General Anesthetic Isoflurane Modulates Inositol 1,4,5-Trisphosphate Receptor Calcium Channel Opening

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ABSTRACT

Background: Pharmacological evidence suggests that inhalational general anesthetics induce neurodegeneration *in vitro* and *in vivo* through overactivation of inositol trisphosphate receptor ($InsP_3R$) Ca^{2+}-release channels, but it is not clear whether these effects are due to direct modulation of channel activity by the anesthetics.

Methods: Using single-channel patch clamp electrophysiology, the authors examined the gating of rat recombinant type 3 $InsP_3R$ ($InsP_3R$-3) Ca^{2+}-release channels in isolated nuclei (N = 3 to 15) from chicken lymphocytes modulated by isoflurane at clinically relevant concentrations in the absence and presence of physiological levels of the agonist inositol 1,4,5-trisphosphate ($InsP_3$). The authors also examined the effects of isoflurane on $InsP_3R$-mediated Ca^{2+} release from the endoplasmic reticulum and changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

Results: Clinically relevant concentrations (approximately 1 minimal alveolar concentration) of the commonly used general anesthetic, isoflurane, activated $InsP_3R$-3 channels with open probability similar to channels activated by 1 μM $InsP_3$ ($P_o \approx 0.2$). This isoflurane modulation of $InsP_3R$-3 P_o depended biphasically on $[Ca^{2+}]_i$. Combination of isoflurane with subsaturating levels of $InsP_3$ in patch pipettes resulted in at least two-fold augmentations of $InsP_3R$-3 channel P_o compared with $InsP_3$ alone. These effects were not noted in the presence of saturating $[InsP_3]$. Application of isoflurane to DT40 cells resulted in a 30% amplification of $InsP_3R$-mediated $[Ca^{2+}]_i$ oscillations, whereas $InsP_3$-induced increase in $[Ca^{2+}]_i$ and cleaved caspase-3 activity were enhanced by approximately 2.5-fold.

Conclusion: These results suggest that the $InsP_3R$ may be a direct molecular target of isoflurane and plays a role in the mechanisms of anesthetic-mediated pharmacological or neurotoxic effects. **(Anesthesiology 2014; 121:528-37)**

THE inositol 1,4,5-trisphosphate receptor ($InsP_3R$) is an intracellular Ca^{2+}-release channel found mostly on the membrane of the endoplasmic reticulum (ER). Activation of $InsP_3R$ by inositol 1,4,5-trisphosphate ($InsP_3$) causes Ca^{2+} release from the ER lumen into the cytoplasm, where it acts as a second messenger to regulate many physiological processes such as cell survival and neurogenesis.[1–4] $InsP_3R$ overactivation also regulates some pathological processes,[1,5] especially apoptosis and neurodegeneration.[6–8] $InsP_3R$ channel activity can be regulated by a diverse array of interacting proteins,[4] including neurodegenerative-associated proteins such as huntingtin-associated protein 1 and Alzheimer's disease-mutant presenilin 1 and presenilin 2.[9,10]

More than 260 million patients worldwide receive surgeries with general anesthesia each year. The diversity of the molecular mechanisms of general anesthetics is still not clear. Activation of $InsP_3Rs$ may play important roles in anesthetic-mediated regulation of intracellular Ca^{2+} homeostasis and some physiological and pathological processes.[11–14] General anesthetics, especially isoflurane, may precondition cells and

What We Already Know about This Topic

- A number of effects of volatile anesthetics, including neurotoxicity and preconditioning, have been attributed to anesthetic-induced activation of inositol trisphosphate receptor calcium-release channels. How anesthetics modulate this receptor is not clear.
- Using patch clamp technology, the effect of isoflurane on inositol trisphosphate receptor function, calcium release, and cell apoptosis was evaluated in membrane preparations.

What This Article Tells Us That Is New

- At a dose of 1 minimum alveolar concentration, isoflurane activated inositol trisphosphate receptor, and this activation was accompanied by an increase in intracellular calcium. Moreover, cell death was increased by isoflurane.
- The data support the premise that isoflurane modulates inositol trisphosphate receptor calcium-release channel and this activity may underlie a variety of effects of isoflurane, including neurotoxicity.

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provide cytoprotection by moderately activating $InsP_3Rs$ and Ca^{2+} release from the ER.[12] Isoflurane may also cause apoptosis and neurodegeneration by causing abnormal Ca^{2+} release from the ER *via* overactivation of $InsP_3R$.[3,11,13,15–17] Sensitized $InsP_3R$ activity in Alzheimer's and Huntington diseases seems to render neurons vulnerable to isoflurane-induced Ca^{2+} release from the ER and subsequent apoptosis.[15,17,18] Isoflurane increases levels of β-site amyloid β-precursor protein-cleaving enzyme[16] and aggregation of mutated huntingtin proteins[17] *via* activation of $InsP_3R$. General anesthetics may cause neuronal apoptosis by disruption of intracellular Ca^{2+} homeostasis.[18–21] Despite these strong associations of anesthetic exposure and $InsP_3R$ activation in normal and pathological conditions, there is a lack of evidence to support direct activation of the $InsP_3R$ by isoflurane.

