Isoflurane Preconditions Hippocampal Neurons against Oxygen–Glucose Deprivation

Role of Intracellular Ca\(^{2+}\) and Mitogen-activated Protein Kinase Signaling

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Background: Isoflurane preconditions neurons to improve tolerance of subsequent ischemia in both intact animal models and in in vitro preparations. The mechanisms for this protection remain largely undefined. Because isoflurane increases intracellular Ca\(^{2+}\) concentrations and Ca\(^{2+}\) is involved in many processes related to preconditioning, the authors hypothesized that isoflurane preconditions neurons via Ca\(^{2+}\)-dependent processes involving the Ca\(^{2+}\)-binding protein calmodulin and the mitogen-activated protein kinase–ERK pathway.

Methods: The authors used a preconditioning model in which organotypic cultures of rat hippocampus were exposed to 0.5–1.5% isoflurane for a 2-h period 24 h before an ischemia-like injury of oxygen–glucose deprivation. Survival of CA1, CA3, and dentate neurons was assessed 48 h later, along with interval measurements of intracellular Ca\(^{2+}\) concentration (fura-2 fluorescence microscopy in CA1 neurons), mitogen-activated protein kinase p42/44, and the survival-associated proteins Akt and GSK-3β (in situ immunostaining and Western blots).

Results: Preconditioning with 0.5–1.5% isoflurane decreased neuron death in CA1 and CA3 regions of hippocampal slice cultures after oxygen–glucose deprivation. The preconditioning period was associated with an increase in basal intracellular Ca\(^{2+}\) concentration of 7–15%, which involved Ca\(^{2+}\) release from intraphosphatidylserine stores in the endoplasmic reticulum, and transient phosphorylation of mitogen-activated protein kinase p42/44 and the survival-associated proteins Akt and GSK-3β. Preconditioning protection was eliminated by the mitogen-activated extracellular kinase inhibitor U0126, which prevented phosphorylation of p44 during preconditioning, and by calmidazolium, which antagonizes the effects of Ca\(^{2+}\)-bound calmodulin.

Conclusions: Isoflurane, at clinical concentrations, preconditions neurons in hippocampal slice cultures by mechanisms that apparently involve release of Ca\(^{2+}\) from the endoplasmic reticulum, transient increases in intracellular Ca\(^{2+}\) concentration, the Ca\(^{2+}\)-binding protein calmodulin, and phosphorylation of the mitogen-activated protein kinase p42/44.

ISCHEMIC preconditioning or ischemic tolerance (IP/IT) is a phenomenon in which an intervention induces long-lasting resistance to the effects of subsequent severe ischemia. IP/IT can be created in a number of tissues, including the brain. 1 IP/IT in the brain occurs in at least two temporal profiles: one in which protection can be induced within seconds to minutes and one in which tolerance develops over hours to days. The latter may require protein synthesis. Examples of extensively studied preconditioning stimuli include sublethal hypoxia, brief periods of global ischemia, cortical spreading depression, and inflammation. It has been proposed that the many known triggers of IP/IT all involve physiologic processes that, if more prolonged or more severe, can cause cellular damage. 2

Volatile anesthetics can precondition the brain against ischemic injuries, both when applied minutes before an injury or a day or more before. Although anesthetic preconditioning (APC) after exposure to volatile anesthetics was first described in the heart, 3 APC has recently been observed in the brains of intact rodents 4–6 and in in vitro models of cerebral ischemia. 7,8 The mechanisms involved in APC in the brain have not been extensively investigated. Proposals for the mechanism of preconditioning with volatile anesthetics in the brain include induction of nitric oxide production 4,5 and activation of adenine triphosphate-sensitive potassium channels. 7

