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Effects of Isoflurane and Hypothermia on Glutamate Receptor–mediated Calcium Influx in Brain Slices

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Background: **To understand how volatile anesthetics protect neurons during cerebral ischemia, we studied the effects of isoflurane on cerebral glutamate receptor–mediated calcium influx. Calcium influx *via* these key excitatory receptors may mediate pain transmission, memory, and the pathophysiologic sequelae of cerebral anoxia or ischemia. Because cerebral protection by hypothermia may involve a decrease in glutamate receptor activity, we also examined the interaction of temperature and isoflurane on glutamate receptor inhibition.**

Methods: **We measured glutamate receptor–mediated changes in cytosolic calcium in 300-μm–thick rat cortical brain slices. Temperature was varied to 28, 34, 37, or 39°C and isoflurane partial pressure to 0.016–0.019 atm (equivalent to 1.16 minimum alveolar concentration [MAC], adjusted for temperature and age). Brain slices were loaded with fura-2 to permit measurement of cytosolic free calcium. Calcium changes due to the glutamate receptor agonist N-methyl-D-aspartate (NMDA) (50 μM), to ischemia levels of L-glutamate (1.0 mM) or to simulated ischemia (1.0 mM glutamate, 100 μM NaCN, and 3.5 mM iodoacetate) was then measured. Slice lactate dehydrogenase leakage and adenosine triphosphate were measured as indices of cellular integrity.**

Results: **Isoflurane reduced both L-glutamate and NMDA-mediated calcium fluxes by approximately 60%. Neither the activity of the NMDA receptor nor its inhibition by isoflurane was altered by temperature. The rate of calcium influx during ischemia was significantly reduced both by temperature and by isoflurane ($P < 0.05$). Adenosine triphosphate loss and lactate dehydrogenase leakage were reduced by isoflurane during simulated ischemia by 37% and 73% ($P < 0.05$), respectively.**

Conclusions: **(1) At 1.16 MAC, isoflurane potently inhibits glutamate receptors and delays cellular injury induced by simulated ischemia, and (2) hypothermia does not reduce the intrinsic activity of cortical glutamate receptors but delays calcium accumulation during simulated ischemia. Isoflurane reduces the severity of key pathophysiologic events in an *in vitro* model of simulated cerebral ischemia. (Key words: Anesthetics, volatile: isoflurane. Animals: rat. Brain, ischemia: temperature. Experimental techniques: brain slices. Ions, calcium: intracellular. Receptors: glutamate; N-methyl-D-aspartate.)**

BRAIN injury from cerebral ischemia is caused by a complex and incompletely understood cascade of events that include hyperactivation of glutamate receptors, increase of intracellular calcium, and depletion of high-energy phosphate compounds.[1] Although it has been argued for many years that volatile general anesthetics should provide protection from cerebral ischemia,[2] the mechanisms by which they protect neurons and other cells in the central nervous system have not been clearly established. An understanding of these mechanisms may clarify why the protective effects of volatile anesthetics have been demonstrated in some studies but not in others.[3–6] This knowledge also may lead to the development of anesthetics with superior cerebroprotective properties.

Reduction in cerebral metabolic rate has been assumed to be central to the protection afforded by anesthetics.[2,7] However, agents such as isoflurane and sevoflurane, which are potent cerebral metabolic suppressants,[8,9] are no better than halothane in protecting against injury from focal cerebral ischemia.[10,11] This indicates that factors other than metabolic suppression are essential to cerebral protection. An untested possibility is a direct inhibition of cerebral glutamate receptors by volatile anesthetics. These receptors play a key role in mediating cellular injuries from hypoxia and ischemia, probably because their activation during ischemia leads to large increases in cytosolic free calcium concentration ($[Ca^{2+}]_c$). An increase in $[Ca^{2+}]_c$ produces a myriad of disturbances, including inhibition of mitochondrial adenosine triphosphate (ATP) production and activation of proteases, lipases, and nitric oxide synthase.[1,12] Volatile anesthetics, at clinical concentrations, have been shown to depress glutamate re-

