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Isoflurane Neuroprotection in Hypoxic Hippocampal Slice Cultures Involves Increases in Intracellular Ca^{2+} and Mitogen-activated Protein Kinases

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Background: **The volatile anesthetic isoflurane reduces acute and delayed neuron death *in vitro* models of brain ischemia, an action that the authors hypothesize is related to moderate increases in intracellular calcium concentration ($[Ca^{2+}]_i$). Specifically, the authors propose that during hypoxia, moderate increases in $[Ca^{2+}]_i$ in the presence of isoflurane stimulates the Ca^{2+}-dependent phosphorylation of members of the mitogen-activated protein kinase (MAP) kinase Ras-Raf-MEK-ERK pathway that are critical for neuroprotective signaling and suppression of apoptosis.**

Methods: **Death of CA1, CA3, and dentate neurons in rat hippocampal slice cultures was assessed by propidium iodide fluorescence 48–72 h after 60–75 min of hypoxia. $[Ca^{2+}]_i$ in CA1 neurons was measured with fura-2 and fura-2 FF. Concentrations of the survival-signaling proteins Ras, MEK, MAP kinase p42/44, and protein kinase B (Akt) were assessed by immunostaining, and specific inhibitors were used to ascertain the role of Ca^{2+} and MAP kinases in mediating survival.**

Results: **Isoflurane, 1%, decreased neuron death in CA1, CA3, and dentate gyrus neurons after 60 but not 75 min of hypoxia. Survival of CA1 neurons required an inositol triphosphate receptor–dependent increase in $[Ca^{2+}]_i$ of 30–100 nM that activated the Ras-Raf-MEK-ERK (p44/42) signaling pathway. Isoflurane also increased the phosphorylation of Akt during hypoxia.**

Conclusions: **Isoflurane stimulates the phosphorylation of survival signaling proteins in hypoxic neurons. The mechanism involves a moderate increase in $[Ca^{2+}]_i$ from release of Ca^{2+} from inositol triphosphate receptor–dependent intracellular stores. The increase in $[Ca^{2+}]_i$ sets in motion signaling *via* Ras and the MAP kinase p42/44 pathway and the antiapoptotic factor Akt. Isoflurane neuroprotection thus involves intracellular signaling well known to suppress both excitotoxic and apoptotic/delayed cell death.**

ISOFLURANE substantially reduces neuron death in organotypic cultures of rat hippocampus for up to 2 weeks after oxygen and glucose deprivation, suggesting that isoflurane may have the intrinsic capacity to mitigate both acute and delayed neuron death.[1] In intact rodents, isoflurane may delay but not prevent apoptosis after severe ischemic brain injuries,[2,3] suggesting that isoflurane may alter key events in the early injury process. Compared with nitrous oxide–fentanyl anesthesia, isoflurane results in less death of hippocampal neurons 5 days after forebrain ischemia in rats, but after 3 months, this difference is absent.[4] Identification of the basis for this early, but not late, neuroprotection is of interest because it may be possible to devise treatments to extent the benefit further into the postischemic recovery period.

Most of the mechanisms proposed for the protective qualities of isoflurane observed in *in vitro* models have focused on the actions of isoflurane on ion channels that contribute to excitotoxic death. Inhibition of *N*-methyl-D-aspartate (NMDA) receptors and potentiation of γ-aminobutyric acid receptors by isoflurane are important antiexcitotoxicity mechanisms.[5,6] Direct effects of anesthetics on the proteins of excitatory and inhibitory receptors are presumed to mediate these antiexcitotoxic effects, consistent with strong experimental evidence that isoflurane directly inhibits recombinant glutamate receptors and calcium channels and augments the activity of γ-aminobutyric acid receptors.[7]

In contrast, relatively less work has been done on the effects of volatile anesthetics on apoptotic cell death. Two studies have provided evidence that volatile anesthetics reduce apoptosis in hypoxic neurons,[8,9] but whether decreased apoptosis is a consequence of reduced excitotoxic injury, reduced apoptosis signaling, or increased antiapoptosis signaling is not known. The reduction of apoptosis by volatile anesthetics may involve alterations in intracellular calcium signaling, which is a major regulator of proapoptotic and antiapoptotic events. Isoflurane prevents large increases in intracellular calcium concentration ($[Ca^{2+}]_i$) in hypoxic neurons, enabling $[Ca^{2+}]_i$ to remain in a survivable range.[5] Even with oxygen present, isoflurane induces small to moderate increases in $[Ca^{2+}]_i$.[10] These moderate increases in $[Ca^{2+}]_i$ may be critical to survival of hypoxic neurons in that they initiate adaptive responses to hypoxic conditions. Moderate increases in $[Ca^{2+}]_i$ trigger important survival signals, including phosphorylation of the antiapoptotic factor Akt,[11] the mitogen-activated protein kinase (MAP) kinase ERK (p42/44),[12] and the transcription of brain-derived neurotrophic factor.[13] Further, changes in gene expression mediated by hypoxia-inducible factor 1α are potentiated by Ca^{2+} *via* calmodulin and the p42/44 MAP kinase pathway.[14] Increases in $[Ca^{2+}]_i$ of 50–200 μM abrogate serum or growth factor withdrawal–induced apoptosis in cultured mammalian neurons[15] and may be a general stimulus for activation of cell

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survival signals.[16] In addition, moderate increases in $[Ca^{2+}]_i$, mediated by NMDA receptors,[17–19] brief hypoxia,[20] or calcium ionophores[21] may be central to all forms of ischemic preconditioning.

The purpose of this study was to test the hypothesis that alterations in $[Ca^{2+}]_i$ homeostasis during hypoxia are central to the *in vitro* neuroprotective effects of isoflurane. To do this, we measured the effects of isoflurane on $[Ca^{2+}]_i$, cell survival, and expression of survival-related proteins in hippocampal slice cultures neurons during and after hypoxia. Specific inhibitors of Ca^{2+}-related signaling pathways were then used to probe the survival significance of the signaling patterns. We show that intracellular signaling pathways and moderate increases in $[Ca^{2+}]_i$ are required for isoflurane protection of hypoxic hippocampal neurons.

