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Volatile Anesthetics Depress Ca^{2+} Transients and Glutamate Release in Isolated Cerebral Synaptosomes

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Background: **The current study was performed to determine whether volatile anesthetics may include as part of their action in the central nervous system the depression of presynaptic transmitter release by alteration in intrasynaptic $[Ca^{2+}]$ ($[Ca^{2+}]_i$).**

Methods: **Guinea pig cerebrocortical synaptosomes were studied at 37°C suspended in control buffer solution containing 1.3 mM external $[Ca^{2+}]$ ($[Ca^{2+}]_e$). Spectrofluorometric assays were used to monitor $[Ca^{2+}]_i$ with the Ca^{2+}-sensitive fluorophore Fura-2 and to monitor glutamate release with an enzyme-coupled assay that produced the fluorescent product nicotinamide adenine dinucleotide phosphate. To activate the increase in $[Ca^{2+}]_i$ and glutamate release, synaptosomes were depolarized by abruptly increasing external $[K^+]$ from 5 to 35 mM. Responses were determined in solutions equilibrated with approximately 1 or 2 minimum alveolar concentration (MAC) isoflurane, enflurane, or halothane and also in solutions with decreased $[Ca^{2+}]_e$ (0.025, 0.05, 0.1, 0.2, 0.4, and 0.6 mM).**

Results: **Although they had no action on basal behavior, the anesthetics depressed the K^+-depolarization–induced increase in both $[Ca^{2+}]_i$ and glutamate release in a dose-dependent fashion. The $[Ca^{2+}]_i$ transient was inhibited by 13–21% per MAC, and glutamate release was depressed 14–28% per MAC. The depression of both $[Ca^{2+}]_i$ and glutamate release caused by 2.5% isoflurane, 3.4% enflurane, and 1.5% halothane could be reproduced by a reduction in $[Ca^{2+}]_e$ to 0.2–0.4 mM.**

Conclusions: **In this setting, isoflurane, enflurane, and halothane decrease $[Ca^{2+}]_i$ in a manner consistent with inhibition of Ca^{2+} entry, possibly by specific voltage-gated neuronal Ca^{2+} channels. This decrease in $[Ca^{2+}]_i$ is sufficient to account for all or most of the associated decrease in glutamate release. (Key words: Anesthetics, volatile: enflurane; halothane; isoflurane. Brain: synaptosomes. Ions: calcium. Neurotransmitters: glutamate.)**

THE primary effect of volatile anesthetics has been attributed to alteration of synaptic transmission in the central nervous system. Although some detailed descriptions have been made of anesthetic effects on postsynaptic neuronal sites, particularly the anesthetic enhancement of γ-aminobutyric $acid_A$–mediated inhibitory Cl^- currents,[1,2] fewer studies have defined presynaptic effects. In the absence of decreased postsynaptic glutamate sensitivity, the observed decrease in glutamate-mediated excitatory postsynaptic potentials by anesthetics in central neurons has been attributed a presynaptic action, possibly mediated by inhibition of Ca^{2+} entry.[3–5] In isolated cerebrocortical synaptosomes, Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCC) leads to an increase in intrasynaptic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and, subsequently, exocytotic secretion of the excitatory neurotransmitter glutamate.[6–9] In mammalian neuronal systems a variety of VGCC (N-, P- and Q-type) appear to contribute to synaptic release processes in various tissues.[9–16] The ability of volatile anesthetics to depress Ca^{2+} currents in the myocardium has been recognized for more than a decade,[17,18] particularly *via* the dihydropyridine-sensitive long-lasting, high-threshold (L-type) VGCC.[19,20] Recently, the N-type VGCC in hippocampal cells has been shown to be depressed by volatile agents.[21] In contrast, the P-type VGCC, which appears to mediate a large fraction of synaptosomal Ca^{2+} entry in the central nervous system,[16] may be far less affected by the volatile anesthetic agents.[22] To determine whether presynaptic Ca^{2+} entry

This article is accompanied by a Highlight. Please see this issue of ANESTHESIOLOGY, page 28A.

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is significantly altered by volatile anesthetics and is associated with depressed transmitter release, we examined the effects of isoflurane, enflurane and halothane on voltage-activated Ca^{2+} influx and glutamate release from isolated cerebral cortex synaptosomes. Depending on the correlation between Ca^{2+} entry and glutamate release, these observations might also provide insights into other processes that may be altered by anesthetics, such as Ca^{2+} elimination (by the plasmalemmal Ca^{2+}-adenosine triphosphatase or Na^+–Ca^{2+} exchanger), intracellular Ca^{2+} mobilization, and Ca^{2+} interaction with vesicular release proteins (such as synaptotagmin, synapsin, and syntaxin[23]).

Materials and Methods

Synaptosome Preparation

For each day's experiments, synaptosomes were prepared by a modification of the method of Nicholls[24] and Bowman *et al.*[9] Following protocols approved by the University of Virginia Animal Research Committee, 250–350-g Dunkin-Hartley guinea pigs were killed by cervical dislocation, or by cardiac excision after anesthesia with 0.1 g/kg pentobarbital given by intraperitoneal injection. Cerebral cortexes (~1 g) were rapidly excised, cooled to 0°C, homogenized in 9 ml 0.3 M sucrose, and centrifuged at 1,500*g* for 10 min. The pellet obtained was centrifuged at 1,500*g* for 10 min again after resuspension in approximately the original volume of 0.3 M sucrose. The combined supernatants were centrifuged at 9,000*g* for 20 min and the pellet (crude synaptosome fraction) was resuspended in 5 ml 0.3 M sucrose. Aliquots (2 ml) of this suspension were layered on 5 ml 0.8 M sucrose and centrifuged at 9,000*g* for 30 min. Particles dispersed in 0.8 M sucrose solution were diluted with an equal volume of ice-cold incubation medium containing (in millimolar concentrations) NaCl 125, KCl 5, $NaHCO_3$ 5, Na_2HPO_4 1.2, $MgCl_2$ 1, glucose 10, and hydroxyethylpiperazine-ethane sulfonic acid 20, at *p*H 7.4 and centrifuged at 16,000*g* for 20 min. The pellet (purified synaptosome fraction) was resuspended in incubation medium.