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ceptor–mediated calcium influx in cultured hippocampal neurons at low glutamate concentrations (50 μM).[13] Whether this depression results in the attenuation of calcium influx at the 1 mM concentration of glutamate in the penumbra region of cerebral infarcts[12] or whether this effect can prevent ATP loss during hypoxia or ischemia has not been investigated. Because isoflurane does not reduce ischemia-induced glutamate release after cerebral ischemia,[14] effects on postsynaptic glutamate receptors are of interest. The effects of anesthetics on glutamate receptors are of additional interest because suppression of central glutamate receptors is a plausible mechanism for the state of anesthesia.[15]

Reduction in brain temperature by only several degrees can lead to dramatic improvement in neurologic outcome from focal cerebral ischemia,[16,17] possibly by reducing ischemia-induced release of excitatory neurotransmitters.[18] Conversely, hyperthermia can profoundly increase ischemic brain injury.[19,20] Because anesthetic administration can impair body temperature regulation, hypothermia has been a confounding factor in studies in which brain temperature has not been carefully controlled.[10] Whether volatile anesthetics augment the protective effects of hypothermia by further decreasing the activation of glutamate receptors has not been specifically studied.

The goals of this study were to determine in brain slices (1) whether isoflurane, at clinically relevant concentrations, prevents glutamate receptor hyperactivation at glutamate concentrations found in the ischemic penumbra; (2) whether hypothermia potentiates suppression of glutamate receptors by isoflurane; and (3) if isoflurane prevents calcium influx, ATP loss, and cell damage in conditions similar to complete ischemia *in vitro*.

Materials and Methods

These studies were approved by the University of California, San Francisco Committee on Animal Research and conformed to relevant National Institutes of Health guidelines.§

Preparation of Brain Slices

Cortical brain slices were prepared from 8–20-day-old rats anesthetized with 2% halothane in oxygen. After decapitation, cortices were rapidly dissected, glued with cyanoacrylate to a holder, and immersed in 1–3°C artificial brain extracellular fluid (ABECF) (Earle's balanced salts, millimolar composition: NaCl 116, $NaHCO_3$ 25, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.9, NaH_2PO_4 0.9, and glucose 10, *p*H 7.40, bubbled with 5% CO_2–95% O_2). Slices 300–350 μm thick were then prepared with a vibrating tissue slicer. Slices were transferred to vials of gassed ABECF containing 1 μM Fura-2 acetoxymethyl ester to permit measurements of $[Ca^{2+}]_c$. After 1 h, dye-loaded slices had fluorescence signals that were five to ten times background fluorescence. However, to permit maximal recovery from slicing trauma, the slices were not studied until 2 h after slicing. During this period, slices were maintained at room temperature (≈ 25°C) in oxygenated ABECF.

For study, slices were tied by their edges to a nylon mesh holder and mounted in a capped fluorometer cuvette containing 2 ml gassed ABECF and a stir bar. O_2 partial pressure in the cuvette remained at greater than 200 mmHg during study. Slices maintained stable $[Ca^{2+}]_c$ and ATP concentration for more than 30 min. The cuvette was mounted in the thermostatically controlled sample chamber of a fluorometer (F-2000, Hitachi, Tokyo, Japan). A fine-gauge thermocouple wire was inserted into the cuvette fluid and monitored continuously. Slices were maintained at the desired temperature (±0.2°C) for 10 min before study. Dye leakage and photobleaching were minimal.

Measurements of Cytosolic Calcium Concentration

Change in $[Ca^{2+}]_c$ in brain slices was determined by dual-excitation fluorescence spectroscopy. Slices were alternately excited with 340- and 380-nm light, and emitted light intensity at 510 nm was recorded. The ratio of these two signals was used to determine the change in $[Ca^{2+}]_c$, based on calibration experiments done in other slices. We calibrated changes in $[Ca^{2+}]_c$ relative to fluorescence changes observed during an *in vivo* dye titration with calcium–ethyleneglycol-bis-(β-aminoethyl ether)-tetraacetic acid buffers. Fluorescence ratio signals corresponding to dye saturation (approximately 3,000 nM) and zero calcium levels were determined by exposing slices first to 10 μM ionomycin (a calcium ionophore) in 1.8 mM Ca^{2+} buffer and then to calcium-free buffer with 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-tetraacetic acid. This calibration determined the relation between fluorescence ratio and $[Ca^{2+}]_c$ concentration from 0 to 3,000

§ National Institutes of Health: Guide for Care and Use of Laboratory Animals. Publication 85-23. Revised. Bethesda, National Institutes of Health, 1985.