There are strong reasons to hypothesize that Ca\(^{2+}\) plays a critical role in most forms of neuronal ischemic preconditioning or tolerance, including tolerance induced by volatile anesthetics. Preconditioning stimuli related to increases in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in the brain include N-methyl-D-aspartate receptor activation, 9 opening of voltage-gated Ca\(^{2+}\) channels (e.g., from depolarization during spreading depression), and application of low concentrations of Ca\(^{2+}\) ionophores. 10 Other stimuli include increases in [Ca\(^{2+}\)]\(_i\) elicited by cytokines, hormones (e.g., erythropoietin) and neuromodulators (adenosine via A\(_3\) receptor activation) as reviewed in Dirnagl et al. 2 Volatile anesthetics also increase [Ca\(^{2+}\)]\(_i\) in neurons. 11,12 These increases in [Ca\(^{2+}\)]\(_i\) could plausibly activate mitogen-activated protein (MAP) kinases, 13,14 stimulate nitric oxide production by nitric oxide synthase, 15 and modulate Ca\(^{2+}\)-sensitive potassium channels. Alterations in gene expression are also probably involved in APC, because Ca\(^{2+}\) is known to activate the transcription factor CREB. 16 Further, substantial evidence shows that transient increase in the MAP kinase–ERK pathway is involved in the development of IP/IT in the brain and heart, 17–19 and because the ERK pathway is activated by increases in [Ca\(^{2+}\)]\(_i\), it is possible that it plays a role in APC as well. The MAP kinase–ERK pathway is involved in acute neuroprotection with isoflurane in a calcium-dependent mechanism. 20

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Received from the Severinghaus-Radiometer Research Laboratories, Department of Anesthesiology and Perioperative Care, University of California at San Francisco, San Francisco, California. Submitted for publication October 14, 2004. Accepted for publication May 11, 2005. Supported by grant No. R01 GM 52212 from the National Institutes of Health, Washington, D.C. to Dr. Bickler.

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Ca\textsuperscript{2+}, MAP KINASES, AND PRECONDITIONING

Anesthesiology, V 103, No 3, Sep 2005

In this study, we used organotypic slice cultures of rat hippocampus as a model to examine the mechanisms underlying APC. Based on the considerations outlined above, we tested the hypothesis that APC is based on anesthetic-induced increases in [Ca\textsuperscript{2+}]\textsubscript{i} that, via the Ca\textsuperscript{2+}-sensing protein calmodulin, activate the MAP kinase-ERK pathway. In our model of APC, we preconditioned hippocampal slice cultures with 0.5 or 1.5% isoflurane for a 2-h period. One day later, hippocampal slice cultures underwent 1 h of oxygen–glucose deprivation (OGD), and cell death in CA1, CA3, and dentate neurons were measured 2 or 3 days after preconditioning and after OGD for assessment of survival-associated proteins.

Materials and Methods

Study Design: Preconditioning in Organotypic Cultures of Hippocampus

Figure 1 shows the basic study design. Slice cultures of hippocampus were exposed to gas-phase 0.5 or 1.5% isoflurane (isoflurane in air plus 5% CO\textsubscript{2}) for 2 h and returned to standard culture conditions for the next 24 h. At that time, the cultures were exposed to combined oxygen and glucose deprivation for 1 h (simulated ischemia). The percentage of dead and living neurons remaining in CA1, CA3, and dentate regions of the slices were determined serially at 2 and 3 days after the simulated ischemia. Measurements of [Ca\textsuperscript{2+}]\textsubscript{i} in CA1 neurons within the cultures were made in separate groups of slices during simulated preconditioning conditions. Other cultures were processed as above and frozen at the times indicated in figure 1 for determination of expression levels of MAP kinases p42/p44, the antiapoptotic protein Akt, and the Akt substrate protein GSK-3\textbeta.

Preparation of Hippocampal Slice Cultures

All studies were approved by the University of California at San Francisco (UCSF) Committee on Animal Research and conform to relevant National Institutes of Health guidelines.

