	64 ± 11§
3 μM Ionomycin (3)	1.3	236 ± 98	833 ± 101‡

Synaptosomes loaded with fura-2 acetoxymethyl ester were incubated in the absence of external Ca^{2+}. After 4 min, 1.3 mM $CaCl_2$ was added and the intrasynaptosomal Ca^{2+} concentration was allowed to reequilibrate. This was followed by addition of the indicated secretagogue. Intrasynaptosomal [Ca^{2+}] was monitored continuously as described in Materials and Methods, and values for plateau changes were determined.

4-AP-4-aminopyridine; PDBu-β-phorbol 12,13-dibutyrate.

* 0.9 mM.

† Values are mean ± SD.

‡ $P < 0.01$, § $P < 0.05$, *versus* control by Student's unpaired two-tailed *t* test.

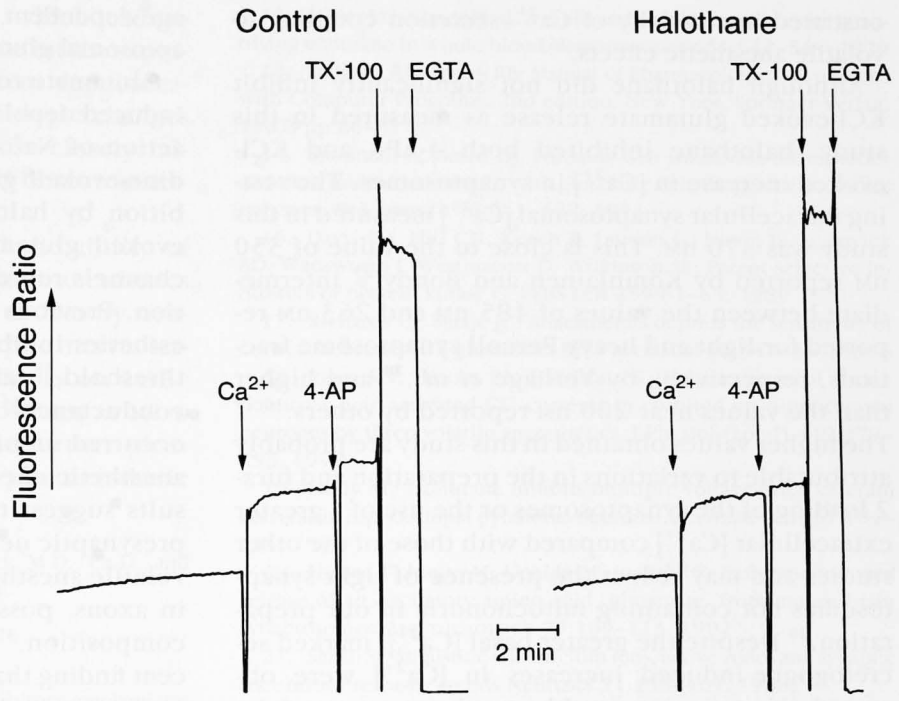

Fig. 7. Effect of halothane on changes in free intrasynaptosomal [Ca^{2+}] produced by 4-aminopyridine (4-AP) monitored by fura-2 fluorescence. Synaptosomes were loaded with fura-2 acetoxymethyl ester, and the fluorescence ratio at excitation wavelengths 340 and 380 nm was monitored at 510 nm in the absence or presence of 0.9 mM halothane. The following additions were made: 1.3 mM $CaCl_2$, 1 mM 4-AP, 6.2 mM Triton X-100 (TX-100), and 7.7 mM ethyleneglycol-bis-(β-aminoethyl ether) tetraacetic acid (EGTA). For calibration, Triton X-100 was added to release the intracellular fura-2 (maximal signal), and ethyleneglycol-bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid was added to chelate Ca^{2+} (minimal signal).

neurotoxins.[3] Heterogeneity in presynaptic mechanisms, including Ca^{2+}–secretion coupling, has been demonstrated in the differential release of glutamate, cholecystokinin-8 and norepinephrine from synaptosomes in response to varying [Ca^{2+}].[23] Any of these physiologic steps involved in neurotransmitter release are potential targets for presynaptic anesthetic effects.