Measurements of intracellular Ca^{2+} and glutamate release were performed using a luminescence spectrofluorometer (LS-50, Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England). During each experiment, the cuvette temperature was thermostatically regulated at 37°C with magnetic stirring of the synaptosome suspension to ensure adequate mixing.

Ca^{2+} Measurements

The free Ca^{2+} concentration inside the synaptosomes ($[Ca^{2+}]_i$) was measured using the Ca^{2+}-sensitive fluorophore Fura-2 and conventional excitation wavelength ratio methods.[9,25] The synaptosomal suspension was preincubated for 5 min at 37°C, followed by the addition of 100 μM $CaCl_2$, 16 μM bovine serum albumin, and 1 μM Fura-2/AM (Sigma Chemical, St. Louis, MO), which diffuses into the synaptosomes and remains there after hydrolysis of the acetoxymethyl (AM) groups. After 35 min, the synaptosomes were centrifuged for 2 min and washed twice with 2 ml fresh medium to eliminate residual Fura-2/AM. An aliquot of Fura-2/AM–loaded synaptosomes (containing ~1.34 mg protein) was then placed in a cuvette containing 2 ml medium to which $CaCl_2$ was added to achieve a final $[Ca^{2+}]$ of 1.3 mM. Following standardized protocols, fluorescence was determined at 510 nm with an excitation wavelength switched between 340 or 380 nm; data points were collected every 1.89 s. One hundred seconds after the addition of Ca^{2+}, $[K^+]$ was increased to 35 mM (by addition of 20 μl 3 M KCl) to depolarize the synaptosomes and initiate Ca^{2+} influx. After 180 s, synaptosomes were made soluble with 1% (weight/volume) Triton X-100 to permit Ca^{2+} entry and saturation of Fura-2/AM, followed by addition of ethyleneglycol-bis-(β-aminoethyl ether) tetraacetic acid to a final concentration 7.5 mM to bind Ca^{2+}, thereby generating the maximum and minimum 340/380 fluorescence ratios, respectively. Based on the maximum and minimum ratios, a computer program then calculated the synaptosomal $[Ca^{2+}]_i$ from fluorescence ratio measured during the 280-s experimental period. In control experiments, this technique was found to be insensitive to the anesthetics. Seven to 13 $[Ca^{2+}]_i$ assays on different synaptosomal preparations were carried out for each anesthetic concentration.

Glutamate Release

Glutamate release was measured under identical conditions used for Ca^{2+} influx, except for omission of Fura-2/AM loading and the addition of an enzyme-coupled assay using glutamate dehydrogenase as described by Nicholls and coworkers.[7,26] In the presence of glutamate dehydrogenase (50 U/ml), nicotinamide adenine dinucleotide phosphate (NADPH) and ketoglutarate are produced from $NADP^+$ (1 mM) and glutamate. NADPH fluorescence was excited at 340 nm and monitored at 460 nm (emission wavelength) as a measure of glutamate release. As in the Ca^{2+} assay, glutamate

Fig. 1. Calibration for glutamate relea
(*A*) Fluorescence intensity (FI) respo
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system using 50 U/ml glutamate dehyd
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duction of fluorescent product nico
amide adenine dinucleotide phosph
(NADPH) from $NADP^+$ (1 mM) in the
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netics, when K_m of the enzyme for
substrate (where K_m = the substrate
centration at which 50% of the maxim
velocity is obtained for a constant enz
concentration) exceeds the subst
concentration by >20-fold, the initi
initial slope of FI increase at 460 nm
squares = an additional experiment.

release was activated by increas
s after the addition of Ca^{2+}) the
of KCl increased the osmolari
~20%, but control experiments
amount of NaCl was added show
in $[Ca^{2+}]_i$ nor detectable glutam
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to a final value. For a standard en
of substrate utilization ($-dS/d
in this case glutamate) is given

$$-dS/dt = [S] \times V_m \times /(K$$

where P = the product and K_m
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Thus the initial rate of NADPH
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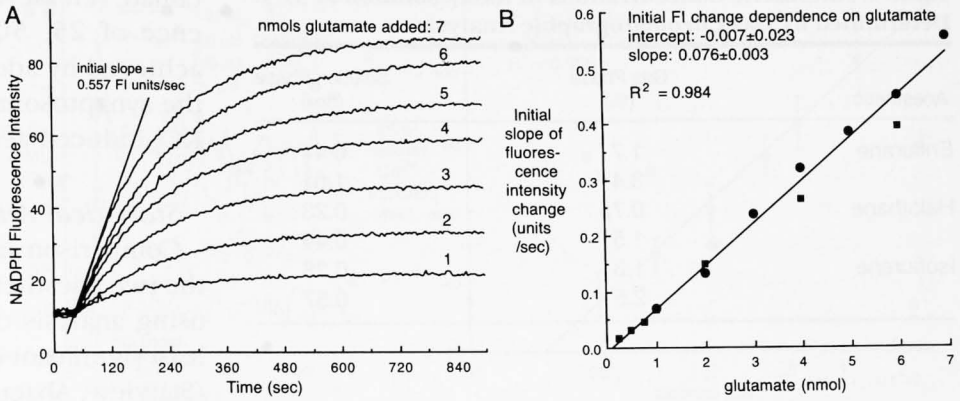

Fig. 1. Calibration for glutamate release. (*A*) Fluorescence intensity (FI) response at 460 nm (excitation at 340 nm) *versus* time with the sudden addition of glutamate (1–7 nmol) to the standard medium at 40 s, using an enzyme-coupled assay system using 50 U/ml glutamate dehydrogenase. FI increases because of the production of fluorescent product nicotinamide adenine dinucleotide phosphate (NADPH) from $NADP^+$ (1 mM) in the medium. According to standard enzyme kinetics, when K_m of the enzyme for the substrate (where K_m = the substrate concentration at which 50% of the maximum velocity is obtained for a constant enzyme concentration) exceeds the substrate concentration by >20-fold, the initial rate of enzyme reaction will be proportional to the substrate concentration. (*B*) The initial slope of FI increase at 460 nm for the addition of various amounts of glutamate. Circles = the slopes determined from *A*; squares = an additional experiment. The response is linear over the range studied.

release was activated by increase in K^+ to 35 mM 100 s after the addition of Ca^{2+} to the solution. The addition of KCl increased the osmolarity of the solution by ~20%, but control experiments in which an equivalent amount of NaCl was added showed neither an increase in $[Ca^{2+}]_i$ nor detectable glutamate release.