Organotypic cultures of the hippocampus were prepared by standard methods\textsuperscript{21,22} as modified by Sullivan et al.\textsuperscript{23} Briefly, Sprague-Dawley rats (aged 8–12 days; Simonsen Laboratories, Gilroy, CA) were anesthetized with 1–2% halothane and given an intraperitoneal injection of ketamine (10 mg/kg) and diazepam (0.2 mg/kg). The rats were decapitated, and the hippocampi were removed and placed in 4°C Gey’s Balanced Salt Solution (UCSF Cell Culture Facility). Next, the hippocampi were transversely sliced (400 μm thick) with a tissue slicer (Siskiyou Design Instruments, Grants Pass, OR), and stored in Gey’s Balanced Salt Solution containing 0.038 mg/ml ketamine at 4°C for 1 h.\textsuperscript{24} The slices were then transferred onto 30-mm-diameter membrane inserts (Milllicell-CM; Millipore, Bedford, MA), and put into six-well culture trays with 1.5 ml slice culture medium per well. The slice culture medium consisted of 50% Minimal Essential Medium (Eagles with Earle’s balanced salt solution; UCSF Cell Culture Facility), 25% Earles balanced salt solution (UCSF Cell Culture Facility), 25% heat-inactivated horse serum (Hyclone Laboratories, South San Francisco, CA) with 6.5 mg/ml glucose, and 5 mM KCl. Slices were kept in culture for 7–14 days before study.

Simulation of Ischemia with In Vitro OGD and Assessment of Cell Death in Cultured Hippocampal Slices

In vitro ischemia was simulated by anoxia combined with glucose-free media (OGD). Before hypoxia, the slices were washed three times with glucose-free Hank’s balanced salt solution. The cultures were then placed into a 24 airtight Billups-Rothenberg Modular Incubator chamber (Del Mar, CA) through which 95% N\textsubscript{2}–5% CO\textsubscript{2} gas, preheated to 37°C, was passed at 5–10 l/min. The temperature of the chamber was kept at 37°C both by passing preheated gas through the chamber and by placing a heat lamp over the chamber. The temperature inside the chamber was monitored with a thermocouple thermometer. After 10 min of gas flow, the chamber was sealed and placed in a 37°C incubator. The partial pressure of oxygen was approximately 0–0.2 mmHg, measured with a Clark-type oxygen electrode. After the injury, the culture tray was removed from the chamber, the anoxic glucose-free Hank’s balanced salt solution was aspirated from the wells, and standard (oxygenated) slice culture media was added.

Cell viability was assessed with propidium iodide (PI) fluorescence (Molecular Probes, Eugene, OR). PI, a highly polar fluorescent dye, penetrates damaged plasma membranes and binds to DNA. Before imaging, slice culture media containing 2.3 μM PI was added to the wells of the culture trays. After 15 min, the slices were examined with a Nikon Diaphot 200 inverted microscope (Nikon Corporation, Tokyo, Japan), and fluoress...
cent digital images were taken using a SPOT Jr. Digital Camera (Diagnostic Instruments Inc., Sterling Heights, MI). Excitation light wavelength was 490 nm, and emission was 590 nm. The sensitivity of the camera and intensity of the excitation light was standardized so as to be identical from day to day. PI fluorescence was measured in the dentate gyrus, CA1, and CA3 regions of the hippocampal slices. Slices were discarded if they showed more than slight PI fluorescence in these regions after 7–10 days in culture. Slices were imaged before OGD (signal assumed to represent 0% cell death) and at 2 and 3 days after OGD. In previous studies, we found that maximum post-OGD death consistently occurs at approximately day 2 or 3 and declines over the next 11 days.23 Serial measurements of PI fluorescence intensity were made in predefined areas (manually outlining CA1, CA3, and dentate separately) for each slice using NIH Image-J software (National Institutes of Health, Bethesda, MD). Thus, cell death was followed in the same regions of each slice after simulated ischemia. After the measurement of PI fluorescence on the third post-OGD day, all of the neurons in the slice were killed to produce a fluorescence signal equal to 100% neuron death in the regions of interest. This was done by adding 100 μM potassium cyanide and 2 mM sodium iodoacetate to the cultures for at least 20 min. Twelve to 24 h later, final images of PI fluorescence (equated to 100% cell death) were acquired. Percentage of dead cells at 0, 2, and 3 days after OGD were then calculated based on these values. A linear relation exists between cell death and PI fluorescence intensity.22,24