Materials and Methods

Preparation of Hippocampal Slice Cultures

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

Organotypic cultures of the hippocampus were prepared by standard methods[22,23] as modified by Sullivan *et al.*[1] Briefly, Sprague-Dawley rats (8–14 days old; Simonsen Laboratories, Gilroy, CA) were given an intraperitoneal injection of ketamine (10 mg/kg) and diazepam (0.2 mg/kg) and anesthetized with 1–2% halothane. The rats were decapitated, and the hippocampi were removed and placed in 4°C Gey's Balanced Salt Solution (UCSF Cell Culture Facility, San Francisco, CA) containing 50 μM adenosine and 0.038 mg/ml ketamine. Next, the hippocampi were transversely sliced (400 μm thick) with a tissue slicer (Siskiyou Design Instruments, Grants Pass, OR) and stored in the Gey's Balanced Salt Solution at 4°C for 1 h.[24] The slices were then transferred onto 25-mm-diameter membrane inserts (Millicell-CM; Millipore, Bedford, MA), and put into six-well culture trays with 1.2 ml slice culture medium per well. The slice culture media for the first 48 h consisted of 50% Minimal Essential Medium (Eagles with Earle's Balanced Salt Solution; UCSF Cell Culture Facility), 25% Earle's balanced salt solution (UCSF Cell Culture Facility), and 25% heat-inactivated horse serum (Hyclone Laboratories, South San Francisco, CA) with 6.5 mg/ml glucose, 50 μM adenosine, and 5 mM KCl; subsequent media lacked adenosine. Slices were kept in culture for 7–14 days before study. Slices were discarded if they showed more than slight propidium iodide (PI) fluorescence (see section on assessment of cell death) at the beginning of the survival studies.

Experiment Design

Cultures were exposed to hypoxia by placing them 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 by both 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 0.1–0.2 mmHg, measured with a Clark-type oxygen electrode. After hypoxia, the culture tray was removed from the chamber, briefly opened to restore oxygenation, and returned to the incubator. For cultures treated with isoflurane, gas flowed through a calibrated vaporizer before entering the Billups-Rothenberg chamber. The hypoxic gas and isoflurane entered the chamber at the same time. In experiments involving inhibitors of MAP kinases or protein kinase B/Akt, the compounds were added to the culture media just before the start of hypoxia, and the media was replaced at the end of the hypoxia. A 10-μM concentration of the MEK1/2 inhibitor U0126 was chosen because this is the minimal concentration required to produce inhibition of phosphorylation of MAPK p42/44 in slice cultures[12] and in addition has been shown to prevent MAPK signaling in cultured neurons.[25]

Assessment of Cell Death

Cell viability was assessed 2 and 3 days after hypoxia with PI (Molecular Probes, Eugene OR), a highly polar fluorescent dye that penetrates damaged plasma membranes and binds to DNA. 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 digital images of fluorescence 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 imaged prior to hypoxia to obtain a PI image assumed to represent 0% cell death. We found that maximum posthypoxia death occurred after 2 or 3 days and declined during the next 11 days.[1] Serial measurements of PI fluorescence intensity were made in predefined areas (manually outlining CA1, CA3, and dentate separately) for each slice using NIH Image software (free software from the US National Institutes of Health, Washington, DC). Thus, cell death was followed in the same regions of each slice after hypoxia. After the measurement of PI fluorescence on the third day after hypoxia,

all of the neurons in the slice were killed with the application of 100 μM potassium cyanide and 2 mM sodium iodoacetate to the cultures for 20–30 min to produce a fluorescence signal equal to 100% neuron death in all regions of interest. Twelve to 24 h later, final images of PI fluorescence (equated to 100% cell death) were acquired. The percentage of dead cells at 2 and 3 days after hypoxia were then calculated based on these values, because a linear relation exists between cell death and PI fluorescence intensity.[23,24]

Measurements of $[Ca^{2+}]_i$

In separate groups of slices, $[Ca^{2+}]_i$ was measured before and after the period of hypoxia and/or isoflurane exposure. Estimates of $[Ca^{2+}]_i$ in CA1 neurons in slice cultures were made using the indicators fura-2 AM or fura-2 FF-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 either 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 (K_D) of fura-2 and fura-2 FF 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 K_D values for fura-2 and fura-2 FF were 311 nM and 8.1 μM, respectively, similar to published values.[26] Fura-2 was used to measure $[Ca^{2+}]_i$ in the range from 0–600 nM, and fura-2 FF was used when larger increases in $[Ca^{2+}]_i$ were expected, *i.e.*, after hypoxia. 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.[27] Estimates of $[Ca^{2+}]_i$ 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 methodology. Five to eight slices were pooled for each assay and repeated

