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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 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 inositol triphosphate–sensitive 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 adenosine 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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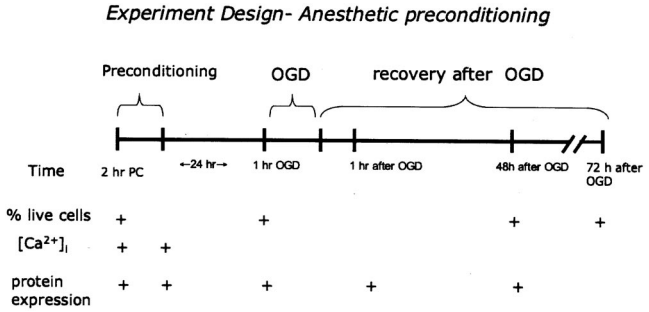

Fig. 1. Preconditioning study design and time line. Hippocampal slice cultures (7–10 days *in vitro*) were preconditioned for 2 h with 0.5 or 1.5% isoflurane. One day later, they were subjected to 1 h of combined oxygen–glucose deprivation (OGD). Cell death (propidium iodide fluorescence) and intracellular Ca^{2+} concentration was measured at the times indicated. In separate cultures, slices were frozen before and after preconditioning and after OGD for assessment of survival-associated proteins.

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 $[Ca^{2+}]_i$ that, *via* the Ca^{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 that injury.

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_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 $[Ca^{2+}]_i$ 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 p42/44, the antiapoptotic protein Akt, and the Akt substrate protein GSK-3β.

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[21,22] as modified by Sullivan *et al.*[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.[24] The slices were then transferred onto 30-mm-diameter membrane inserts (Millicell-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 2-l airtight Billups-Rothenberg Modular Incubator chamber (Del Mar, CA) through which 95% N_2–5% CO_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 fluores-

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 $[Ca^{2+}]_i$

In separate groups of slices, $[Ca^{2+}]_i$ was measured before, during, and after 1% isoflurane exposure. Estimates of $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ as described previously.[26] Estimates of $[Ca^{2+}]_i$ with this technique are accurate to approximately $\pm$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