In this study, we report for the first time that clinically relevant concentrations of isoflurane directly modulate the activity of the $InsP_3R$ channel and sensitize the channel to basal levels of $InsP_3$, resulting in $InsP_3R$-mediated Ca^{2+} release from the ER and amplification of $InsP_3$-induced $[Ca^{2+}]_i$ signals and induction of cell apoptosis. These results suggest that the $InsP_3R$ may be one of the molecular mechanisms of anesthetic-mediated pharmacological and toxic effects in neurodegeneration.

Materials and Methods

Cell Culture

DT40 Chicken Lymphocytes. DT40 cells lacking the genes for all three isoforms of $InsP_3Rs$ (DT40-KO, RIKEN Cell Bank No. RCB 1467, Ibaraki, Japan) and DT40-KO cells stably overexpressing the rat type 3 $InsP_3R$ (DT40-R3)[22] were used in this study. Although the work was conducted in a chicken lymphocyte cell line, the channel studied is the rat recombinant $InsP_3R$ channel. We elected to use $InsP_3R$-3 instead of other $InsP_3R$ subtypes because $InsP_3R$-3 has been shown to have relatively higher opening probability activated by $InsP_3R$ agonists and is a useful *in vitro* model to examine the $InsP_3R$ activation by its agonists.[22] Given the high level of primary sequence homology among mammalian $InsP_3Rs$, and the substantial similarities in the major features of the regulation of endogenous and recombinant $InsP_3R$ channels by cytoplasmic $InsP_3$ and Ca^{2+} (biphasic activation and inhibition by Ca^{2+}; and monotonic, saturable activation by $InsP_3$),[4] we expect that our findings that isoflurane affects Ca^{2+} and $InsP_3$ regulation of $InsP_3R$ channels have relevance for similar regulations of neuronal $InsP_3R$ channels, and thereby have relevance for Ca^{2+} homeostasis in neurons. Cells were maintained in suspension culture in Roswell Park Memorial Institute 1640 medium containing 10% fetal calf serum, 1% chicken serum, penicillin (100 units/ml), streptomycin (100 μg /ml), and glutamine (2 mM) at 37°C in a 95% air and 5% CO_2 humidified incubator.

Electrophysiology

There is no way to directly observe single-channel activity of the $InsP_3R$ in the native ER membrane because performing patch clamp electrophysiology on the ER directly has not been achieved. Because the outer nuclear membrane is continuous with the ER membrane topologically, and because there is no evidence to suggest that the biochemical properties of the outer nuclear membrane are distinct from those of the rest of the ER, nuclear patch clamping of the ER membrane is a tool to record ER-localized ion channel activities, including the $InsP_3R$.[4] We have examined the effects of isoflurane on $InsP_3Rs$ located on the outer nuclear membrane. Preparation of isolated nuclei from DT40 cells was performed as described.[10,22–26] Cells were washed twice with phosphate buffered saline and suspended in a nuclear isolation solution containing 150 mM KCl, 250 mM sucrose, 1.5 mM 2-mercaptoethanol, 10 mM Tris·HCl, 0.05 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Complete; Roche Molecular Biochemicals, Indianapolis, IN), adjusted to pH 7.3 with KOH. Isolated nuclei were placed in a recording chamber containing a standard bath solution: 140 mM KCl, 10 mM HEPES, and 0.5 mM 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, ≈200 nM free Ca^{2+}, adjusted to pH 7.3 with KOH. During the nuclear isolation protocol and the introduction of nuclei onto the stage of the patch clamp microscope, any endogenous $InsP_3$ is washed away. Selected intact nuclei were patch clamped and single-channel activities were recorded in the on-nucleus configuration. Recording pipettes with resistances of 8 to 10 MΩ were used. The pipette solution contained 140 mM KCl, 0.5 mM adenosine triphosphate, and 10 mM HEPES (pH 7.3), with varying concentrations of isoflurane, $InsP_3$, and Ca^{2+}. The free Ca^{2+} concentration was varied by addition of an appropriate Ca^{2+} chelator, as previously described.[27] Single-channel currents were amplified using an Axopatch-200B amplifier (Molecular Devices, Downingtown, PA), filtered at 1 kHz, and digitized at 5 kHz with an ITC-16 interface (Instrutech, Port Washington, NY) and Pulse+ Pulse Fit software (HEKA Electronik, Bellmore, NY). All recordings were performed at room temperature with the pipette electrode at –40 mV relative to the reference bath electrode. Single-channel analyses were performed on recordings exhibiting only a single channel using QuB (University of Buffalo, Buffalo, New York) and Igor Pro (WaveMetrics, Lake Oswego, OR) softwares. Figures were generated using Igor Pro software and Adobe Illustrator (Adobe Systems Incorporated, San Jose, CA).