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nM. This ratio method avoided errors in background correction and is independent of fura-2 loading, dye loss, and photobleaching, and is most accurate for measuring changes in $[Ca^{2+}]_c$. These calibrations were corrected for the change in the dissociation constant for fura-2 with temperature.[21] Absolute resting calcium concentrations were determined in more extensive calibrations in other slices, as described by Bickler.[22]

Measurement of Glutamate Receptor Activity

The activity of glutamate receptors was determined by measuring changes in $[Ca^{2+}]_c$ mediated by receptor-linked ion channels. Calcium fluxes were verified as coming predominately from receptor linked ion channels in preliminary experiments: 1 μM tetrodotoxin, 1 μM conotoxin (N-type calcium channel antagonist), or nimodipine (L-type calcium channel antagonist) did not alter calcium influx during N-methyl-D-aspartate (NMDA) application by more than 5%. These studies ruled out calcium influx mechanisms other than those due to the NMDA receptor. Dizocilpine (50 μM), a specific NMDA antagonist, prevented detectable calcium influx during application of 50 μM NMDA.

Experimental Design

Response to Agonists. Fura-loaded slices were transferred to cuvettes containing gassed ABECF and placed in the thermostatically controlled cuvette holder of the fluorometer. To measure calcium influx at glutamate concentrations found in the penumbra of cerebral infarcts, L-glutamate was injected into the cuvette (1.0 mM final concentration). To assess the activity of the NMDA subtype of the glutamate receptor, 50 μM NMDA was used instead. Slices were exposed to NMDA twice, once during a trial in which control response to NMDA was determined and again after 1 h washout and recovery in fresh ABECF. Slices showing responses less than 75% of the mean response in the first control were rejected. Slices were studied again, either with a second control application of agonist or during application of agonist in the presence of isoflurane. This procedure was used to reduce the effect of interslice variability in NMDA responses.

Response to Simulated Ischemia. The rate of change of $[Ca^{2+}]_c$ during simulated cerebral ischemia was evaluated in a separate group of slices exposed to glutamate (1.0 mM), cyanide (100 μM), and iodoacetate (3.5 mM). This procedure was performed in an attempt to duplicate biochemically the conditions of high glutamate concentration and blockade of both aerobic and anaerobic ATP production as in complete ischemia. The rate of change of $[Ca^{2+}]_c$ was then evaluated during a 200–300-s period.

Determination of Anesthetic Concentrations

Saturated isoflurane solution (2.5 ml liquid isoflurane in 15 ml ABECF at 25°C) was introduced into cuvettes by syringe injection 100 s before agonist addition. The actual partial pressure of isoflurane resulting in the cuvette was determined by gas chromatographic analysis of ABECF samples from cuvettes at 28°C and 37°C. Isoflurane was extracted into air in a constant-temperature water bath. The air was then injected into a Gow Mac Gas Chromatograph (Bridgewater, NJ) calibrated with an isoflurane standard. Isoflurane loss was 12% at 28°C and 30% at 37°C during mock experiments. These loss estimates were used to adjust the amount of isoflurane added to cuvettes to produce the desired partial pressure, assuming a linear relation between loss rate and temperature. Adult rat minimum alveolar concentration (MAC) for isoflurane was assumed to be 0.014 atm at 37°C[23] and to decrease with temperature, as determined by Eger *et al.*[24] for halothane. This decrease with temperature is almost identical to the decrease in isoflurane vapor pressure with decreasing temperature, except at 28°C, where MAC is 17% less than predicted by changes in vapor pressure alone. We also corrected MAC values for the known effects of age on halothane potency in young rats.[25] We assumed that age produces similar percentage changes in halothane and isoflurane MAC and that this effect is independent of temperature. Isoflurane concentrations in the cuvettes at 1.16 MAC were approximately 325–375 μM, depending on temperature.

Measurements of Brain Slice Adenosine Triphosphate Concentrations and Lactate Dehydrogenase Leakage during "Ischemia"

To test the hypothesis that isoflurane delays ATP loss during *in vitro* "ischemia," ATP levels were measured in brain slices exposed to cyanide and iodoacetate with or without 0.018 atm (1.16 MAC) isoflurane at 37°C. ATP levels were measured with a bioluminescence assay (firefly luciferin-luceriferase, Analytical Luminescence Laboratory, San Diego, CA) and a luminometer. Total slice protein was measured with a standard spectrophotometric assay (Enhanced Protein Assay, Pierce, St. Louis, MO).