Because volatile anesthetics have been found to affect the activity of purified protein kinase C *in vitro*,[24,25] the role of protein kinase C in the inhibition of glutamate release by halothane was examined. The protein kinase C activator PDBu potentiates 4-AP–evoked glutamate release from synaptosomes by a mechanism that is thought to involve facilitation of membrane depolarization by phosphorylation and inhibition of K^+ channels.[7] However, 4-AP–evoked glutamate release was sensitive to inhibition by halothane even in the absence of PDBu. Furthermore, halothane remained an effective inhibitor of glutamate release in the presence of Ro 31-8220, a potent and selective inhibitor of protein kinase C.[14] These data indicate that the inhibition of glutamate release by halothane is not attributable to inhibition of protein kinase C.

Halothane did not inhibit glutamate release significantly when evoked by direct synaptosome depolarization with KCl, which circumvents 4-AP–induced K^+ channel blockade and Na^+ channel–mediated depolarization, or by ionomycin, a Ca^{2+} ionophore that circumvents voltage-dependent Ca^{2+} channels. In fact, ionomycin-evoked glutamate release was enhanced by halothane, an effect that may involve a direct interaction between halothane and ionomycin to allow more Ca^{2+} influx. These findings suggest that the volatile anesthetic-sensitive step(s) in neurotransmitter release involve(s) membrane depolarization steps proximal to Ca^{2+} influx. The observation that ionomycin-evoked glutamate release is not inhibited by halothane suggests that Ca^{2+}–secretion coupling or other aspects of synaptic vesicle exocytosis are not important targets for the inhibition of 4-AP–evoked glutamate release by halothane.

Although neuronal voltage-dependent Ca^{2+} channels are inhibited by volatile anesthetics (at somewhat higher concentrations than those used here),[18] inhibition of Ca^{2+} influx by this mechanism is apparently not involved in the effect of halothane on 4-AP–evoked glutamate release, because KCl-evoked glutamate release (which also depends on Ca^{2+} influx through voltage-dependent Ca^{2+} channels) was insensitive to halothane in our assay. These data also implicate a target proximal to Ca^{2+} influx and imply that synaptic vesicle exocytotic mechanisms are relatively insensitive to volatile anesthetic effects. Experiments with electropermeabilized adrenal chromaffin cells have also dem-

onstrated insensitivity of Ca^{2+}–secretion coupling to volatile anesthetic effects.[26]

Although halothane did not significantly inhibit KCl-evoked glutamate release as measured in this study, halothane inhibited both 4-AP– and KCl-evoked increase in $[Ca^{2+}]$ in synaptosomes. The resting intracellular synaptosomal $[Ca^{2+}]$ measured in this study was 370 nM. This is close to the value of 350 nM reported by Komulainen and Bondy,[27] intermediate between the values of 485 nM and 263 nM reported for light and heavy Percoll synaptosome fractions, respectively, by Verhage *et al.*,[28] and higher than the values near 200 nM reported by others.[10,29] The higher values obtained in this study are probably attributable to variations in the preparation and fura-2 loading of the synaptosomes or the use of a greater extracellular $[Ca^{2+}]$ compared with those of the other studies and may reflect the presence of light synaptosomes not containing mitochondria in our preparation.[28] Despite the greater basal $[Ca^{2+}]$, marked secretogogue-induced increases in $[Ca^{2+}]$ were observed that are comparable to those reported by others.[10,27] The observation that halothane inhibited the increase in $[Ca^{2+}]$ produced by depolarization with 30 mM KCl suggests that halothane is capable of inhibiting voltage-dependent Ca^{2+} channels in synaptosomes. This effect was not likely mediated by an effect on the Na^+/Ca^{2+} exchanger, the Na^+, K^+ adenosine triphosphatase, or the Ca^{2+} adenosine triphosphatase, because basal intrasynaptosomal $[Ca^{2+}]$ was not affected.