Measurement of Cytoplasmic Ca^{2+} Concentration ($[Ca^{2+}]_i$)

DT40-KO or DT40-R3 cells were seeded onto glass coverslips coated with 0.01% poly-L-ornithine for 1 h before measurements. Cells were loaded with 2.5 μM acetoxymethyl ester form of calcium sensitive dye Fura-2 (Molecular Probes, Grand Island, NY) for 30 min at room temperature in Hanks' balanced salt solution (Sigma, Pittsburgh, PA) containing 1.8 mM $CaCl_2$ and 0.8 mM $MgCl_2$, pH 7.4. Coverslips were then placed in a sealed perfusion chamber (Warner Instruments,

Hamden, CT) and continuously perfused at room temperature with Hanks' balanced salt solution. Fura-2 was alternately excited at 340 and 380 nm, and emitted fluorescence (510 nm) was collected and recorded using a charged-coupled device-based imaging system running IPLab v3.7 software (Biovision Technologies, Exton, PA). The data are presented as the ratio of fluorescence intensities recorded at 340 and 380 nm excitations (F340/F380) during baseline and isoflurane application. F340/F380 ratios were recorded from more than 30 cells in at least three separate experiments. For analyses of $[Ca^{2+}]_i$ oscillations, ratio images were collected from more than 60 cells during 20 min in at least four separate experiments. The percentage of cells with obvious $[Ca^{2+}]_i$ responses (a single transient increase or sustained oscillations), peak $[Ca^{2+}]_i$, area under the $[Ca^{2+}]_i$ *versus* time curve, and oscillation frequency were determined and analyzed as described previously.[28] Hanks' balanced salt solution samples were collected from inflow and outflow tubes of the recording chamber to determine the anesthetic concentration to which the cells were exposed. High-performance liquid chromatography measurements showed that isoflurane was consistently maintained at 0.4 mM (not shown). This concentration corresponds to a minimum alveolar concentration of approximately 1.

Detection of Caspase-3 Activity

Proteolytic activation of caspase-3 was measured in lysates from DT40 wild-type (DT40-wt), DT40-KO, and DT40-R3 cells after exposure to isoflurane using previously described protocols.[13] Caspase-3 activity was measured using Ac-DEVD-AFC hydrolysis kit (Caspase-3; Calbiochem, Billerica, MA). In brief, DT40-KO or DT40-R3 cells grown in six well plates were exposed to isoflurane (2.4%) for 24 h which resulted in 0.8 mM isoflurane in the culture medium[29] and reliably produced isoflurane toxicity in this anesthesia exposure cell model,[13] harvested *via* trypsinization, and washed with phosphate buffered saline. The cell pellet was gently resuspended in CelLytic M lysis buffer with protease inhibitor cocktail (Sigma). Lysate was centrifuged and the resultant supernatant was used for the assay. Ac-DEVD-AFC, the caspase substrate, was added at a final concentration of 50 μM and the samples were incubated for 45 min at 37°C. Ac-DEVD-AFC hydrolysis was monitored by fluorescence emission of the released AFC (excitation, 400 nm; emission, 500 nm) using a multi-wavelength excitation dual-wavelength-emission fluorometer (Delta RAM; Photon Technology International, Edison, NJ). Under these excitation and emission conditions, Ac-DEVD-AFC hydrolysis produced a yellow-green fluorescence as suggested by the manufacturer (Calbiochem).

Statistical Analysis

Statistical analyses of data were done with IGOR Pro software (Wavemetrics). All data are presented as mean ± SEM. Sample size for the electrophysiological and Ca^{2+} imaging experiments were used based on previous experiences.[27,30] Experimenters were not blinded to conditions. To avoid error introduced by subjectivity, all electrophysiological experiments in which nuclear membrane patches were successfully isolated were recorded and analyzed.

Statistical comparisons between data points were done using two-tailed tests. All statistically significant differences between data points stated were established using unpaired *t* tests (with Bonferroni correction for multiple comparisons within a family of inferences). All lacks of statistically significant differences between data points stated were established by ANOVA. Unless a more stringent *P* value is stated, *P* value less than 0.05 was used for rejecting the null hypothesis.