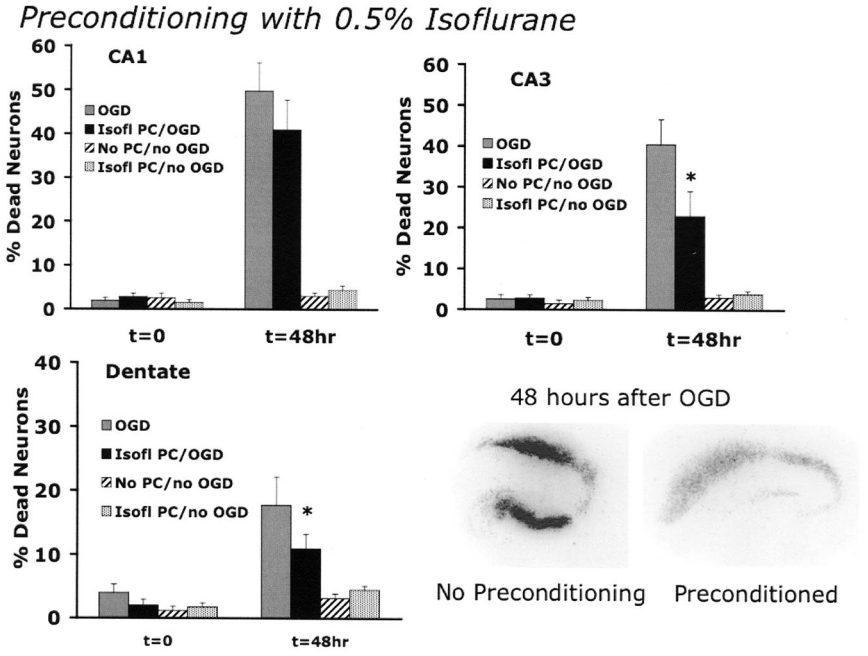

Fig. 2. The effects of 0.5% isoflurane preconditioning (2 h at 37°C) on the percentage of dead hippocampal neurons 2 days after oxygen–glucose deprivation. Isofl PC/no OGD = isoflurane preconditioning only, not followed by OGD; Isofl PC/OGD = preconditioned followed by OGD 24 h later; No PC/no OGD = mock preconditioning with vehicle only, no subsequent OGD; OGD = oxygen–glucose deprivation only, no preconditioning. The number of slices in each group are as follows: 11 slices in the control OGD group, 13 in the isoflurane PC/OGD group, 13 in the No PC/no OGD group, and 14 in the Isoflurane PC/no OGD group. * Significant reduction in cell death in the preconditioned/OGD group compared with the nonpreconditioned/OGD group.

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 CA 3 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.[23]

Intracellular Ca^{2+} during Preconditioning

In slice cultures grown on special culture inserts, we measured $[Ca^{2+}]_i$ 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, $[Ca^{2+}]_i$ in CA1 neurons increased during 10 min of isoflurane application and returned to baseline during washout. In figure 4, averaged traces of $[Ca^{2+}]_i$ are shown. The increase in $[Ca^{2+}]_i$

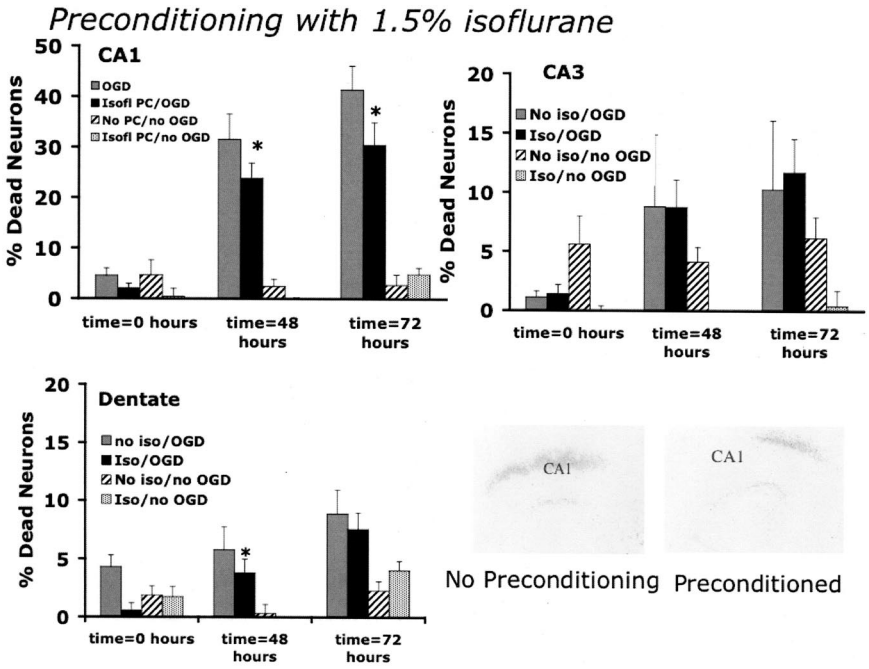

Fig. 3. The effects of 1.5% isoflurane preconditioning on the percentage of dead hippocampal neurons 2 and 3 days after oxygen–glucose deprivation (OGD). See figure 1 legend for a description of the abbreviations. The number of slices in each group was 12 or 13.

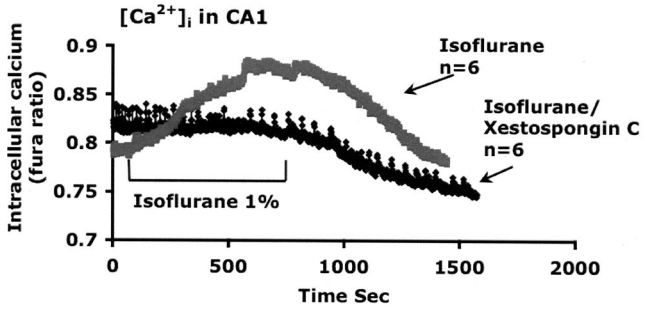

Fig. 4. One percent isoflurane reversibly increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in CA1 neuron cell bodies in hippocampal slice cultures. Each trace is an average of six experiments, each done on a separate slice culture. Xestospongin C, an inositol triphosphate receptor antagonist, was present 30 min before isoflurane exposure.