Because of the enzyme-coupled aspect of this assay, it requires time for reaction. The sudden increase in glutamate due to addition or release results in a measurable fluorescence signal that increases exponentially to a final value. For a standard enzyme reaction, velocity of substrate utilization ($-dS/dt$, where S = substrate, in this case glutamate) is given by:

$$-dS/dt = [S] \times V_{max}/(K_m + [S]) = dP/dt \qquad (1)$$

where P = the product and K_m = the substrate concentration at which 50% of the maximum velocity (V_{max}) is obtained for a constant enzyme concentration. Because the glutamate concentration is much lower than K_m of glutamate dehydrogenase (~120 μM), the equation may be simplified to:

$$dP/dt = -dS/dt = ([S] \times V_{max})/K_m. \qquad (2)$$

Thus the initial rate of NADPH production is proportional to the glutamate concentration ([S]), and any sudden increase in glutamate can be estimated from the initial slope of NADPH appearance. To assess both the linearity of the assay as well as the validity of the assumption, the fluorescence intensity (FI) was measured in response to the sudden increase in glutamate caused by the addition of a specified amount (0.25–7 nmol) to the incubation medium, which contained 50 U/ml glutamate dehydrogenase and 1 mM $NADP^+$ (fig. 1A). As predicted from enzyme kinetics, the increase in FI was an exponential function of time after the addition of glutamate with a time constant of ~190 s at each glutamate concentration, the final FI stabilizing after 800–900 s. To 7 nmol added glutamate, the initial slope was a linear function of the added glutamate and the resulting [glutamate] (fig. 1B). Unlike the calibration curves, which achieve a stable FI once the added glutamate is metabolized, the initial and subsequent ongoing synaptosomal glutamate release activated by KCl depolarization results in a slow, ongoing increase in FI after 900 s. Consequently, absolute value of FI at any point cannot be used as a measure of initial or total glutamate release. However, because the initial slope of FI is a linear function of added glutamate to 7 nmol, the initial glutamate released can still be estimated from the initial slope. Although a deconvolution of the FI response based on the time constant of the reaction permitted an estimate of ongoing glutamate release, this was not routinely performed. To provide for any variation in each assay, the change in FI slope resulting from the addition of 6 nmol glutamate at 900 s was used an internal calibration for that particular assay (see fig. 4, for example). Five to eight glutamate assays on different synaptosomal preparations were carried out for each anesthetic concentration. We also verified that this method of glutamate determination is insensitive to the volatile anesthetics.

Anesthetic Administration

Anesthetic effects were determined by incubation of the synaptosomes for 5 min before study in an assay

Table 1. Anesthetic Concentrations in Assay Solution at 37°C Determined by Gas Chromatographic Analysis

Anesthetic	Gas Phase (%)	Solution Phase (mM)
Enflurane	1.7	0.44
	3.4	1.01
Halothane	0.75	0.23
	1.5	0.44
Isoflurane	1.3	0.28
	2.5	0.57

solution preequilibrated with the specified anesthetic concentration. Solutions were preequilibrated for 20 min by bubbling with anesthetic-containing filtered air from a calibrated vaporizer, whereas control solutions were bubbled with filtered air only. Incubation and assays were carried out in cuvettes in which an appropriate concentration of anesthetic-containing air continually flowed through the head space to prevent loss of anesthetic from the medium to the atmosphere. The Ca^{2+} influx or glutamate release assay were performed with approximately equipotent anesthetic concentrations: 0.75 or 1.5% halothane; 1.3 or 2.5% isoflurane; 1.7 or 3.4% enflurane; representing one or two times human minimum alveolar concentration (MAC), or ~0.8 and ~1.6 times guinea pig MAC.[27] The aqueous phase anesthetic concentrations at 37°C as determined by gas chromatography are listed in table 1 and represent 89–105% of the value predicted from published partition coefficients.[28]

To decrease the amount of Ca^{2+} entry and thereby assess the relation between the Ca^{2+} transient and glutamate release, assays were also performed in the presence of 25, 50, 100, 200, 400, and 600 μM Ca^{2+}, achieved by addition of smaller quantities of $CaCl_2$ to the synaptosome-containing assay solution before the KCl-induced activation.

Statistical Analysis

Comparison among the absolute values of control and the two anesthetic concentrations and were performed using analysis of variance and the Fisher's protected least significant difference test for planned comparisons (Statview, Abacus Software, Berkeley, CA). Significance of the concentration dependence of the effects was determined by calculating the slope and its estimated error (least-squares regression analysis) using the absolute values at 0, 1, and 2 MAC of anesthetic. Values were also calculated as percent of control for any given days experiments (one to three assays), and tested *versus* 100% by an unpaired *t* test.

Results

Ca^{2+} Dependence of Synaptosomal Responses

The increase in $[Ca^{2+}]_i$ as well as glutamate release from these isolated synaptosomes was clearly dependent on $[Ca^{2+}]$ in the external medium ($[Ca^{2+}]_e$). The depolarization induced by increasing $[K^+]$ from 5 to 35 mM produced a characteristically rapid peak (<10 s) of $[Ca^{2+}]_i$ of 100–150 nM above the basal concentration (120–200 nM), followed by a partial decrease to a stable plateau phase of 50–80 nM above the basal $[Ca^{2+}]_i$ (fig. 2A). In nominally Ca^{2+}-free solutions, there was never an increase in $[Ca^{2+}]_i$. In su