Results

Activation of $InsP_3R$ by Isoflurane

Previous studies indicated that $InsP_3Rs$ are involved in isoflurane-mediated increases in $[Ca^{2+}]_i$ in PC12 cells, DT40 chicken B lymphocytes, Huntington's striatal neuronal cell lines, and primary cortical neurons.[11,13,16,31] To test whether isoflurane might directly modulate the activity of the $InsP_3R$, we measured single $InsP_3R$ channel currents in native ER membranes by nuclear patch clamp electrophysiology.[4,10,23,24,26,28] Homo-tetrameric $InsP_3R$-3 channel activities were recorded by patch clamping outer membranes of nuclei isolated from DT40-KO cells stably expressing the rat type 3 $InsP_3R$.[10,22,32] This isoform has ligand regulation and permeation properties similar to other $InsP_3R$ isoforms, but with robust gating that provides sensitive detection of modulation of channel activity in patch clamp electrophysiology. $InsP_3R$-3 channel activity was observed with 2 μM Ca^{2+} and either 1 or 10 μM $InsP_3$ in the pipette solution (fig. 1, A–C) in 90% or more of patches (table 1; 18 of 20 and 31 of 34 for 1 and 10 μM $InsP_3$, respectively). Surprisingly, with 400 μM isoflurane in the pipette solution, $InsP_3R$ channel activity could be elicited in the absence of $InsP_3$ (fig. 1D), although with low open probability (P_o) and in less than 20% of patches (table 1; 15 of 85). The channels activated by isoflurane were identified as $InsP_3R$ by their sensitivity to the $InsP_3R$ antagonists heparin (100 μg/ml) or xestospongin C (1 μM) (fig. 1, E and F; table 1). The mean open probability, open duration (t_o), and closed time (t_c) of the $InsP_3R$-3 channels activated by 400 μM isoflurane were similar to those of channels activated by subsaturating 1 μM $InsP_3$ (fig. 1, G–I), suggesting that isoflurane activates $InsP_3R$-3 channels with similar kinetics to the endogenous agonist $InsP_3$ but with lower efficacy. Interestingly, the concentration dependence of the modification of $InsP_3R$-3 channel activity by isoflurane was biphasic: at low isoflurane concentration (<400 μM), increases in isoflurane concentration increased channel P_o, but channel P_o decreased as isoflurane concentration was increased beyond 400 μM. (fig. 2). Thus, $InsP_3R$-3 channels are activated within a narrow range of isoflurane concentration. This is different from $InsP_3$ activation of the channel, in which channel P_o increases with

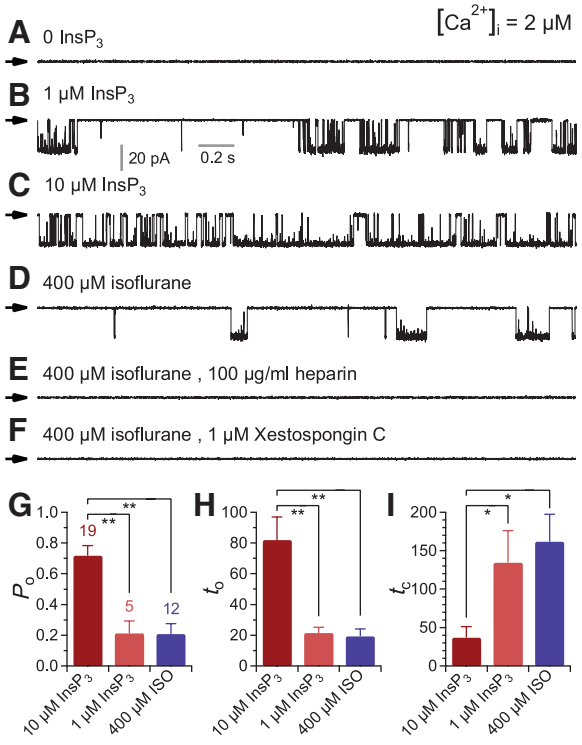

Fig. 1. Isoflurane activates single inositol 1,4,5-trisphosphate receptor ($InsP_3R$) channel gating. (*A–F*) Typical single-channel current traces in membrane patches from nuclei isolated from DT40-KO cells stably expressing rat type 3 $InsP_3R$. Concentrations of $InsP_3$, isoflurane, and $InsP_3R$ inhibitors used are as indicated. Currents shown were recorded at room temperature with applied potential (V_{app}) = –40 mV, and *arrows* indicate the closed channel current level. Concentration of free Ca^{2+} ($[Ca^{2+}]_i$) = 2 μM in all the experiments unless stated otherwise. (*G–I*) Bar graphs showing $InsP_3R$ gating characteristics: mean open probability (P_o), open duration (t_o), and closed time (t_c), respectively, in various inositol 1,4,5-trisphosphate ($InsP_3$) or isoflurane (ISO) concentrations. *Error bars* indicate SEM, and number of experiments analyzed is tabulated.**, * *P* (*t* test with Bonferroni correction) <0.005 and 0.05, respectively, for the quantities connected by the brackets.