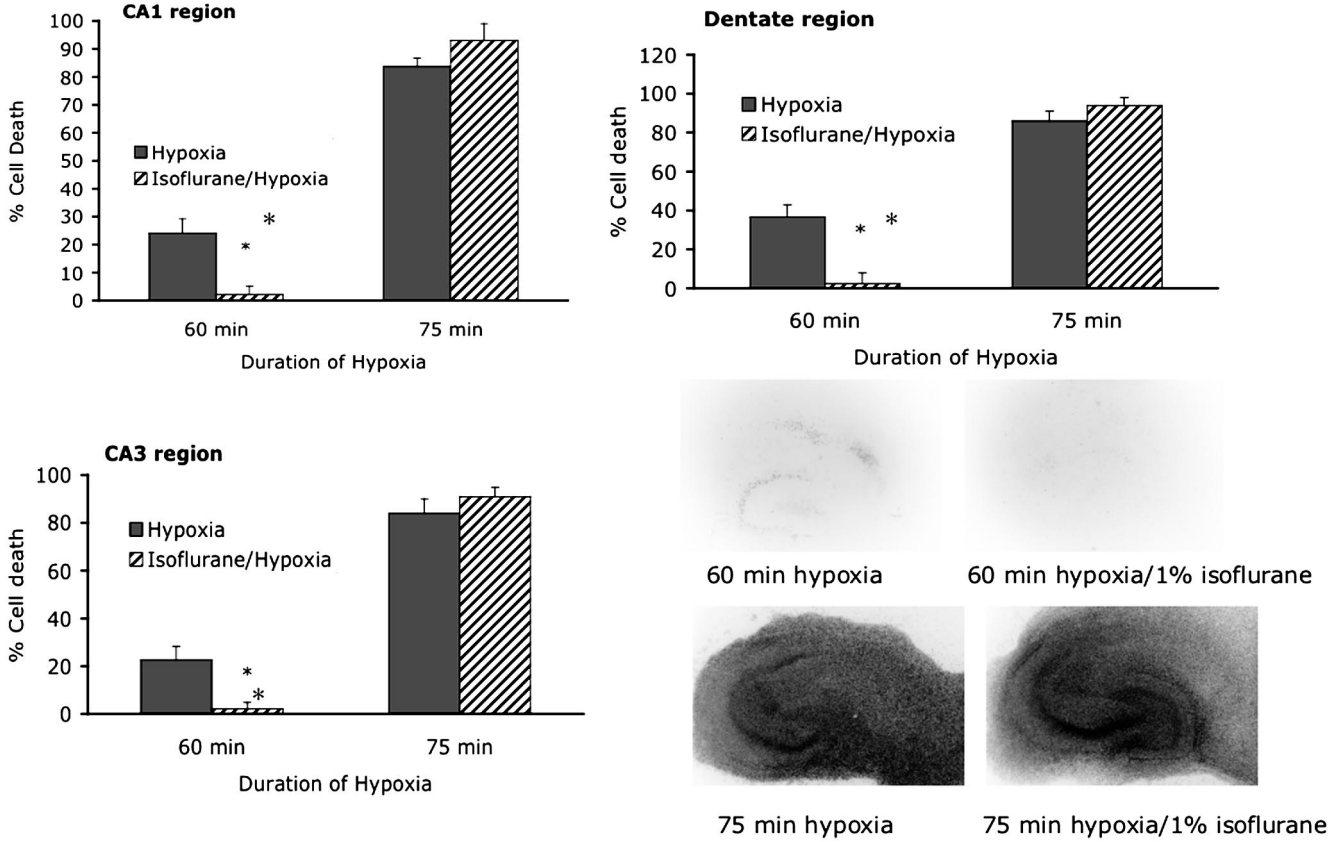

Fig. 1. Isoflurane reduced cell death after 60 but not 75 min of hypoxia. Percentage of dead CA1, CA3, and dentate neurons 48 h after 60 and 75 min of hypoxia. In the isoflurane/hypoxia groups, 1% isoflurane (in 95% air–5% CO_2) was present during the entire period of hypoxia. * Significant reduction in cell death compared with hypoxia group; $P < 0.05$. The number of slices in each group was 10–15. Examples of propidium iodide fluorescence in slice cultures at 48 h after hypoxia are also shown at the *lower right*. *Dark areas* indicate fluorescence in the nuclei of dead neurons.

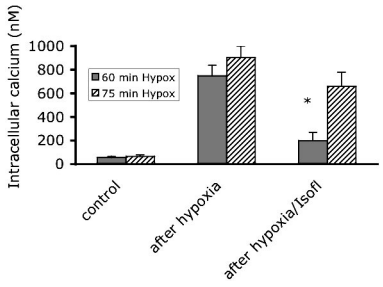

Fig. 2. Intracellular calcium measurements (made with fura-2; see Materials and Methods) from the CA1 neuron cell body region of hippocampal slice cultures before hypoxia (control, n = 12 cultures) and after 60 or 75 min of hypoxia with (n = 6) and without (n = 6) 1% isoflurane present during the entire period of hypoxia. * Isoflurane present during hypoxia reduced intracellular calcium concentration in CA1 neurons compared with the hypoxia group ($P < 0.01$, analysis of variance).

three to four times. Samples were obtained both before and 24 h after hypoxia. Protein content in each sample was measured 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 and quantified by image analysis software (NIH Image). *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 MAPK p42/44 (Thr 202/204 phosphorylation) were obtained from Cell Signaling Technology (Beverly, MA). Relative protein concentrations in the *in situ* preparations were measured with a microscope, a digital camera, and NIH Image software.

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 $P < 0.05$.

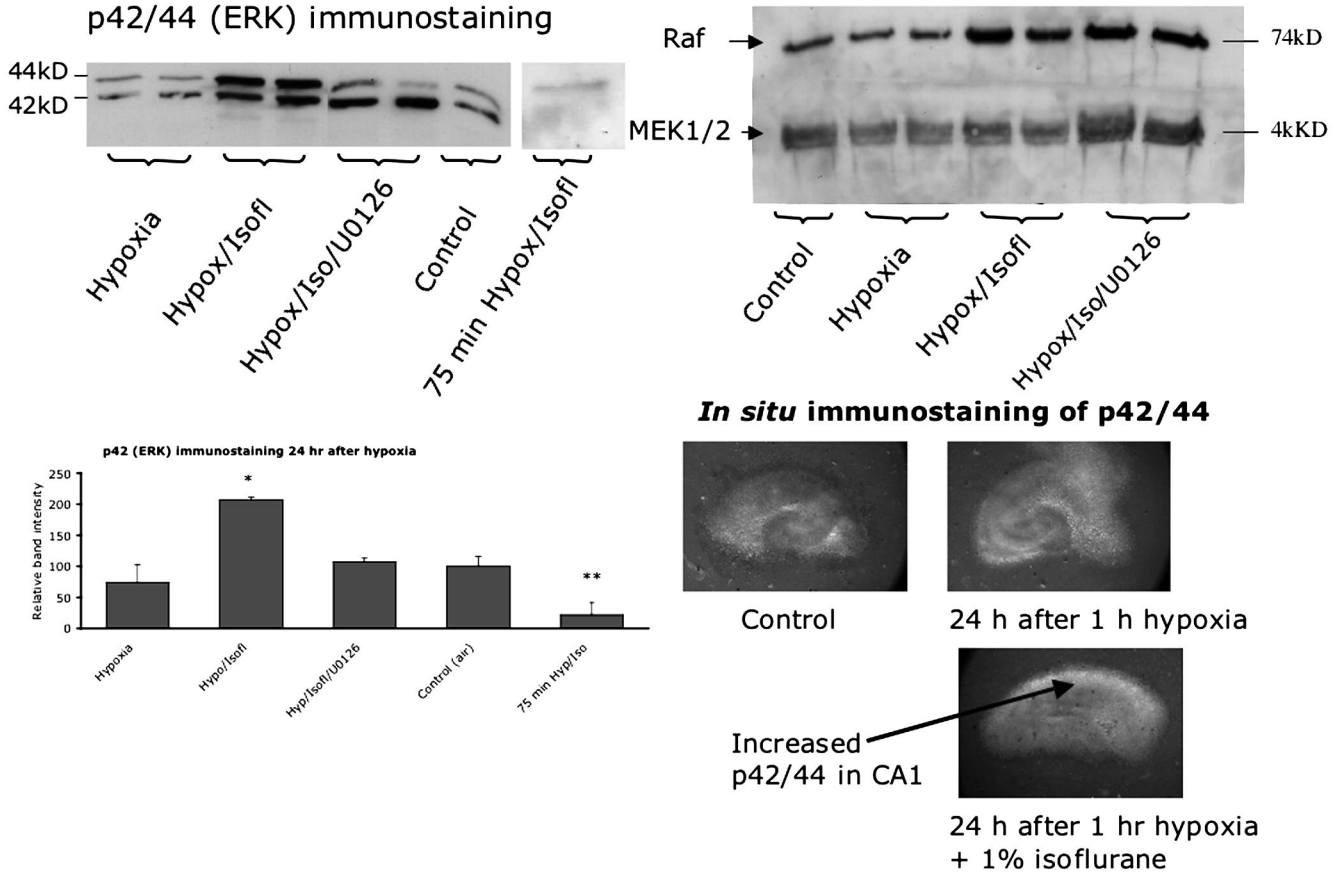

Fig. 3. Isoflurane increases signaling *via* Raf-MEK-ERK 24 h after hypoxia. Western blots and *in situ* immunostaining (negative images) of several mitogen-activated protein kinase signaling molecules in hippocampal slice cultures exposed to hypoxia (24 h after 60 min of 95% N_2–5% CO_2) and hypoxia plus 1% isoflurane with and without the MEK1/2 inhibitor U0126 (10 μM). Molecular weights of bands are indicated along sides of gels. The *lower left bar graph* shows the average staining intensity of the p44 band in four gels. Note that isoflurane present during 75 min of hypoxia (same group of slices, separate immunoblot from 60-min hypoxia group) is not associated with increased p42/44 concentrations.