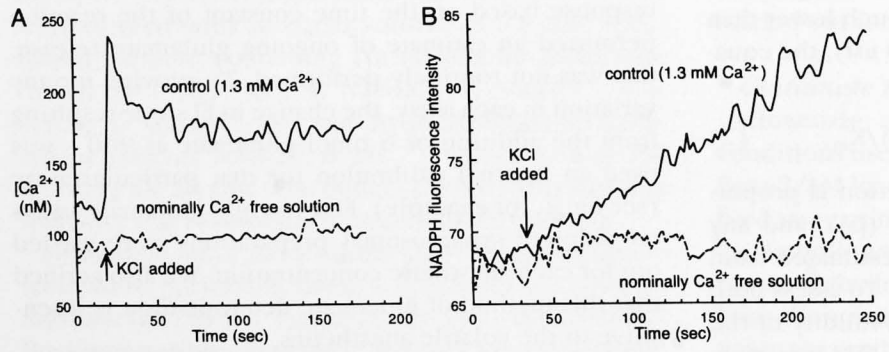

Fig. 2. Intrasynaptosomal $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and glutamate release in response to depolarization of synaptosomes (1.34 mg protein) by increasing K^+ from 5 to 35 mM. (*A*) The $[Ca^{2+}]_i$ changes as determined by the Fura-2/AM fluorescence ratio method. In the presence of 1.3 mM Ca^{2+}, a large initial transient was observed on the increase in external $[K^+]$. The large initial transient subsequently decreased to a plateau that was typically half that of the initial transient. In the absence of added Ca^{2+} (nominally Ca^{2+}-free), no Ca^{2+} transient is observed. (*B*) Nicotinamide adenine dinucleotide phosphate (NADPH) fluorescence response to added KCl in the presence of 1.3 mM Ca^{2+} and in nominally Ca^{2+}-free solution. The initial slope of the fluorescence response in 1.3 mM Ca^{2+} gives an estimate of the initial glutamate release of 1.5 nmol, based on the equivalence of its slope to one fourth of the slope observed on addition of the internal standard of 6 nmol (done at 900 s and not shown in the tracing; see fig. 4, for example).

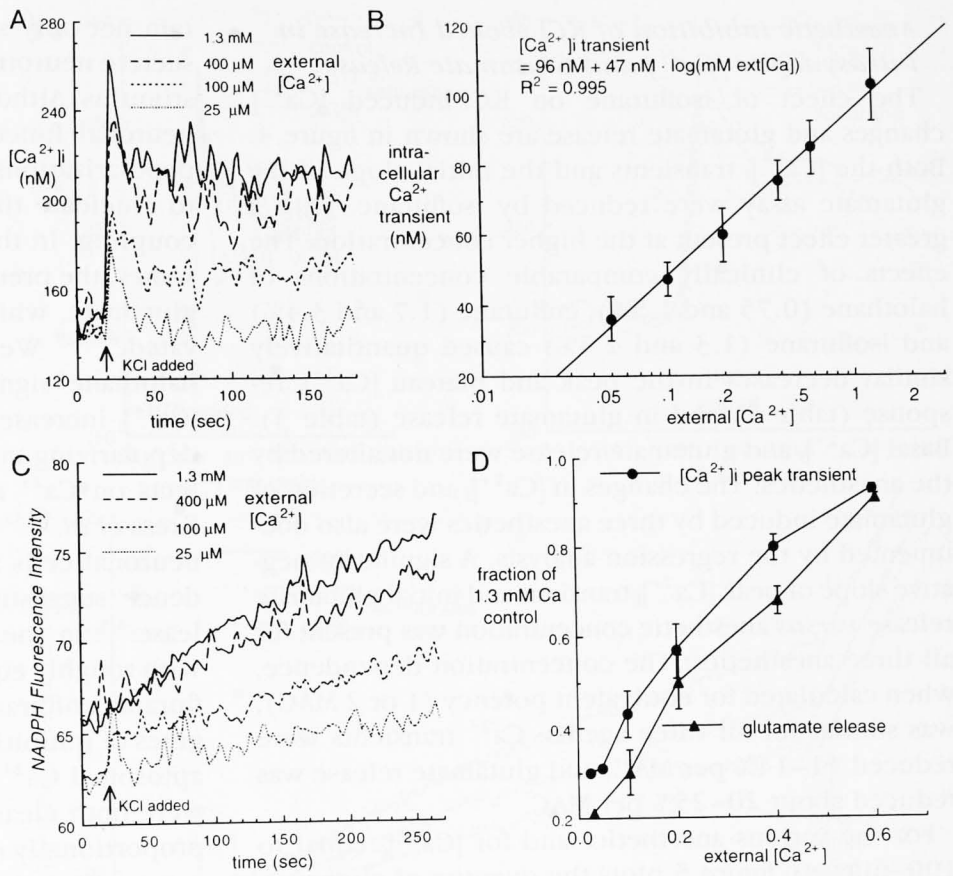

Fig. 3. Intrasynaptosomal $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and the glutamate release in response to KCl depolarization in the presence of varied $[Ca^{2+}]$ in the external medium ($[Ca^{2+}]_e$). (*A*) Changes in $[Ca^{2+}]_i$ from one synaptosomal preparation in the presence of the varying $[Ca^{2+}]_e$ indicated. (*B*) The dependence of the net $[Ca^{2+}]_i$ transient on $[Ca^{2+}]_e$ (ext[Ca]). Each point represents the mean of three to five measurements. (*C*) Changes in nicotinamide adenine dinucleotide phosphate (NADPH) fluorescence intensity (FI) representing glutamate release with varied $[Ca^{2+}]_e$, in which the decreased slope of the FI increase is proportional to the magnitude of glutamate release. (*D*) Fractional change in the Ca^{2+} transient and glutamate release plotted as a fraction of the control level observed in presence of 1.3 mM $[Ca^{2+}]_e$.

never an increase in $[Ca^{2+}]_i$. In such cases, the contaminant $[Ca^{2+}]_e$ in the absence of a Ca^{2+} chelating agent was typically in the range of 2–4 μM.

In the presence of external Ca^{2+}, the release of glutamate was evident from the increase in NADPH FI after KCl-induced depolarization, where the initial slope is proportional to the amount of the initial glutamate release (fig. 2B). The slope is subsequently maintained by ongoing glutamate release. Based on the FI slope resulting from addition of 6 nmol glutamate (tracing at 900 s not shown), the initial glutamate release was estimated as 1.5 nmol glutamate from the initial slope of the curve. In 30–40% of experiments, a modest baseline of glutamate release before depolarization was present as evidenced by a slightly positive slope of FI. In these cases, KCl-independent release of glutamate was subtracted from KCl-dependent release before tabulation. Depolarization in nominally Ca^{2+}-free solution did not activate glutamate release, indicating that Ca^{2+} influx is responsible for the observed increase.

