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Isoflurane Inhibits Multiple Voltage-gated Calcium Currents in Hippocampal Pyramidal Neurons

*Robert E. Study, M.D., Ph.D.**

Background: **The mechanisms by which volatile anesthetics produce general anesthesia are unknown. Voltage-gated calcium currents in central neurons are potential target sites for general anesthetics because they are involved in the regulation of excitability and are essential for synaptic transmission.**

Methods: **Freshly isolated rat hippocampal pyramidal neurons were studied using the whole-cell patch clamp method. Calcium currents were isolated from other voltage-activated currents by blocking sodium and potassium channels. Calcium current subtypes were studied using the specific blockers nitrendipine and ω-conotoxin GVIA.**

Results: **Isoflurane inhibited multiple voltage-gated calcium currents in hippocampal neurons. Isoflurane inhibited both the high- and low-voltage-activated calcium current in a clinically relevant concentration range, giving half-maximal inhibition of the peak high-voltage-activated current (measured at current maximum) at about 2% gas phase concentration, and the sustained current (measured at the end of an 800-ms depolarization) at about 1%. Isoflurane also accelerated both components of the two-component exponential decay of the high-voltage-activated current. Studies using specific channel blockers showed that the calcium current contained contributions from T, L, N, and other channels, including probably P channels. Isoflurane inhibited all of these in clinically relevant concentrations, although detailed analysis of the effects on the individual channel types was not attempted.**

Conclusions: **Given the importance of calcium currents in the regulation of excitability in central neurons and the involvement of P and N channels in neurotransmitter release, this effect may represent an important action of volatile anesthetics in producing general anesthesia. (Key words: Anesthetics, volatile: isoflurane. Brain, hippocampus: calcium currents; neurons.)**

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* Instructor, Department of Anaesthesia, Harvard Medical School; Attending Anesthesiologist, Brigham and Women's Hospital.

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Address reprint requests to Dr. Study: Brigham and Women's Hospital, 75 Francis Street, Boston, Massachusetts 02115.

THE mechanisms by which volatile anesthetics produce general anesthesia are unknown. However, the last few years have seen significant progress in uncovering some of their effects at the cellular level. It generally is believed that a decrease in intrinsic excitability or synaptic excitation and/or an increase in synaptic inhibition underlies general anesthesia, but only recently has significant progress been made in defining what changes occur.[1] Volatile anesthetics, which have relatively low potency and steric specificity compared with most other centrally acting drugs, might be expected to have multiple actions on the brain that contribute to the general anesthetic state.[2] Several groups have reported that volatile anesthetics enhance and/or mimic the action of the inhibitory neurotransmitter GABA,[3–5] as well as inhibit the action of the excitatory neurotransmitter glutamate at NMDA-type receptors.[6] Either of these would decrease excitability in central neurons. The voltage-gated channels directly responsible for electrical excitability and neurotransmitter release are particularly attractive candidates for general anesthetic action: An increase in potassium channel activity or an inhibition of sodium or calcium channels could reduce cellular excitability and thereby produce an anesthetic state, for example. Sodium channels, which are the prime contributors to the action potential of most neurons, are little affected at clinically relevant concentrations of anesthetics and thus would not appear to be a primary site of action.[7–9] Anesthetic enhancement of potassium currents in brain has been reported,[10] but this is not a typical finding since others have reported inhibition.[11–13] Calcium currents, however, may be important sites of anesthetic action, because they are involved in the regulation of excitability and the release of neurotransmitters. An inhibition of calcium currents in central neurons could help explain the observations that synaptic transmission in various systems is inhibited by general anesthetics[14] and that inhibition

of excitatory postsynaptic currents observed in spinal neurons appears to be presynaptic.[15] Therefore, investigation of the effects of general anesthetics on calcium channels in the brain may provide a clue to how general anesthetics work.

Complicating the study of calcium currents in central neurons is the variety of calcium channel types, which apparently serve different functions. Nonneuronal excitable cells such as cardiac muscle have one or two types of calcium channel: the L channel, underlying a "slow inward" current, and the T channel, underlying a small transient current that may regulate action potentials in some cardiac myocytes.[16] Neurons exhibit a much richer variety of calcium channels. There is now evidence for four types, and at least five have been proposed.[17–19] These include a low-voltage-activated, fast inactivating channel, called the T channel, which appears to be involved in regulating action potential firing patterns[19,20] and four types of high-voltage-activated channels. One of the latter, the L channel, is slowly inactivating and is similar, if not identical, to that responsible for the slow inward current in nonneuronal cells.[19,21] The other types exist predominantly or exclusively in neurons. They include the N channel, the P channel, and at least one other type, which so far has been revealed only from the residual calcium current after all known channels are blocked.[18,22] There is strong evidence suggesting that at least P and N channels mediate the release of central neurotransmitters,[23–25] although some contribution of L-type channels may be present.[26]

It is important to investigate general anesthetic actions in central neurons, not only because they show differences in calcium channels from peripheral tissues, but also because the central nervous system (CNS) is the site of general anesthesia. Nevertheless, there is little information on the effects of volatile anesthetics on calcium currents in central neurons. A brief communication describing halothane inhibition of a slow inward current in hippocampal slices has appeared,[27] but no detailed study of calcium current inhibition by a volatile anesthetic in central neurons has been reported. The current study describes the effects of isoflurane, the most widely used volatile anesthetic, on calcium currents in hippocampal pyramidal neurons. Acutely dissociated rat hippocampal pyramidal neurons were chosen because they have been shown to contain all of the known classes of calcium channels, and they allow more accurate recording of calcium currents with whole-cell patch-clamp than do cultured neurons or hippocampal slice because of better space clamping.

Methods and Materials

This study received approval from the Harvard Medical Area Standing Committee on Animals. Hippocampal neurons were prepared from rats of either sex at postnatal days 13–18 by a method modified from those of Kay and Wong[28] and Kaneda *et al.*[29] Rats were killed by decapitation and brains were quickly removed and placed in ice-cold incubation solution containing (in mM): NaCl 120, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, d-glucose 25, and PIPES (piperazine-10, N'-bis{2-ethanesulfonic acid}) 20. Slices of whole hippocampus 0.5 mm thick were prepared with a McIlwain-type tissue chopper and returned to this solution. Trypsin (1.5 mg/ml; Sigma Type XI, St. Louis, MO) was added to this solution, and the tissue was incubated at 37°C for 60 min with 100% O_2 bubbling to agitate the slices gently. After this incubation, the slices were transferred to the same medium without trypsin and incubated with oxygen bubbling for at least 15 min. Viable cells could be obtained from these slices for about 6 h. After this incubation, neurons were isolated by trituration in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, cat. #380-2430AJ) by passing the slices two or three times through Pasteur pipettes fire-polished to decreasing tip size and placed in a 35-mm plastic tissue culture plate coated with poly-d-lysine (Sigma). After a settling period of 15 min, the medium was changed to recording medium (composition below). Neurons were identified easily by visual appearance and maintained their basic morphology, with the exception of having shortened processes (usually 2–5 times the cell body diameter). Pyramidal neurons could be identified visually and have been shown by others to behave electrophysiologically as such.[30] All recordings reported here were done on such cells.