Lactate dehydrogenase (LDH), a soluble cytosolic enzyme, leaks from injured neurons and glial cells dur-

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ing hypoxia.[26] LDH levels were measured in slice supernatant solutions collected during the ATP assays. LDH activity was measured fluorometrically by monitoring the disappearance of NADH (excitation 340 nm and emission 460 nm) in a reaction with pyruvate (2 mM) and NADH (1 mM) at *p*H 7.0.

Statistics

An analysis of variance with a Student-Newman-Keuls *post hoc* test was used to determine whether isoflurane depressed the activity of glutamate receptors. A paired Student's *t* test was used for single paired comparisons. The significance of regressions was tested with y using the *t* test method of the Statview statistical package (Abacus Concepts, Berkeley, CA), and differences between regression lines were compared with a *t* test according to Glantz Stanton.[27]

Results

Effects of Isoflurane on Resting Cell Calcium

Basal $[Ca^{2+}]_c$ in cortical brain slices at 37°C was 190 ± 31 nM (n = 6). Isoflurane (1.16 MAC, 0.016–0.019 atm) increased $[Ca^{2+}]_c$ in cortical brain slices at all four temperatures studied (22, 37, 78, and 87 nM above baseline, respectively), as shown in figure 1. At all temperatures, the increase in $[Ca^{2+}]_c$ caused by isoflurane was gradual and required 100–150 s to reach maximal. The increase in $[Ca^{2+}]_c$ was fully reversible if the slice was returned to anesthetic-free ABECF. Dantrolene (10 μM), an inhibitor of calcium release from endoplasmic reticulum, prevented increases in resting $[Ca^{2+}]_c$ in 6 of 8 slices exposed to 1.16 MAC isoflurane at 37°C.

Effects of Isoflurane on Glutamate-mediated Calcium Influx

Isoflurane, at 1.16 MAC and 37°C, decreased calcium influx due to 1.0 mM L-glutamate to 63 ± 24% of control (mean ± SD, n = 18, unpaired *t* test, $P < 0.05$). An example of this decrement in calcium influx is shown in figure 2.

Effects of Temperature and Isoflurane on NMDA-mediated Calcium Influx

Figure 3 shows an example of the decrement in NMDA-mediated calcium influx caused by 1.16 MAC isoflurane. This reduction in influx was significant at 34, 37 and 39°C ($P < 0.05$); *P* was 0.08 at 28°C (fig. 4). Hypothermia (28 or 34°C) or hyperthermia (39°C) did not affect the activity of glutamate receptors in cortical brain slices, as measured by NMDA-mediated changes in $[Ca^{2+}]_c$. Neither peak calcium levels nor the temporal pattern (rate of calcium increase during NMDA application or rate of return to baseline) of calcium changes due to NMDA were affected by altering the temperature from 37°C (analysis of variance).

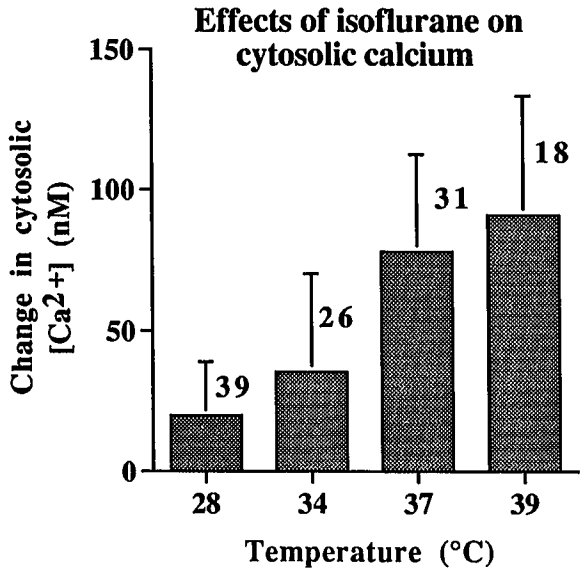

Fig. 1. Effect of isoflurane on basal cytosolic free calcium concentrations ($[Ca^{2+}]_c$) in cortical brain slices at various temperatures. Values represent mean increases in $[Ca^{2+}]_c$ during a 2-min exposure to isoflurane (1.16 MAC). Error bars = 1 SD; n values are shown above bars. All changes in $[Ca^{2+}]_c$ were significantly different from preisoflurane levels.