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 $[Ca^{2+}]_i$ as those shown in figure 4 were observed, as was recovery toward baseline (data not shown). The increases in $[Ca^{2+}]_i$ 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 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.

Figure 6 shows the impact of the calmodulin inhibitor calmidazolium and the MEK inhibitor U0126 on isoflurane preconditioning. Slices were preconditioned with 1.5% isoflurane either with or without the inhibitors present. Preconditioning was not seen in slices exposed to either of these agents. It is unlikely that toxicity of these inhibitors were responsible for these findings because they did not increase cell death in slices subjected to OGD or in slices exposed only to the inhibitors and not to OGD.

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-3 kinase 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 precondition-

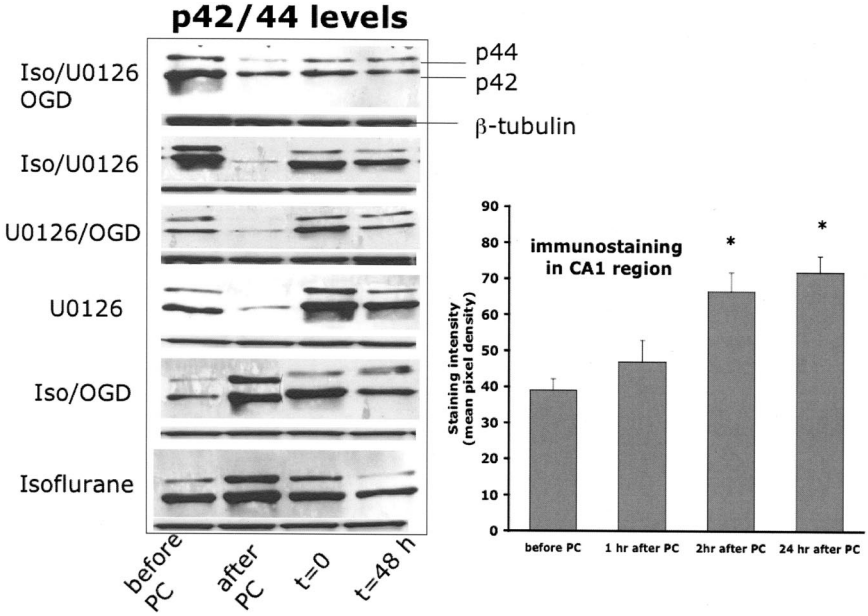

Fig. 5. Phosphorylated ERK1/2 (p42/44) levels in slice cultures preconditioned with 1.5% isoflurane. At *left* are representative Western blots from whole slice cultures before preconditioning (PC), immediately after 2 h of PC, 24 h later (just before oxygen–glucose deprivation [OGD] or mock OGD), and 2 days after OGD. The position of p44, p42, and a gel-loading control protein (β-tubulin) are the same for each experimental group. At *right* is immunostaining density (NIH Image) with p42/44 antibodies in the CA1 region of slice cultures. *Bars* represent mean ± SE of six slices. * $P < 0.05$ compared with control.

