ISOFLURANE INDUCED IMPAIRMENT OF SYNAPTIC TRANSMISSION IN HIPPOCAMPAL NEURONS OF THE GUINEA PIG IN VITRO

by

Peter Miu

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The University of British Columbia
Vancouver, Canada

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Abstract

The effects of anaesthetic applications of isoflurane on 82 CA_1 neurons were studied in in vitro preparations (guinea pigs) using intracellular recording techniques. Various parameters of their excitability such as membrane electrical properties, action potentials and their afterhyperpolarizing potentials as well as synaptic transmission were determined during bath perfusion of clinically relevant concentrtaions of isoflurane. Concentrations of isoflurane were detected in the bath with ^{19}fluorine nuclear magnetic resonance techniques, and were found to range between 0.02 and 0.3 mM. No consistent effects on the membrane properties were observed. When synaptic activity was blocked by tetrodotoxin, isoflurane induced a hyperpolarization (3-5 mV) without affecting input conductance which was computed from the voltage responses to injections of hyperpolarizing current pulses and the slopes of current-voltage relations for each cell. Responses to depolarizing pulses revealed that the threshold, amplitude and duration of the evoked spikes were not greatly altered, although repetitive spike firing was suppressed in a dose-dependent manner by isoflurane. Similarly, the amplitude and duration of the long-lasting hyperpolarizations following the elicitation of multiple (4 or 5) spikes were reduced in a reversible and dose-dependent manner. Reductions in amplitude and duration of excitatory and inhibitory postsynaptic potentials evoked by electrical stimulation of stratum radiatum were observed; these effects also were strictly dependent on the dose, as well as on duration of the application. These investigations have revealed that isoflurane interferes with synaptic transmission in the hippocampal slice preparation and suggest that presynaptic actions on transmitter release, in addition to postsynaptic effects contribute to the mechanisms of the anaesthetic state induced by isoflurane.

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FOR HER LOVE, PATIENCE, AND SUPPORT

THROUGHOUT MY POSTSECONDARY EDUCATION

AND LAST BUT NOT LEAST

MY BEST FRIEND MAHSA

FOR BELIEVING IN ME

(SDIMBYAH)

1. INTRODUCTION

1.1 Historical background

In the early 1800s, surgical procedures were performed only with great risk to the patient. Relief from pain during such operations was attempted by administering alcohol, narcotics such as hashish, and opiates. In desperation, the patient was either rendered unconscious by a blow to the head, or was restrained by force (for those who were less fortunate). Although nitrous oxide (laughing gas) was available, its use was 'purely' for entertainment purposes (Goodman and Gilman, 1985; Miller, 1986)

The analgesic property of nitrous oxide was discovered in 1844 by a dentist, Horace Wells. During an exhibition of its exhilarating properties, he observed that a participant accidently injured himself without feeling any pain while under the influence of nitrous oxide. This discovery enabled Wells to perform painless tooth extraction, using nitrous oxide as the sole analgesic agent. Unfortunately, attempts to persuade his colleagues about these therapeutic effects were unsuccessful. William T.G. Morton in 1846 introduced diethylether as the hypnotic agent for surgical procedures, which subsequently became the agent of choice for general anaesthesia. Nitrous oxide later received extensive use in dental and surgical practices in the United States. Since that time, there have been many developments of new and much more specific anaesthetic/analgesic substances (Goodman and Gilman, 1985).

1.2 Physiological definition of consciousness

A fascinating feature of the general anaesthetic classification is the lack of a stringent requirement in the chemical structure of an agent and its anaesthetic activity (SAR). However, the ultimate effect induced by such agents is unconsciousness, i.e., the anaesthetic state which is clinically defined as a collection of anaesthetic effects in humans.

Presently, an exact definition of consciousness is elusive because of the discrepencies in interpretations of consciousness by proponents of various disciplines, e.g., philosophy, psychology, and physiology. Human consciousness can be divided into two distinct components, the level of consciousness (or wakefulness), and awareness. An index of the level of consciousness is alertness which is maintained by the reticular activating system and its connections to the cerebral cortex. Indices for awareness include attention, expectations, learning, and memory. These attributes of awareness are inextricably linked, and dependent on, anatomically defined regions of the brain such as the limbic system and other specialized regions of the cerebral hemispheres. Hence, communication between various brain structures (e.g., the cerebral cortex and the hippocampus) is crucial for mental processes which invariably include memory. Therefore, a generalized suppression of neuronal excitability in the neocortex and reticular activating system, induced by lesioning or drugs, may produce alterations in consciousness (Ropper and Martin, 1983).

1.3 Anatomical correlates of consciousness

1.3.1 Reticular activating system. The level of wakefulness is maintained by the reticular activating system, consisting of nuclei located in the medial tegmental gray matter of the brainstem which extend from the medulla to the posterior diencephalon. Early experiments demonstrated that stimulation of the reticular formation desynchronizes the electrical activity (EEG), consistent with awakening from sleep (Moruzzi and Magoun, 1949). In contrast, lesions of the mesencephalon or diencephalon abolish the EEG activation associated with wakefulness (Lindsley et al, 1949; Lindsley et al, 1950). Subsequent experiments have revealed that cortical arousal is regulated partly by diffuse and nonspecific ascending projections from the reticular formation directly, or indirectly through the intralaminar and

related thalamic nuclei (Starzl et al, 1951a,b). Hence, the attenuation of wakefulness by hypnotic agents has been proposed to be produced by a depression of the reticular activating system (Lindsley et al, 1949; Lindsley et al, 1950; French et al, 1953; Arduini and Arduini, 1954; Mori et al, 1972; Shimoji et al, 1984).

1.3.2 Cerebral hemispheres. Despite the powerful influence of the reticular formation, wakefulness also depends primarily on the overall functional state of the cortex. For example, an ischemic condition of the brain induces an uncoupling between neuronal metabolism and electrical activity thereby resulting in the disappearance of useful cerebral function or coma (Krnjević, 1975). With the development of radioactive glucose detection techniques, various investigators have demonstrated that anaesthesia is induced by an 'uniform' depression of brain metabolic activity; nevertheless, some regions of the brain respond differently in their changes of metabolism (Sokoloff et al, 1977; Savaki et al, 1983; Peschanski et al, 1986). Functional paralysis of cerebral structures by anaesthetics begins with the cortex and descends to basal ganglia, cerebellum, spinal cord, and medulla (Vickers et al, 1984). Other factors which contribute to cortical dysfunction resulting in alterations in wakefulness include lesion, epilepsy, and hypoglycemia.

1.4 Conscious experience

Physical insults such as diseases, and trauma to the brain commonly lead to an permanent, i.e., irreversible, loss of mental awareness of the internal and external milieux, and subsequent attenuation of cognitive functions. Anaesthetic actions are reversible and furthermore, awareness during surgery (Utting, 1982) as well as the reversibility of anaesthesia indicate a less extensive, and possible more selective, assault on cortical functions in contrast to that observed with physical damage. The transient postanaes-

thetic depression of cognitive functions (e.g., learning and memory) is a relatively common observation in patients; however, prolonged inhibition of cerebration (i.e., the residual depression of the central nervous system -- CNS), lasts several hours, and is more prevalent or pronounced in elderly patients (Davenport, 1986). Hence, the effects of anaesthetic state on human cognitive functions may terminate less rapidly than other, more obvious criteria for consciousness, e.g., the level of wakefulness (Miu and Puil, 1988).

1.4.1 Mental processes. Learning and memory are a consequence of associations between sensory experiences and diverse brain structures. The exact regions of the brain in which these processes take place are still a mystery. Nevertheless, learning is assumed to be associated with cerebral hemispheres, despite the observations that in some animal species without cortex, the capability of learning is retained. In humans, impairment in the capacity for new learning is associated with the lesioning or destruction of primarily the neocortex. Furthermore, clinical studies have provided valuable information on the relationship between the location of cortical lesions and the resulting cognitive deficiency. For example, Meyer and Yates (1955) noted evidence of defective learning ability with temporal lobe epilepsy. Later, Scoville and Milner (1957) demonstrated that the temporal lobes and hippocampal formations are involved in learning and retrieval of learned responses from memory.

1.4.2 Molecular mechanism. The molecular mechanism of learning and memory has been the subject of intensive examinations, and the results indicate that learning or conscious experience produces anatomical, biochemical, and electrophysiological changes at synapses. Eccles (1965) hypothesized that conscious experience facilitates growth of pre-existing synapses. This hypothesis was supported by the findings that rats placed in an enriched

environment (i.e., presence of stimulating objects) develop a greater of the cerebral cortex, i.e., in actual cortical weight (Rosenzwieg and Bennett, 1969) as well as a greater numbers of spines per unit of length of dendrite (Globus et al, 1973) than animals placed in an impoverished environment. In addition, other investigations have shown that significantly greater dendritic branching develops in animals exposed to the enriched environment (Volkmar and Greenough, 1972; Greenough and Volkmar, 1973). The transformation from temporary, to more permanent synaptic contacts occurs with repeated activation of the same pathways, hence the concept of consolidation of memory. Cotman and Nieto-Sampedro (1982) concluded that synapses throughout the nervous system have a continuously high turn over rate, and that learning stimulates this ongoing plasticity.

Behavioral plasticity and cognitive processes, manifest as electrical signals, occur in the associative cortex, different from the primary motor and sensory area (Pandya and Seltzer, 1982). Also, the extensive inter-connections between the associative cortex and hippocampus (Teyler and DiScenna, 1984b) suggest that the learned responses are transmitted to the hippocampus where consolidation occurs by a mechanism of long-term potentiation (LTP) (Teyler and DiScenna, 1984a).

1.5 Objectives

Pauling (1961) believed that both consciousness and memory were the result of electrical oscillations in the brain. This view is extended by the proposal that self-consciousness (awareness of internal self) would not occur in the absence of memory (Krnjević, 1974; Popper and Eccles, 1977). Because of the likely possibilities that anaesthetics affect the consolidation and retrieval of learned responses and that their persistent effects produce the slow return of cognitive processes during the postoperative period, we hypothesized that anaesthetic agents may disrupt mnemonic communication by direct actions on neocortical and archicortical structures.

Although the importance of the neocortex in these processes appears paramount (cf. above), the *in vitro* slice preparation of the hippocampal formations in experimental animals confer many advantages over similar preparations of neocortex. For example, the well-defined anatomical organization of the hippocampus allows easy visualization and identification of the perikarya for experimental studies involving intracellular penetration with microelectrodes. The lamellar organization of fibres in the hippocampus facilitates the isolation of selective pathways that can be stimulated, i.e., stimulation in the stratum radiatum evokes orthodromic responses in the CA_1 cells. An important feature of the hippocampus, or at least the slice preparations of hippocampus, is the relative resilience to hypoxic conditions inadvertly incurred by excision of the tissue.

The choice of anaesthetic in these investigations was isoflurane which is a relatively new agent that has come into prominent clinical use during the past 5 years in North America. Hence, the effects of isoflurane were investigated on the membrane electrical properties and activities of pyramidal cells in *in vitro* slice preparations of the hippocampus. The emphasis in these investigations was to determine, using intracellular recording techniques, the actions of isoflurane on the membrane excitabilities and synaptically evoked potentials in the CA_1 neurons. Specifically, we were interested in determining the actions of the anaesthetic on excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs). Furthermore, ^{19}fluorine-NMR techniques were used to estimate the exact concentrations of isoflurane in the solution bathing the hippocampal tissue.

2. METHODS

2.1 Animal preparation

The experiments were performed on in vitro slice preparations of hippocampus obtained from albino guinea pigs of either sex, weighing 200-300 g. An animal was anaesthetized initially with 4% halothane in a closed 2-liter chamber. Following loss of the righting reflex, 4 % (v/v) halothane was administered by mask (O_2 flow rate, 1 liter/min). Tracheotomy was performed in order to assist ventilation, and halothane was subsequently delivered endotracheally. Sometimes, artificial ventilation was employed. The concentration of halothane required for the maintanence of anaesthesia was titrated against spontaneous ventilation and responses of the animal to painful stimuli induced by surgical manipulations. The usual dose of halothane administered at this stage varied from 1 to 2% (v/v). The animal then was placed in a stereotaxic head holder and an extensive bilateral craniotomy was performed. Care was taken during this procedure so as to preserve the integrity of the dura since it was consistently observed that tearing of the dura while removing the skull always led to uncontrollable subdural hematoma. After removal of the dura, the cerebral cortices were aspirated to reveal the underlying hippocampi.