The whole-cell patch clamp technique was used for electrical recordings from neurons.[31] Extra- and intracellular recording media were designed to allow calcium currents to be recorded in isolation and with great stability, with sodium and potassium currents blocked pharmacologically using tetrodotoxin (with low sodium) and tetraethyl ammonium chloride (TEA), respectively. The extracellular recording medium contained (in mM): TEA 145, HEPES 10, $MgCl_2$ 1, d-glucose 6, tetrodotoxin 0.001, and $BaCl_2$ or $CaCl_2$ as indicated (usually 3–5), adjusted to *p*H 7.4 with CsOH and to

osmolarity 315–320 with sucrose or water as needed. Glass micropipettes (uncoated) were pulled to tip resistances of 3–7 MΩ when fire-polished and filled with intracellular recording medium, which contained (in mM): N-methyl-d-glucamine methane sulfonate 110, N-methyl-d-glucamine chloride 10, HEPES 5, BAPTA-Cs_4 (1,2-bis(2-aminophenoxy)ethane N,N,N′,N′-tetraacetic acid, tetracesium) 5, Na_2GTP 2, and MgATP 5. In addition, 20 mM tris-phosphocreatine and 0.05–0.15 mg/ml creatine kinase were added to create an adenosine triphosphate-regenerating system that prevented "rundown" of calcium currents during long recordings. To reduce enzymatic autodigestion of the cells during recording, 0.1 mM leupeptin was added.[30] The *p*H of the pipette solution was adjusted to 7.2 with CsOH and osmolarity adjusted to 315–320 mOsm as above. All experiments were performed at room temperature (21–22°C).

Current and voltage recordings were made *via* an Ag/AgCl wire to an Axopatch 200 amplifier (Axon Instruments, Foster City, CA) filtered at 2 kHz and digitized at 1 kHz using a Lab Master DMA A/D converter (Axon Instruments). Digitized data were recorded and analyzed on a IBM-compatible computer using pClamp software (Axon Instruments). Series resistance as well as junction potential (which was minimal) were corrected using the recording amplifier circuitry, reducing the command voltage error to insignificant levels (less than 3 mV). Capacitative transients were minimized and frequency response was maximized with the resistance and capacitance compensation circuitry of the amplifier.

Recordings were made after the calcium current amplitude remained constant, usually after a 5–10-min period of "runup," during which calcium currents often increased in size, possibly because of the intracellular dialysis of the cells with the adenosine triphosphate-regenerating system and intracellular calcium chelator. Calcium currents were often stable for more than 2 h. Cells that showed rundown of calcium current during analysis were not used. Cells typically were held at −50 mV and hyperpolarized for 3 s at −90 mV to remove inactivation before depolarizing steps were done. This allowed for much better long-term cell stability than continuous holding potentials of −90 mV. Cells with detectable current decay, lack of stability of currents, or discontinuities in current recordings indicating poor space clamp were not used for analysis. Calcium current was separated from the small leak current by subtracting conductance obtained from a linear least-squares fit of the current measured with depolarizations in 10-mV increments from −90 to −40 mV (−60 mV in cells with T currents) on a current-voltage (I-V) plot. Isoflurane reversibly increased a membrane conductance that was slightly outward-rectifying at positive potentials, which caused a small negative shift of apparent reversal potential of calcium current in I-V curves. All measurements of calcium currents (other than in I-V curves) were performed at 0 mV or more negative, where linear leak subtraction was most accurate.

Isoflurane was applied by two methods. In some experiments, isoflurane was applied by exposure of the extracellular solution to the indicated concentrations of anesthetic in humidified air, using a standard anesthetic vaporizer. Anesthetic concentrations in the gas phase delivered to the recording system were measured using a freshly standardized infrared analyzer (Puritan-Bennett, Miami, FL), and the reported concentrations represent gas concentration (vol/vol) settings corrected by standard curves derived at each setting to reflect actual delivered concentrations. In the concentration range used in most experiments, the vaporizer delivered 83% of the concentration setting of the vaporizer. The anesthetic was applied by exposing the extracellular medium in the dish containing the cells to anesthetic in the vapor phase. The chamber was a semiclosed system with a small opening for the recording pipette and a vacuum scavenge system, with the medium kept shallow (about 2 mm depth) to accelerate equilibration with anesthetic. This provided more stable recording conditions for these cells than using bulk flow of anesthetic-containing medium, as has been done by some other investigators using more sturdy cells.[12,32] In addition, the concentration of anesthetic in the medium could be precisely controlled. Resulting concentrations in the liquid phase near the cells were measured in early experiments with gas chromatography.[8] Using this method, the concentration of anesthetic in solution was half-equilibrated in about 2 min. The equilibrated concentration (at 8 min exposure) of isoflurane in the medium was 0.39 mM per 1% in gas phase, nearly identical to that expected from calculations based on the Ostwald solubility coefficient for Ringer's solution corrected for temperature (0.384 mM per 1%).[33,34] Experimental observations using this method were done after 8 min or more exposure to anesthetic. Results are reported in terms of vapor phase concentrations (in %vol/vol) at equilibrium.