If instead of 1.3 mM or nominally zero Ca^{2+}, an intermediate $[Ca^{2+}]_e$ was used, $[Ca^{2+}]_i$ transients of intermediate amplitude were obtained, and glutamate release was also reduced below the control (1.3 mM) concentration. Figure 3A presents the effects of 25–400 μM $[Ca^{2+}]_e$ on the amplitude of the $[Ca^{2+}]_i$ transient and plateau initiated by the K^+-induced depolarization. A modest reduction occurred with 400 μM $[Ca^{2+}]_e$, with smaller $[Ca^{2+}]_i$ transients evident in the presence of the lower $[Ca^{2+}]_e$. In spite of various complicating considerations, the decrease in the $[Ca^{2+}]_i$ transient was a simple linear function of the log of $[Ca^{2+}]_e$ (fig. 3B). This relation would be anticipated if the $[Ca^{2+}]_i$ transients were a linear function of inward Ca^{2+} current amplitude, and assuming current can be approximated by a simple conductance model, current will be proportional to the Ca^{2+} equilibrium potential, which is proportional to log ($[Ca^{2+}]_e/[Ca^{2+}]_i$). The glutamate release, as assessed by the initial slope of increasing FI, also decreased as $[Ca^{2+}]_e$ was decreased (fig. 3C). Figure 3D presents the dose-dependent depression in glutamate release, as well as in the $[Ca^{2+}]_i$ transient, for the reductions in $[Ca^{2+}]_e$. With a reduction in $[Ca^{2+}]_e$, glutamate release was typically more depressed than was the $Ca^{2+}{}_i$ transient. Neither the basal $[Ca^{2+}]_i$ nor the basal glutamate release before depolarization was altered by the decreased $[Ca^{2+}]_e$ (data not shown).

Anesthetic Inhibition of KCl-evoked Increase in Intrasynaptic [Ca^{2+}] and Glutamate Release

The effect of isoflurane on KCl-induced $[Ca^{2+}]_i$ changes and glutamate release are shown in figure 4. Both the $[Ca^{2+}]_i$ transients and the initial slope of the glutamate assay were reduced by isoflurane, with a greater effect present at the higher concentration. The effects of clinically comparable concentrations of halothane (0.75 and 1.5%), enflurane (1.7 and 3.4%) and isoflurane (1.3 and 2.5%) caused quantitatively similar decreases in the peak and plateau $[Ca^{2+}]_i$ response (table 2) and in glutamate release (table 3). Basal $[Ca^{2+}]_i$ and glutamate release were not altered by the anesthetics. The changes in $[Ca^{2+}]_i$ and secretion of glutamate induced by three anesthetics were also documented by the regression analysis. A significant negative slope of peak $[Ca^{2+}]_i$ transient and initial glutamate release *versus* anesthetic concentration was present for all three anesthetics. The concentration dependence, when calculated for equivalent potency (1 or 2 MAC), was similar for all three agents: Ca^{2+} transients were reduced 11–14% per MAC, and glutamate release was reduced about 20–25% per MAC.

For the various anesthetics and for $[Ca^{2+}]_e$ equal to 100–600 μM, figure 5 plots the quantity of glutamate release as a fraction of control ($Q_{glut}/Q_{glut-control}$) *versus* the peak $[Ca^{2+}]_i$ transient, also expressed as a fraction of control ($[Ca^{2+}]_i/[Ca^{2+}]_{i-control}$), where the control values were those observed in 1.3 mM $[Ca^{2+}]_e$ in the absence of anesthetic. The mean percent same-day control values are plotted. If glutamate release is a simple linear function of $[Ca^{2+}]_i$, then the points should fall on the line of unity (n = 1). Instead, the fractional reduction in glutamate release is slightly greater than that for $[Ca^{2+}]_i$. Assuming a simple model for Ca^{2+}-dependent glutamate release in which n Ca^{2+} ions induce exocytosis, then:

$$Q_{glut}/Q_{glut\text{-}control} = ([Ca^{2+}]_i/[Ca^{2+}]_{i\text{-}control})^n, \quad (3)$$

where n = the cooperativity of the Ca^{2+}. In general, the points defined for reduced $[Ca^{2+}]_e$ as well as for the three anesthetics fall in the range of the lines defined by n = 1 and n = 2 (fig. 5).

Discussion

Although homogenization of brain tissue destroys neuronal cell bodies, the membranes of nerve endings reseal into synaptosomes, small functional sacs that retain not only synaptic vesicles but also the ability to secrete neurotransmitter in response to a depolarizing stimulus. Although they of course do not reflect intact neuronal function mediated by axonally transmitted depolarizations, synaptosomes have been widely used to elucidate the mechanisms of excitation–secretion coupling. In the mammalian cerebrocortical synaptosomes, the predominant excitatory neurotransmitter is glutamate, which is released when Ca^{2+} entry is activated.[6–8,29] We found that isoflurane, enflurane, and halothane significantly depressed the synaptosomal $[Ca^{2+}]_i$ increase and release of glutamate evoked by a depolarizing concentration of K^+ (35 mM). These effects on Ca^{2+} are consistent with those reported by Kress *et al.*[30,31] in a variety of neuronal and some nonneuronal cells as well as with electrophysiologic evidence suggesting decreased neuronal glutamate release.[4,5] In the current study, solutions equilibrated with roughly equivalent clinical concentrations of isoflurane, enflurane, and halothane produced similar degrees of inhibition. As anticipated, the increase in synaptosomal Ca^{2+} ($[Ca^{2+}]_i$) and the release of glutamate were both clearly dependent on $[Ca^{2+}]_e$ and could be proportionally reduced by decreasing $[Ca^{2+}]_e$. Furthermore, effects on $[Ca^{2+}]_i$ and glutamate release caused by a reduction in $[Ca^{2+}]_e$ to 400 μM (~30% of the 1.3 mM control value) replicated the actions of the anesthetics. Because in nominally Ca^{2+}-free solution, KCl depolarization by itself did not increase $[Ca^{2+}]$, release of Ca^{2+} from any intrasynaptosomal stores present did not appear to be occurring.