Each hippocampus was undercut in one motion with a large scalpel blade and quickly immersed in pre-cooled ($\sim$4°C) artificial cerebrospinal fluid (ACSF) oxygenated with 95/5 % gaseous mixture of O_2/CO_2. The constituents of the ACSF were (in mM): NaCl, 124; KCl, 3.75; KH_2PO_4, 1.25; $MgSO_4.7H_2O$, 2; $CaCl.2H_2O$, 2; dextrose, 10; $NaHCO_3$, 26. Thick (400 μm) transverse slices of the hippocampus were cut with a McIlwain tissue chopper. One slice was 'sandwiched' in nylon mesh and placed in the recording chamber (Fig. 1). The remaining slices were kept at room temper-

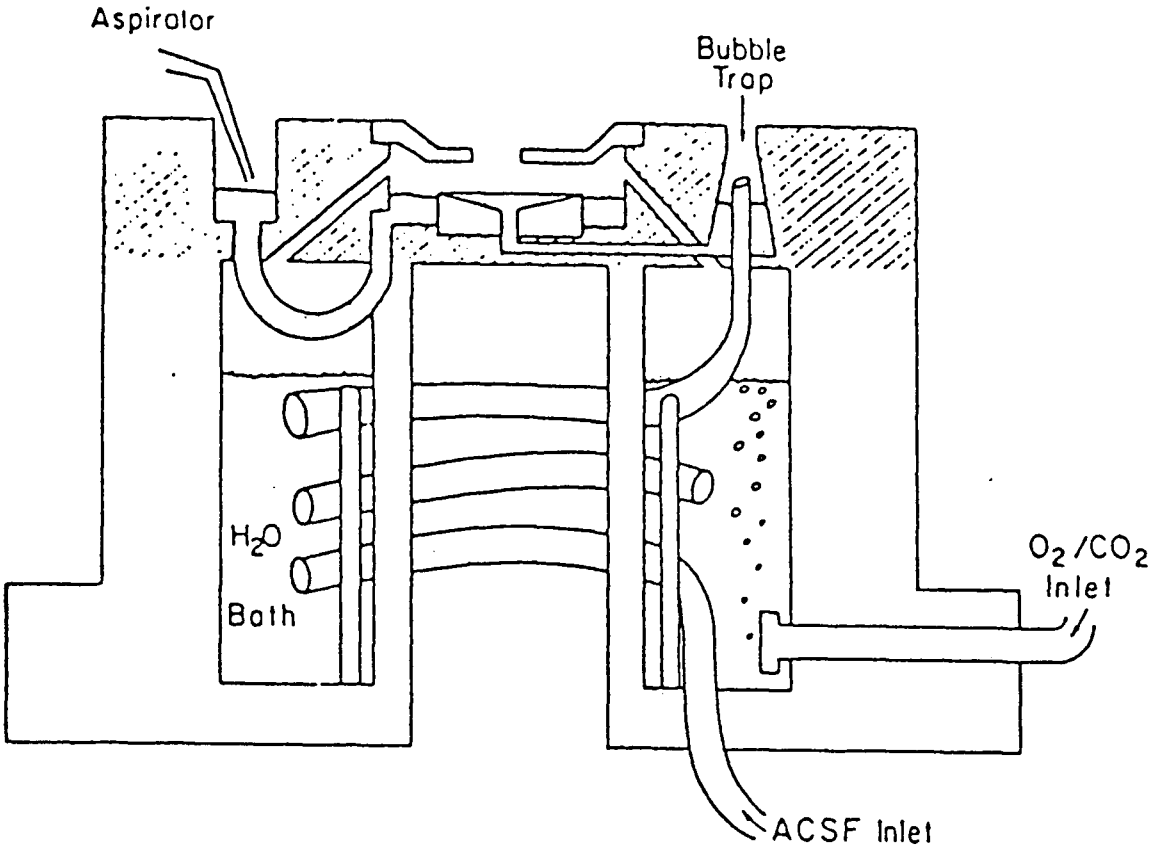


FIG. 1. Schematic drawing of a similar type of perfusion chamber for intracellular recordings.

ature (22-24°C) in ACSF, bubbled continuously with 95/5% of O_2/CO_2 mixture, until needed. The temperature of the bath was maintained at 32°C by a thermoregulator throughout the experiment. The slice was perfused in the recording chamber at a constant rate of 2-3 ml/min with oxygenated ACSF (pH 7.4). Humidified gas (95% O_2/ 5 % CO_2) was passed into the atmosphere immediately above the slice.

2.2 Administration of isoflurane

Since the total volume of the recording chamber was 1 ml, the perfusate was rapidly exchanged within about 1 min, with fresh ACSF solution. Isoflurane was applied in the concentrations indicated by the output settings of several Fluotec-3 vaporizers using 95 % O_2, 5% CO_2 at a flow rate of more than 500 ml/min. The ACSF in several reservoirs consisting of inverted syringes (60 ml volume) was bubbled with the gas mixture for a minimum of 20 min to ensure a maximal equilibration with the anaesthetic at a designated concentration before its application by perfusion to the slice.

2.3 Determinations of isoflurane concentrations

The concentrations of isofurane in the bathing solution were determined using the ^{19}fluorine-nuclear magnetic resonance (^{19}F-NMR) technique. Aliquots (1.6 ml) of the perfusate, pre-equilibrated with a particular concentration of isoflurane indicated by the setting of the calibrated vaporizer output, were collected from the recording chamber with an air-tight syringe and injected into NMR sampling tubes containing 0.3 ml of 20 mM trifluroacetic acid (TFA) (final concentration of TFA in the sampling tube was 2.5 mM) and 0.5 ml of deuterium oxide. The NMR tubes were then tightly capped and analyzed in the Department of Chemistry at room temperature using an NMR spectrometer (270 MHz). Field lock was used with D_2O as the locking signal. Signals were averaged from 3000 scans/sample with the sweep width set to 6000 Hz Sometimes, more signals were averaged to reduce back-

ground noise. Proton-decoupling was used with an offset frequency of 4000 Hz. The ^{19}F-NMR signals generated from TFA (internal standard) and isoflurane were Fourier transformed, and the size of individual peak integrated. The concentration of isoflurane in the perfusate was determined by taking the ratio of the integrals from the internal standard and the second peak, -4.8 ppm, (i.e. tri-fluorine atoms of isoflurane), multiplied by the concentration of the internal standard:

$$[IFL] = IFL/TFA \times [TFA].$$

2.4 Recording techniques and arrangement

Intracellular recordings began approximately 1 hr after placing the slice in the recording chamber. Microelectrode, mounted on a Narishige micromanipulator, was positioned into the CA_1 region of the hippocampal slice under visual guidance with a dissecting microscope. The microelectrode was rapidly advanced into the first 150 μm of the slice because it had been assumed that most of the pyramidal cells near the surface were either damaged or destroyed by the tissue chopper. Thereafter, the microelectrode was lowered with smaller advancing increments (approximately 1 μm steps) until a cell was found. Successful penetration of the recording electrode was achieved by 'buzzing' the cell with the electrode capacitance neutralization control on the amplifier.

Conventional intracellular recording techniques were used with a WPI amplifier (Model M701) connected to an oscilloscope (Tektronic RM 565). CA_1 neurons were impaled with single barrel glass micropipettes (1 mm diameter tubing) containing 3 M KCl or with 4 M potassium acetate (KAc) for experiments on evoked synaptic potentials. The tip resistances of 3 M KCl electrodes, measured in the tissue before penetrating the cell, ranged from 40 to 60 MΩ, whereas 4 M KAc electrodes ranged from 60 to 80 MΩ. Membrane input resistances were estimated with bridge-balance techniques from voltage

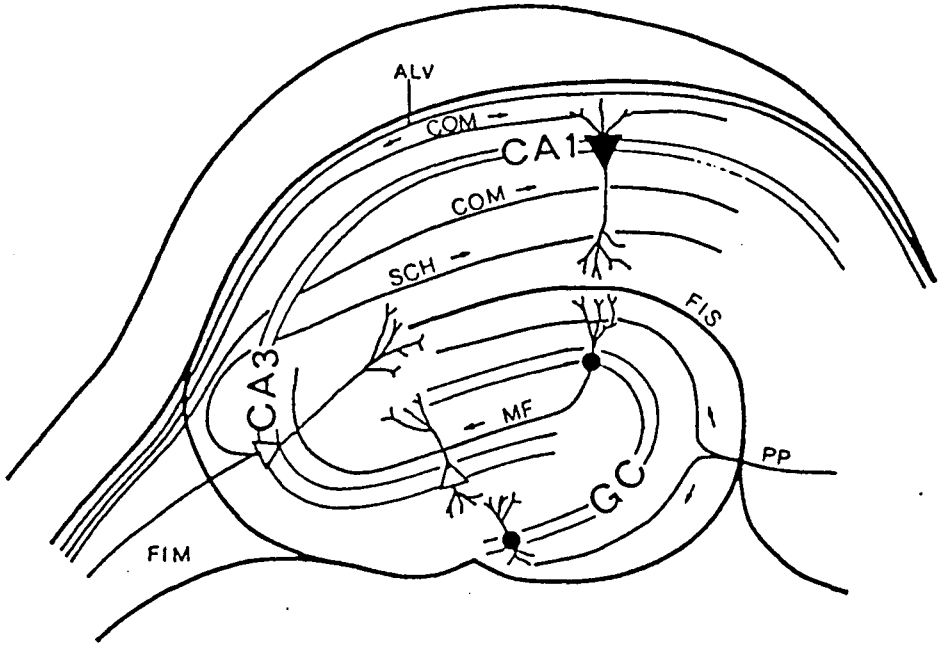
responses to intracellular injections of constant current pulses (100 ms duration delivered at 1 Hz). Resting membrane potential was monitored continuously with a chart recorder, and zero voltage level was determined after withdrawing the electrode from the cell.

Amplified intracellular signals were digitized (PCM-1 digital VCR-instrumentation recorder adaptor) and stored on super VHS tape with a Canon recorder (model VR-HF 600) for off-line playback and analysis by computer. Some of the recorded intracellular signals (in analog form) were analyzed with a waveform recorder (Biomation 805) which has the capability of digitizing the input voltage sweep into 2048 points thus permitting manual control of digital-to-analog conversion for display on the oscilloscope, or reproduction of the sweep on paper of an X-Y pen recorder (Hewlett-Packard 7015B).

For experiments on synaptic potentials, a tungsten bipolar stimulating electrode was place in the stratum radiatum region of the hippocampal slice (Fig. 2). Stimulation frequency was set to 0.2 Hz. and the stimulation strength ranged from 10-15 V.

2.5 Data acquisition

Intracellular voltage signals, played back from Canon tape recorder, were fed into 'ERAT', an interface panel, which converts the input signals in the analog form to the digital form. The data were stored temporarily in the memory bank of MINC-23 computer during the data acquisition. After completion of this phase, the files were transferred and stored for the actual analysis procedures in another computer (PDP 11/44) which has a larger memory capacity.



ALV: Alveus
COM: Commissural fibres
FIM: Fimbria
FIS: Hippocampal fissure
GC: Granule cell body layer
MF: Mossy fibres
PP: Perforant path
SCH: Schaffer collaterals

FIG. 2. Schematic drawing of the hippocampal slice.

2.6 Computer analysis

Individual traces of synaptic transients in the raw data file of MINC were selected, and approximately 17 to 25 traces in each of the three groups (control, isoflurane, and recovery) were chosen from the raw data file for the averaging program. A new file was subsquently created for the averaged traces, and various parameters of the synaptic transients from these averaged traces were analyzed, e.g., amplitude of both EPSPs and IPSPs, and changes in resting potentials.

2.7 Statistics

Most data were tested statistically with one-way analysis of variance (ANOVA) for overall significance. Group comparisons were performed using post hoc comparison test. Sample size of all the experiments was 5 or more, unless otherwise stated. The accepted level of significance is $p<0.05$.