Effects of Temperature and Isoflurane on Calcium Accumulation during Simulated Ischemia

At 37°C, simulated complete ischemia (1.0 mM glutamate, 100 μM cyanide, 3.5 mM iodoacetate) caused $[Ca^{2+}]_c$ to increase to a maximum of 2,150 ± 300 nM (n = 21). At 28°C the maximal change was 1,950 ± 345 nm (n = 18, not significant compared with 37°C). Isoflurane did not decrease the maximal change in $[Ca^{2+}]_c$ during 15 min of simulated ischemia at 28 or 37°C. However, both hypothermia and isoflurane significantly delayed the *rate* of $[Ca^{2+}]_c$ change during simulated ischemia *in vitro* (fig. 5). A regression of the effect of temperature on rate of $[Ca^{2+}]_c$ change during ischemia was significant in both control ($P < 0.001$) and isoflurane groups ($P < 0.01$). Furthermore, the slopes of these regressions were significantly dif-

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ferent from each other ($P < 0.05$), indicating a significant reduction in calcium accumulation by isoflurane.

Effects of Isoflurane on Adenosine Triphosphate Loss and Lactate Dehydrogenase Release during Ischemia

Two minutes of simulated ischemia reduced ATP from 27.4 ± 4 to 5.2 ± 1.2 nmol/mg protein (n = 9 in each group; $P < 0.01$). After 4 or 6 min of ischemia, remaining ATP was present at less than 0.5 nmol/mg protein. Isoflurane (1.16 MAC), when present for 2 min of simulated ischemia, resulted in less ATP loss (8.3 ± 1.3 nmol/mg protein; $P < 0.05$ compared with the 2-min group without isoflurane).

Whereas 2 min of ischemia increased LDH leakage to 3.14 times control (n = 8, $P < 0.01$), isoflurane (1.16 MAC) prevented detectable changes in LDH (0.85 times control; n = 8) during a similar 2-min period of ischemia.

Discussion

The results show that isoflurane decreases the activity of cerebral glutamate receptors and limits the rate of calcium influx and cell damage (ATP loss or LDH leakage) during simulated ischemia *in vitro*. Furthermore, whereas hypothermia does not decrease glutamate receptor–mediated calcium influx in cortical brain slices, hypothermia does decrease the rate of calcium influx during ischemia. The current results therefore offer insights into how volatile anesthetics and hypothermia reduce the severity of key pathologic events during cerebral ischemia. Furthermore, they may explain important aspects of the anesthetic state.

Is Isoflurane a Cerebral Protectant?

A substantial body of evidence indicates that agents that reduce the activity of cerebral glutamate receptors prevent some of the adverse effects of cerebral ischemia.[1,12,28–30] Antagonism of glutamate receptors, by limiting calcium influx, may prevent a well-documented cascade of calcium-dependent injury. The NMDA receptor complex, with its high calcium permeability, has been the most extensively studied. NMDA receptor antagonists such as dizocilpine reduce the calcium influx and improve neurologic outcome after focal ischemia.[29,30] We show that isoflurane diminishes NMDA and glutamate-mediated calcium influx at glutamate concentrations occurring during brain ischemia.