The second method of anesthetic application was used to produce quickly reversible effects with less

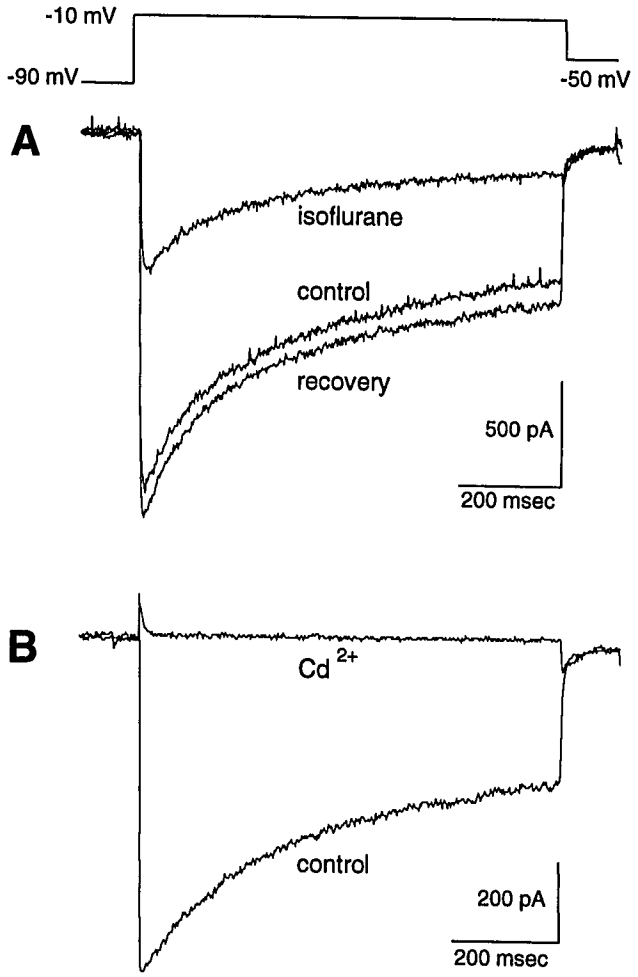

Fig. 1. Isoflurane reversibly inhibits calcium currents in hippocampal neurons. The top trace represents voltage steps to the indicated potential. (*A*) Calcium current before, at 10 min into, and 13 min after exposure to isoflurane applied by exposure of the extracellular solution to the anesthetic at 2.5% gas phase concentration. Depolarization from −90 mV produced an inward current with a biphasic decay. (*B*) Block of inward current by 200 μM $CdCl_2$. The charge carrier in both cells was 3 mM Ba^{2+}.

control of concentration at the cell. In these experiments, anesthetic was applied by pressure (1–2 lbs/in^2) to individual cells by micropipette (2–4 μ tip size). Isoflurane was pre-equilibrated with extracellular medium by bubbling with air containing the indicated concentrations of anesthetic, after which the medium was placed in the micropipette. The anesthetic concentration remained stable in the pipette for at least 1 h. Other drugs, such as calcium channel antagonists, were applied to cells using a continuous flow system that exchanged the entire extracellular solution in the plate. Results are expressed in terms of mean ± SE, and statistical significance is in terms of two-tailed Student's *t* test, using paired observations unless indicated otherwise, or single-factor analysis of variance, where indicated.

Results

Characteristics of the Calcium Current

Calcium currents were found in 122 of 134 neurons in which stable recordings were achieved. Typical currents, shown in figure 1, are induced by depolarization to −10 mV from a holding potential of −90 mV. The current activates quickly and decays with biphasic kinetics. Isoflurane reversibly reduced both transient and sustained components of the calcium current. In every cell in which calcium current was detectable (n = 103), it was inhibited by isoflurane. Onset of the effect was immediate (as quickly as could be measured, within 3 s) when anesthetic was applied by pressure pipette and reversed within several seconds after pressure was turned off. Pressure application of extracellular medium alone had no effect on calcium currents (data not shown). Onset and offset were slower, taking minutes, when anesthetic was applied by exposure to an atmosphere containing isoflurane (see methods and materials). Inward currents were observed with either Ca^{2+} or Ba^{2+} as the charge carrier and were blocked by Co^{2+} (3 mM) or Cd^{2+} (200 μM; fig. 1B). Ba^{2+} was used in initial experiments to rule out any involvement of calcium-dependent inactivation of calcium currents in the observed effects,[35] but most experiments used Ca^{2+}. There was no significant difference in any isoflurane inhibition using either Ca^{2+} or Ba^{2+}, although the transient component of the current was larger using Ca^{2+} (*e.g.*, compare fig. 1 with fig. 4). Currents will be referred to as calcium currents regardless of the charge carrier.

Voltage Dependence of Calcium Currents and Calcium-independent Currents

The current-voltage relationship (I-V curve) for calcium currents is shown in figure 2A. In cells without a low-voltage-activated, or T, current (see below), inward current began to activate at about −30 mV, reaching a maximum at −10 to 0 mV, then declining and reversing at positive potentials. Figure 2A shows the I-

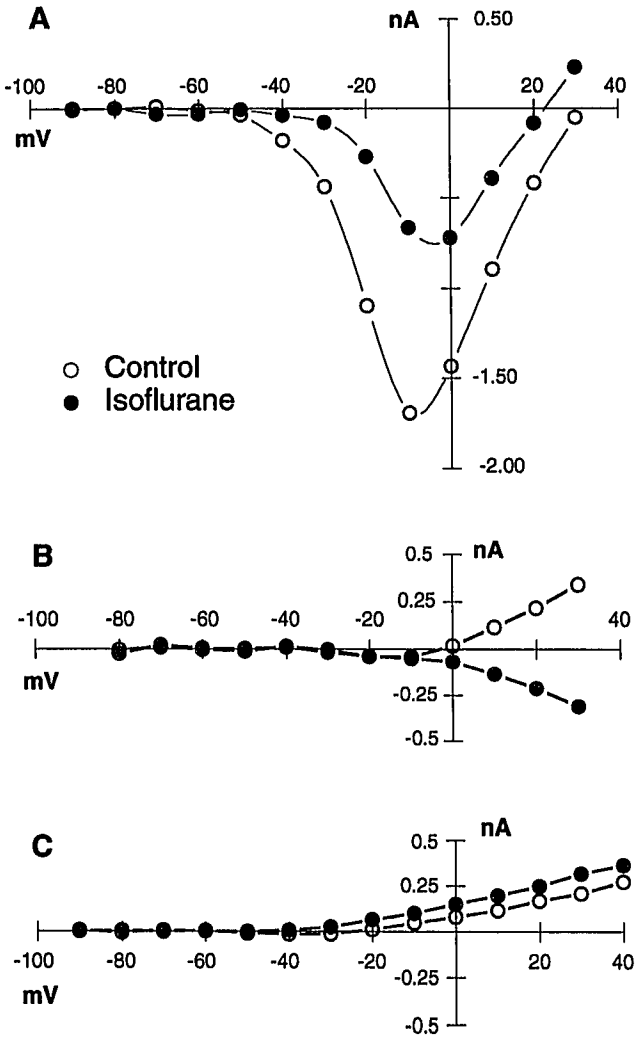

Fig. 2. Current-voltage (I-V) relationships for hippocampal neurons under control conditions (open circles) and with 2.5% isoflurane (filled circles). Current was measured at its peak, 27 ms from the beginning of the depolarization. All traces were leak-subtracted as described in methods and materials. (*A*) I-V relationship for calcium current using 3 mM Ba^{2+} as the charge carrier. (*B*) I-V relationship with 3 mM Ba^{2+} and 200 μM $CdCl_2$. Note slight outward current becomes inward with isoflurane. (*C*) I-V relationship using 0.1 mM Ca^{2+} as the charge carrier. Note that isoflurane increases outward current slightly.