3. RESULTS

3.1 Electrical membrane properties of CA_1 pyramidal cells

Only cells with stable resting potentials and a relative constant input resistance measured over 20 min were selected for isoflurane administration. Stable recordings were obtained from 82 out of 100 CA_1 pyramidal cells. Within 5 to 10 min following cell penetration by the recording electrode, resting potential usually hyperpolarized and eventually stabilized at a more negative value. Concomitantly, input resistance, measured by injecting 100 ms hyperpolarizing pulses, also increased and thereby indicated proper sealing of the membrane around the microelectrode. The mean resting potential obtained from a random sampling of 23 cells was -65.4 ± 1.3 mV. Input resistance was usually greater than 50 MΩ (cf. Fig. 4). Both spontaneous, and single spikes evoked by intracellular injections of depolarizing current, had peak amplitudes of more than 80 mV. The recordings generally lasted 1-5 hours, and most cells were exposed to more than one dose of isoflurane (Table 1).

3.2 Isoflurane concentrations

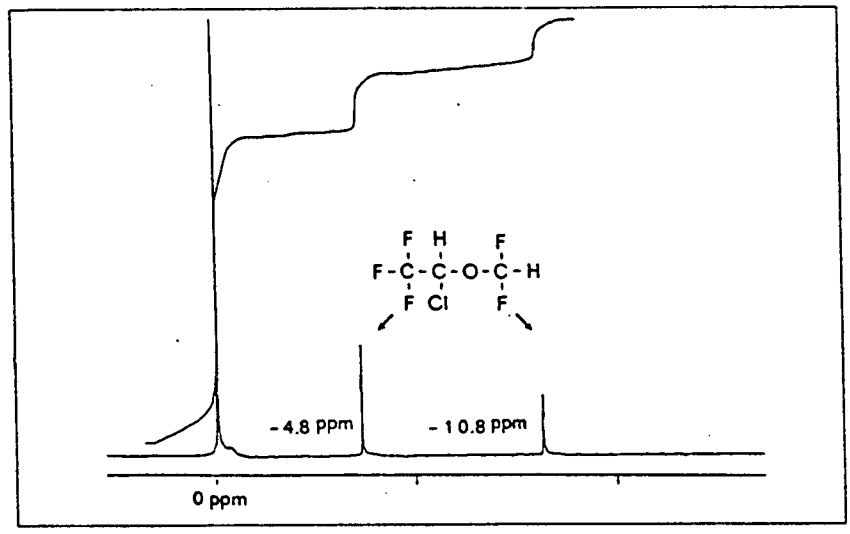
To ascertain the amount of isoflurane that had been dissolved in the perfusate, samples of ACSF containing different concentrations of isoflurane were collected from the recording chamber and analyzed by spectrometer with radiofrequency of 270 MHz. An example of the ^{19}F-NMR spectrum obtained from 4% isoflurane dissolved in ACSF is illustrated in Fig. 3A. The first peak represents the internal standard (2.5 mM TFA). The second and third peaks, downfield from the TFA peak, occurring at -4.8 ppm and -10.8 ppm respectively, represent tri- and di-fluorine atoms of isoflurane. The concentration of isoflurane in the perfusate was calculated by taking the

IFL conc. (Vol. %)	No. of cells	No. of applications
0.5	15	28
1.0	18	28
1.5	5	6
2.0	20	49
3.0	12	20
4.0	12	17
Total	82	148

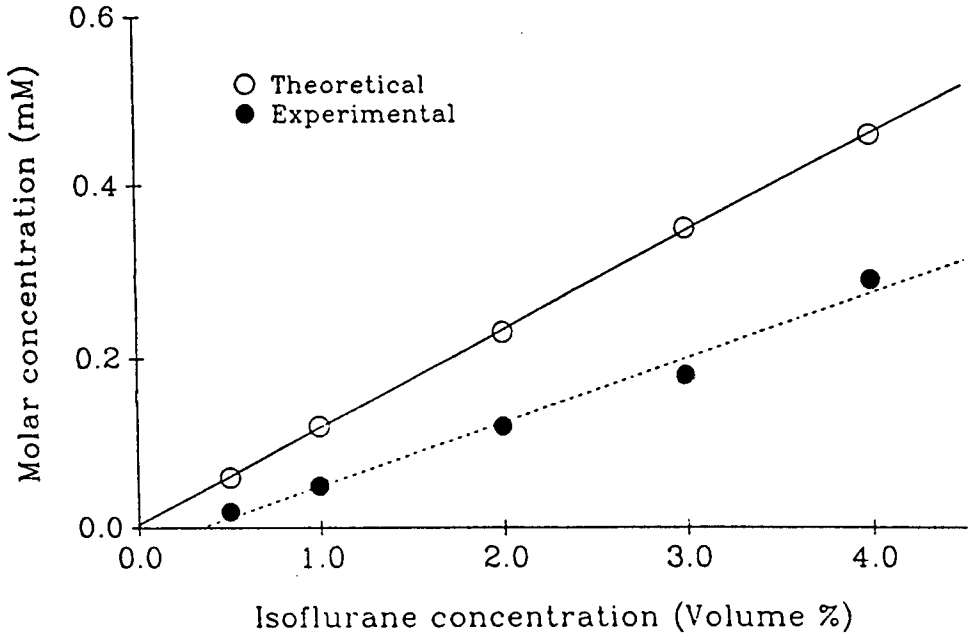
Table 1. Number of isoflurane applications.

FIG. 3. Isoflurane concentrations as determined by ^{19}fluorine nuclear magnetic resonance techniques (^{19}F-NMR). (A) ^{19}F-NMR spectra of ACSF containing 4% isoflurane which was collected from recording chamber. The first peak (0 ppm) represents TFA, internal standard. The second (-4.8 ppm) and third (-10.8 ppm) peaks represent tri- and di-fluorine atoms of isoflurane respectively. Spectra analyzed by spectrometer with radiofrequency 270 MHz. (B) Theoretical and experimental concentrations of isoflurane plotted against vaporizer output settings.

A


F H F
F-C-C-O-C-H
F Cl F
-4.8 ppm
-10.8 ppm
0 ppm

B


Molar concentration (mM)
0.6
0.4
0.2
0.0
Theoretical
Experimental
0.0
1.0
2.0
3.0
4.0
Isoflurane concentration (Volume %)

ratio of the integrals under the peaks (cf. Methods), and the vaporizer output settings from 0.5-4% were subsequently converted to give corresponding NMR concentrations which ranged from 0.02 to 0.3 mM (Fig. 3B).

Also included in Fig. 3B is the calculated or theoretical values. Comparison of the theoretical values with those empirically determined reveals that 40 % of isoflurane was lost in the perfusate. This can be attributed to difficulties in the collection procedure, e.g. improper sealing of the sampling tube, leakage before actual measurement, etc. Also, the large concentration difference between the solution containing isoflurane and the atmosphere above the orifice of the recording chamber may facilitate rapid evaporation. This process is, of course, facilitated by the large concentration gradient existing between the atmosphere and the perfusate containing dissolved isoflurane. In addition, the lipid solubility of the slice preparation is greater than that of the ACSF.

3.3 Spikes and membrane potentials

Spontaneous firing in 20 pyramidal cells, exposed to approximately 6 min of various concentrations of isoflurane, were analyzed, and the results are summarized in Table 2. Isoflurane concentrations ranging from 0.5 to 4% were applied. At low doses, three different responses were observed, i.e., reduction, no change, and augmentation in the rate of spontaneous firing. The percentage of cells that responded to 0.5 % isoflurane applications with a reduction in the rate of spontaneous firing was 33 % (Table 2). With increasing isoflurane concentrations, however, the number of cells with a reduced firing rate was greater and the reduction reached a maximum value of 60% in the presence of 3 % isoflurane. In addition, the number of cells with unaltered firing rates was decreased with increasing anaesthetic concentrations. That is, with applications of 3 and 4%, isoflurane either enhanced or decreased the rate of spontaneous firing. The amplitudes and durations

IFL conc. (Vol. %)	Cell no.	Spontaneous firing	No. applications resulted in reduced firing (%)
0.5	1	-	
	4	-	
	2	+	
	3	+	
	2	0	
	3	0	33
1.0	4	-	
	6	-	
	1	+	
	5	+	
	1	0	40
2.0	3	-	
	10	-	
	11	-	
	12	-	
	13	-	
	14	-	
	7	+	
	7	+	
	7	0	
	8	0	
	9	0	55
3.0	6	-	
	12	-	
	17	-	
	15	+	
	16	+	60
4.0	6	-	
	13	-	
	20	-	
	18	+	
	19	+	60

-: reduction
+: augmentation
0: no change

Table 2. Effects of isoflurane on spontaneous firing of CA_1 pyramidal neurons.

of single spikes were unaltered by isoflurane administration (data not shown).

Various concentrations of isoflurane were applied to 50 pyramidal cells. The changes in their resting potentials measured at 6 min of isoflurane perfusion are summarized in Fig. 4A. The columns in this figure represent the percentage of the total number of applications for the particular application. Isoflurane administration in low concentrations did not elicit a change in membrane potential in many neurons. However, the majority of neurons were depolarized by low doses, whereas doses of 2% and higher hyperpolarized most cells. The only exception was the 1% applications where a majority of the cells responded to isoflurane with no change in membrane potential. In several experiments, 1 μM tetrodotoxin (TTX) was added to the perfusate to eliminate the influences of released synaptic transmitter on the resting potential. The results showed that 12 out of 12 cells were hyperpolarized by isoflurane in concentrations of 1.0-4.0% (data not shown). One cell responded to 4 % isoflurane application with membrane hyperpolarization while the other cell depolarized.

3.4 Input resistances and voltage-current relations

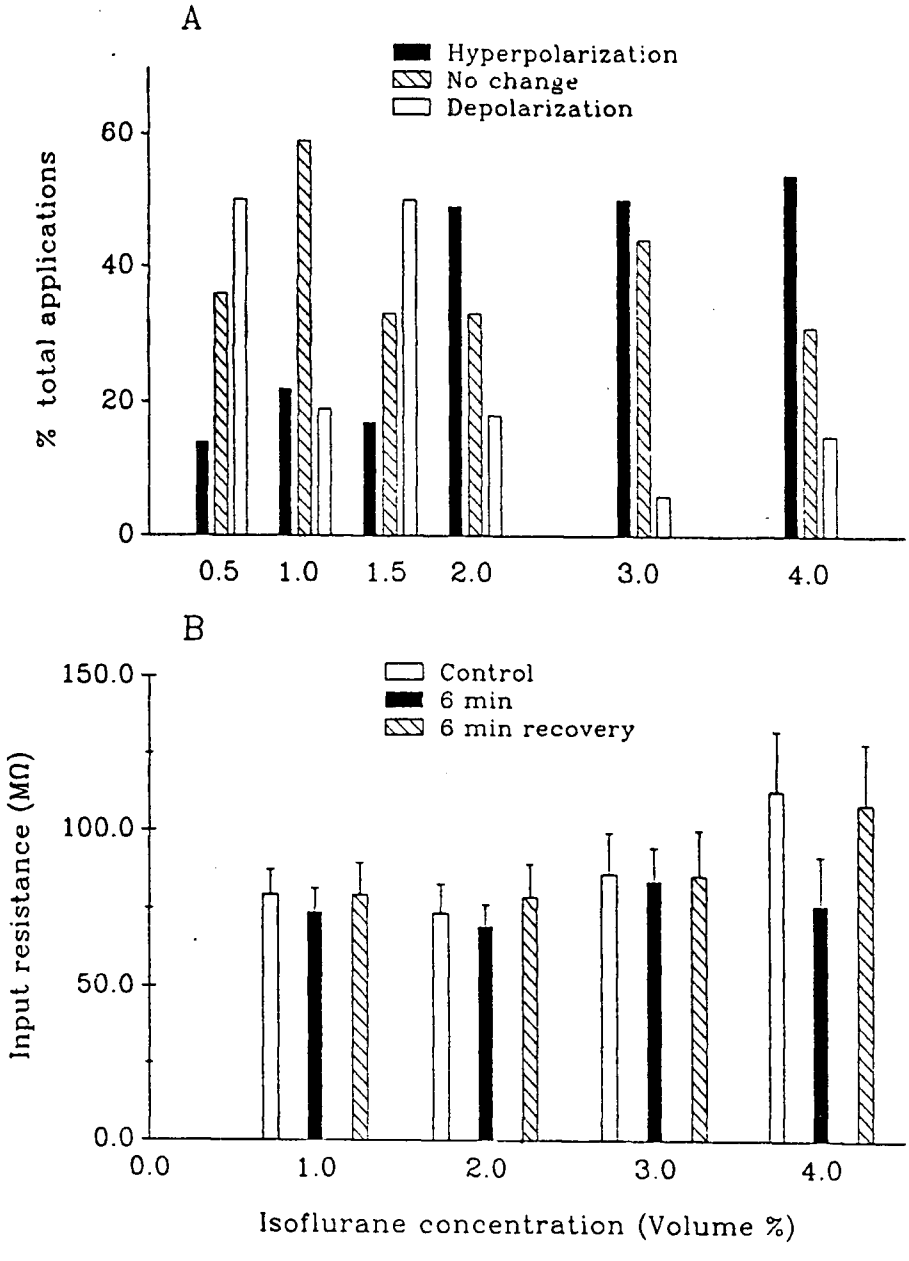
Instantaneous membrane input resistance was measured by injecting hyperpolarizing current pulses at 1 Hz through a bridge balanced recording electrode into the soma of CA_1 pyramidal cells. Subsequent voltage deflection was measured, and membrane input resistance was calculated using the Ohm's law:

$$V = IR$$

where V is membrane voltage response to current injection, I is the current injected, and R is the input resistance.