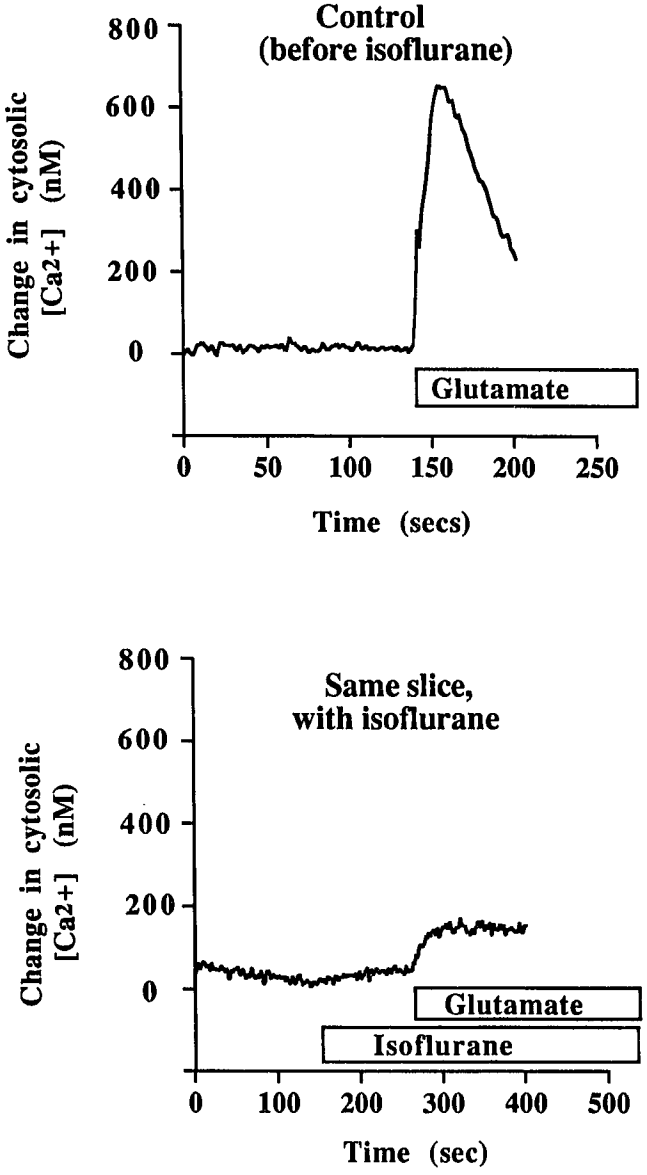

Fig. 2. Effect of isoflurane (1.16 MAC) on glutamate-mediated calcium influx in a cortical slice at 37°C. Box = period during which ischemia levels of glutamate (1.0 mM) were present. Basal cytosolic free calcium concentration ($[Ca^{2+}]_c$) changed during the exposure to isoflurane.

The current results therefore predict that isoflurane reduces key pathologic events under *in vivo* conditions and provide a mechanism other than hypothermia[31] for the cerebral protective properties of volatile anesthetics.

However, isoflurane does not completely prevent glutamate-mediated changes in $[Ca^{2+}]_c$. Because isch-

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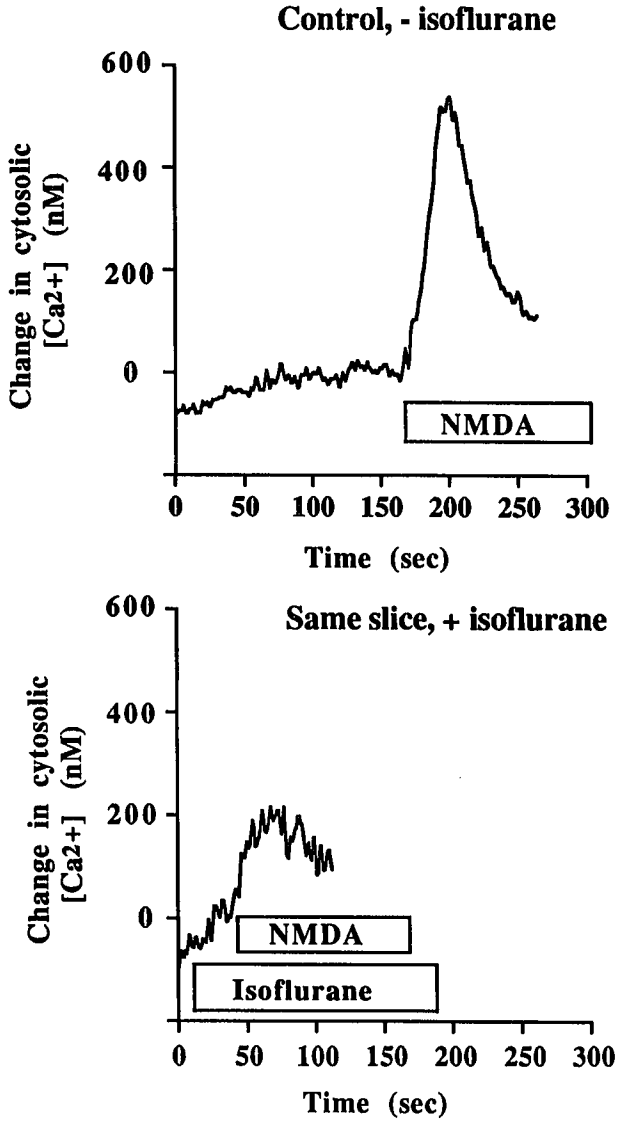

Fig. 3. Effect of isoflurane on changes mediated by 50 μM N-methyl-D-aspartate in cytosolic calcium concentration in a cortical brain slice at 37°C.