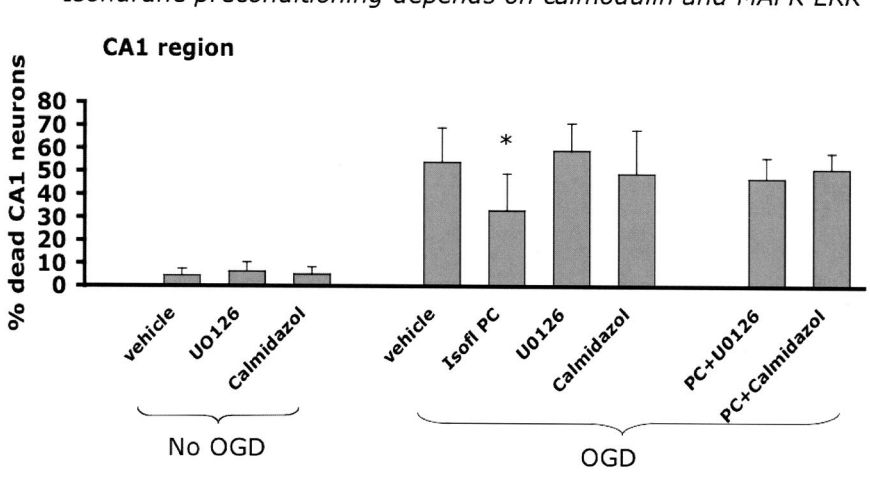

Fig. 6. The effects of the mitogen-activated extracellular kinase (MEK) inhibitor U0126 and the calmodulin inhibitor calmidazolium on preconditioning with 1.5% isoflurane in the CA1 region. OGD = oxygen–glucose deprivation; PC + calmidazol = cell death in cultures preconditioned with 10 μM calmidazolium present; PC + U0126 = cell death in cultures preconditioned in the presence of the MEK inhibitor. * Reduction in cell death compared with nonpreconditioned cultures. The number of cultures in each group was 12–15.

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 preconditions neurons in hippocampal slice cultures by mechanisms that seem to involve intracellular Ca^{2+} 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[29,30] 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 $[Ca^{2+}]_i$ 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

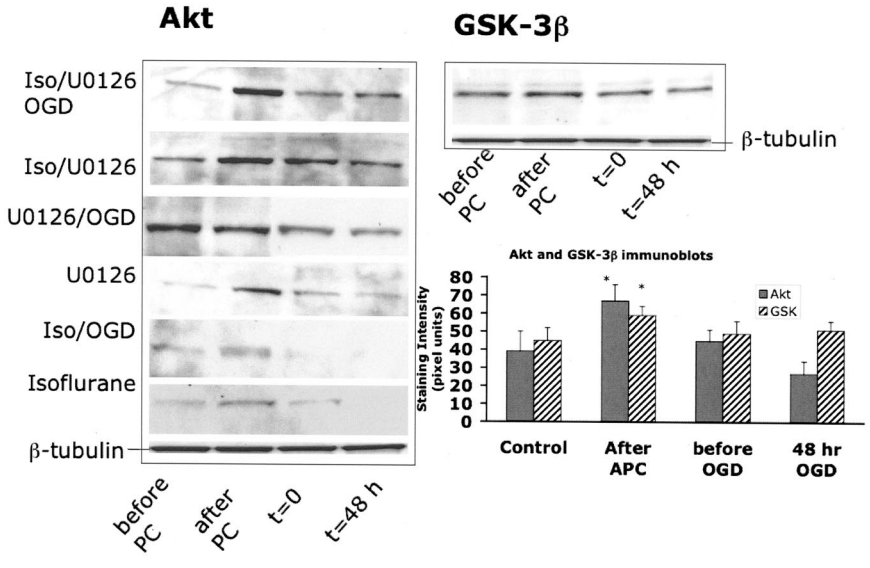

Fig. 7. Western blots of Akt and GSK-3β in slice cultures preconditioned with 1.5% isoflurane before and after preconditioning and before and 48 h after oxygen–glucose deprivation (OGD). APC = anesthetic preconditioning; Isoflurane = preconditioning only, no subsequent OGD; Iso/OGD = preconditioned with isoflurane, OGD 24 h later; Iso/U0126 = isoflurane and the mitogen-activated extracellular kinase (MEK) inhibitor were present during preconditioning and there was no subsequent OGD; Iso/U0126/OGD = isoflurane and a MEK inhibitor were both present during preconditioning and were followed by OGD 24 h later; PC = preconditioning; U0126 = the MEK inhibitor was present during a 2-h period 24 h before mock OGD; U0126/OGD = the MEK inhibitor was present during mock preconditioning and was followed by OGD 24 h later. *Bar graph* shows mean staining intensity of Akt and GSK (n = 6 slices). * Groups different from control ($P < 0.05$).