Isoflurane, in concentrations ranging from 1.0 to 3.0%, did not reduce input resistance significantly (Student t-test). However, input resistance

FIG. 4. Isoflurane actions on passive electrical membrane properties of hippocampal CA_1 pyramidal cells. (A) Distribution of pooled resting potential responses (82 neurons) to various doses of isoflurane. Resting potentials recorded after 6 min of isoflurane perfusion. KCl electrode. (B) Input resistance values of CA_1 cells, obtained from small (<10 mV) voltage responses to hyperpolarizing current pulses. Means ± SEM.


A
Hyperpolarization
No change
Depolarization
% total applications
60
40
20
0
0.5
1.0
1.5
2.0
3.0
4.0
B
Control
6 min
6 min recovery
Input resistance (MΩ)
150.0
100.0
50.0
0.0
0.0
1.0
2.0
3.0
4.0
Isoflurane concentration (Volume %)

was reduced significantly with the 4% dose. It is conceivable that the observed reduction in input resistance may not be a direct consequence of anaesthetic action since large spontaneous depolarization of unknown origin occurred in 2 out of 7 cells studied, thereby contributing to a large decrease (i.e., rectification) in the mean input resistance. These data are summarized for 19 cells in Fig. 4B.

In addition to the instantaneous input resistance measurements, voltage-current relations were also examined in the presence of isoflurane. A total of 7 cells were exposed to 12 min applications of 2% isoflurane by perfusion. Membrane voltage deflections corresponding to increasing hyperpolarizing current pulses were measured and calculated using the Ohm's law decribed earlier. The data were pooled and summarized in Fig. 5A which shows that the slope resistances were not altered significantly. The magnitude of the reduction in slope resistance was approximately the same as that measured by the steady-state voltage response to constant current injection (cf. Fig. 4B).

The voltage value at the point where the control slope intercepts with the slope obtained during anaesthesia is 20 mV, which when added to the average resting potential of these cells gives -75.5 mV. Since the theoretical reversal potential for potassium is approximately -80 mV, the close approximation of the empirical value suggests that perhaps isoflurane may alter resting K^+-conductance (g_K). The voltage-current relations were also studied in the presence of 1 μM tetrodotoxin (TTX). Isoflurane was applied 4 times to 2 cells, and the results are shown in Fig. 5B. In contrast to the previous voltage-current relations, there is slight membrane rectification with large hyperpolarizing current injections. Nonetheless, a significant alteration in slope resistance also was not observed with TTX-blockade of endogenous synaptic activities. In Fig. 6, 6 min of 3%

FIG. 5. Voltage-current (V/I) relationships of CA_1 pyramidal cells. Open circles represent the peak voltage responses in ACSF, and closed circles represent the peak voltage responses recorded after 12 min of 2% isoflurane perfusion. (A) Steady-state voltage responses to hyperpolarizing current injections (7 neurons) in the absence of TTX (1 μM). (B) Steady-state voltage responses to hyperpolarizing current injections (4 neurons) in the presence of TTX (1 μM). Means (2 to 5 cells each) ± SEM are given in neurons that were injected with identical current pulses.

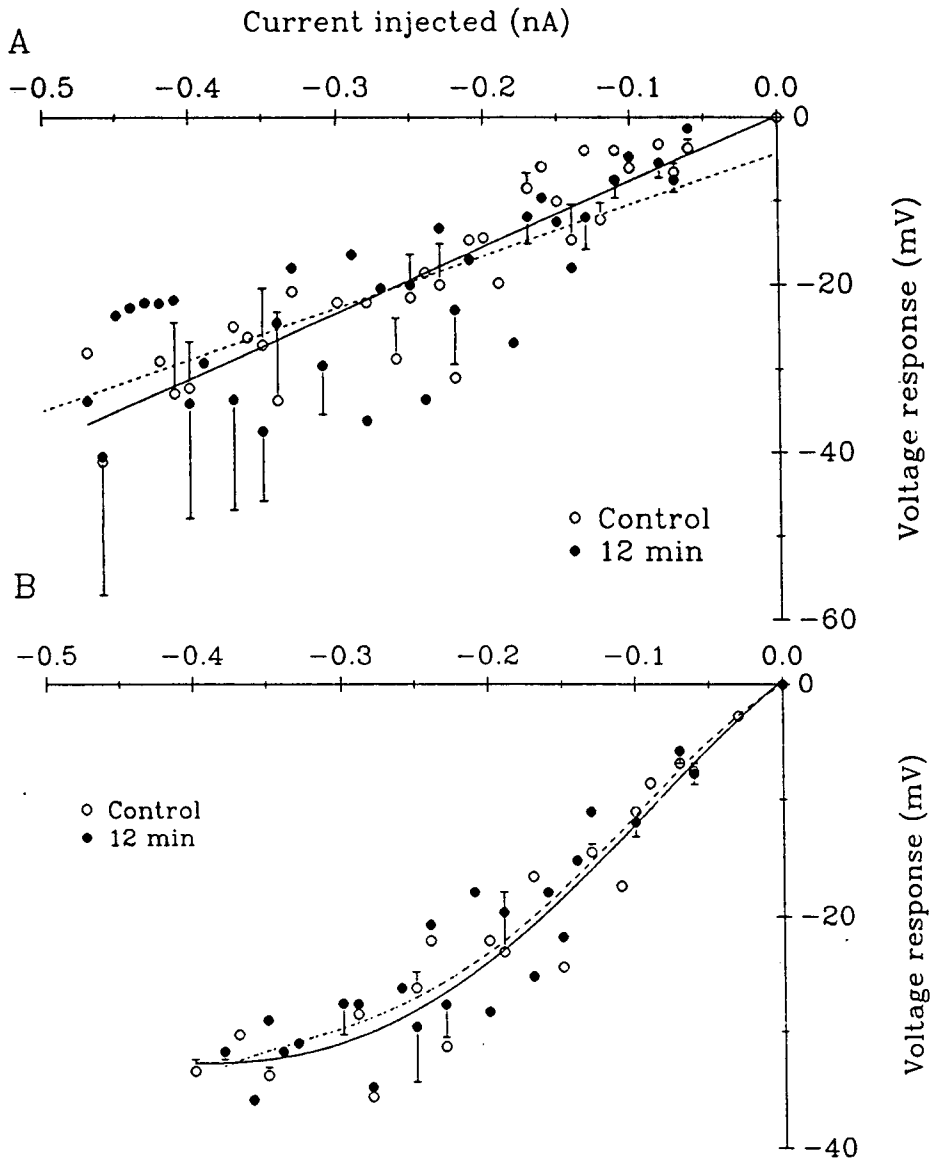
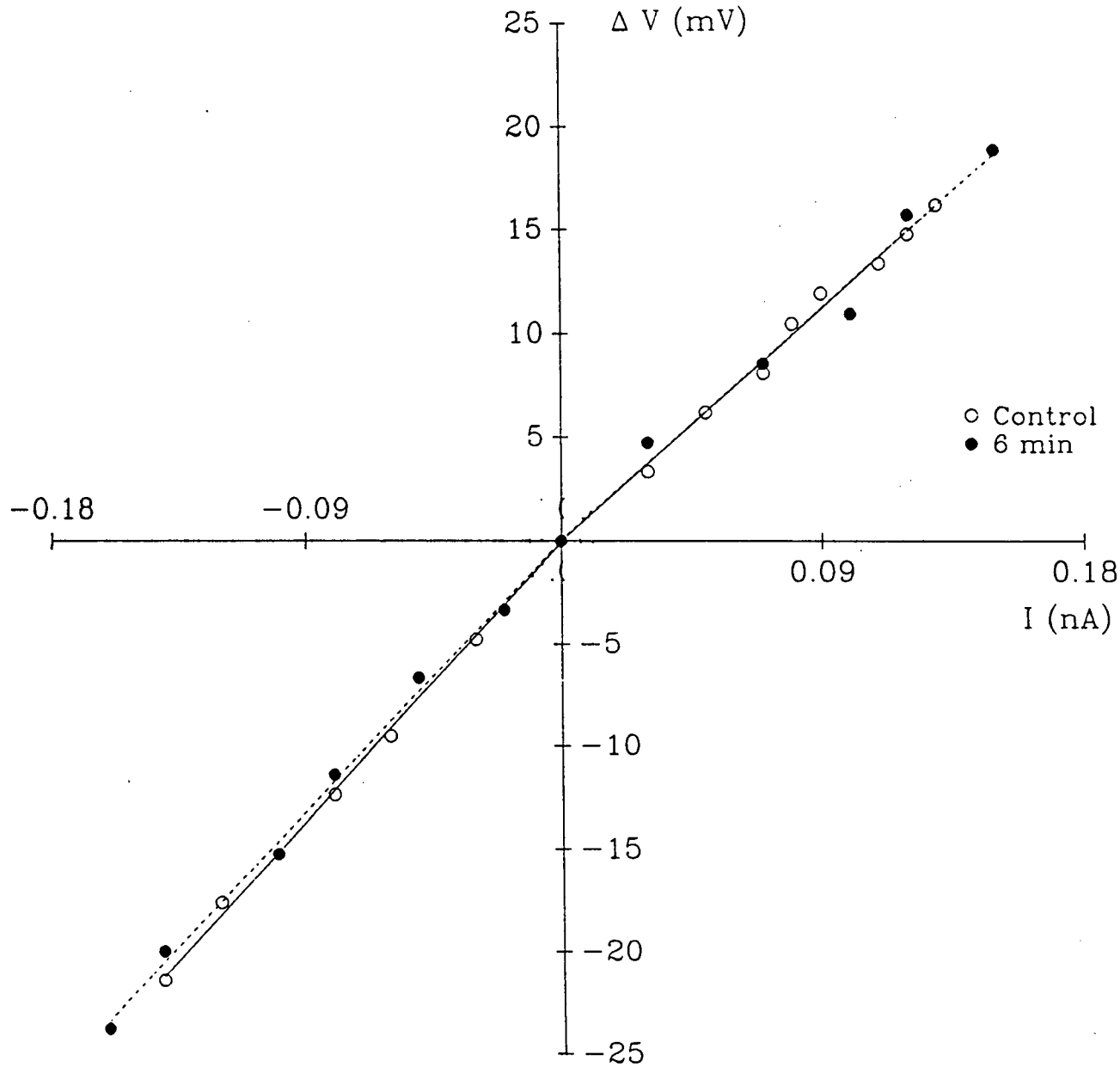

A
Current injected (nA)
−0.5
−0.4
−0.3
−0.2
−0.1
0.0
0
−20
−40
−60
Voltage response (mV)
Control
12 min
B
−0.5
−0.4
−0.3
−0.2
−0.1
0.0
0
−20
−40
Voltage response (mV)
Control
12 min

FIG. 6. Voltage-current (V/I) relation of one CA_1 pyramidal cell, in the presence of 1 μM TTX. Open circles represent the peak voltage responses in control ACSF, and closed circles represent the peak voltage responses recorded after 6 min of 3% isoflurane perfusion.