V curve for this current measured at its maximum amplitude (which will be referred to as the peak current). In this cell, isoflurane (2.5%) produced an approximately 60% inhibition of the current measured at its peak. When the current is measured during the more sustained phase at the end of the 800-ms depolarizing pulse (which will be referred to as the sustained current), the inhibition was greater, about 75%. Figure 2A is typical in that isoflurane inhibited calcium current at all potentials.

Isoflurane also reversibly increased a membrane conductance that was linear until near 0 mV and then became slightly outward-rectifying. This outward rectification appears to be responsible for the apparent negative shift in reversal potential in the presence of isoflurane, as seen in figures 2A and 5. This conductance was preserved in the presence of Cd^{2+} or in low Ca^{2+} medium and thus could be examined in the virtual absence of calcium current. This calcium-independent effect of isoflurane could be adequately subtracted out using linear leak subtraction as described in methods, allowing the calcium current to be measured accurately. Linear subtraction of the conductance in each case showed little deviation from linearity at up to 0 mV. In the presence of Cd^{2+}, the outward rectification caused by isoflurane was reversed (fig. 2B), but in low (0.1 mM) Ca^{2+} medium, it was retained (fig. 2C). In the latter case, the error caused by linear leak subtraction at 0 mV was less than 5% in control and about 10% in the presence of 2.5% isoflurane when compared to the average values of calcium current at 0 mV (1.2 nA). Therefore, all measurements used voltages of 0 mV or more negative, and most used −10 mV or less. Preliminary experiments suggest that this conductance increase induced by isoflurane is at least partially due to Cl^-, as has been reported by Yang *et al.*[4],†

Concentration Dependence and Effect on Decay Kinetics

The inhibition of calcium current by isoflurane was concentration-dependent and occurred in the range in which anesthesia occurs (fig. 3). Concentration-response experiments (averaged from five cells) showed that the sustained current was more sensitive than current measured at peak response. Inhibition of the sustained current was half-maximal at about 1% isoflurane, whereas the peak current required about 2% to reach half-maximal effect. This difference is consistent: at 2.5% isoflurane (the most often used concentration), the sustained current was reduced by 94.4 ± 4.0%, compared with 70.1 ± 3.1% for the peak current, a significant difference (n = 15 cells, $P \ll 0.0001$).

Anesthetic inhibition of calcium currents could be manifested not only as a reduction of calcium current

† Study R: Unpublished results. 1994.

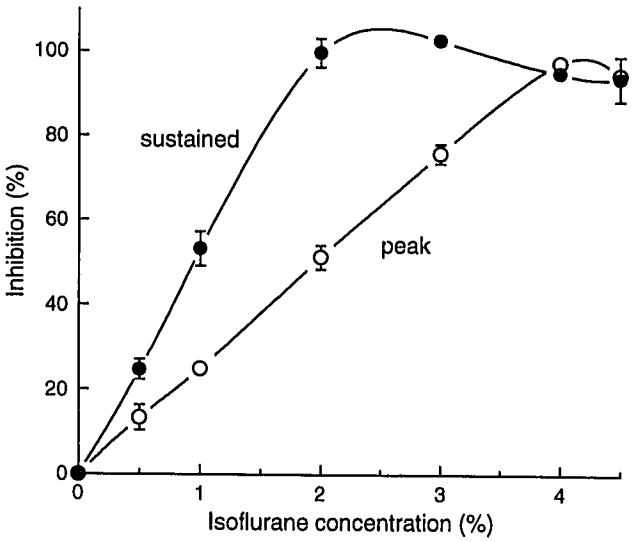

Fig. 3. Concentration-response curves for isoflurane inhibition of calcium current in hippocampal neurons. Open circles represent current measured at peak response (27 ms after depolarization); closed circles represent sustained current measured at the end of an 800-ms depolarization. Data represent mean ± SEM for five cells, using depolarization from −90 mV to 0 mV. All measurements are significantly different from control ($P < 0.01$ or better). Isoflurane was applied by exposure of the extracellular medium to the indicated concentrations in air. The charge carrier was 5 mM Ca^{2+}.

magnitude but also in an acceleration of its decay. The biphasic decay that was apparent in cells recorded from a holding potential of −80 to −90 mV was confirmed by its consistent fit to the simple two-exponential model $I = a + b(\exp\{-t/t_T\}) + c(\exp\{-t/t_S\})$, where a, b, and c are amplitudes and t_T and t_S are the time constants of decay of the transient and the sustained components of the current, respectively (fig. 4). The average time constant of decay of the rapid transient component was 58 ± 6 ms, whereas the more sustained component was 402 ± 47 ms when voltage steps to −10 mV were used (n = 9 cells). These decays were dependent on the test potential, tending to be longer with increasingly depolarized steps (up to 0 mV), but always fitting a two-component exponential model. When the effect of isoflurane on the decay kinetics of the two components of calcium current was examined, a small but consistent acceleration of decay was seen for both. The acceleration of decay using depolarizations from −90 to −10 mV (Ca^{2+} and Ba^{2+} as charge carriers) averaged 28.9 ± 5.4% (n = 9 cells, $P < 0.005$) for the transient component and 21.6 ± 9.9% (n = 9 cells, $P < 0.05$) for the more sustained component. There was no difference in effect using Ca^{2+} or Ba^{2+} (data not shown), and so it is unlikely to be a function of calcium-dependent inactivation.