Results

Isoflurane Reduces Cell Death after 60 but Not 75 Minutes of Hypoxia

Isoflurane, 1%, reduced cell death in CA1, CA3, and dentate neurons 48 h after 60 but not 75 min of hypoxia (fig. 1). In slice cultures without isoflurane, 60 min of hypoxia caused the death of 24% of CA1 neurons, 23% of CA3 neurons, and 37% of dentate neurons. Isoflurane, 1%, reduced these percentages by 20–30%, to less than 3% death in each region. In contrast, after 75 min of hypoxia, 80–100% of neurons were dead in CA1, CA3, and dentate regions, and isoflurane did not provide protection. Examples of the degree and distribution of cell death in slice cultures 48 h after hypoxia are also shown in the images in figure 1. Peak cell death in hippocampal slice neurons occurred 48–72 h after hypoxia, similar to injuries involving oxygen and glucose deprivation.[1]

In a separate group of slice cultures, we measured $[Ca^{2+}]_i$ in CA1 neurons just after 60 and 75 min of hypoxia. These estimates of $[Ca^{2+}]_i$, made with fura-2, were corrected for background fluorescence changes that may occur during hypoxia because of changes in endogenous fluorescent compounds such as reduced nicotinamide dinucleotide. Isoflurane, 1%, reduced the increase in $[Ca^{2+}]_i$ that occurred in CA1 neurons during 60 but not 75 min of hypoxia (fig. 2). Therefore, the protective effects of isoflurane in hypoxic CA1 neurons after 60 min of hypoxia was correlated with a smaller increase in $[Ca^{2+}]_i$ at the end of the hypoxic period.

Isoflurane Increases Signaling through the Ras-Raf-ERK-MEK MAP Kinase Signaling Pathway during Hypoxia and Is Essential for Neuroprotection

A screen of several intracellular signaling pathways in cultured cortical and hippocampal neurons revealed that the MAP kinase p42/44 pathway (also known as the Ras-Raf-MEK-ERK pathway) was strongly up-regulated in neurons exposed to a combination of hypoxia and isoflurane (unpublished data, Christian S. Fahlman, Ph.D., UCSF, San Francisco, California, August 2004). Accordingly, we performed Western blots and *in situ* immunostaining of several components of this pathway. Figure 3 shows that although hypoxia alone had a small effect on the concentration of Raf, MEK and ERK, the combination of isoflurane and 60 min of hypoxia produced a large increase in phospho-ERK (p42/44) immunostaining. One upstream component of this pathway (Raf) showed a similar increase in the presence of 60 min of hypoxia and isoflurane, whereas phospho-MEK 1/2, which is just upstream of ERK (p42/44), remained unchanged. The MEK1/2 inhibitor U0126 decreased the phosphorylation of p44 in slices exposed to isoflurane and hypoxia. Further, *in situ* immunostaining of intact slice cultures showed that in CA1 neurons, isoflurane present during hypoxia resulted in increased p42/44 for 24 h after hypoxia. Taken together, the data in figure 3 show that isoflurane substantially increases signaling *via* the Ras-Raf-MEK-ERK pathway during 60 min of hypoxia. In contrast, the more severe injury of

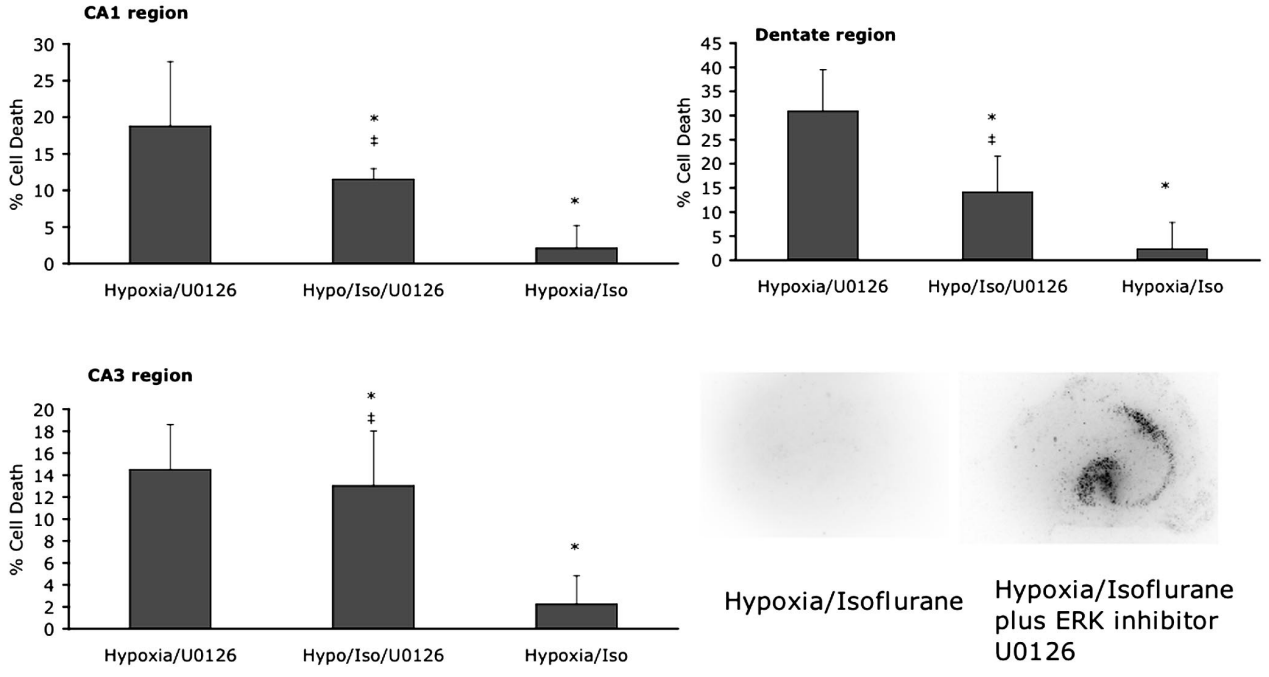

Fig. 4. Isoflurane neuroprotection requires ERK (p42/44). The MEK1/2 inhibitor U0126 (10 μM) antagonizes isoflurane protection of CA1, CA3, and dentate neurons. There were 9–12 slices in each treatment group. The *lower right panel* shows an example of a propidium iodide fluorescence image showing dead CA1, CA3, and dentate neurons in a culture treated with the MEK1/2 inhibitor U0126 (10 μM).