25
Δ V (mV)
20
15
10
5
−0.18
−0.09
0.09
0.18
I (nA)
−5
−10
−15
−20
−25
Control
6 min

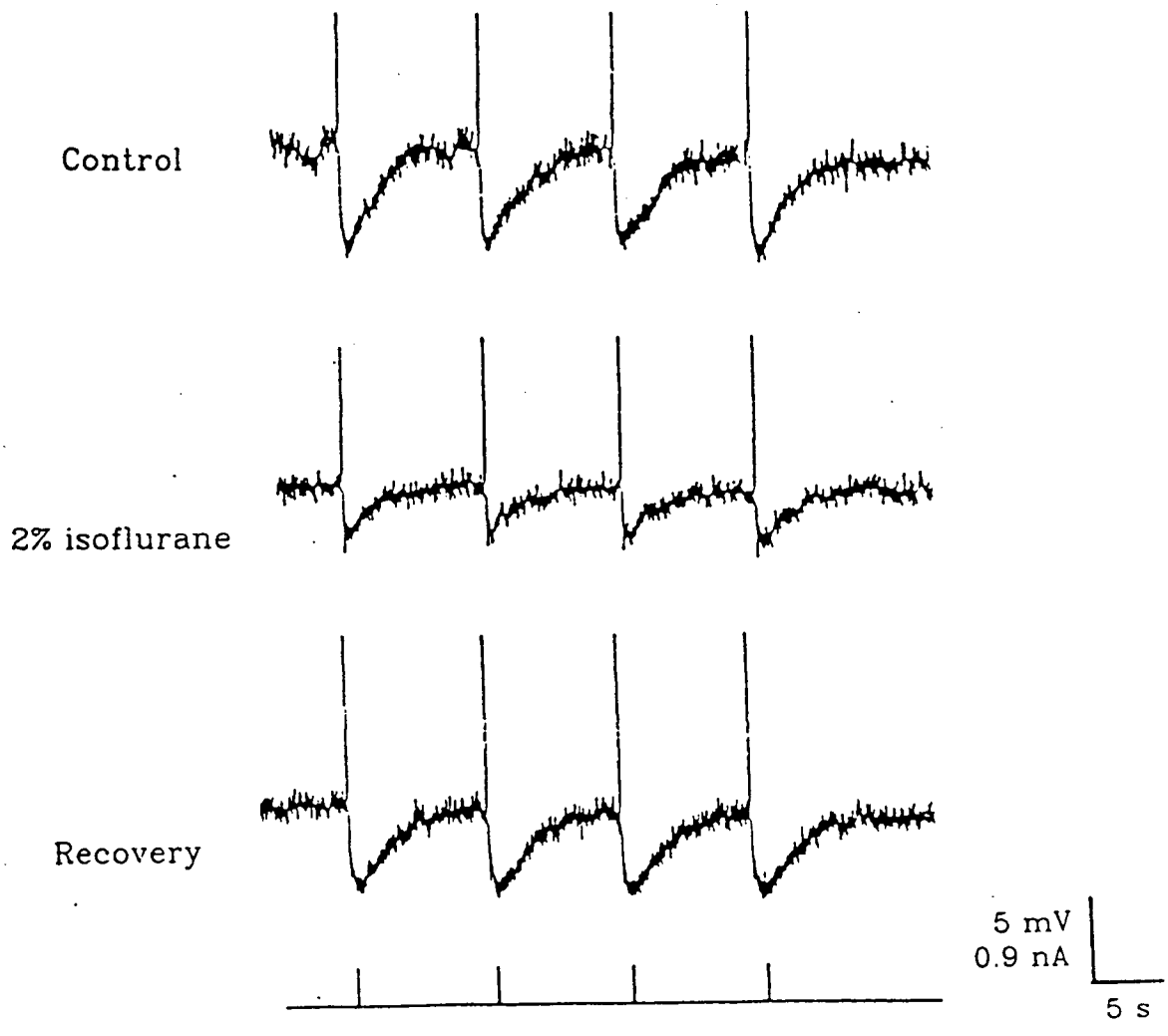
isoflurane perfusion, again, did not alter membrane slope resistance significantly. With injections of depolarizing current pulses, a slight outward rectification was observed. This figure further illustrates that this cell did not show anomalous rectification in either the depolarizing or hyperpolarizing direction, when compared to the two previous cells in which 2 % isoflurane had been administered. Hence, the electrical properties of the membrane were assumed to obey Ohm's law.

3.5 Postspike afterhyperpolarizing potentials (AHPs)

AHPs were generated by injecting suprathreshold depolarizing current pulses once every 10 s to elicit multiple spikes, i.e. usually 4 or 5 spikes. The spikes in each train were usually more than 80 mV amplitude. The average peak amplitude of the AHPs in ACSF was 3.1 ± 0.2 mV (n=35) (cf. Table 3), and the average duration of the AHPs was 4.1 ± 0.4 mV (n=28) (cf. Table 4). Both the amplitudes and durations of the AHPs were measured in 20 cells exposed to multiple applications of various concentrations of isoflurane. In the representative cell of Fig. 7, the amplitudes of the AHPs were reduced at 6 min of 2% isoflurane perfusion. Full recovery to control values was observed in this cell, and 17 other neurons at 6 min after returning the perfusion to normal ACSF. A summary for these cells is given in Fig. 8A, and Table 3 (Tables 3 and 4 represent the actual values of AHP amplitude and duration, in mV and seconds). As indicated in the Figure, the peak amplitude was slightly reduced at 3 min of isoflurane perfusion. However, profound reductions in the peak amplitude of the AHPs were observed at 6 min of isoflurane perfusion. The magnitude of reduction, obtained with application of 2-4 % isoflurane, was dose-dependent and statistically significant (Student t-test).

In contrast, dose-dependent reductions in AHP durations were not observed with increasing isoflurane concentrations (Fig. 8B, and Table 4).

FIG. 7. Postspike afterhyperpolarizations (AHPs). AHPs were elicited by passing depolarizing current pulses (0.1 Hz) of 4 nA, 100 ms in a CA_1 neuron. 6 min perfusion of 2% isoflurane reduced both the amplitudes and durations of AHPs. Full recovery to control values was observed at ~6 min after returning the perfusion to normal ACSF. KCl electrode recording.


Control
2% isoflurane
Recovery
5 mV
0.9 nA
5 s

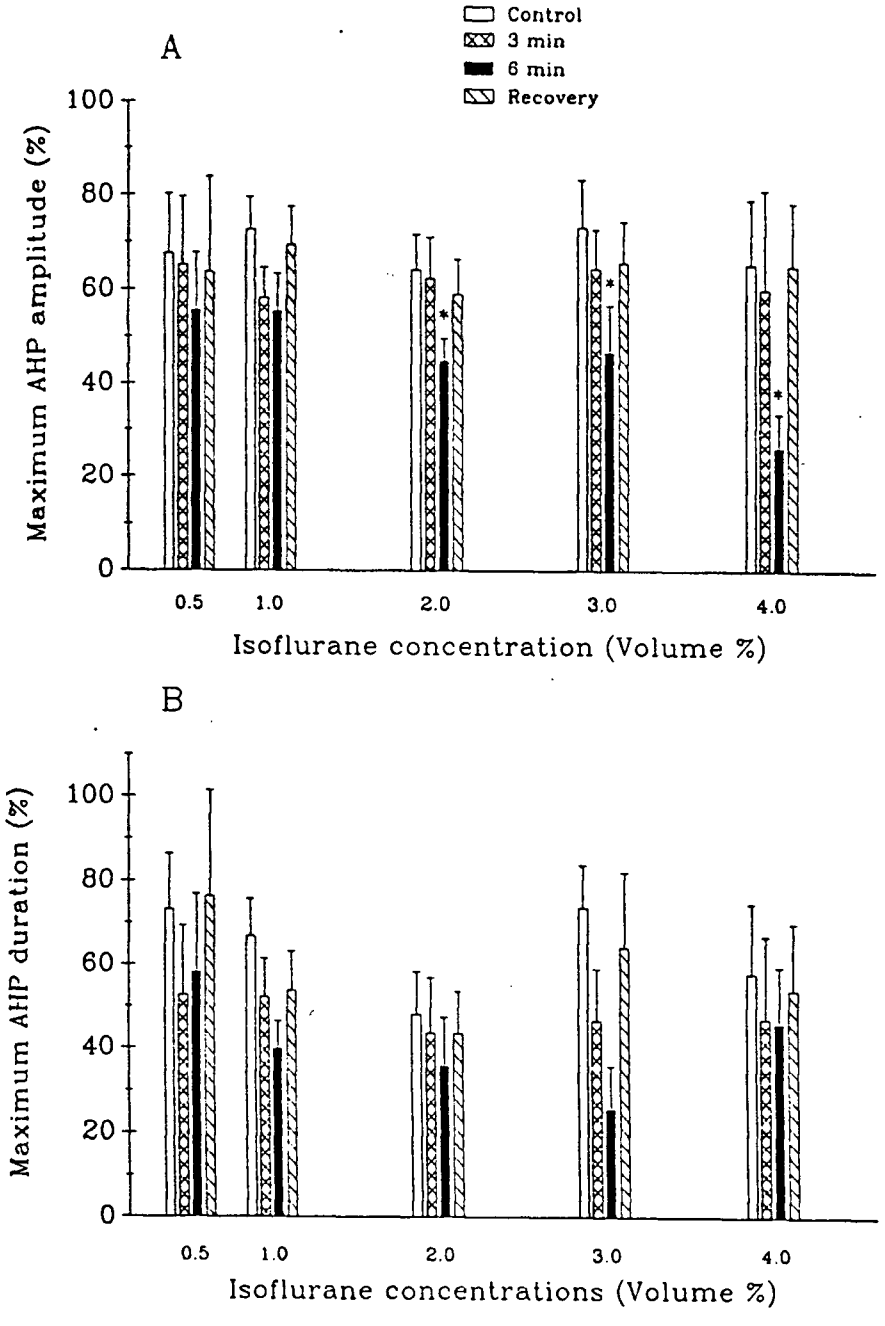
AHP amplitude (mV ± SEM, no. of applications)				
IFL (Vol. %)	Perfusion time (min) Control	Isoflurane 3	6	Recovery 6
0.5	3.0 ±0.6 (5)	2.9 ±0.7 (5)	2.5 ±0.5 (5)	2.9 ±0.9 (4)
1.0	2.7 ±0.3 (10)	2.1 ±0.2 (10)	2.0 ±0.3 (9)	2.5 ±0.3 (10)
2.0	4.0 ±0.5 (10)	3.9 ±0.5 (10)	2.8 ±0.3 (10)	3.7 ±0.5 (10)
3.0	2.9 ±0.4 (5)	2.6 ±0.3 (5)	1.9 ±0.4 (5)	2.6 ±0.3 (5)
4.0	2.6 ±0.5 (5)	2.4 ±0.8 (4)	1.1 ±0.3 (4)	2.6 ±0.5 (5)

Table 3. Effects of isoflurane on the amplitudes of AHPs of CA_1 pyramidal neurons.

IFL (Vol. %)	AHP duration (s ± SEM, no. of applications)			
	Perfusion time (min)			
	Control	Isoflurane 3	Isoflurane 6	Recovery 6
0.5	2.7 ±0.5 (4)	1.9 ±0.6 (4)	2.1 ±0.7 (4)	2.8 ±0.9 (4)
1.0	4.8 ±0.6 (6)	3.8 ±0.7 (5)	2.9 ±0.5 (9)	3.9 ±0.7 (5)
2.0	4.7 ±1.0 (9)	4.3 ±1.3 (9)	3.5 ±1.1 (9)	4.3 ±1.0 (8)
3.0	3.5 ±0.5 (5)	2.2 ±0.6 (5)	1.2 ±0.5 (4)	3.1 ±0.9 (5)
4.0	3.8 ±1.1 (4)	3.1 ±1.3 (3)	3.0 ±0.9 (4)	3.5 ±1.1 (4)

Table 4. Effects of isoflurane on the durations of AHPs of CA_1 pyramidal neurons.