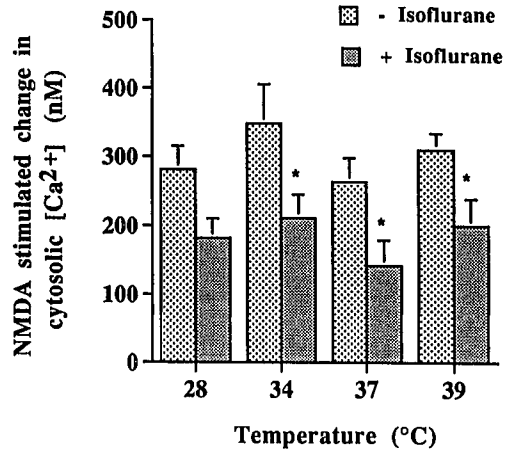

Fig. 4. Effect of isoflurane (1.16 MAC, corrected for age and temperature) on increases mediated by 50 μM N-methyl-D-aspartate in cytosolic free calcium concentration ($[Ca^{2+}]_c$) at various temperatures. Boxes = mean ± SD of 15–20 slices. *Significant difference between control (−isoflurane) and isoflurane-exposed slices. At 28°C, $P = 0.08$.

emia involves calcium entry from multiple sources and is not prevented completely even if a substantial fraction of glutamate receptors are blocked,[32,33] it is perhaps not surprising that isoflurane does not result in a more profound decrease in the maximum extent of calcium change during simulated complete ischemia. Although the current results predict that isoflurane will delay calcium influx in cerebral ischemia by reducing the activity of glutamate receptors, it is not clear whether isoflurane will necessarily prevent calcium from rising to levels that result in cell injury.

Does Isoflurane Exert a Direct Effect on Glutamate Receptors during Ischemia?

Our results do not permit us to conclude with certainty that the inhibition of glutamate receptor–me-

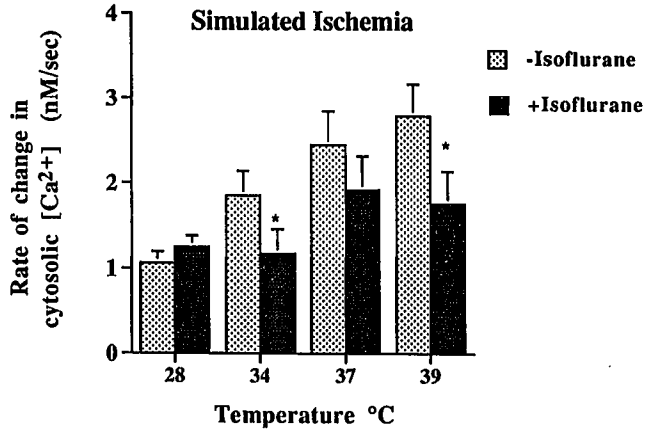

Fig. 5. Influence of temperature and isoflurane (1.16 MAC, 0.018 atm) on rate of change in cytosolic calcium during simulated ischemia (1.0 mM glutamate, 100 μM NaCN, 3.5 mM iodoacetate) in cortical brain slices. Boxes = mean and SD for 18–24 slices. *Significant difference from control value. Regression of changes *versus* temperature were significant for both control and isoflurane groups ($P < 0.001$); regressions also were significantly different from each other ($P < 0.05$).

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diated calcium influx by isoflurane is a direct effect on the glutamate receptor because we did not exclude several effects that could have indirectly influenced the activity of the glutamate receptor. These effects include activation of protein kinases (which could then phosphorylate the NMDA receptor) by elevations in basal $[Ca^{2+}]_c$, activation of a calcium-sensitive potassium conductance,[34] and activation of other potassium channels that would hyperpolarize the neurons[15] and influence the voltage-dependent NMDA channel activation. A reduction in the single-channel activity of NMDA receptors in excised membrane patches from hippocampal neurons was reported by Yang and Zorumski,[35] but without a detailed analysis of effects at clinically relevant isoflurane concentrations.