$[InsP_3]$ until the saturating $[InsP_3]$ is reached. Beyond that, further increase in $[InsP_3]$ does not enhance channel activity any more.[32]

$[Ca^{2+}]_i$ Dependence of Isoflurane-induced $InsP_3R$ Channel Activity

Gating of $InsP_3Rs$ is under complex allosteric regulation by $InsP_3$ and $[Ca^{2+}]_i$. In general, $InsP_3R$ channel activity is biphasically regulated by $[Ca^{2+}]_i$ with maximum channel P_o observed over a broad range of $[Ca^{2+}]_i$ in the presence of saturating (10 μM) $InsP_3$. In the presence of subsaturating $[InsP_3]$, sensitivity of the channel to inhibition by high $[Ca^{2+}]$ is enhanced, resulting in a narrower P_o *versus* $[Ca^{2+}]_i$ dependence.[4,32] Thus, we investigated the $[InsP_3]$ and $[Ca^{2+}]_i$ dependencies of isoflurane-activated $InsP_3R$ channel activity. At low (0.4 or 0.9 μM) or high (6 and 10 μM) $[Ca^{2+}]_i$, 400 μM of isoflurane-activated channel P_o was substantially lower (P_o < 0.006) than that observed in 2 μM $[Ca^{2+}]_i$, (P_o = 0.2; fig. 3), resulting in a narrow biphasic $[Ca^{2+}]_i$ dependence.

Isoflurane Modulates $InsP_3R$ Channel Sensitivity to $InsP_3$

Although isoflurane activated $InsP_3R$-3 channel activity in the absence of $InsP_3$ in the pipette solution, the sensitivity of isoflurane activation of the channel to the competitive inhibitor heparin suggests that isoflurane may activate the $InsP_3R$-3 by sensitizing the channel to low $[InsP_3]$ generated locally in the patched nuclear membrane. To test this, we recorded $InsP_3R$ channel activity in DT40-R3 cells with 400 μM isoflurane, 2 μM Ca^{2+}, and a range of $[InsP_3]$ in the pipette solution. As expected, $InsP_3$ enhanced channel P_o in a dose-dependent manner (fig. 4, A–D). At very low (0.1 and 0.5 μM) $[InsP_3]$, 400 μM isoflurane potentiated channel activity to a level higher than by either agonists alone (fig. 4E). In contrast, at higher (1 μM) $[InsP_3]$, 400 μM isoflurane no longer potentiated channel activity significantly (fig. 4E). In saturating (10 μM) $[InsP_3]$, 400 μM isoflurane did not enhance channel activity measurably (fig. 4E). These results suggest that isoflurane increases the functional sensitivity of the $InsP_3R$-3 to $InsP_3$ only at low, subsaturating $[InsP_3]$ (<0.1 μM).

Isoflurane Modulates $InsP_3R$-mediated $[Ca^{2+}]_i$ Signaling and Apoptosis

Our single-channel recordings indicate that isoflurane at clinically relevant concentrations potentiates the activity of the $InsP_3R$-3 at low levels of $[InsP_3]$ that can exist

Table 1. Channel Detection Rate (Pd) of Isoflurane-activated Type 3 $InsP_3R$

	Isoflurane (0.4 mM)	$InsP_3$ (μM)	Heparin (100 μg/ml)	Xestospongin C (1 μM)	Channel Detection Rate (Pd)	Patches (n)
Type 3 $InsP_3R$	–	0	–	–	0/14(0)	14
	+	0	–	–	15/85(0.18)	85
	–	1	–	–	18/20(0.9)	20
	–	10	–	–	31/34(0.92)	34
	+	–	+	+	0/40(0)	40
	+	–	–	+	0/40(0)	40
$InsP_3R$ knockout	–	10	–	–	0/10(0)	10
	+	0	–	–	0/40(0)	40

$InsP_3$ = inositol 1,4,5-trisphosphate; $InsP_3R$ = inositol 1,4,5-trisphosphate receptor.

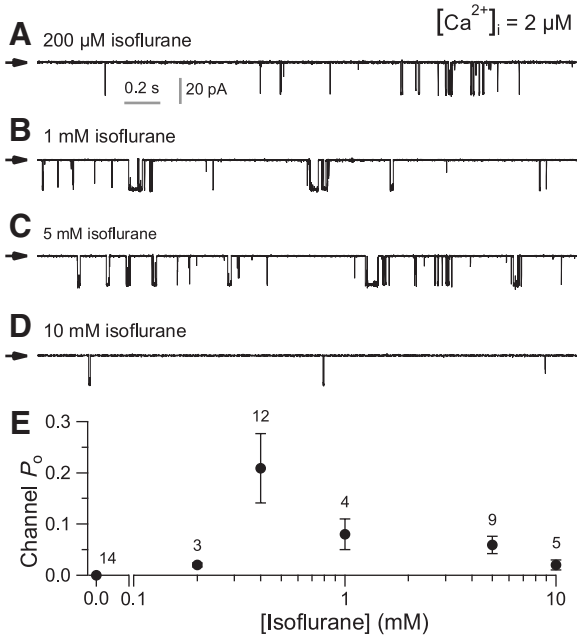

Fig. 2. Dependence of inositol trisphosphate receptor ($InsP_3R$) channel activity on isoflurane concentration. (*A–D*) Typical single-channel current traces of type 3 ($InsP_3R$-3) channel activity in various isoflurane concentrations as indicated. Currents shown were recorded at room temperature with applied potential (V_{app}) = – 40 mV, and *arrows* indicate the closed channel current level. Concentration of free Ca^{2+} ($[Ca^{2+}]_i$) = 2 μM. See figure 1D for single $InsP_3R$ channel current trace in 400 μM isoflurane. (*E*) Isoflurane concentration dependence of $InsP_3R$-3 channel open probability P_o. *Error bars* indicate SEM, and number of experiments analyzed is tabulated.