FIG. 8. Effects of isoflurane on AHPs of 18 CA_1 pyramidal neurons. (A) Time- and dose-dependent reduction of the amplitudes of AHPs by isoflurane. AHP amplitudes, measured from 18 CA_1 neurons, were expressed as the percentage of the maximum AHP amplitude obtained by eliciting 4 or 5 spikes. Significant reduction in the amplitudes of AHPs were observed after ~6 min perfusions of 2-4% isoflurane. (B) Reduction of the durations of AHPs by isoflurane. AHP durations, measured from 18 CA_1 neurons, were expressed as the percentage of the maximum AHP duration obtained by eliciting 4 or 5 spikes. Means ± SEM; *, $p<0.05$ by Student t-test.


A
Control
3 min
6 min
Recovery
Maximum AHP amplitude (%)
100
80
60
40
20
0
0.5
1.0
2.0
3.0
4.0
Isoflurane concentration (Volume %)
B
Maximum AHP duration (%)
100
80
60
40
20
0
0.5
1.0
2.0
3.0
4.0
Isoflurane concentrations (Volume %)

Nonetheless, profound reductions in maximum AHP duration were still observed at 6 min with application of 1-3 % isoflurane. These were not statistically significant (Student t-test) because of the large variability in the measurements, which is partly attributable to the spontaneous synaptic activity and electrical 'noise'.

3.6 Synaptic transients

Postsynaptic potentials were generated in 11 pyramidal CA_1 cells by stimulating the afferent fibres in the stratum radiatum (Fig. 2). In all of these cells, subthreshold current strength was used to elicit EPSPs of near maximal amplitude without generating action potentials. At least 20 of these EPSPs were averaged by computer to give means for the control, and for each time interval of isoflurane perfusion, including recovery. The averaged EPSP amplitude of the 11 cells in the control solution was 6.7 ± 0.3 mV (35 averages; cf. Table 5, representing actual values). As illustrated in Fig. 9A, the amplitudes of EPSPs were reduced substantially after 6 min perfusion of 3 % isoflurane; at this time, a slight hyperpolarization ($\sim$2 mV) was observed. The amplitudes of EPSPs recovered at 6 min after returning the perfusion to the control ACSF. More than one dose of isoflurane was applied to 11 cells, and the results indicate that the amplitudes of the EPSPs were reduced significantly and in a dose- and time-dependent manner with increasing doses (0.5-3%) of isoflurane (Fig. 9B). Full recovery, measured after 6 min of returning to ACSF, was observed with low concentrations; however, only partial recovery was observed with high concentrations (Fig. 10).

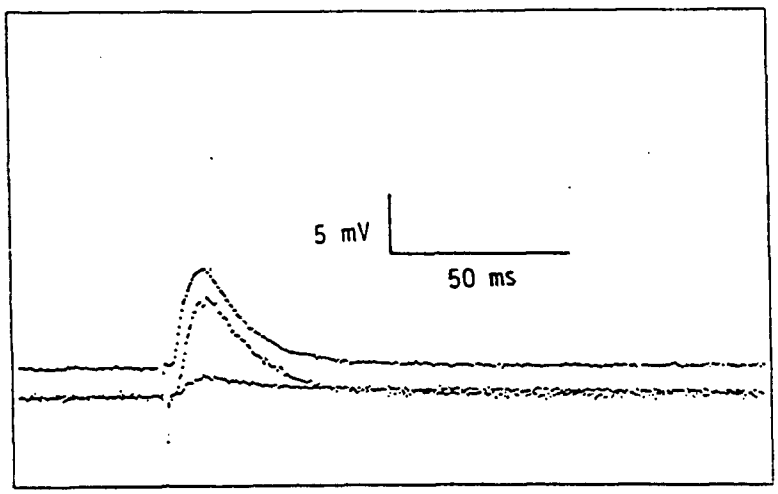
Inhibitory postsynaptic potentials (IPSPs), following EPSPs, were measured using K-acetate electrode in 8 out of 11 cells that received 22 applications of isoflurane in various concentrations. The data show that despite depolarization of the membrane potential, isoflurane at 0.5 % reduced the

IFL (Vol. %)	Evoked EPSP amplitude (mV ± SEM, no. of applications)			
	Perfusion time (min)			
	Control	Isoflurane 3	Isoflurane 6	Recovery 3	Recovery 6
0.5	6.8 ±0.7 (8)	6.0 ±0.8 (8)	5.1 ±0.9 (8)	5.7 ±1.0 (8)	6.4 ±1.1 (7)
1.0	6.4 ±0.6 (8)	5.3 ±0.5 (8)	4.6 ±0.5 (9)	5.6 ±0.8 (9)	6.8 ±0.8 (9)
1.5	6.1 ±0.6 (6)	4.7 ±0.8 (6)	3.3 ±0.7 (6)	3.6 ±0.6 (6)	4.7 ±0.6 (6)
2.0	5.8 ±0.5 (7)	3.9 ±0.3 (7)	2.8 ±0.4 (7)	2.9 ±0.2 (7)	4.3 ±0.4 (6)
3.0	8.5 ±0.6 (5)	6.3 ±1.2 (5)	3.1 ±1.2 (4)	4.0 ±1.4 (4)	6.0 ±1.5 (4)
4.0	9.8 (1)	6.6 (1)	2.2 (1)	2.7 (1)	6.1 (1)

Table 5. Effects of isoflurane on the excitatory postsynaptic potentials of CA_1 pyramidal neurons.

FIG. 9. Isoflurane reduces the amplitudes of EPSPs in a dose- and time-dependent manner. (A) Control, 6 min of 3% isoflurane, and recovery traces (~25 computer averaged traces) were superimposed. Perfusion of ~6 min of 3% isoflurane drastically reduced the peak amplitude of EPSPs and associated cell hyperpolarization (2 mV). Partial recovery to control peak amplitude was observed at ~6 min after returning the perfusion to normal ACSF. Orthodromic stimulation (10-15 V, 0.2 Hz) of the Schaffer collaterals was used. KAc electrode. (B) Dose-response curve obtained from 11 CA_1 neurons exposed to various doses of isoflurane. The peak amplitudes of the EPSPs were expressed as percentage of maximum peak EPSP amplitude. Open circles represent 3 min of isoflurane perfusion, and closed circles represent 6 min of isoflurane perfusion. Means ± SEM; a, significantly different from control; b, significantly different from 3 min of isoflurane perfusion; $p<0.05$ (*Post hoc* multiple comparison). Overall significance determined by ANOVA.

A


5 mV
50 ms

B

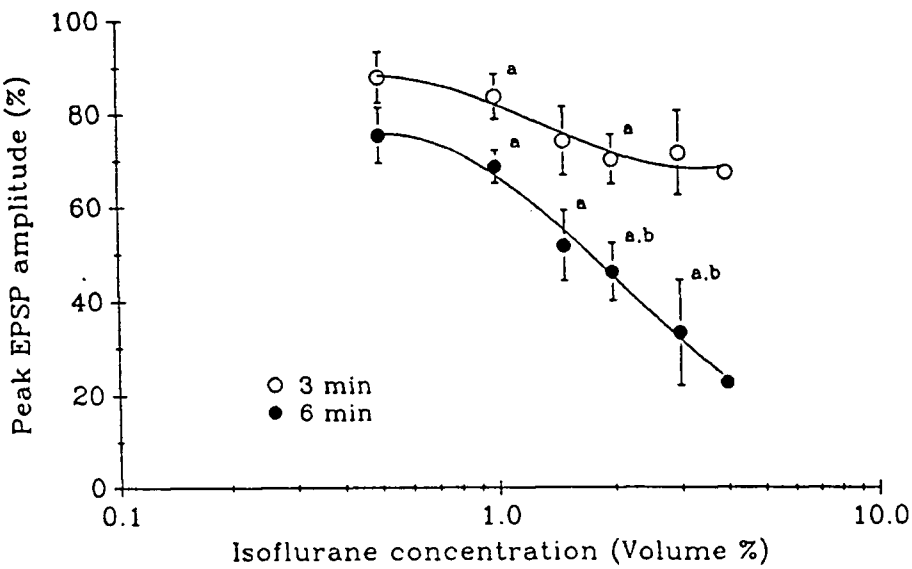
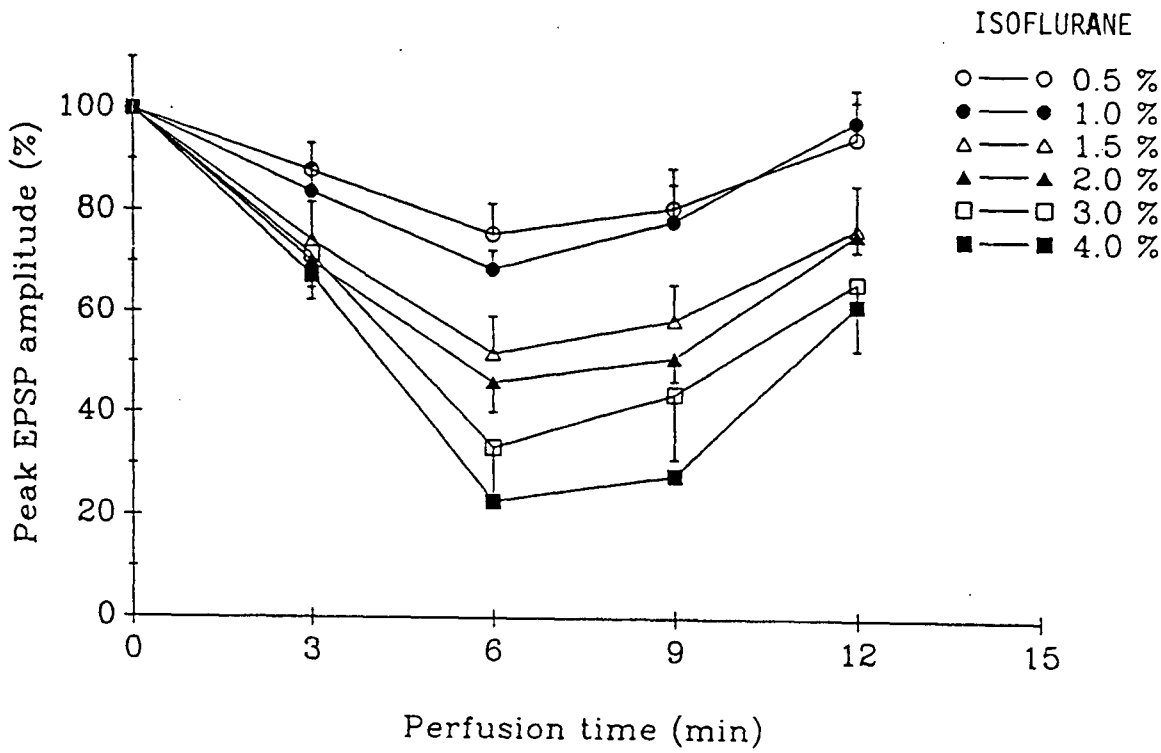
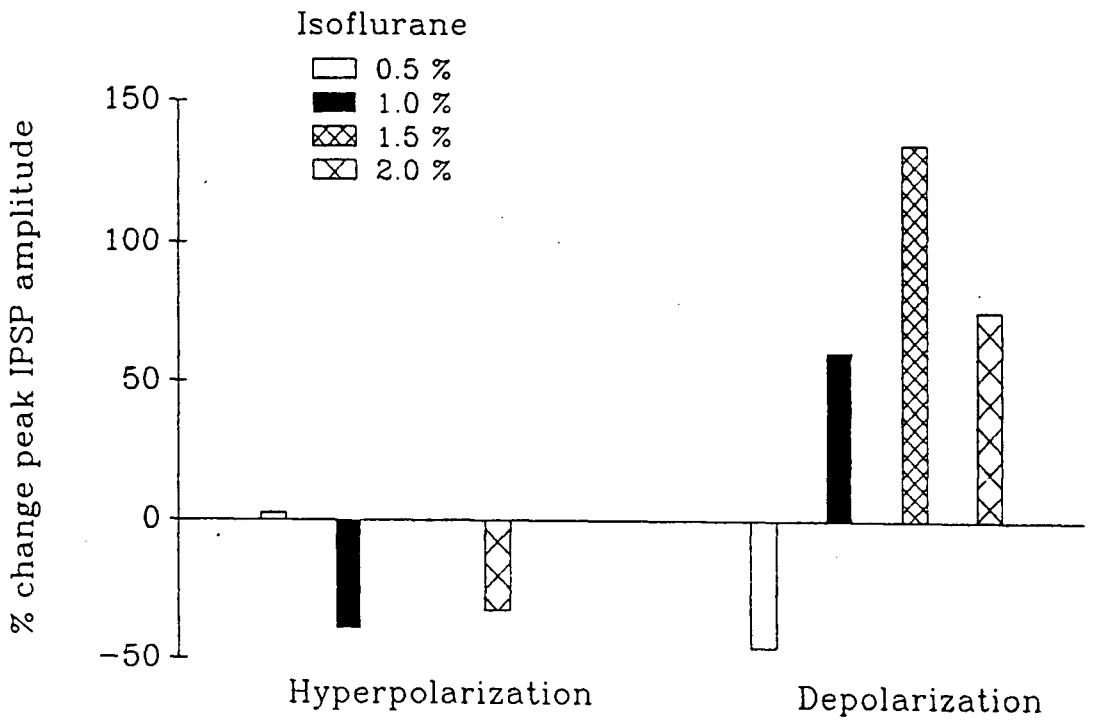