Isoflurane Inhibition of Calcium Channel Subtypes

Calcium currents in central neurons are the result of the activation of several types of voltage-gated channels with different but overlapping biophysical properties and probably different functions. The low-voltage-activated transient, or T-type current, can be separated from the others by its unique voltage dependence, but the other types overlap in their biophysical properties, requiring pharmacologic separation.[19] Hippocampal

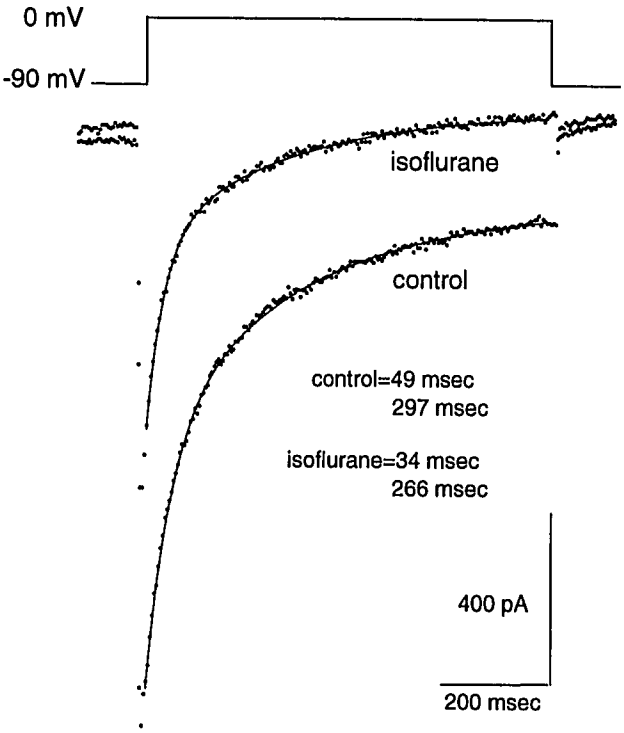

Fig. 4. Calcium currents are well fit with a double-exponential curve with and without isoflurane (2.5%), and the rate of decay is accelerated by isoflurane. Current traces are shown as their digital sampling points (every other point shown for clarity). The continuous lines represent best fits to the equation $I = a + b(\exp\{-t/t_T\}) + c(\exp\{-t/t_S\})$, where a, b, and c are amplitudes and t_T and t_S are the time constants of decay of the transient and the sustained components of the current. Isoflurane inhibited the peak and sustained currents and shortened the decay time constants of both components. Times indicated are time constants of the two components of the decay using this method. This cell had no low-voltage-activated transient (T-type) current. The charge carrier was 5 mM Ca^{2+}.

pyramidal cells appear to contain all the known calcium channel classes,[18,22,36–38] including the channel(s) underlying this resistant current. The concentration-response relationship for isoflurane (fig. 3), where 100% inhibition is achieved, suggests that all of the channel types present are inhibited. To show more directly that all of the known types of calcium channels are present in this preparation and are inhibited by isoflurane, experiments were performed to determine the contribution of calcium channel types by their biophysical and pharmacologic properties.

The T channel underlies a transient, low-voltage-activated current. This current is largely inactivated at potentials less negative than −70 mV, and thus is easily separated from the other calcium currents, all of which are high-voltage-activated. Figure 5A shows a typical T current. It was observed in only a minority (19 of 122) of the cells studied, probably because its expression appears to decrease with increasing age of the rat in this type of preparation.[39,40] The T current was studied using depolarizing steps from a holding potential of −90 to −50 or −40 mV, where almost all of the activatable current is T-type. This current can be seen in the I-V curve as the increase in the range of −60 to −30 mV, as seen in figure 5B. Twelve of these cells were tested with isoflurane (2.5%), which this current by 75 ± 5% ($P < 0.005$), similar to its potency against the high-voltage-activated current. Some contamination of high-voltage-activated currents is present at these potentials. Comparison of the relative activation of these high-voltage-activated currents at −40 mV and −10 mV (fig. 2A), as well as the residual sustained current as in figure 5A, suggests that about 10–15% of the transient current measured with jumps from −90 mV to −40 mV is due to high-voltage-activated currents. Because this current was not detectable in the majority of cells, more detailed analysis was not attempted.

The other known calcium currents are high-voltage-activated, requiring depolarizations more positive than −30 mV for significant activation and showing a maximum activation at about −10 to 0 mV (fig. 2). The lack of a T current in most of the cells studied allowed analysis of these currents without contamination from this type. Specific blockers exist for three of the channel types known to exist in central neurons. The effect of supramaximal concentrations of the snail toxin fraction ω-conotoxin GVIA (ω-CgTx), a blocker of N-type channels, and nitrendipine, a blocker of L-type channels, is summarized in table 1. (The new specific P-type channel blocker, ω-Aga-VIA, was not generally available

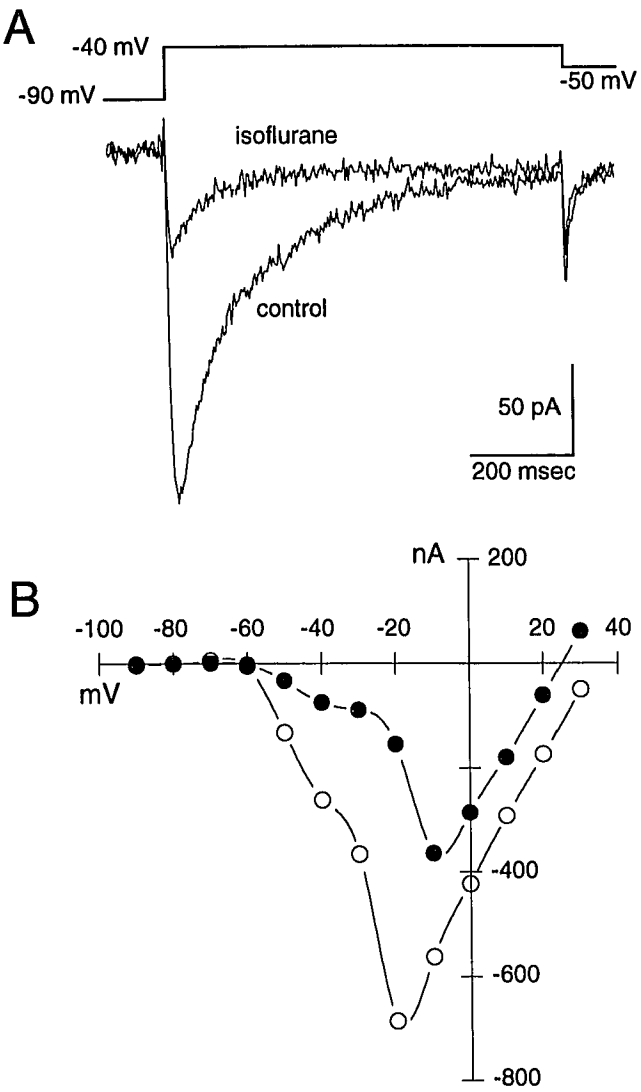

Fig. 5. Isoflurane inhibits the low-voltage-activated transient calcium current (T-type). (*A*) Current traces from a voltage step of −90 to −40 mV, where the high-voltage-activated currents are not significantly activated. (*B*) Current-voltage relationship for total current in a cell with a substantial component of T-type of current. Note that an inward current hump activating at about −60 mV represents this current. Open circles represent control; filled circles are in the presence of 2.5% isoflurane. The charge carrier was 5 mM Ca^{2+}.