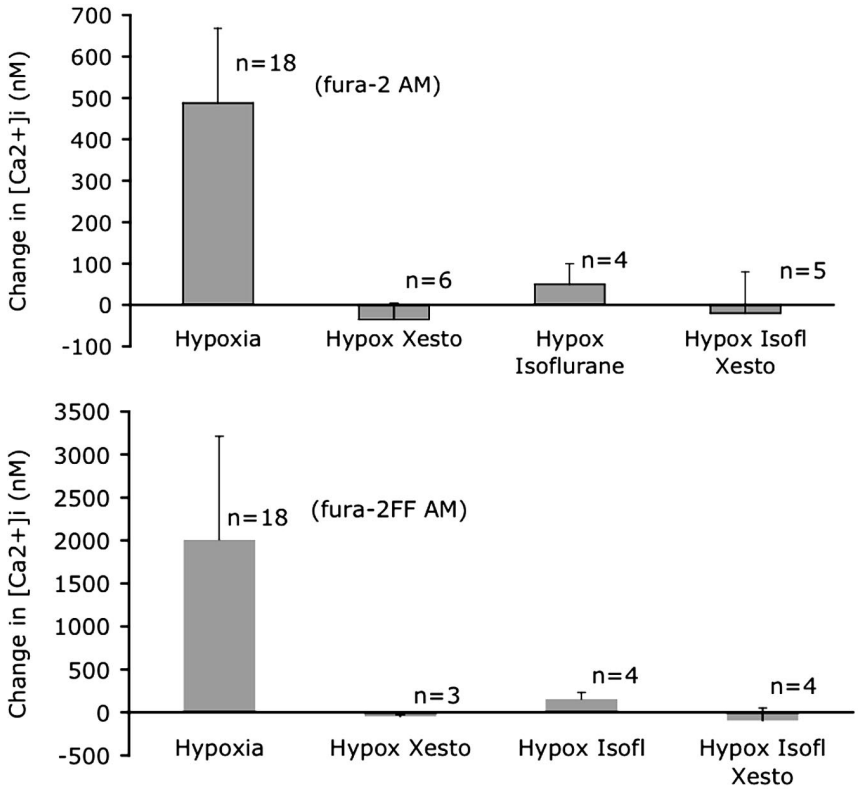

Fig. 5. Intracellular calcium concentration ($[Ca^{2+}]_i$) changes in CA1 neurons during hypoxia. Isoflurane reduced the increase in $[Ca^{2+}]_i$ normally seen in hypoxic neurons and the inositol triphosphate (IP_3) receptor antagonist decreases $[Ca^{2+}]_i$ to below baseline levels. The *upper panel* shows measurements with the high-affinity calcium indicator fura-2, and the *lower panel* shows corresponding measurements with fura-2 FF, a low-affinity indicator. Fura-2, because of its high calcium affinity, is more appropriate for estimates of resting cell $[Ca^{2+}]_i$. Conversely, fura-2 FF, with a dissociation constant (K_D) of approximately 8 μM, is better for posthypoxia estimates of $[Ca^{2+}]_i$ (see text).

75 min of hypoxia, which was associated with extensive cell death (fig. 1), resulted in minimal p42/44 immunostaining (fig. 3, separate immunoblot shown in upper left).

To test the hypothesis that isoflurane neuroprotection in slice cultures depends on the Ras-Raf-MEK-ERK pathway, we used the MEK inhibitor U0126 (10 μM), a compound we previously found to inhibit phosphorylation of p42/44 in our slice cultures.[21] U0126 eliminated isoflurane neuroprotection at 48 h after 60 min hypoxia (fig. 4). Further, U0126 did not increase death after hypoxia compared with hypoxia alone (fig. 1), making it unlikely that the reversal of isoflurane protection was caused by U0126 toxicity.

Isoflurane Promotes Maintenance of Moderate Increases in $[Ca^{2+}]_i$ during and after Hypoxia and Increases Calcium-dependent Signals

Moderate increases in $[Ca^{2+}]_i$ are linked to the activation of the MAP kinase p42/44 pathway and other survival signals.[14,21] We therefore hypothesized that the moderate increases in $[Ca^{2+}]_i$ seen in hypoxic neurons in the presence of isoflurane are causally related to the phosphorylation of MAP kinases and subsequent reductions in cell death. To pursue this possibility, we used the compound xestospongin C as a tool to eliminate the hypoxia- and/or anesthetic-induced release of Ca^{2+} from inositol triphosphate (IP_3) receptor–dependent intracel-

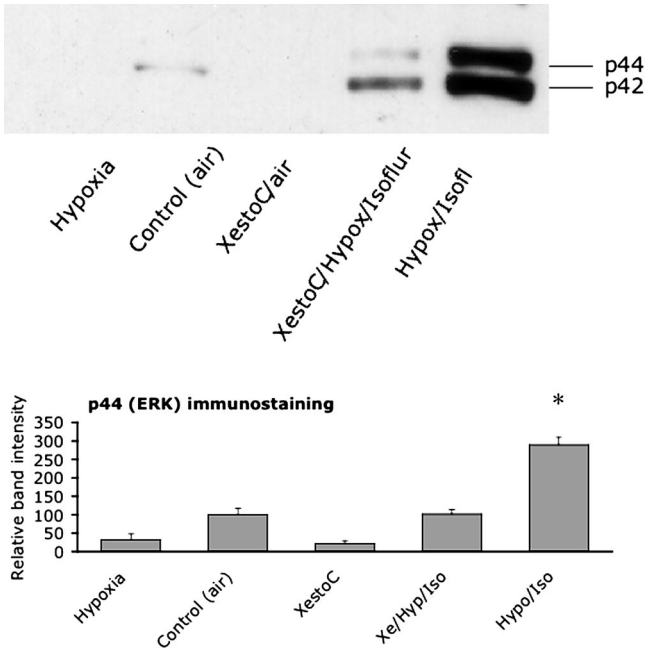

Fig. 6. Phosphorylation of mitogen-activated protein kinase p42/44 in hypoxic slice cultures exposed to 1% isoflurane was prevented by the IP_3 receptor antagonist xestospongin C (1 μM). Xestospongin C also prevented increases in intracellular calcium concentration during hypoxia and blocked the neuroprotective effects of isoflurane (figs. 4 and 6). The *bar graph* shows mean staining intensity of three blots. * Significant increase compared with control.

lular stores.[28] In CA1 neurons, a significant portion of the increase in $[Ca^{2+}]_i$ during hypoxia was due to release from intracellular stores (endoplasmic reticulum) because xestospongin C prevented the increase in $[Ca^{2+}]_i$ during hypoxia (fig. 5). The same was seen in neurons exposed to xestospongin C in the presence of hypoxia combined with 1% isoflurane. Importantly, figure 5 also shows that the IP_3 antagonist xestospongin C actually results in average net decreases in neuron $[Ca^{2+}]_i$ compared with before hypoxia. This was observed with both a high-affinity and a low-affinity Ca^{2+} indicator in separate cultures.