Peak EPSP amplitude (%)
100
80
60
40
20
0
0.1
1.0
10.0
Isoflurane concentration (Volume %)
3 min
6 min
a
a
a
a
a,b
a,b

FIG. 10. Time course of isoflurane action and recovery on evoked EPSPs. Dose-dependent partial recovery was observed in CA_1 neurons exposed to high anaesthetic concentrations. Means ± SEM.


ISOFLURANE
0.5 %
1.0 %
1.5 %
2.0 %
3.0 %
4.0 %
Peak EPSP amplitude (%)
100
80
60
40
20
0
0
3
6
9
12
15
Perfusion time (min)

peak amplitudes of IPSPs, whereas in concentrations of 1 % or more, the peak IPSP amplitude was enhanced (Fig. 11). The reduction in IPSP amplitude with 0.5 % administration indicates possible physiological anaesthetic action since depolarization increases Cl^- electrochemical gradients, thereby facilitating Cl^- influx. The time course of recovery from these effects was similar to that observed in the case of isoflurane-depression of EPSPs.

FIG. 11. Effects of isoflurane on inhibitory postsynaptic potentials (IPSPs). Responses of the amplitudes of IPSPs to various concentrations of isoflurane were categorized by membrane potential response following 6 min perfusion.


Isoflurane
0.5 %
1.0 %
1.5 %
2.0 %
% change peak IPSP amplitude
150
100
50
0
−50
Hyperpolarization
Depolarization

4. DISCUSSION

4.1 Isoflurane concentrations

Knowledge of the concentration administered is important for comparing these results with the experimental findings reported by other investigators, as well as to correlate them to clinical observations. In the present investigations, the concentrations of isoflurane applied to the hippocampal slices are in a range similar to those used clinically. For clinical applications, typical MAC values for anaesthesia range between 0.5% and 2.0% (Goodman and Gilman, 1985; Miller, 1986). These values correspond to the vaporizer outputs of 0.6-2.3% for isoflurane or 0.4-1.5 % for halothane. Using chromatographic methods, some investigators have found that 0.2-1.0 mM isofurane in the liquid phase corresponded to 0.6-3.4 % isoflurane in the gas phase (Yoshimura et al, 1985; Takenoshita and Takahashi, 1987). Noninvasive ^{19}fluorine-NMR techniques also have been used for in vivo studies to assess anaesthetic concentraions in the brain (Wyrwicz et al, 1983; Romppainen et al, 1986; Mills et al, 1987; Litt et al, 1987). Accordingly, endotracheal applications of 1 % isoflurane resulted in 0.1-0.5 mM in the rabbit brain (Wyrwicz et al, 1983).

The values of 0.02-0.3 mM isoflurane obtained in our experiments, with vaporizer outputs of 0.5-4%, can be attributed to loss of anaesthetics into the atmosphere above the recording chamber, and/or a result of other technical difficulties associated with collecting the samples (e.g., incomplete sealing of NMR sampling tubes). However, the bath concentrations of isoflurane applied to our hippocampal slices were probably sufficient to give final tissue concentrations similar to those found in the CSF of patients anaesthetized with isoflurane (Goodman and Gilman, 1985; Yoshimura et al, 1985; Bazil et al, 1987). For instance, Bazil et al (1987) reported that

the distribution of a chemically very similar anaesthetic agent, halothane, in animals anaesthetized at a 1% vaporizer setting is fairly uniform - 32.3 nmol/mg lipid in whole brain, and 35.1 nmol/mg lipid in a slice preparation of the hippocampus. Since the lipid solubility is similar between isoflurane and halothane, the distribution of isoflurane in the hippocampal slices is, therefore, assumed to be similar to that in whole brain. Hence, the effects of isoflurane on the membrane electrical properties of CA_1 pyramidal cells presently observed have clinical 'relevance', i.e., some bearing on the actual mechanism of the general anaesthetic state.

4.2 Postsynaptic modifications

4.2.1 Resting potentials and membrane conductances. The present investigations have demonstrated inconsistent changes in resting potentials of guinea pig CA_1 cells. However, in agreement with previous reports, a hyperpolarization was consistently observed in the presence of TTX. Furthermore, isoflurane had no effect on the input conductances either in the absence or presence of TTX.

The inconsistency in resting potentials observed in the absence of TTX was probably related to endogenous excitatory and/or inhibitory afferent activities. The slicing technique invariably alters the balance between excitatory and inhibitory influences impinging on a target cell. Consequently, each recording will reflect the net tonic influence predominating in each neuron following slicing. For example, in the presence of a strong tonic inhibitory influence, the resting potential is likely to be 'clamped' close to the reversal potential of IPSPs. Addition of TTX would be expected to abolish spontaneous presynaptic action potentials responsible for the tonic excitatory and/or inhibitory influences. In view of the above, the effects of isoflurane on resting membrane potential would be more accurately reflected in recordings with TTX present in the perfusion media. Under

these conditions, the impaled cell was, therefore, dissociated from synaptic bombardments caused by presynaptic action potentials. It was clear that isoflurane hyperpolarized cells without concomitant changes in their input conductances. The lack of isoflurane effect on membrane conductance, measured in the soma, does not necessarily mean that anaesthetic actions do not occur on the membrane electrical properties of CA_1 cells, as reported by others. It is conceivable that isoflurane may alter membrane conductances in the dendritic area which presumably would not be detected due to the cable properties of the dendrites, i.e., opening of ionic channels thereby creating a distal shunt in the axonal membrane.

Previous investigations have demonstrated that intravenous agents (e.g., pentobarbital) and volatile anesthetics in clinical doses reduce cellular excitability by increasing membrane conductance and producing a hyperpolarization. These effects have been observed in the α-motoneurons (Whitney and Glenn, 1986; Takenoshita and Takahashi, 1987) and hippocampal pyramidal cells (Nicoll and Madison, 1982; Berg-Johnsen and Langmoen, 1987; O'Beirne et al, 1987). In contrast, there are some reports of no change in the input conductances or the resting potentials of rat CA_1 pyramidal cells (Yoshimura et al, 1985; Brooks et al, 1986). Nicoll and Madison (1982) postulated that the increased input conductance produced by anaesthetics like halothane, diethylether, and pentobarbital, may be mediated by potentiating a g_K. In support with this proposal, volatile anaesthetics (Chalazonitis, 1967), pentobarbital (Sato et al, 1967), and metabolic inhibitors (e.g., 2,4-dinitrophenol) (Godfraind et al, 1970; Godfraind et al, 1971) reduce neuronal excitability, presumably mediated by an increase in g_K. Recently, the role of g_K in the regulation of neuronal excitability during anoxia has been confirmed by the findings that reversal of the electro-chemical gradient for Cl^- had no effect on anoxia-induced events

(Hansen et al, 1982), and that the anaesthetic evoked hyperpolarization was markedly enhanced in potassium-free media (Fujiwara et al, 1987).

4.2.2 Afterhyperpolarizing potentials. In the present investigation, isoflurane reduced the amplitudes of the AHPs in a dose-dependent manner. This effect is consistent with other observations of isoflurane-actions in human sympathetic ganglia (Puil et al, 1988) and neocortical cells (El-Beheiry and Puil, 1988) in which the amplitudes of AHPs also were suppressed.

The AHPs are prolonged hyperpolarizing undershoots, rebounding after spike-like depolarizations. Associated with the hyperpolarizations are an increase in membrane conductances. The mechanism for this phenomenom was first determined in spinal motoneurons (Krnjević and Lisiewicz, 1972; Krnjević et al, 1978; Zhang and Krnjević, 1986), and later in various other neurons, e.g., sympathetic (Kuno et al, 1983; Pennefather et al, 1985; Tokimasa, 1985), inferior olivary (Llinas and Yarom, 1981), olfactory cortex (Constanti and Sim, 1987), and hippocampal pyramidal cells (Kandel and Spencer, 1961; Schwartzkroin, 1975; Alger and Nicoll, 1980; Hotson and Prince, 1980).

In the hippocampal pyramidal cells, these AHPs were attributed to the activation of a Ca^{2+}-dependent K^{+}-conductance (g_K) since like motoneurons (Krnjević et al, 1978), they could be abolished by intracellular injection of EGTA (Schwartzkroin and Stafstrom, 1980), removal of external Ca^{2+} (Brown and Griffith, 1983), or the addition of external divalent cations (e.g., Cd^{2+}, Mn^{2+}, and Co^{2+}) which interfered with Ca^{2+} entry (Alger and Nicoll, 1980; Hotson and Prince, 1980; Wong and Prince, 1981; Brown and Griffith, 1983; Lancaster and Adams, 1986; Segal and Baker, 1986). The genesis of AHPs is strongly dependent on the extracellular $[K^{+}]$ levels (Alger and Nicoll, 1980). Hence, anaesthetics that interfere with Ca^{2+} metabolism, for example, will alter the AHPs.

Our findings further support this possibility since AHPs were diminished by isoflurane. Nevertheless, it is feasible that isoflurane may act at other sites which are involved in the Ca^{2+}-dependent g_K, for example, Cl^- and K^+ channels. Blockade of Cl^- conductances by anaesthetics, however, is unlikely or at least may be masked since other investigations have demonstrated that anaesthetics potentiate IPSPs (Nicoll et al, 1975; Gage and Robertson, 1985; Proctor et al, 1986; Harrison et al, 1987). El-Beheiry and Puil (1988) observed that, in the neocortical cells, Cl^- conductances contribute slightly to the genesis of AHPs since addition of bicuculline only slightly reduced the amplitudes of AHPs. Thus far, we are not aware of any evidence showing that anaesthetics directly block K^+ currents in mammalian CNS. In support of this view is the observation that action potential durations were not altered during applications of isoflurane. Isoflurane-induced reductions in the amplitudes of AHPs occur at membrane potentials where there would be no contribution of membrane voltage. Such an influence might be expected from the observations that Ca^{2+}-activated K^+ channels show a voltage dependency.

Applications of pentobarbital (O'Beirne et al, 1987) and ethanol (Carlen et al, 1982) increase the AHP amplitudes in hippocampal CA_1 pyramidal cells. These findings, however, are not necessarily incompatible with our results using isoflurane. The inconsistency suggests that different agents may alter calcium metabolism in a different manner, for example: changes in the disposition of intracellular Ca^{2+} (Krnjević, 1974; Ho and Harris, 1981) or in Ca^{2+}-currents (Blaustein and Ector, 1975; Blaustein, 1976; Heyer and Macdonald, 1982; Krnjević and Puil, 1988).

In the present investigation, isoflurane is not likely to have produced alterations in g_K's other than the Ca^{2+}-dependent g_K since input conductances were not reduced significantly. Furthermore, isoflurane applica-

tions had no effects on the amplitudes and durations of spontaneous and evoked spikes which suggests that Na^+-channels are unlikely postsynaptic sites of anaesthetic action. The failure of isoflurane applications to affect input conductance may suggest possible involvement of other postsynaptic g_K's or alterations in presynaptic mechanisms.