The observation that halothane is more effective in the inhibition of the 4-AP–evoked rather than the KCl-evoked intrasynaptosomal $[Ca^{2+}]$ increase despite comparable secretogogue-induced $[Ca^{2+}]$ increases indicates different mechanisms for the halothane effects, and argues against the sole involvement of Ca^{2+} channel blockade in the effect of halothane on 4-AP–evoked release. The absence of a significant effect of halothane on KCl-evoked glutamate release, despite its inhibition of KCl-evoked $[Ca^{2+}]$ increase, suggests that the synaptosomal KCl-activated Ca^{2+} channels inhibited by halothane may not be closely coupled to glutamate release. Two pertinent observations support this interpretation. First, the N-type Ca^{2+} channel antagonist ω-conotoxin GVIA inhibits KCl-evoked increase in $[Ca^{2+}]$ in synaptosomes by 20%[30] without affecting glutamate release.[29] Second, P-type Ca^{2+} channels appear to be relatively insensitive to inhibition by volatile anesthetics,[31] and may represent the noninactivating voltage-dependent Ca^{2+} channels that are coupled to synaptosomal glutamate release.[29]

Glutamate release evoked by 4-AP– or veratridine-induced depolarization of synaptosomes requires the action of Na^+ channels.[3] Because 4-AP– or veratridine-evoked glutamate release is sensitive to inhibition by halothane, whereas KCl- or ionomycin-evoked glutamate release is not, presynaptic Na^+ channels represent a potential site of halothane action. Previous studies have shown that volatile anesthetics inhibit axonal conduction,[32] axonal firing threshold,[33] and axonal Na^+ channel gating and conductance[34–36]; however, these effects generally occurred at higher anesthetic concentrations than anesthetic effects on synaptic transmission.[1] Our results suggest that the Na^+ channels present in the presynaptic nerve terminal may be more sensitive to volatile anesthetic effects than Na^+ channels present in axons, possibly because of a different isozymic composition.[37] These data are consistent with the recent finding that central nervous system Na^+ channels are suppressed by clinically relevant concentrations of general anesthetics.[38] Further studies are required to determine the precise mechanism(s) of volatile anesthetic inhibition of synaptosomal glutamate release, because only indirect conclusions regarding the mechanism(s) involved can be drawn from the present data.

Several methodologic considerations are relevant to the interpretation of the data from this study. First, synaptosomes are a heterogeneous preparation of nerve endings derived from a variety of neuron types. Thus, the biochemical changes measured in this system are an average of those occurring in a population and may reflect different regulatory and modulatory mechanisms, and different pharmacologic responses. Second, the neurotransmitter content of synaptosomes depends on the brain region(s) used in their preparation. Glutamate is the dominant neurotransmitter released by cerebrocortical synaptosomes, but the release of other amino acid neurotransmitters, peptides and catecholamines can also be measured.[3] It is possible that the release of other neurotransmitters, that demonstrate different Ca^{2+} release sensitivities, may differ in their responses to various anesthetics. Third, the use of chemical secretogogues to induce neurotransmitter release does not precisely mimic release evoked by electric stimulation. This is particularly true of KCl-evoked and veratridine-evoked release, which may result in nonphysiologic inactivation of Na^+ and Ca^{2+} channels,

as well as of ionomycin-evoked release, which leads to very high and nonlocalized increases in intrasynaptosomal [Ca^{2+}]. By producing only transient depolarizations, 4-AP appears to mimic the physiologic changes produced by electric stimulation most closely, although its action at K^+ channels could obscure anesthetic effects at this potential target.

In summary, we have demonstrated the ability of clinically relevant concentrations of volatile anesthetics to inhibit 4-AP–evoked release of the excitatory neurotransmitter glutamate from presynaptic nerve terminals. Analysis of the effects of anesthetics on 4-AP–evoked glutamate release from synaptosomes *in vitro* should provide a useful model for the further pharmacologic characterization of presynaptic anesthetic mechanisms.

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