The observation that the decrease in the $[Ca^{2+}]_i$ transient and greater decrease in glutamate release caused by the anesthetics can be closely duplicated by decreasing $[Ca^{2+}]_e$ has important implications. Because the relation between the reduced $[Ca^{2+}]_i$ transient and glutamate release observed in the presence of the anesthetics can be seen in their absence with decreased $[Ca^{2+}]_e$, it is likely that the intrasynaptosomal mechanisms responsive to Ca^{2+}, which ultimately progress to vesicle exocytosis, are not markedly altered by the anesthetics. If the synaptosomal proteins that bind Ca^{2+} and then foster vesicle fusion with the membrane were directly depressed (or enhanced) by anesthetics, then for a given $[Ca^{2+}]_i$ transient, the glutamate release should be less than (or greater than) that observed when the transient was depressed by altering $[Ca^{2+}]_e$. If either the anesthetics or decreased $[Ca^{2+}]_e$ altered the resting $[Ca^{2+}]_i$ before depolarization, then the behavior of various Ca^{2+} sensitive regulatory enzymes might in-

Fig. 4. The effects of isoflurane on the
induced Ca^{2+} transient and glutamate
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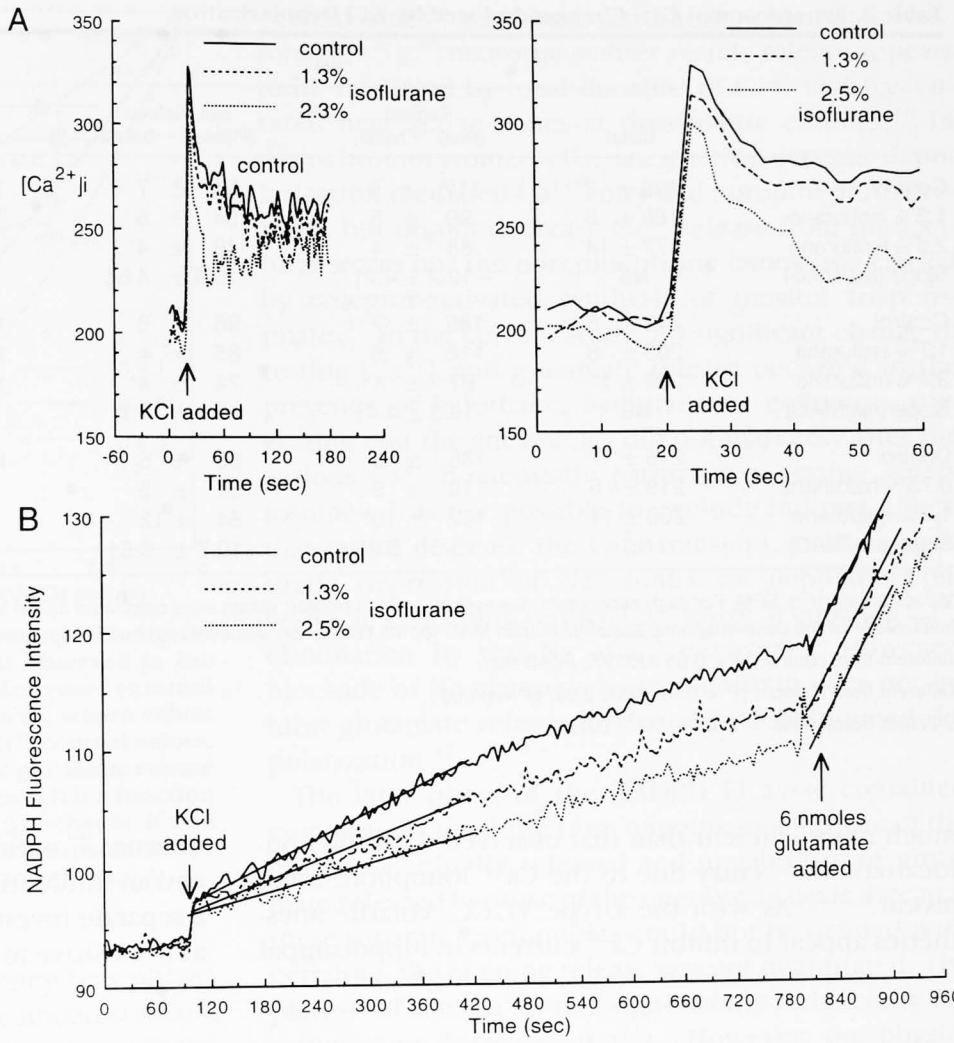

Fig. 4. The effects of isoflurane on the KCl-induced Ca^{2+} transient and glutamate release. (*A*) Ca^{2+} transients in the absence and presence of isoflurane in the extrasynaptosomal medium, where isoflurane concentrations in solution have been equilibrated with either 1.3 or 2.5% isoflurane. Shown are responses at slow (*left*) and fast (*right*) time scales, respectively. (*B*) Changes in nicotinamide adenine dinucleotide phosphate (NADPH) fluorescence intensity (FI), representing the initial glutamate release in the presence of isoflurane. The small initial step in FI seen on KCl addition was occasionally seen with certain preparations and represented a mixing artifact, not glutamate-dependent production of NADPH.

fluence subsequent exocytosis. However, no such change in $[Ca^{2+}]_i$ behavior was noted. Consequently, the observed glutamate release from the synaptosomes can be largely explained by presuming the anesthetics mediate their action predominately by decreasing the abrupt increase in $[Ca^{2+}]_i$ responsible for activating the exocytotic cascade. Whether this interpretation can be applied to intact neurons requires verification.

The reduction of $[Ca^{2+}]_i$ transient is associated with a slightly greater decrease in the quantity of glutamate release ($Q_{glut}/Q_{glut\text{-}control}$). The suggestion has been made that the process of exocytosis shows cooperativity in its Ca^{2+} dependence such that two or more Ca^{2+} must bind to specific sites.[11,13,32,33] Such a dependence is typical of that observed previously, in which transmitter release is reduced in proportion to some power function of the reduction in $[Ca^{2+}]$. The current observations using decreased $[Ca^{2+}]_e$ are consistent with the presence of some degree of cooperativity ($n > 1$), and the anesthetics do not appear to markedly alter the degree of cooperativity.