during the course of this study.) Because a residual current remains unblocked by all known calcium channel blockers, isoflurane inhibition was studied indirectly, by subtraction of the isoflurane inhibition in the presence and absence of specific channel blockers. Figure 6 shows a typical cell in which N- and L-type currents, as well as current insensitive to blockers of

Table 1. Inhibition of High-voltage-activated Calcium Current in Hippocampal Pyramidal Neurons by ω-Conotoxin GVIA and Nitrendipine

	% Inhibition (n)		
	0.5 μM ω-CgTx	10 μM Nitrendipine	ω-CgTx (0.5 μM) + 10 mM Nitrendipine
Peak current	56 ± 8 (8)	51 ± 7 (5)	70 ± 12 (3)
Sustained current	65 ± 6 (8)	67 ± 9 (5)	82 ± 12 (3)

The value from each cell is the average of two to five observations; n refers to the number of cells. All numbers are significantly different from control with $P < 0.05$ or less, each cell treated as a single value. The effects of the two drugs in combination is not equivalent to the sum of the two added individually, because the drugs were tested on different cells, with different proportions of calcium current.

ω-CgTx = ω-conotoxin GVIA.

these channel types, could be seen. Isoflurane (2.5%) substantially inhibited the total calcium current in this cell, as expected (fig. 6A). Nitrendipine, a specific blocker of L channels, was added to eliminate current from L channels (fig. 6B). The concentration used (10 μM) has been shown to be supramaximal and have only a small amount of N-channel block in these cells.[18] In this cell, nitrendipine reduced the sustained component more than the transient component (48% *vs.* 15%). In the presence of nitrendipine, isoflurane reduced the remaining current by approximately the same proportion. This suggests not only that nitrendipine-resistant current is inhibited by isoflurane but also that the nitrendipine-sensitive current is inhibited as well. By comparing the inhibition by nitrendipine in the absence and presence of isoflurane, an estimate of the inhibition of the nitrendipine-sensitive current by isoflurane can be made. In this cell, isoflurane inhibited 65% of the nitrendipine-sensitive current when measured at peak and 100% when measured at the end of the 800-ms depolarization. After recovery from isoflurane, addition of ω-CgTx in supramaximal concentration (0.5 μM)[18] to nitrendipine showed that a substantial component of the nitrendipine-resistant current was N-type (76% of peak and 75% of sustained current; fig. 6C) and that most of the isoflurane inhibition of calcium current in the presence of nitrendipine was therefore of the N-type. Previous studies have shown that most of the current remaining after nifedipine and ω-CgTx is P-type.[18,22] This remaining current also was inhibited by isoflurane (fig. 6C). Therefore, it is likely that some of the isoflurane effect on this residual current involves P channels, but it remains to be determined directly. Because of the existence of calcium current resistant to all known blockers and because of the scarcity of ω-Aga-VIA, no attempt was made to isolate the P current and study its response to isoflurane. Figure 7 shows another cell in which most of the calcium current is ω-CgTx-sensitive. Most of the isoflurane in-

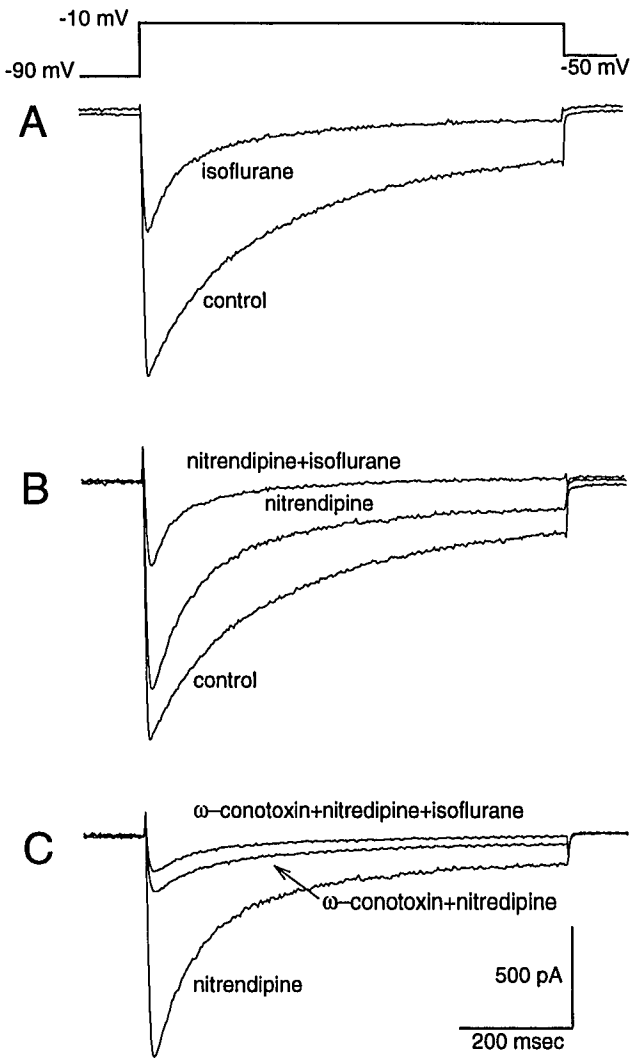

Fig. 6. Isoflurane (2.5%) inhibition of calcium currents in the presence and absence of nitrendipine and ω-conotoxin GVIA. Isoflurane (2.5%) was applied by puffer pipette. (*A*) Isoflurane inhibits the high-voltage-activated current resulting from a depolarization from −90 to −10 mV. (*B*) The same cell before and after a supramaximal concentration of 10 μM nitrendipine was added to block L channels, leaving N- and P-type (as well as other undefined calcium channels). (*C*) The same cell with 0.5 μM ω-conotoxin GVIA added to nitrendipine, to eliminate N and L channels. This cell had no T current. The charge carrier was 5 mM Ca^{2+}.