Next, we investigated whether the stimulation of IP_3 receptor–mediated Ca^{2+} release was required for activation of the Ras-MEK pathway. The increase in p44 concentrations mediated by isoflurane during hypoxia depended on Ca^{2+} release from intracellular stores, because the IP_3 antagonist xestospongin C prevented the increase in p44 immunostaining (fig. 6).

Finally, we examined the link between Ca^{2+} release from intracellular stores and isoflurane-mediated neuroprotection. As shown in figure 7, xestospongin C prevented isoflurane from decreasing cell death after hypoxia. This was observed in CA1, CA3, and dentate neurons. The presence of xestospongin C in hypoxic cultures did not significantly increase cell death, making it unlikely that the reversal of isoflurane protection was due to toxicity of xestospongin C.

Isoflurane Activates Antiapoptotic Proteins during Hypoxia

Intracellular calcium signaling has many important effects on cell survival, including modulation of the antiapoptotic protein kinase B-Akt pathway; moderate elevation of $[Ca^{2+}]_i$ increases Akt phosphoactivation and promotes survival in slice cultures.[21] Accordingly, levels of immunostaining of two phosphorylation sites of Akt and a downstream target of Akt (glycogen synthase kinase) were measured in slice cultures before and after hypoxia with or without 1% isoflurane. Western blots of phospho-Akt are shown in figure 8, indicating that isoflurane present during hypoxia increased the phosphorylation of Akt at one of two critical phosphorylation sites (threonine 308, target of PDK2[29]). Another phosphorylation site on Akt, S473, did not show increased phosphorylation, but a target of activated Akt, glycogen synthase kinase, was phosphorylated in the presence of isoflurane ($P < 0.05$). In this case, the phosphorylation was not dependent on IP_3 receptors because phosphorylation occurred equally strongly in the presence of isoflurane and xestospongin C. This suggests that although the combination of hypoxia and isoflurane acti-

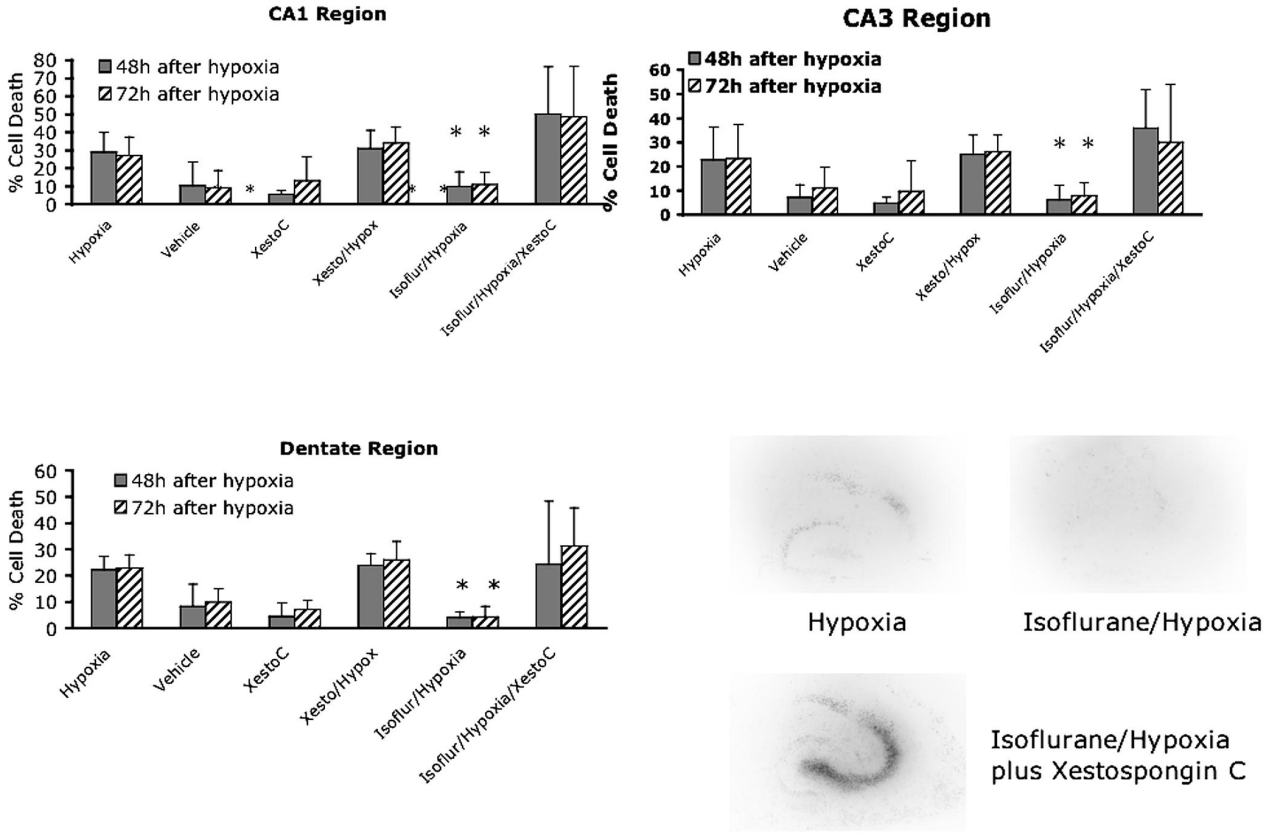

Fig. 7. Effects of xestospongin C on isoflurane neuroprotection in slice cultures. The *lower right* shows examples of cell death (*dark staining*) in cultures in which xestospongin C was present during hypoxia combined with isoflurane. There were 9–13 slices in each group. * $P < 0.05$ compared with hypoxia, xestospongin–hypoxia, and isoflurane–hypoxia–xestospongin groups.