Effects of Temperature on the Activity of the Glutamate Receptor Complex

Hypothermia is known to be a potent cerebral protectant.[16–18] Our results show that glutamate receptors, like many other ligand and voltage-gated ion channels, are affected minimally by mild hypothermia,[36–39] perhaps because the energy of activation of channel opening and ligand binding is very low, so that ion flux through the aqueous pore is minimally altered by temperature. However, between 24°C and 14°C the activity of the NMDA receptor has been shown to be depressed threefold.[40] Clearly, mechanisms other than a direct effect of temperature on glutamate receptor activity must account for the protective effects of mild hypothermia. The most important of these other factors is probably diminished glutamate release in ischemic brain tissue.[18] The results of our study document another effect: hypothermia delays calcium influx during simulated ischemia. This effect may be attributable to reduced ATP depletion mediated by a hypothermia-related reduction in metabolic rate.

Of interest, reducing the temperature to 28°C caused a 57% reduction in calcium accumulation rate during simulated ischemia, but isoflurane reduced this rate of change at 37°C by only 22% (fig. 5). This finding is consistent with the profound degree of cerebral protection afforded by hypothermia[16–18] and the mild degree by volatile anesthetics.[31]

Effects of Isoflurane on Basal Cytosolic Calcium

We demonstrate that isoflurane at clinically relevant concentrations increases basal $[Ca^{2+}]_c$. This finding is in agreement with several other studies in many cell types, including neurons.[34,40] However, this phenomenon has not been previously shown to be temperature dependent. Our studies differed from others in that we studied brain slices rather than isolated cultured neurons or other cells. Furthermore, we show that a possible mechanism for increases in $[Ca^{2+}]_c$ is release from intracellular stores, because this increase was blocked with dantrolene. Our data do not permit us to speculate on whether the isoflurane-induced increase in basal $[Ca^{2+}]_c$ is somehow protective (*i.e.*, by activating kinases that act to decrease neurotransmission[34]) during ischemia.

Inhibition of Glutamate Receptors as a Mechanism for General Anesthesia

Our results support the hypothesis that inhibition of glutamatergic neurotransmission by volatile anesthetics in the spinal cord and brain is a cause of the anesthetic state. Previous data attest this claim: (1) glutamatergic pathways comprise a very important, if not dominant, set of excitatory pathways in brain[41]; (2) pain transduction in the spinal cord involves glutamatergic neurotransmission and is inhibited by volatile anesthetics[42]; (3) long-term potentiation, the cellular substrate of memory (or amnesia) is mediated, at least in hippocampus, by glutamatergic pathways[41]; and (4) drugs that act at glutamate receptors, such as ketamine and dizocilpine either are potent anesthetics or dramatically alter the MAC for isoflurane.[43]

Do Brain Slices Accurately Predict Effects in Intact Brain?

Extrapolation of results from brain slices to the intact brain may be difficult, as experiment using brain slices have several important limitations. First, there is a variable injury layer in each slice that may not behave as normal brain during hypoxia or glutamate exposure, even though resting calcium levels appear normal. Second, brain slices probably do not accumulate lactic acid and other metabolites to the same extent during anoxia or ischemia as intact brain, although in our model the intracellular compartment was acidified by about 0.6 *p*H units.[44] Thus, conditions in the slice environment may not completely duplicate those of *in vivo* ischemia. However, brain slices may be more suitable models of the intact brain than cultured neurons because of their normal synaptic structure. An additional advantage is that brain slices are much more sensitive than cultured neurons to metabolic insults; as shown by Dubinsky and Rothman,[45] 100 μM NaCN produces a small increase in $[Ca^{2+}]_c$ in dissociated hippo-

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campal neurons, whereas in brain slices,[31] $[Ca^{2+}]_c$ increases to very high levels.

We conclude that isoflurane, at clinically relevant concentrations, reduces the severity of key pathologic events during simulated cerebral ischemia, including ATP loss, LDH leakage, and calcium influx mediated by glutamate receptors. Hypothermia, though not reducing glutamate receptor activity, reduces the rate of calcium influx in simulated ischemia.

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