Measurements of \( [Ca^{2+}] \)

In separate groups of slices, \( [Ca^{2+}] \), was measured before, during, and after 1% isoflurane exposure. Estimates of \( [Ca^{2+}] \) in CA1 neurons in slice cultures were made using the indicator fura-2/AM and a dual excitation fluorescence spectrometer (Photon Technology International, South Brunswick, NJ) coupled to a Nikon Diaphot inverted microscope. Slice cultures were incubated with 5–10 μM of the indicator dye for 15–30 min before measurements. Cultures for these measurements were grown on Nunc Anopore (Nalge Nunc, Rochester, NY) culture tray inserts because of their low autofluorescence at fura-2 excitation wavelengths. Slit apertures in the emission light path were adjusted to restrict measurement of light signals to those coming from the CA1 cell body region. Calibration of \( [Ca^{2+}] \) was done by using the dissociation constant of fura-2 determined in vitro with a \( Ca^{2+} \) buffer calibration kit (Molecular Probes). The calibration process involved using the same light source, optical path, and filters as used with the slice culture measurements. The dissociation constant for fura-2 was 311 nM, similar to published values.25 Background fluorescence (i.e., fluorescence in the absence of fura) was subtracted from total fluorescence signals before calculation of \( [Ca^{2+}] \) as described previously.26 Estimates of \( [Ca^{2+}] \) with this technique are accurate to approximately ±10 nM.

Immunostaining and Western Blots of Slice Cultures

Western blots of proteins from culture homogenates were performed with standard methods. Five to eight slices were pooled for each assay, and each study was repeated three or four times. Samples were obtained at the times indicated in figure 1. Protein content in each sample was measured (Bradford protein assay with Coomassie blue) and adjusted so that equal amounts of protein were applied to each lane. Protein bands were visualized after incubation with biotinylated secondary antibodies followed by an enhanced chemiluminescence assay. The intensity of immunostaining was analyzed by scanning the photographic images and using image analysis software (NIH Image) to quantify the staining intensity. In situ immunostaining of the activated forms of Akt and MAP kinase ERK (p42/44) were done in hippocampal cultures fixed with 4% chilled paraformaldehyde. Antibodies to p-Akt (Ser 473 phosphorylation) and those to MAP kinase p42/44 (Thr 202/Tyr 204 phosphorylation) were obtained from Cell Signaling Technology (Beverly, MA). As with the Western blots, appropriate biotinylated secondary antibodies were used to stain the proteins of interest. Relative protein levels in the in situ preparations were measured with a microscope, a digital camera, and NIH Image software. This procedure involved outlining regions of interest in each slice and determining the mean gray value within that region as an index of staining intensity.

Statistical Analysis

The percentage survival of neurons in the different regions of the slices is generally not normally distributed. Therefore, the Kruskal–Wallis test followed by the Mann–Whitney U test (JMP; SAS Institute, Cary, NC) was used to compare the means of different treatment groups. T tests or analyses of variance were used to compare other group means, and allowance was made for multiple comparisons. Differences were considered significant for \( P < 0.05 \).