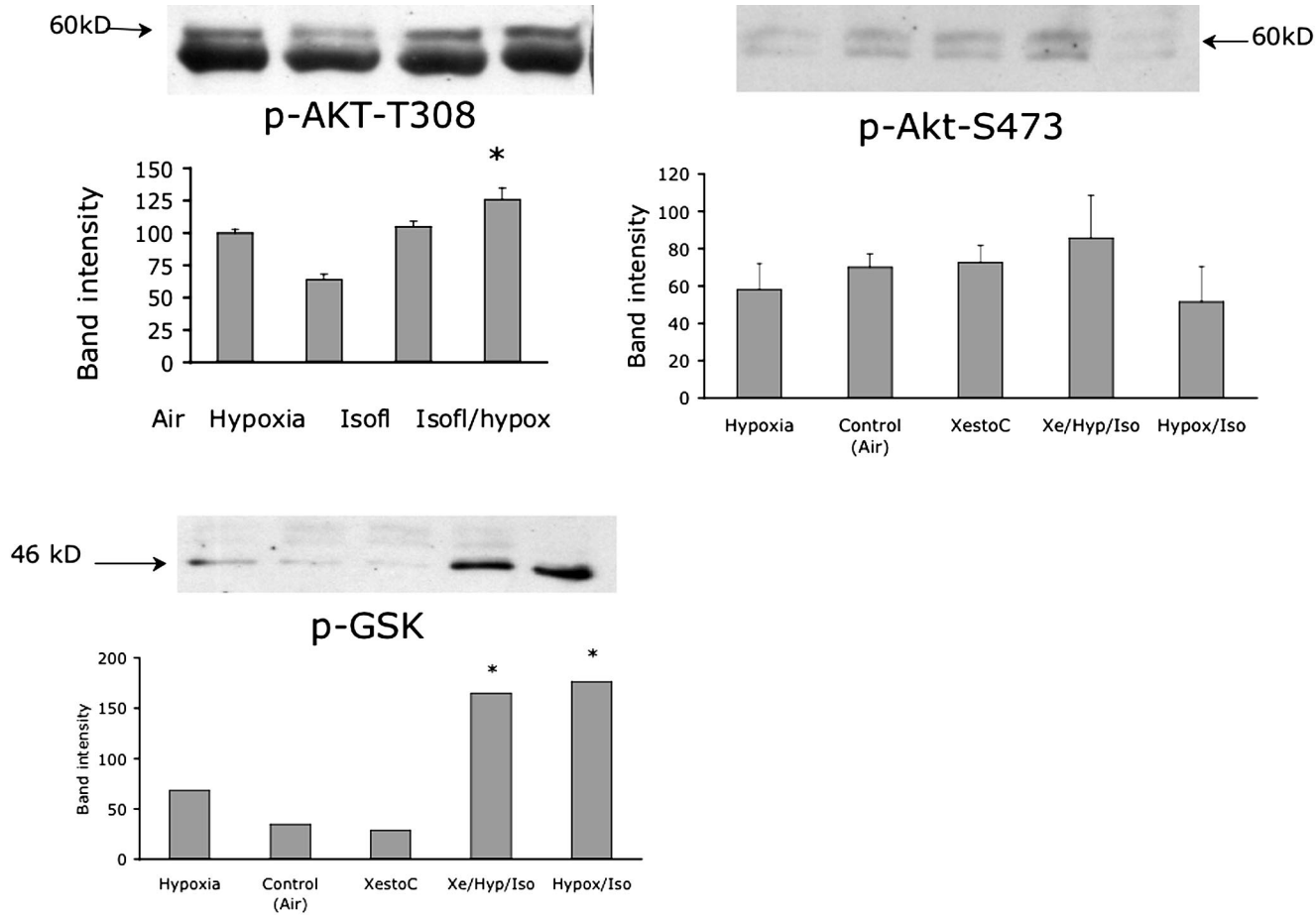

Fig. 8. Isoflurane neuroprotection is associated with phosphorylation of Akt at T308 (* $P < 0.05$) but not at S473 during hypoxia. The phosphorylation of the Akt target glycogen synthase kinase (pGSK) was also increased by isoflurane during hypoxia. The *bar graphs* show mean staining intensity from three to four Western blots.

vates the survival factor Akt, its activation does not depend only on increases in $[Ca^{2+}]_i$ mediated by IP_3 receptors.

Discussion

This study reports several novel findings: (1) Isoflurane neuroprotection from hypoxia-induced death requires that $[Ca^{2+}]_i$ during hypoxia increases moderately; (2) the presence of isoflurane during hypoxia increases the phosphorylation of the Ras-MEK-ERK pathway and is required for neuroprotection; and (3) isoflurane increases the concentration of active (phosphorylated) Akt, an antiapoptotic protein, during hypoxia. Therefore, the findings of this study point to phosphorylation of key signaling molecules as crucial to the neuroprotective effect of isoflurane. We suggest that moderate increases in $[Ca^{2+}]_i$ set in motion events that regulate this pattern of phosphorylation.

Increased $[Ca^{2+}]_i$ Is Required for Neuroprotection with Isoflurane

A proposed intracellular signaling scheme involving the interaction of isoflurane and Ca^{2+} during hypoxia is presented in figure 9. During hypoxia, the presence of isoflurane results in $[Ca^{2+}]_i$ remaining within 30–100 nM of resting levels (figs. 2 and 5), whereas without it, $[Ca^{2+}]_i$ increases to near 2 μM. Confirmed by both a high-affinity and a low-affinity Ca^{2+} indicator, $[Ca^{2+}]_i$ during hypoxia normally increases to levels associated with cell death. We propose that isoflurane, by inhibiting NMDA receptors[30] and by decreasing glutamate release,[31] allows $[Ca^{2+}]_i$ to increase moderately to a neuroprotective window of $[Ca^{2+}]_i$ that triggers the elaboration of survival signals (fig. 9). Our data suggest that an increase in $[Ca^{2+}]_i$ is required for survival, because when increases in $[Ca^{2+}]_i$ during hypoxia are prevented by xestospongin C (fig. 5), isoflurane protection is eliminated and neurons die (fig. 7). A large increase in $[Ca^{2+}]_i$ has been recognized as playing a central role in excitotoxic neuronal death after anoxic/ischemic injuries, whether from glutamate receptors or from TRPM7 cation channels.[32–34] However, the importance of Ca^{2+} as a signal molecule necessary for normal synaptic health and long-term survival of neurons is increasingly clear as well. A substantial body of evidence shows that moderately increased $[Ca^{2+}]_i$ may be a criti-