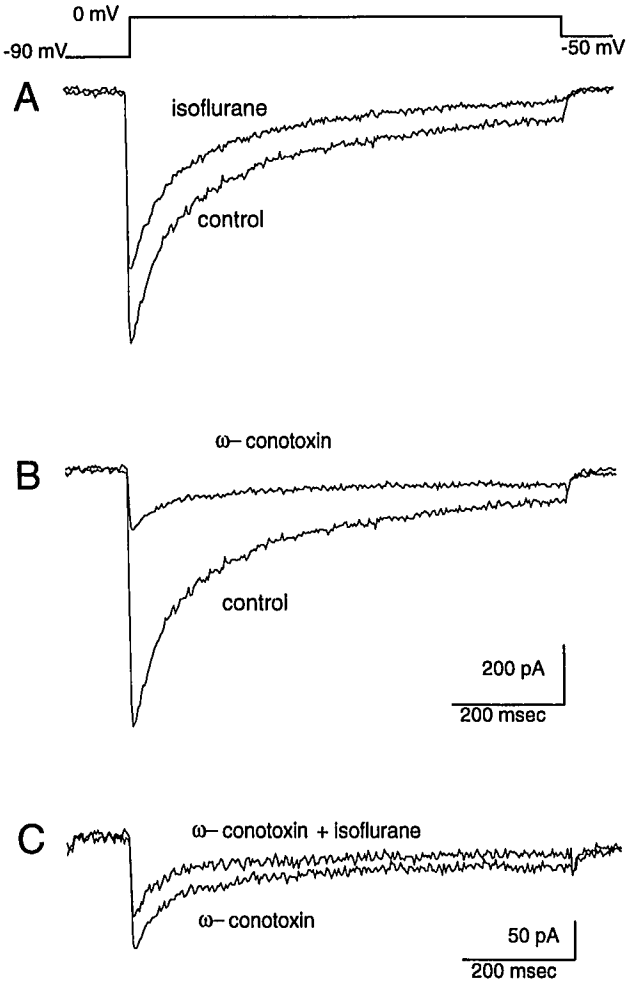

Fig. 7. Isoflurane inhibition of calcium current in the presence and absence of 0.5 μM ω-conotoxin GVIA (ω-CgTx). (*A*) Isoflurane inhibition of calcium current in the absence of ω-CgTx. (*B*) Inhibition of most of the calcium current in this cell with ω-CgTx. (*C*) Inhibition of the ω-CgTx-resistant current with isoflurane. Isoflurane (2.5%) was applied from a pressure pipette. The charge carrier was 5 mM Ca^{2+}.

hibition in this cell is therefore of N current. Through comparison of isoflurane inhibition in the absence and presence of ω-CgTx, it was shown that 2.5% isoflurane inhibited the ω-CgTx-sensitive current by 26% measured at peak current and 29% measured at the end of an 800-ms pulse.

Table 2 summarizes the effect of isoflurane on the specific channel subtypes. The low-voltage-activated transient current was somewhat more sensitive than the transient (peak current) components of the high-voltage-activated subtypes. The total high-voltage-activated current (as shown above) and the nitrendipine-sensitive (L-type) current showed more inhibition of the sustained than did the peak current; this was also apparent for the ω-CgTx-sensitive (N-type) and the residual current (ω-CgTx plus nitrendipine-resistant) but did not reach statistical significance. All of the high-voltage-activated channel subtypes show roughly equal sensitivity to isoflurane at 2.5%.

Discussion

The results reported here show that isoflurane reversibly inhibits the calcium current in isolated rat hippocampal pyramidal neurons. The inhibition of the high-voltage-activated current by isoflurane occurs in a clinically relevant concentration range, where 50% inhibition is found at about 1% for the sustained component of the current and 2% for the peak current. This is about 1 and 2 MAC, respectively, in humans and near the ED_{50} for loss of righting reflex in rodents.[41] The sensitivity of the low-voltage-activated T current was similar. Isoflurane also affects the kinetics of the high-voltage-activated current, causing an acceleration of decay of both the transient and the sustained components. Isoflurane inhibits all the voltage-gated calcium channels that are present in hippocampal pyramidal neurons; 100% inhibition can be obtained at clinically realistic concentrations (about 3–4% gas phase concentration). Although all currents arising from specific channel types were not isolated, the results show that the calcium current in these neurons is comprised of T, L, and N components, as well as others that probably include P-type. The inhibition of calcium currents is not a property unique to isoflurane; halothane and enflurane are also effective†. Isoflurane was chosen for detailed investigation because of its lower solubility, resulting in faster onset and offset of its action.

The type of calcium current most readily isolated is the low-voltage-activated, or T current, which can be distinguished by its biophysical properties alone. There is substantial evidence that the T current is involved in the control of repetitive firing of neurons[20,42,43] and may be altered in epilepsy, because in some areas of the brain it is blocked by anticonvulsants.[42,43] This current was only detectable in a minority (16%) of cells. The incidence seen in the cells studied here agrees with published reports.[39,44] Inhibition by anesthetics of a T-type current in central neurons has not been reported previously. Volatile anesthetics also inhibit the T current in rat dorsal root ganglion neurons[32] and

Table 2. Inhibition by Isoflurane of Subtypes of Calcium Current in Hippocampal Pyramidal Neurons

		% Inhibition			
		High-voltage-activated (n)			
	Low-voltage-activated T-type (n)	Total	ω-CgTx-sensitive (N-type)	Nitrendipine-sensitive (L-type)	ω-CgTx + Nitrendipine-resistant
Peak	75 ± 5 (12)	40 ± 5 (10)	34 ± 8 (6)	38 ± 7 (4)	44 ± 9 (6)
Sustained	*	84 ± 6 (10)	68 ± 14 (6)	93 ± 15 (4)	71 ± 10 (6)

All numbers are significantly different from control with $P < 0.05$ or better; inhibition of the T-type current was significantly different from that of each of the peak high-voltage-activated currents at $P < 0.01$ or better; n refers to the number of cells. Peak current measured as total high-voltage-activated and as L-type are different from the sustained component ($P < 0.05$). Peak current for N-type and ω-CgTx + nitrendipine-resistant current was not significantly different from sustained ($P > 0.10$) using Student's unpaired t test. Effects on peak and sustained currents do not significantly differ between conditions for the high-voltage-activated currents ($P > 0.25$, one-way analysis of variance). Inhibition of total high-voltage-activated current is from the same cells that were tested with ω-conotoxin GVIA (ω-CgTx) or nitrendipine. Inhibition of nitrendipine-sensitive and conotoxin-sensitive current was measured by subtraction of the inhibition by nitrendipine (10 μM) or ω-CgTx (0.5 μM) in the presence of isoflurane from that in its absence.