Neurotransmitter release from neurons is mediated by Ca^{2+} entry into nerve terminals, activating a complex of proteins that cause fusion of the membrane of the transmitter-containing synaptic vesicle with the cell membrane, resulting in exocytosis.[23,34] Ca^{2+} entry appears to be mediated by specific VGCC that are located near the active synaptosomal release zone of the neuronal membrane[32] and that are insensitive to the Ca^{2+}-entry blockers such as the dihydropyridines classically active in the cardiovascular system.[35] Glutamate exocytosis appears to be coupled to Ca^{2+} entry through several VGCC types including the N-, P- and Q-types, which are sensitive to ω-CTx-GVIA, ω-Aga IVA, and ω-CTx-MVIIC, respectively.[9,11,13–16,36,37] Although the exact VGCC may vary with the neuron,[38] Ca^{2+} entry through VGCC appears to be of major importance because the depolarization-coupled glutamate release is

Table 2. Synaptosomal Ca^{2+} Changes Induced by KCl Depolarization

	nM				Percent of Same Day Control		
	Basal	Evoked (Peak − basal)	Net Plateau (Plateau − basal)	n	Evoked	Plateau	n
Control	190 ± 8	112 ± 6	64 ± 7	18			
1.3% isoflurane	180 ± 6	99 ± 5	54 ± 5	11	84 ± 7¶	83 ± 6¶	6
2.5% isoflurane	177 ± 14	88 ± 4	43 ± 4*	8	74 ± 5**	67 ± 11¶	5
Slope (nM/MAC)	NS	−12.2 ± 4.2†	−10.3 ± 4.6‡				
Control	219 ± 6	136 ± 7	96 ± 6	19			
1.7% enflurane	203 ± 8	118 ± 6	85 ± 4	13	86 ± 6¶	92 ± 3**	6
3.4% enflurane	206 ± 11	97 ± 4*	74 ± 4*	12	62 ± 8**	65 ± 6**	5
Slope (nM/MAC)	NS	−19.3 ± 4.4†	−5.3 ± 4.7				
Control	215 ± 7	135 ± 6	95 ± 5	16			
0.75% halothane	219 ± 6	119 ± 9	91 ± 5	8	79 ± 7¶	84 ± 3**	4
1.5% halothane	200 ± 11	102 ± 10*	84 ± 12	7	65 ± 5*	67 ± 5**	4
Slope (nM/MAC)	NS	−15.5 ± 5.2§	−10.7 ± 3.6†				

Values are mean ± SEM. For each experiment, the evoked and net plateau values were calculated as the total measured $[Ca^{2+}]_i$ at peak or plateau minus the basal level. Slope of the dose–response assumes human MAC for the calculation; values would be 25% greater using guinea pig MAC. NS = not significant.

Different from control: *$P < 0.05$ ANOVA, PLSD test.

Different from 0 slope: †$P < 0.01$, ‡$P < 0.05$, §$P < 0.0001$.

Different from 100%: ¶$P < 0.05$, **$P < 0.01$.

much more efficient than that observed with the non-localized Ca^{2+} entry due to the Ca^{2+} ionophore ionomycin.[8,24,29] As with the L-type VGCC, volatile anesthetics appear to inhibit Ca^{2+} currents in hippocampal neurons,[39] with a prominent effect observed on N-type and an unidentified, possibly P- or Q-type, VGCC.[21] In a separate investigation, P-type VGCC were interpreted as insensitive to volatile anesthetics,[22] although the au-

Table 3. Synaptosomal Glutamate Release

	Basal Release (nmol)	Initial Release (nmol)	n	Initial Release (% same day control)	n
Control	0.88 ± 0.11	1.62 ± 0.17	10		
1.3% isoflurane	0.63 ± 0.10	1.34 ± 0.17	8	86 ± 1§	8
2.5% isoflurane	0.42 ± 0.11	0.96 ± 0.12*	5	67 ± 1§	5
Slope (nmol/MAC)	NS	−0.33 ± 0.12†			
Control	0.46 ± 0.17	1.65 ± 0.25	8		
1.7% enflurane	0.42 ± 0.19	1.33 ± 0.27*	6	81 ± 4§	6
3.4% enflurane	0.58 ± 0.21	0.83 ± 0.12*	7	59 ± 2§	7
Slope (nmol/MAC)	NS	−0.41 ± 0.15†			
Control	0.84 ± 0.29	1.30 ± 0.09	5		
0.75% halothane	0.80 ± 0.15	0.93 ± 0.05*	7	72 ± 1§	7
1.5% halothane	0.64 ± 0.14	0.77 ± 0.04*	7	57 ± 2§	7
Slope (nmol/MAC)	NS	−0.26 ± 0.04‡			

Values are mean ± SEM. To reduce the variability in glutamate release due to variation in synaptosomal preparations, the initial release in the presence of anesthetic was expressed as a percent of the same day control. Slope of the dose–response assumes human MAC for the calculation; values would be 25% greater using guinea pig MAC. NS = not significant.

Different from control: *$P < 0.05$ ANOVA, Fisher PLSD test.

Different from 0 slope: †$P < 0.02$, ‡$P \leq 0.0001$.

Different from 100%: §$P < 0.01$.

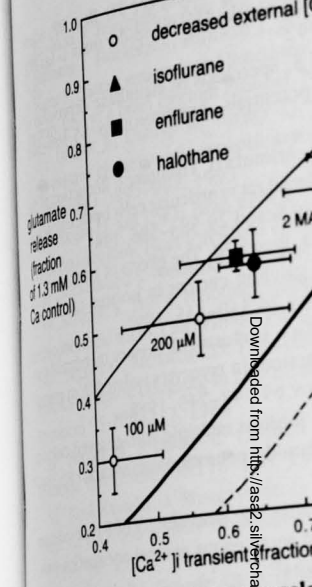

Fig. 5. The change in glutamate rele
intrasynaptosomal $[Ca^{2+}]$ ($[Ca^{2+}]_i$) t
presence of 1 or 2 MAC anesthetics o
$[Ca^{2+}]$ (600, 400, 200, and 100 μM, as
are expressed as a fraction of the 1.
The lines indicate the response antic
is strictly a linear function of $[Ca^{2+}]$
of a higher power of $[Ca^{2+}]_i$ ($n = 2$
or three Ca^{2+} are required to bind
activation of glutamate release, acc

thors' assumption that anesthet
is higher at room temperature m
the dose-requirement. Nevert
cooperativity of Ca^{2+} in mediati
the 10% depression observed a
more profound actions on pre
lease.