Results

Preconditioning with 0.5 and 1.5% Isoflurane Reduces Death in Hippocampal Neurons

Both a relatively low (0.5%) and relatively high (1.5%) clinical concentration of isoflurane preconditioned neurons in hippocampal slice cultures. Results with 0.5% isoflurane are shown in figure 2, and those for 1.5% isoflurane are shown in figure 3. The degree of preconditioning varied with isoflurane concentration and cell
region. With 0.5% isoflurane, protection was statistically significant in CA3 and dentate but not CA1 \((P = 0.10)\). In the slices preconditioned with 1.5% isoflurane, CA1 and dentate neurons were protected, but those in CA3 were not. The magnitude of the reduction in cell death achieved with preconditioning was approximately 15–40%, which is modest compared with the 70–80% reduction in cell death achieved when isoflurane is present at the same time as the OGD injury.\(^2\)\(^3\)

**Intracellular \(\text{Ca}^{2+}\) during Preconditioning**

In slice cultures grown on special culture inserts, we measured \([\text{Ca}^{2+}]_i\) in CA1 neurons during application of perfusion solution equilibrated with 1% isoflurane. In this system, approximately 30% of the isoflurane is lost between the reservoir bottle and the microscope stage slice-holding chamber, as measured with a gas chromatograph in the laboratory of Edmund Eger, M.D. (Professor, Department of Anesthesia and Perioperative Care, UCSF). Therefore, the isoflurane concentration reaching the CA1 neurons was approximately 0.7%, between the two concentrations examined in the survival studies. In each of six slices examined, \([\text{Ca}^{2+}]_i\) increased during 10 min of isoflurane application and returned to baseline during washout. In figure 4, averaged traces of \([\text{Ca}^{2+}]_i\) are shown. The increase in \([\text{Ca}^{2+}]_i\)
after 10 min of isoflurane was statistically significant ($P < 0.001$). In several slices, isoflurane perfusion was continued for up to 1 h. Similar increases in $[\text{Ca}^{2+}]_i$ as those shown in figure 4 were observed, as was recovery toward baseline (data not shown). The increases in $[\text{Ca}^{2+}]_i$ produced by isoflurane were almost completely prevented in slices pretreated with 10 nM xestospongin C, a highly specific inositol triphosphate (IP$_3$) receptor antagonist. Anesthetic-induced IP$_3$ receptor–dependent release of $\text{Ca}^{2+}$ from the endoplasmic reticulum is consistent with previous reports.27

Figure 5 shows that isoflurane preconditioning is associated with significant increases in immunostaining of p42/44 in whole slice cultures and in the CA1 region as well. The labels next to the Western blots refer to the compounds present during preconditioning (isoflurane or U0126) and whether an injury of OGD followed 24 h later. Samples taken at $t = 0$ were obtained just before the OGD. In Western blots, the largest increases in immunostaining in preconditioned slices occurred in the p44 band (isoflurane and Iso/OGD groups). A mitogen-activated extracellular kinase (MEK) inhibitor greatly reduced the phosphorylation of p42/44 during preconditioning (those slices treated with U0126). In slices treated with the MEK inhibitor, p42/44 levels detected with Western blots returned toward baseline after 24 h, although in situ immunostaining indicated a sustained increase in p42/44 staining 24 h after preconditioning, perhaps reflecting regional rather than global expression of this phosphoprotein in the slice.

Preconditioning was associated with increased phosphorylation of the antiapoptotic protein Akt (fig. 7). This increase in immunostaining was not altered by the MEK inhibitor U0126. Akt phosphorylation did not persist 24 h after preconditioning. Similarly, increased phosphorylation of the Akt-activated protein GSK-3β was also seen after preconditioning, but this increase was not seen 24 h later. The role of Akt in preconditioning neuroprotection was evaluated in slice cultures treated with the phosphatidylinositol-3kinase inhibitor LY294002, which is an effective means of decreasing Akt phosphorylation in slice cultures (Jonathan Gray, Department of Anesthesia, UCSF; unpublished observations). The presence of LY294002 during preconditioning...
ing prevented a reduction in cell death observed 48 h after the injury (fig. 8).