* No sustained component.

clonal pituitary cells.[12] The dorsal root ganglion T current is extremely sensitive to halothane and isoflurane, with an EC_{50} for halothane of about 100 μM in aqueous phase, below that required for general anesthesia (about 0.2 MAC[41]). The T current of clonal pituitary cells shows a much lower sensitivity to halothane, about 1 mM (roughly 2 MAC).[12] In the hippocampal neurons studied here, the inhibition by isoflurane (75% inhibition at 2.5%, or 0.975 mM) suggests that the T current sensitivity is close to the latter and well within the clinical range. The importance of this inhibition to general anesthesia is unclear; however, given the sensitivity of the T current to some anticonvulsants, it could be related to the known anticonvulsant activity of most general anesthetics.[45]

Investigating the contribution of the various high-voltage-activated channel types to the calcium current requires pharmacologic separation. Complete isolation of current from each channel type is not yet possible in these cells, because some current remains even after maximal blockade of all the known channels.[18] However, the existence of at least three high-voltage-activated channel types could be demonstrated pharmacologically, and the inhibition of each by isoflurane could be inferred. Inhibition of L channels was shown by subtraction of the isoflurane effect in the absence and presence of nitrendipine. This type of channel is present in a wide variety of neurons and nonneuronal cells and appears to be involved in the regulation of neuronal excitability.[19] Volatile anesthetics inhibit L currents in myocardial cells,[46] in which the effect may be related to the negative inotropic effect of volatile anesthetics. A similar effect has been shown in PC12 cells.[47] Although the hippocampal L channels may be somewhat different from those in the periphery, the response to volatile anesthetics is similar.

N channels also were inhibited by isoflurane. The N current represents the activity of a channel type that appears to be specific to neurons and some secretory cells.[19,48] It is, like the L current, high-voltage-activated but tends to decay more during a sustained pulse. A significant proportion of the calcium current in the hippocampal neurons studied here is N current, as evidenced by the effect of the specific blocker ω-CgTx. Isoflurane inhibited the N current with potency similar to that for the L-type. To date, little is known about anesthetic effects on N currents in any preparation. Gross and MacDonald[49] showed inhibition by pentobarbital of a transient component of high-voltage-activated current in dorsal root ganglion neurons, but their identification of it as N-type was based on kinetic criteria, now known to be inaccurate.[50] Inhibition of a dihydropyridine-resistant high-voltage-activated current by halothane was seen by Kress *et al.*[47] in clonal pituitary cells, but the channel type was not determined.

In hippocampal neurons treated with both ω-CgTx and nitrendipine to eliminate N and L channels, a small proportion of the current remained. This current also was inhibited by isoflurane. There is growing evidence that channels that are resistant to ω-CgTx and dihydropyridines also play an important role in synaptic transmission in the CNS.[51,52] The recently described P channel,[53] which is specific to neurons, is widely distributed

in the brain.[54] The spider toxin, ω-Aga-VIA, has been shown to block P channels, which are the dominant contributor to the dihydropyridine- and ω-CgTx-resistant current in hippocampal pyramidal cells.[22] This agent, which is not widely available, may prove useful in future studies to investigate the inhibition of P channels by anesthetics.

It is generally agreed that synaptic transmission is especially sensitive to general anesthetics.[14,55] The findings reported here that a volatile anesthetic inhibits multiple types of calcium currents in central neurons may point to a mechanism for this. An inhibition of neurotransmitter release could contribute to, or be responsible for, general anesthesia, and this could be due to calcium channel inhibition. Presynaptic inhibition by halothane in the CNS has been shown in at least two studies.[15,56] It is not certain which calcium current type (or types) is most responsible for synaptic transmission in the CNS. L currents are common in central neurons, but a major role in synaptic transmission appears unlikely.[19,51,57] Attempts to modify synaptic transmission in the CNS with agents that affect the L channel have been largely unsuccessful, and these agents do not have general anesthetic properties. In contrast, there is growing evidence that N and P channels are important for synaptic transmission in the CNS. N channels have been shown to be important for the release of neurotransmitters in the peripheral nervous system[24] and the CNS,[58,59] including the hippocampus.[60,61] P channels also appear to be important for neurotransmission in the CNS.[22,62] Inhibition of N and P currents by volatile anesthetics is therefore a possible means by which they produce presynaptic inhibition and possibly general anesthesia. However, such presynaptic inhibition might affect excitatory as well as inhibitory synaptic transmission, and so does not *a priori* imply neuronal inhibition of excitation.

Agents with such low potency and steric specificity as volatile anesthetics may have several actions relevant to general anesthesia. Not only calcium current inhibition but also chloride current enhancement at the $GABA_A$ receptor complex has been reported in these cells[4] and may be represented by the reversible conductance increase seen in the experiments reported here but subtracted out to measure calcium current. These two effects occur in the same concentration range; therefore, both may be important in generating anesthesia. Other actions of anesthetics in the brain may not be directly related to general anesthesia but may be a result of inhibition of calcium channels, such as cerebral vasodilation and protection from ischemia. Of particular interest is the recent report that a blocker of N-type calcium channels affords effective protection from global cerebral ischemia in rats.[63] A broad range of actions does not imply total nonspecificity, however: sodium currents are relatively resistant to the effects of general anesthetics.[7-9]

Exactly how volatile anesthetics might inhibit the activity of calcium channels is not clear. The inhibition may represent a direct action on a channel protein, an action through membrane lipid alterations, or an indirect action, perhaps through a second-messenger system. An action through a G-protein, cyclic nucleotide, or phosphatidyl inositol product is worth consideration, because calcium currents are known to be highly regulated by these systems depending on the channel and cell type.[64] Anesthetics also might affect the ability of the L channel to be phosphorylated, thereby inhibiting its activity. This mechanism might apply to other calcium channels, although less is known about the role of phosphorylation in their function. Further studies of hippocampal calcium current regulation and its alteration by volatile anesthetics will be required before the site, mechanism, and significance of this inhibition can be determined.

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