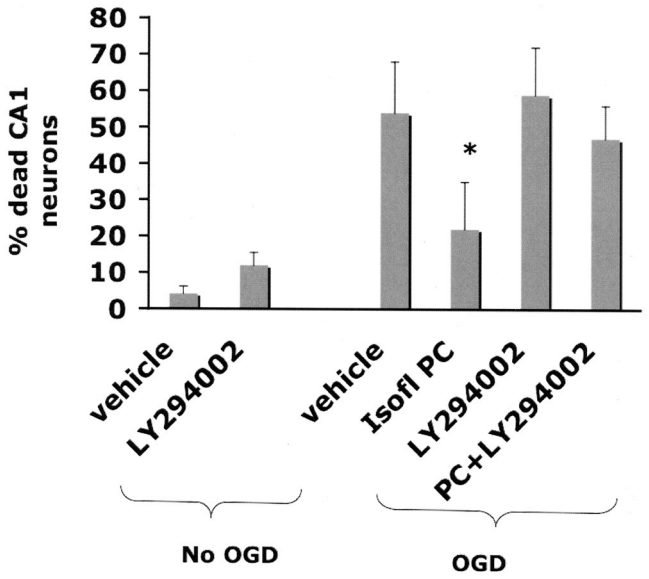

Fig. 8. The effects of the phosphatidylinositol-3 (PI_3) kinase inhibitor LY294002 (50 μM) on preconditioning neuroprotection with 1.5% isoflurane in the CA1 region. Isofl PC = slices preconditioned with isoflurane 24 h before oxygen–glucose deprivation (OGD); PC + LY294002 = cultures preconditioned with isoflurane in the presence of the PI_3 kinase inhibitor. * Reduction in cell death ($P < 0.05$) compared with nonpreconditioned cultures. The number of cultures in each group was 6–10.

role for Ca^{2+} in preconditioning is supported by our observations that APC is associated with moderate and reversible increases in $[Ca^{2+}]_i$ in CA1 neurons and by the observation that calmidazolium, an inhibitor of calmodulin, prevented preconditioning. Calmodulin is a key Ca^{2+}-binding/sensing protein that is involved in a myriad of Ca^{2+}-dependent processes. It is specifically tied to both the activation of the MAP kinase–ERK pathway in preconditioning in the brain[10,17] and to activation of Ca^{2+}-calmodulin dependent protein kinase II activation in heart preconditioning.[31] Calmodulin also activates nitric oxide synthase. The nitric oxide system seems to be important in preconditioning both with *N*-methyl-D-aspartate receptor activation[15] and in APC with isoflurane and sevoflurane.[4,5] Finally, moderate increases in $[Ca^{2+}]_i$, 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 PI_3 kinase, a major regulator of Akt. Akt seems to play a role in isoflurane preconditioning because LY294002, a compound that prevents the phosphorylation of Akt by PI_3 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 $[Ca^{2+}]_i$. Among the interesting possibilities that remain to be investigated are that changes in $[Ca^{2+}]_i$ are linked *via* a calmodulin-dependent protein kinase (*e.g.*, 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.[32] 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.[33] 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 Dirnagl *et al.*,[2] most, if not all, preconditioning stimuli are toxic in high doses or when they are sustained for long periods.

Sources of Ca^{2+} Involved in Anesthetic Preconditioning

The release of Ca^{2+} from intracellular stores (*i.e.*, the endoplasmic reticulum) is likely the source of Ca^{2+} involved in APC. Isoflurane increases $[Ca^{2+}]_i$ in cortical brain slices, hippocampal brain slices, and isolated cortical and hippocampal neurons.[12] This process does not require extracellular Ca^{2+} but is inhibited by dantrolene and azumolene, both ryanodine receptor antagonists.[12] The IP_3 receptor antagonist xestospongin C also antagonizes this release (fig. 4). The ryanodine and IP_3 receptor complex is the major avenue for intracellular signal–mediated Ca^{2+} release in neurons.[35] It is not known whether anesthetics such as isoflurane act directly on this complex to release Ca^{2+} or if other messengers such as 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 $[Ca^{2+}]_i$ 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

slice cultures,[23] do not replicate the temporal loss of anesthetic neuroprotection observed in intact rodents.[36] 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.

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