Discussion

We found that both 0.5 and 1.5% isoflurane precondition neurons in hippocampal slice cultures by mechanisms that seem to involve intracellular Ca\(^{2+}\)/MAPK and the MAP kinase–ERK pathway. Preconditioning was also associated with increased levels of the antiapoptotic protein kinase Akt (protein kinase B). Although APC was statistically significant in several cell regions and with both 0.5 and 1.5% isoflurane, the magnitude of the protection was modest, with reductions in cell death of approximately 15–40%. In similar studies with isoflurane present at the same time as OGD, we found reductions in cell death of 65–90%.23,28 The greater potency of isoflurane in protecting neurons when it is present at the time of the simulated ischemia may reflect the direct effects of isoflurane on excitatory ion channels and on excitatory neurotransmitter transporters as opposed to those involved in preconditioning. The reason for the differences in the capacity for isoflurane to precondition neurons in different regions of the hippocampus was not evident. We can speculate that although 0.5% isoflurane may be capable of preconditioning dentate neurons, a higher concentration (or a longer preconditioning duration) is required to precondition CA1 neurons. Regional differences in the expression of the signaling molecules involved in preconditioning, regional differences in calcium homeostasis, or regional differences in sensitivity to hypoxic injury are among the possible factors, but none of these were examined in this investigation.

Ca\(^{2+}\) and Preconditioning in the Brain

The proposal that increases in $[\text{Ca}^{2+}]_i$ are a necessary component of APC in the brain is consistent both with what is known of other forms of ischemic preconditioning and tolerance and also with previously proposed mechanisms for APC in the brain and heart. An essential
Role for \( \text{Ca}^{2+} \) in preconditioning is supported by our observations that APC is associated with moderate and reversible increases in \([\text{Ca}^{2+}]_i\) in CA1 neurons and by the observation that calmodulin, an inhibitor of calmodulin, prevented preconditioning. Calmodulin is a key \( \text{Ca}^{2+} \) -binding/sensing protein that is involved in a myriad of \( \text{Ca}^{2+} \) -dependent processes. It is specifically tied to both the activation of the MAP kinase–ERK pathway in preconditioning in the brain and to activation of \( \text{Ca}^{2+} \) -calmodulin dependent protein kinase II activation in heart preconditioning. Calmodulin also activates nitric oxide synthase. The nitric oxide system seems to be important in preconditioning both with \( N \)-methyl-D-aspartate receptor activation and in APC with isoflurane and sevoflurane. Finally, moderate increases in \([\text{Ca}^{2+}]_i\), such as those observed in the presence of isoflurane, could plausibly be linked to increased phosphorylation of the antiapoptotic protein Akt, because nitric oxide is an activator of PI3 kinase, a major regulator of \( \text{Ca}^{2+} \)  -dependent processes. It is specifically tied to both the activation of the MAP kinase–ERK pathway in preconditioning in the brain and to activation of \( \text{Ca}^{2+} \) -calmodulin dependent protein kinase II activation in heart preconditioning. Calmodulin also activates nitric oxide synthase. The nitric oxide system seems to be important in preconditioning both with \( N \)-methyl-D-aspartate receptor activation and in APC with isoflurane and sevoflurane. Finally, moderate increases in \([\text{Ca}^{2+}]_i\), such as those observed in the presence of isoflurane, could plausibly be linked to increased phosphorylation of the antiapoptotic protein Akt, because nitric oxide is an activator of PI3 kinase, a major regulator of Akt. Akt seems to play a role in isoflurane preconditioning because \( \text{LY294002} \), a compound that prevents the phosphorylation of Akt by PI3 kinase, eliminated isoflurane preconditioning neuroprotection in CA1 neurons (fig. 8).

This study examined only several possible signaling mechanisms during preconditioning that are related to moderate increases in \([\text{Ca}^{2+}]_i\). Among the interesting possibilities that remain to be investigated are that changes in \([\text{Ca}^{2+}]_i\) are linked via a calmodulin-dependent protein kinase (e.g., \( \text{Ca}^{2+} \)-calmodulin dependent protein kinase II) to the regulation of other protein kinases and phosphatases that modulate inhibitory and excitatory ion channels and neurotransmitter receptors. In the context of brain ischemia, such actions may be important in limiting neurotoxicity from glutamate and other excitotoxins. It is also plausible that this type of signaling process is involved in the mechanism of anesthetic action of isoflurane.