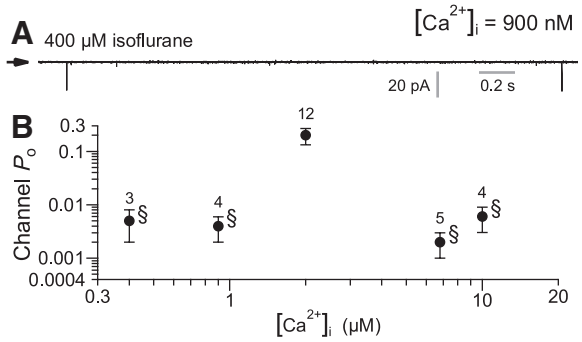

Fig. 3. Ca^{2+} dependence of isoflurane-activated type 3 inositol trisphosphate receptor ($InsP_3R$-3) channel. (*A*) Typical single-channel current traces of $InsP_3R$ channels activated by 400 μM isoflurane in free Ca^{2+} concentration ($[Ca^{2+}]_i$) of 900 nM. Currents shown were recorded at room temperature with applied potential (V_{app}) = –40 mV, and *arrow* indicates the closed channel current level. (*B*) $[Ca^{2+}]_i$ dependence of $InsP_3R$-3 channel open probability (P_o) in 400 μM isoflurane. *Error bars* indicate SEM, and number of experiments analyzed is tabulated. Note the logarithmic channel P_o axis. Low channel P_o (<0.01, marked with §) were observed in all $[Ca^{2+}]_i$ examined other than 2 μM. There is no statistically significant difference among these low channels P_o (P > 0.05, ANOVA test). These channel P_o were so low that the number of active channel(s) (*N*) in the membrane patch cannot be accurately determined. Thus, the data plotted are effectively the NP_o value, which may be an overestimate of the actual P_o value.

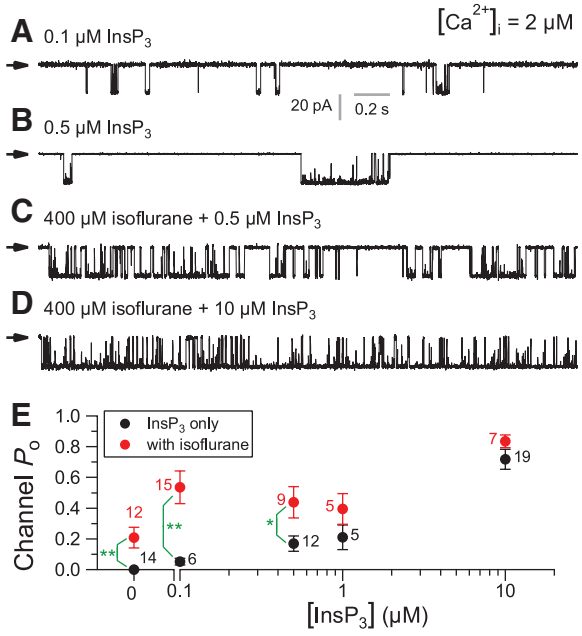

Fig. 4. Effects of 400 μM isoflurane on the activity of inositol trisphosphate receptor ($InsP_3R$) channel in various $[InsP_3]$. (*A–D*) Typical single-channel current traces of $InsP_3R$ channels in various $[InsP_3]$ in the absence and presence of 400 μM isoflurane, as indicated. Currents shown were recorded at room temperature with applied potential (V_{app}) = –40 mV, and *arrows* indicate the closed channel current level. See figure 1, A–C, for $InsP_3R$ channel current traces in 0, 1, and 10 μM $InsP_3$ with no isoflurane, and figure 1D for $InsP_3R$ channel current trace in 400 μM isoflurane only. (*E*) Statistically similar $InsP_3R$ channel activities (P > 0.05, ANOVA test) were observed in 100 nM ≤ $[InsP_3]$ ≤ 1 μM in the absence of isoflurane. With 400 μM isoflurane, statistically similar $InsP_3R$ channel activities (P > 0.05, ANOVA test) were also observed in 0 ≤ $[InsP_3]$ ≤ 1 μM. Importantly, 400 μM isoflurane significantly increased $InsP_3R$ channel activity at low $[InsP_3]$ (0, 100, and 500 nM). *Error bars* indicate SEM, and number of experiments analyzed is tabulated. **, *P (t test) <0.005 and 0.05, respectively, for the quantities connected by the brackets. $InsP_3R$ channel P_o in saturating 10 μM $InsP_3$ were significantly higher (P < 0.05, t test with Bonferronic correction) than those in subsaturating $[InsP_3]$, in the presence or absence of 400 μM isoflurane. However, $InsP_3R$ channel activity in saturating (10 μM) $InsP_3$ was not enhanced by 400 μM isoflurane (P > 0.05, t test).