Although association does no
results are consistent with a sch
of VGCC by volatile anesthetic
of neurotransmitter release. H
diated alterations in other aspe
ulation have been described
the observed effects.[40,41] Volat
shown to interfere with the s
sine triphosphatase[42] as well
however, the role of these
$[Ca^{2+}]_i$ and influencing neurot
ronal cells remains undefine
polarization with $[K^+]$ of 55
tivate Ca entry only through
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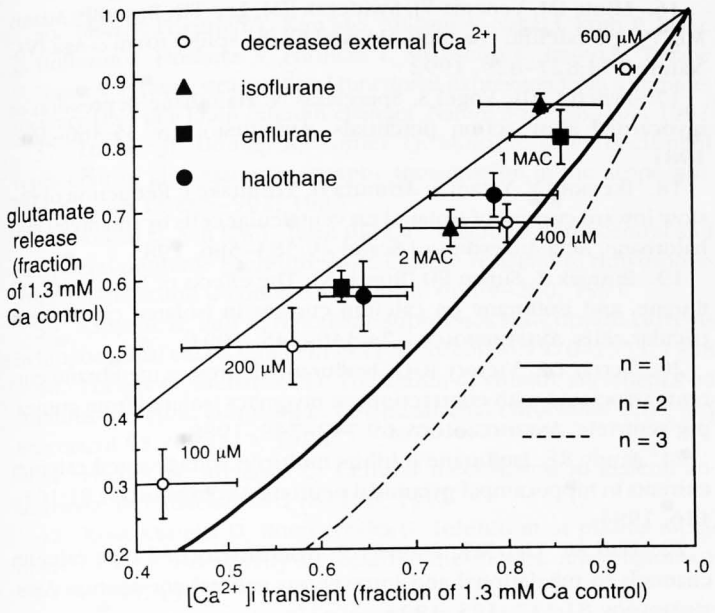

Fig. 5. The change in glutamate release *versus* the change in intrasynaptosomal $[Ca^{2+}]$ ($[Ca^{2+}]_i$) transient observed in the presence of 1 or 2 MAC anesthetics or with decreased external $[Ca^{2+}]$ (600, 400, 200, and 100 μM, as indicated), where values are expressed as a fraction of the 1.3 mM Ca^{2+} control values. The lines indicate the response anticipated if glutamate release is strictly a linear function of $[Ca^{2+}]$ (n = 1) or if it is a function of a higher power of $[Ca^{2+}]_i$ (n = 2 or n = 3)—that is, if two or three Ca^{2+} are required to bind to a site responsible for activation of glutamate release, according to equation 3.

thors' assumption that anesthetic potency (gas phase) is higher at room temperature may have underestimated the dose-requirement. Nevertheless, because of the cooperativity of Ca^{2+} in mediating release,[11,13,32,33] even the 10% depression observed at ~1 MAC may result in more profound actions on presynaptic transmitter release.

Although association does not indicate causation, the results are consistent with a scheme in which inhibition of VGCC by volatile anesthetics accounts for inhibition of neurotransmitter release. However, anesthetic-mediated alterations in other aspects of cellular Ca^{2+} regulation have been described that could contribute to the observed effects.[40,41] Volatile anesthetics have been shown to interfere with the sarcolemmal Ca^{2+}-adenosine triphosphatase[42] as well as Na^+–Ca^{2+} exchange,[43] however, the role of these processes in regulating $[Ca^{2+}]_i$ and influencing neurotransmitter release in neuronal cells remains undefined. In synaptosomes, depolarization with $[K^+]$ of 55 mM or less appears to activate Ca entry only through VGCC, and does not activate the Na^+–Ca^{2+} exchange pathway.[44] Although $Ca^{2+}{}_i$ stores in neuronal endoplasmic reticulum or "calciosomes" may also contribute to changes in neuronal $[Ca^{2+}]_i$,[45] neurotransmitter vesicle release appears to be mediated by local domains of Ca^{2+} that has entered near active zones at presynaptic endings.[46] In pheochromocytoma cells, anesthetics depress depolarization mediated Ca^{2+} entry and norepinephrine release, but do not decrease Ca^{2+} release from intracellular stores nor the norepinephrine exocytosis caused by receptor-activated synthesis of inositol trisphosphate.[31] In the current study, no significant change in resting $[Ca^{2+}]_i$ and glutamate release occurred in the presence of halothane, isoflurane or enflurane, suggesting that the anesthetics did not markedly alter the various Ca^{2+} homeostatic pathways in resting synaptosomes. It is not possible to exclude indirect effects that might decrease the Ca^{2+} transient, such as anesthetic depression of Na^+ influx on depolarization, which in turn could reduce Ca^{2+} entry or enhance Ca^{2+} elimination by the Na^+–Ca^{2+} exchanger. However, blockade of Na channels by tetrodotoxin does not inhibit glutamate release mediated by KCl-induced depolarization.[47]

The later phase of the NADPH FI assay contained components resulting from ongoing metabolism of the glutamate initially released and metabolism of glutamate released because of the ongoing analysis. Because these separate components could not be defined with certainty, the ongoing release was not quantitated. The plateau of the $[Ca^{2+}]_i$ was significantly reduced by anesthetics or decreased $[Ca^{2+}]_e$. However, the physiological relevance of the sustained depolarization and the associated ongoing glutamate release is unclear.

Although glutamate release stimulated by the KCl depolarization represents an artificial situation that imperfectly reflects *in situ* synaptic behavior, the anesthetic depression of glutamate release is consistent with the reported depression by halothane of the presynaptic glutamate release that generates excitatory postsynaptic potentials in thalamic[5] and hippocampal CA1 neurons.[48] It is unclear to what degree a 15–25% decrease in glutamate release, observed with 1 MAC anesthetic, could by itself interfere with the capacity of neurons to integrate and communicate information. However, when such an action on presynaptic endings is combined with the enhancement of γ-aminobutyric $acid_A$–mediated inhibitory activity also caused by the volatile anesthetics,[1,2] the resulting effect should be more profound. Such combined actions might cause a greater alteration in behavior of individual neurons as well as in entire neural networks, and may also explain the

differences in the quality of the anesthetic state as well as the neurophysiologic behavior produced by the volatile agents when compared with more pure γ-aminobutyric acid$_A$–activating agents (barbiturates and benzodiazepines).[49]

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