Although APC was associated with a substantial immediate increase in MAP kinase p42/44 immunostaining, some increased staining was still evident 24 h later. A similar but more dramatic phenomenon was observed by Zheng and Zuo\(^6\) with respect to APC-associated upregulation of MAP kinase p38 in intact rodents. The phosphorylated form of that protein was increased for at least 14 days after preconditioning with 2% isoflurane. In the study of Zheng and Zuo, increased expression of p42/44 (ERK1/2) was not observed, in contrast to our finding here and in studies of acute isoflurane neuroprotection in hippocampal slice cultures.\(^{20}\) Sustained activation of MAP kinase signaling pathways is also interesting in view of the fact that sustained activation of MAP kinase pathways is associated with neuronal injury, not protection.\(^{35}\) Transient up-regulation of MAP kinases, such as that observed after preconditioning (fig. 5), is more clearly linked to neuroprotective signal transduction.\(^{34}\) As stressed by Dimagl et al.,\(^2\) most, if not all, preconditioning stimuli are toxic in high doses or when they are sustained for long periods.

**Sources of \( \text{Ca}^{2+} \) Involved in Anesthetic Preconditioning**

The release of \( \text{Ca}^{2+} \) from intracellular stores (i.e., the endoplasmic reticulum) is likely the source of \( \text{Ca}^{2+} \) involved in APC. Isoflurane increases \([\text{Ca}^{2+}]_i\) in cortical brain slices, hippocampal brain slices, and isolated cortical and hippocampal neurons.\(^{12}\) This process does not require extracellular \( \text{Ca}^{2+} \) but is inhibited by dantrolene and azumolene, both ryanodine receptor antagonists.\(^{12}\) The \( \text{IP}_3 \) receptor antagonist xestospongin C also antagonizes this release (fig. 4). The ryanodine and \( \text{IP}_3 \) receptor complex is the major avenue for intracellular signal–mediated \( \text{Ca}^{2+} \) release in neurons.\(^{35}\) It is not known whether anesthetics such as isoflurane act directly on this complex to release \( \text{Ca}^{2+} \) or if other messengers such as \( \text{IP}_3 \) or reduced nicotinamide dinucleotide are required.

**Limitations of In Vitro models of Neuroprotection and Preconditioning**

The slice culture model used in this study has significant advantages over other *in vitro* models, including preserved synaptic structure, neuron–glia relations, and the possibility of measuring survival and \([\text{Ca}^{2+}]_i\) in different cell types. Like all *in vitro* models, it has limitations that may restrict extrapolation to intact animals. One significant limitation is that *in vitro* models, even

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*Fig. 8. The effects of the phosphatidylinositol-3 (PI3) kinase inhibitor LY294002 (50 \( \mu \text{M} \)) on preconditioning neuroprotection with 1.5% isoflurane in the CA1 region. Isofl PC = slices preconditioned with isoflurane 24 h before oxygen–glucose deprivation (OGD); PC + LY294002 = cultures preconditioned with isoflurane in the presence of the PI3 kinase inhibitor. *Reduction in cell death (\( P < 0.05 \)) compared with nonpreconditioned cultures. The number of cultures in each group was 6–10.*
slice cultures, do not replicate the temporal loss of anesthetic neuroprotection observed in intact rodents. It is not yet clear whether APC provides durable neuroprotection in either in vivo or in vitro models. Another possible limitation of APC is that, at least in our in vitro model, it seemed weakly neuroprotective compared with conditions when isoflurane is present during the simulated ischemia. Further studies in both in vitro and in vivo models are needed to clarify the durability and power of APC.

The authors thank Jen Schuyler, B.S. (Technician, Department of Anesthesia, University of California at San Francisco, San Francisco, California), for technical assistance.

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