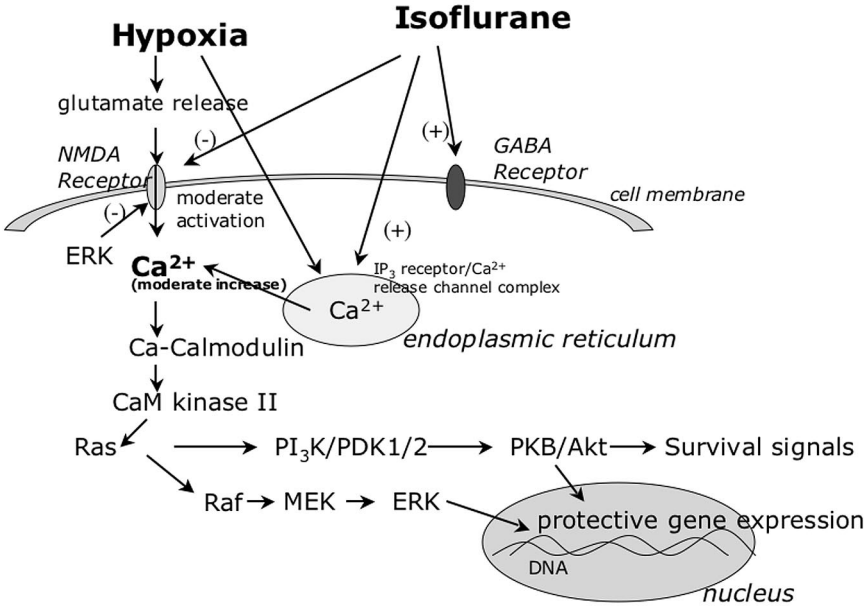

Fig. 9. Proposed scheme of how moderate increases in intracellular calcium concentration ($[Ca^{2+}]_i$) in hypoxic neurons in the presence of isoflurane initiates survival signaling. During hypoxia, release and accumulation of glutamate from energy-stressed neurons normally causes a large increase in $[Ca^{2+}]_i$, an action opposed by isoflurane *via* inhibition of *N*-methyl-D-aspartate (NMDA) receptors and augmentation of γ-aminobutyric acid (GABA) receptors and other processes. At the same time, isoflurane causes the release of Ca^{2+} from intracellular stores, with the net result being a moderate increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ activates the calcium-binding protein calmodulin and the calcium-dependent kinase CaM kinase II as well as Ras. Ras in turn activates both the mitogen-activated protein kinase (MAPK) signaling family and protein kinase B.[29] MAPK p42/44 (ERK) is both a transcription factor and negative regulator of Ca^{2+} influx *via* NMDA receptors.[41] IP_3 = inositol triphosphate; PKB = protein kinase B.

cal survival signal, including data from our slice culture model.[21] Conversely, excessively low $[Ca^{2+}]_i$ is detrimental during or after brain ischemia because it deprives neurons of defensive mechanisms and trophic influences critical for survival[35,36] and promotes neuronal apoptosis.[37] We propose that the neuroprotective actions of isoflurane may be summarized as keeping $[Ca^{2+}]_i$ not only within survivable limits during hypoxia but into a zone associated with active survival signaling.

Isoflurane and Ca^{2+}-dependent Regulation of Cell Fate

Our data show that during hypoxia, the presence of isoflurane is associated with increased concentrations of proteins related to cell survival, including increases concentrations of the neuroprotective factors MAP kinase p42/44 and Akt. The MAP kinase p42/44 pathway is important in neuroprotective signaling *via* membrane-associated growth factor receptors.[38] Activated Akt is a potent inhibitor of apoptosis in neurons[39] and requires increases in Ca^{2+} for its up-regulation,[11,40] although as shown in figure 8, the phosphorylation of the Akt target glycogen synthase kinase apparently does not require IP_3 receptor–mediated increases in $[Ca^{2+}]_i$. We did not test whether phosphorylation of Akt at T308 is dependent on IP_3 receptors. The MAP kinase p42/44 is also involved with isoflurane protection, similar to the role of the persistent increase in MAP kinase p38 concentrations associated with preconditioning reported in intact rats.[8] Further, MAP kinase p42/44 links growth factor receptors for nerve growth factor and brain-derived neurotrophic factor to neuroprotective effects such as NMDA receptor modulation and reduction of glutamate excitotoxicity.[41] MAP kinase p42/44 also phosphorylates hypoxia-inducible factor 1α, a transcription factor required for the adaptation of cells to acute and chronic hypoxia.[14] It is not clear whether the moderate increase in $[Ca^{2+}]_i$ during hypoxia is sufficient for the protective actions of isoflurane or whether additional factors independent of $[Ca^{2+}]_i$ changes are required. However, small increases in $[Ca^{2+}]_i$ in slice cultures are strongly neuroprotective if present just before oxygen and glucose deprivation.[21] Thus, the effects of isoflurane on signaling pathways are consistent with known neuroprotective processes in other models and contexts.

The protective actions of isoflurane on signaling pathways and on apoptosis regulators are not in conflict with earlier proposals that isoflurane either inhibits excitotoxic[31,42] or augments inhibitory processes.[6] The signaling mechanisms described here interact with processes involved in excitotoxicity. For example, activation of the MAP kinase ERK pathway *via* growth factor receptors results in decreased NMDA receptor activity and excitotoxicity.[41] The possibility that one neuroprotective effect of isoflurane is related to second-messenger modulation of NMDA receptors and TRPM7 cation channels is currently under investigation.

Limitations of Isoflurane Neuroprotection and Delayed Apoptosis

The neuroprotective effects of isoflurane were present after 60 but not 75 min of hypoxia (Fig. 1). Beyond noting that isoflurane did not increase p42/44 concentrations after 75 min of hypoxia, we did not investigate the mechanisms for this difference. It is possible that this or other deleterious events relate to the lack of sustained neuroprotection observed in rodent models of relatively severe cerebral ischemia[2,3] and may mean, as pointed out by Warner,[43] that anesthetic neuroprotection may be one of decreasing the effective severity of an injury.

The antiapoptotic signaling found here and more directly observed in other studies[8,9] suggests that isoflurane will provide sustained protection for mild injuries. More study is needed in both *in vitro* and *in vivo* animal models to determine the effects of anesthetics on the time course of cell death, cell death processes, and regeneration of lost neurons. Whether anesthetics can, under some conditions, produce durable neuroprotection in animal models or clinically relevant neuroprotection in humans is an important unanswered question.[44]

Conclusions

Isoflurane and hypoxia interact to trigger neuroprotective signals in hippocampal neurons. This seems to be a result of moderate increases in $[Ca^{2+}]_i$ during hypoxia when 1% isoflurane is present. The moderate increases in $[Ca^{2+}]_i$ are related to phosphorylation of the Ras-Raf-MEK-ERK pathway, which is important in neuroprotective signaling. Preventing isoflurane-induced increases in $[Ca^{2+}]_i$ and interfering with the ERK pathway during hypoxia blocks neuroprotection. Furthermore, isoflurane protection is associated with increased activity of the antiapoptotic factor protein kinase B/Akt, which is consistent with the reduction of apoptosis by isoflurane observed in other *in vitro* studies.

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