in unstimulated cells in basal conditions. To determine whether this effect influences intracellular Ca^{2+} signaling, we measured $[Ca^{2+}]_i$ in individual DT40-KO or DT40-R3 cells kept in complete growth medium. Application of 400 μM isoflurane resulted in a significant transient increases in $[Ca^{2+}]_i$ in the DT40-R3 cells that were absent in DT40-KO cells lacking $InsP_3R$ expression (fig. 5, A–C).

To further study the potentiating effects of isoflurane on intracellular Ca^{2+} signaling in weakly stimulated cells, the cells were perfused with complete growth medium containing a low concentration (50 ng/ml) of anti-immunoglobulin M antibody to weakly stimulate the B-cell receptor to generate low, but higher than basal levels of $InsP_3$.[33]

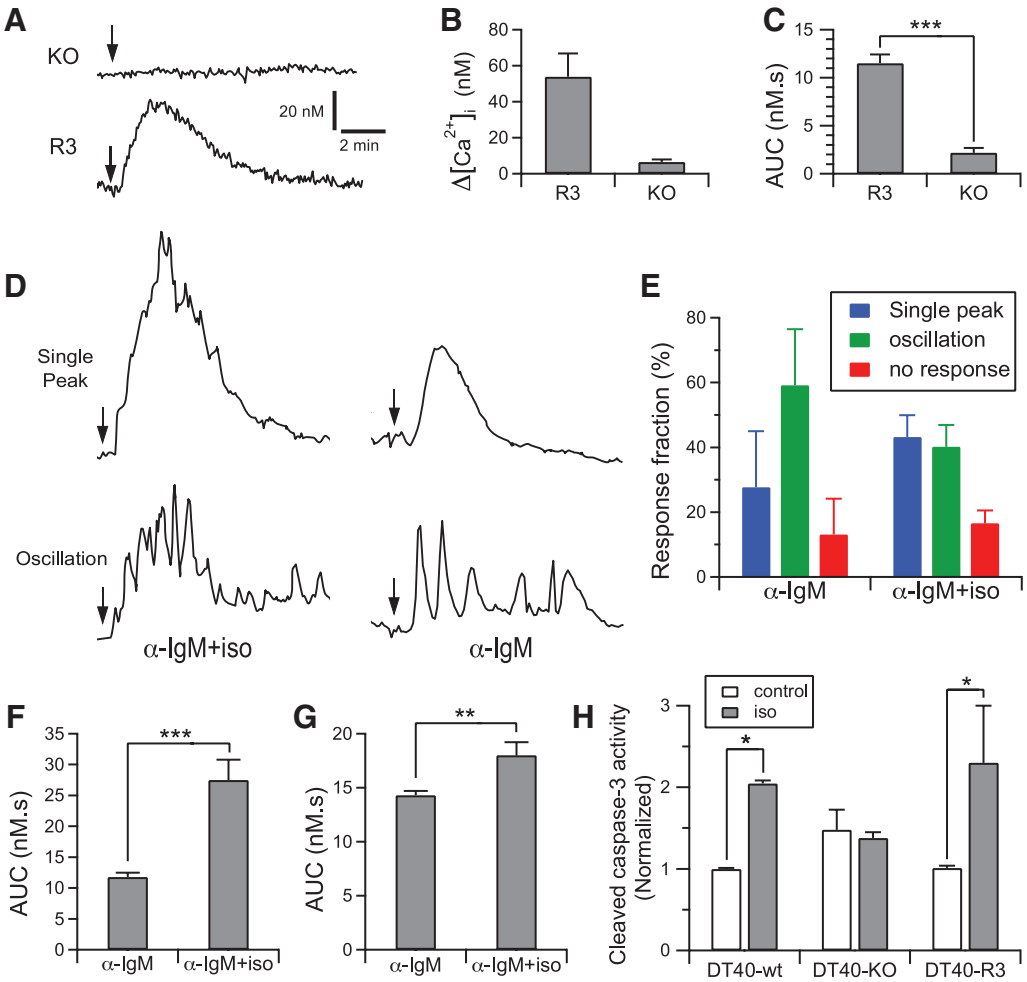

Fig. 5. Isoflurane amplifies cytosolic Ca^{2+} signals in DT40 cells by sensitizing inositol trisphosphate receptor ($InsP_3R$) activation and induces cell apoptosis. (*A*) Representative single-cell Ca^{2+} responses to 400 μM isoflurane (Iso) in DT40 cells with triple knock out of $InsP_3R$ (DT40-KO) or DT40-KO transfected with rat recombinant type 3 $InsP_3R$ (DT40-R3) cells. *Arrows* mark the addition of iso to the complete growth medium. (*B*) Peak amplitudes and (*C*) area under curve (AUC) for DT40-KO and DT40-R3 cells responding to isoflurane. (*D*) Representative single-cell Ca^{2+} responses and oscillations induced by addition (marked by *arrows*) of 50 ng/ml anti-immunoglobulin M antibody (α-IgM) or 50 ng/ml α-IgM plus isoflurane in Dulbecco's Modified Eagle Medium. (*E*) Fraction of cells with various type of response to stimulation by α-IgM or α-IgM+iso in the medium. (*F*) AUC of cells with single Ca^{2+} peak response triggered by α-IgM or α-IgM+iso. (*G*) AUC of cells with Ca^{2+} oscillations in response to stimulation by α-IgM or α-IgM+iso. (*H*) Isoflurane significantly increased caspase-3 activity only in DT40 wild-type (DT40-wt) cells or in DT40 expressing only $InsP_3R$ (DT40-R3), but not in DT40 cells with triple knock out of $InsP_3R$ (DT40-KO). All calcium measurement data are summary of at least 141 cells (N ≥ 141, *B* and *C*) or 212 cells (N ≥ 212, *E–G*) from four separate experiments. The N values for caspase-3 activity are the average from three separate experiments (N = 3). *Error bars* indicate SEM, and number of experiments analyzed is tabulated. *, **, ***Significant difference ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, *t* test).*Significant difference ($P < 0.05$, *t* test) between quantities connected by the brackets in the bar graphs.