4.3 Presynaptic modifications

4.3.1 Axonal conduction. The variability in resting potentials and, in particular, the reductions in the amplitudes of EPSPs observed in the present investigations suggest possible alterations in axonal conduction produced by anaesthetics.

Sowton and Sherrington (1905) showed that clinical concentrations of volatile anaesthetics block synaptic transmission but have a relatively minor influence on axonal conduction. This observation has been subsequently confirmed in other studies on using sympathetic nerve axons (Larrabee and Posternak, 1952), lateral olfactory tract fibres (Richards et al, 1975), and perforant path of the hippocampus (Richards and White, 1975). Berg-Johnsen and Langmoen (1986) recently demonstrated that the amplitude and conduction velocity of action potentials in the unmyelinated fibres were reduced by isoflurane in a dose-dependent manner, within the stratum radiatum layer of the hippocampus.

The apparent contradiction in the anaesthetic actions on axonal conduction may be attributed to the high anaesthetic doses applied to the slice preparations in the investigations of Berg-Johnsen and Langmoen (1986) because isoflurane was delivered directly from the vaporizer output (see above and Wyrwicz et al, 1983; Bazil et al, 1987). Moreover, Bazil (1987) observed that for similar levels of anaesthesia in in vivo and in vitro preparations, anaesthetic concentrations in the equilibrating gas were lower than those used for the in vivo preparations. Hence, high concentrations of

anaesthetic may have reduced both axon terminal excitability and conduction velocity, presumably by enhancing inactivation of a Na^+-conductance (Saint et al, 1986; Quastel and Saint, 1986). The bath concentrations of isoflurane achieved in our experiments were less than 0.3 mM which suggests that anaesthetic actions on the transmitter release mechanism are more likely, at least in the isoflurane dose range of 0.5 to 2%.

4.3.2 Synaptic transmission. The findings that isoflurane may affect neuronal transmission by interfering with synaptic potentials are generally consistent with those reported for other anaesthetics observed in mammalian spinal cord (Brooks and Eccles, 1947; Somjen and Gill, 1963; Loyning et al, 1969), sympathetic ganglion (Larrabee and Posternak, 1952), cuneate nucleus (Galindo, 1969), and olfactory cortex (Richards, 1973). Recent investigations have shown that halothane induces a uniform suppression of both EPSPs and IPSPs in rat spinal motoneurons (Takenoshita and Takahashi, 1987). However, Yoshimura et al (1985) found that clinical doses of isoflurane, halothane, and enflurane had no effect on the amplitudes of EPSPs of hippocampal CA_1 neurons, although these agents selectively suppressed the peak amplitudes of IPSPs. In the same type of preparation, injectable anaesthetics including barbiturates (Nicoll et al, 1975), etomidate (Proctor et al, 1986), and alphaxalone (Harrison et al, 1987) have been reported to potentiate IPSPs. Accordingly, and using voltage-clamp techniques, Gage and Robertson (1985) demonstrated that halothane applications reversibly increase the time constant of decay of hippocampal inhibitory postsynaptic currents. A reduction in IPSP amplitude was observed in the present investigation but only with an isoflurane concentration of less than 1%. However, and in concert with the above findings of Gage and Robertson and others, concentrations of isoflurane more than or equal to 1 % enhanced the amplitudes of IPSPs.

The dose-dependent suppression and augmentation of the peak IPSP amplitude by isoflurane suggest possible contamination of the amplitudes of the IPSPs by the ionic mechanism generating the EPSPs. That is, high doses of the anaesthetic reduced the amplitude of EPSPs thereby unmasking the full amplitudes of IPSPs. However, changes in the amplitudes of IPSPs may be measured more exactly during applications of isoflurane in low anaesthetic doses that do not significantly alter the amplitudes of EPSPs (Yoshimura et al, 1985). Hence, we may speculate that the excitatory phase of anaesthesia observed clinically may be a consequence of the suppression of IPSPs preceding that of EPSPs.

4.4 Mechanism of anaesthesia

4.4.1 Membrane effects. The dose-dependent effect of isoflurane on responses such as synaptic transients, AHPs, and membrane polarization suggest a possible role of concentration-dependent anaesthetic actions in the membrane. A good correlation between anaesthetic potency and lipid solubility has led to the conclusion that the principal target site of the anaesthetic agents is in the hydrophobic region of membrane bound protein or in the lipid bilayer (La Bella, 1981; Franks and Lieb, 1982). Moreover, anaesthesia (or hypnosis) results when an equivalent molar concentration of a particular agents is achieved within the cell membrane (Meyer, 1937; Mullins, 1954; Seeman, 1972) or when equivalent volumes of cell membrane are occupied by the anaesthetic molecules (Richard, 1978; Koblin, 1979). Accumulation of isoflurane in the membrane can be inferred from present observation on the dose-dependent suppression of the peak amplitudes of synaptic transients and AHPs, e.g., the greater the dose, the greater the depression of the amplitudes of EPSPs.

The time required for isoflurane to accumulate in the plasma membrane is another important determining factor for the concentration dependent drug

action. This was also demonstrated indirectly by the observations that the degree of peak amplitude reduction in EPSPs and AHPs was greater at 6 min than that at 3 min of isoflurane administration. An augmentation in the drug effect with prolonged perfusion time was, nevertheless, an expected phenomena because of the high lipophilicity of the agent and time-dependent accumulation of isoflurane in the plasma membrane. Thus from the synaptic transient studies, partial recovery of peak EPSP amplitude after high isoflurane concentrations indicates an accumulation of an appreciable number of isoflurane molecules in the lipid bilayer. Consequently, a longer time would be required for the anaesthetic molecules to overcome the 'barrier' of lipophilicity and exist the plasma membrane. In contrast, full recovery after low concentrations of isoflurane indicates a rapid termination of anaesthetic action on membrane proteins or ionic channels.

4.4.2 Transmitter release. It is well established that depolarization of the presynaptic terminals leads to transmitter release via inward movement of Ca^{2+} through voltage-dependent calcium channels (Hodgkin and Keynes, 1957; Katz and Miledi, 1967a,b; Katz and Miledi, 1969; Augustine et al, 1987). Most of these investigations were performed using presynaptic terminals of the squid giant synapse (Katz and Miledi, 1969; Katz and Miledi, 1967b; Llinas et al, 1981; Augustine and Eckert, 1984; Augustine et al, 1987), but relatively few studies have been performed in the mammalian CNS because of the inaccessibility of these terminals to direct electrophysiological analysis using present techniques. Nevertheless, Ca^{2+}-dependent synaptic transmission has been demonstrated in the guinea-pig olfactory cortex (Richards and Sercombe, 1970) and hippocampus (Dingledine and Somjen, 1981).

Using radioactive labelled Ca^{2+} (^{45}Ca), anaesthetic doses of barbiturates have been shown to reduce ^{45}Ca uptake into the presynaptic terminals (synaptosomes) isolated from mammalian brain (Blaustein and Ector, 1975; Sohn and Ferrendelli, 1976; Ondrusek et al, 1979) and sympathetic ganglia (Blaustein, 1976). Recently, Krnjević and Puil (1988) demonstrated that halothane reduces Ca^{2+}-inward currents in hippocampal CA_1 pyramidal cells. An accumulation of internal Ca^{2+}, $[Ca^{2+}]_i$, inactivates the voltage-gated calcium conductance (Eckert and Ewald, 1982), resulting in reduced Ca^{2+} influx. There is evidence demonstrating that volatile anaesthetics can, indeed, elevate intracellular free Ca^{2+} by suppressing mitochondrial Ca^{2+}-uptake (Rosenberg, 1973; Sweetman and Esmail, 1975; Krnjević, 1975). Although Ca^{2+}-currents were not directly measured in the present investigation as in the experiments of Krnjević and Puil (1988), the reduction in the amplitudes and durations of AHPs suggests that presynaptic calcium metabolism is perhaps altered by isoflurane. A Ca^{2+}-dependent-g_K has been described in synaptosomes of rat brain inferring its existence in terminal membrane (Bartschat and Blaustein, 1985). In view of these observations, anaesthetics may produce impairment of synaptic transmission by reducing presynaptic Ca^{2+} entry (Blaustein and Ector, 1975; Blaustein, 1976; Ondrusek et al, 1979; Heyer and Macdonald, 1982) and subsequent transmitter release (Haycock et al, 1977). Hence, the attenuation of synaptic transmission during isoflurane anaesthesia is likely to be mediated, at least in part, by a suppression of EPSPs secondary to a reduction in Ca^{2+}-dependent transmitter release.

Rapid recovery from the actions of isoflurane in the hippocampal slices was observed within minutes; however, complete elimination of such agents in an in vivo preparation apparently requires a considerably longer time (Wyrwicz et al, 1983; Litt et al, 1987). This is partly due to the large

amount of lipid in the brain tissue, acting as a 'reservoir' for re-distribution of the anaesthetic molecules. Hence, the long period for recovery of cognitive functions in elderly patients from the effects of general anaesthesia (cf. Introduction) may result from persistence of the anaesthetic in the CNS, thereby prolonging the disruption of Ca^{2+}-dependent synaptic transmission in aged brain (cf. Landfield and Pitler, 1984).

5. SUMMARY AND CONCLUSIONS

(i) The effects of isoflurane on synaptic potentials and membrane excitabilities in hippocampal slice in vitro preparations of guinea pig were investigated in CA_1 neurons using intracellular recording techniques. A relatively new clinical anaesthetic, isoflurane, was applied in the artificial cerebrospinal fluid bathing the in vitro preparations.

(ii) Isoflurane concentrations were detected in the bath with ^{19}fluorine nuclear magnetic resonance techniques (^{19}F-NMR), and were found to range between 0.02 to 0.3 mM. These concentrations are similar to the doses of isoflurane used clinically for producing the general anaesthetic state in humans.

(iii) Stable recordings, lasting 1-5 hours, were obtained from 82 CA_1 pyramidal cells. In a random sampling of 23 of the 82 neurons, the mean resting membrane potential was -65.4 ± 1.3 mV, and input resistances usually were greater than 50 MΩ. Spontaneous activity was sometimes observed. In order to study the postsynaptic effects of isoflurane administration without interference from endogenous synaptic activity, tetrodotoxin (1 μM) was added to the perfusate. The combined application of tetrodotoxin and isoflurane usually hyperpolarized the neurons by 3-5 mV. However, no significant changes in their input resistances were observed.

(iv) The average number of applications of isoflurane to the neurons was 1.8. Doses of isoflurane in the range of 0.02-0.3 mM produced no consistent effects on resting potentials; however, the number of neurons that were depolarized by isoflurane administration was significantly less at high doses than at low doses. Similarly, input resistances which were determined using bridge-balance techniques from

the membrane voltage displacements resulting from intracellular injections of hyperpolarizing current pulses and from the slopes of current-voltage relations, were not significantly altered by (0.5-4%) administrations of isoflurane.

(v) Applications of isoflurane reduced the amplitudes and durations of afterhyperpolarizing potentials following multiple spikes evoked by injections of current pulses. The amplitudes of excitatory and inhibitory postsynaptic potentials generated by subthreshold electrical stimulation of the Schaffer collaterals also were diminished by such administrations. These effects on afterhyperpolarizing potentials and synaptic transients were statistically significant as well as dose- and time-dependent.

(vi) It is concluded that isoflurane anaesthesia does not greatly affect the passive membrane properties of hippocampal CA_1 neurons. The isoflurane-induced reduction in the amplitudes of the postspike afterhyperpolarizations suggest a decrease in their ionic genesis, i.e., via a Ca^{2+}-dependent, K^+-conductance mechanism. Similarly, the interference of excitatory and inhibitory synaptic potentials indicates that isoflurane may decrease Ca^{2+}-dependent transmitter release. These data suggest that the ionic mechanisms of action of isoflurane involve presynaptic sites as well as postsynaptic sites, and directly invoke alterations in intracellular Ca^{2+}-disposition (e.g., binding and uptake) or inward Ca^{2+}-currents.

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