Eighty-two ± 4% of DT40-R3 cells responded to anti-immunoglobulin M antibody with either a single transient increase in $[Ca^{2+}]_i$ or with $[Ca^{2+}]_i$ oscillations (fig. 5, D and E). Whereas the addition of 400 μM isoflurane did not change the percentage of cells responding to anti-immunoglobulin M antibody (fig. 5, D and E), it enhanced the percentage of cells that responded with a single prolonged $[Ca^{2+}]_i$ peak (fig. 5E), consistent with a stronger $InsP_3R$ response. Because of the variable responses of cells (oscillations *vs.* single-peak responses), we quantified the $[Ca^{2+}]_i$ responses in all cells by the areas under the $[Ca^{2+}]_i$-*versus*-time curves. The presence of isoflurane significantly increased the area under curve for cells responding with a single-peak transient (fig. 5F; $P < 0.001$) or with $[Ca^{2+}]_i$ oscillations (fig. 5G; $P < 0.01$). These results suggest that isoflurane, at a clinically used concentration, potentiates low-level $InsP_3$-mediated $[Ca^{2+}]_i$ signaling.

Prolonged exposure to isoflurane is associated with widespread cell death in diverse *in vivo* and *in vitro* systems.[13,16] Because activation of $InsP_3R$-mediated Ca^{2+} signaling has been linked to apoptosis,[34,35] and considering the effects of isoflurane on $InsP_3R$ gating observed here, we assessed the role of this effect in isoflurane-mediated apoptosis. Caspases are a

family of cysteine proteases that play crucial roles in apoptosis. Activation of caspase-3 is a central event in the progression of programmed cell death. We therefore monitored caspase-3 cleavage as a measure of apoptosis. In agreement with our previous observations of cells expressing endogenous $InsP_3R$,[13,16] isoflurane triggered apoptosis in the DT40-R3 cells, but not in the $InsP_3R$-deficient cells (fig. 5H). These observations support a role for exaggerated activation of $InsP_3R$ in isoflurane-induced apoptosis, consistent with previous observations.[11,13,16]

Discussion

We have, for the first time, demonstrated that isoflurane at clinically relevant concentrations modulates the activity of the $InsP_3R$ Ca^{2+}-release channel at the single-channel level. The modulating effects of isoflurane on the $InsP_3R$ regulate Ca^{2+} release from the ER that results in exaggerated $[Ca^{2+}]_i$ signaling. We have also demonstrated, for the first time at the molecular level, that isoflurane causes Ca^{2+} release from the ER *via* this activation of $InsP_3R$ and can therefore affect intracellular Ca^{2+} homeostasis, regulation of cytosolic Ca^{2+} oscillations, and cell survival. These results provide novel insights into possible molecular mechanisms of anesthesia-mediated effects on neurodegeneration and cognitive function.

Inhalational Anesthetic Modulates $InsP_3R$ Activation

It was previously demonstrated that both isoflurane and halothane may increase $[Ca^{2+}]_i$ primarily by inducing Ca^{2+} release from the intracellular Ca^{2+} stores in neurons.[36] Our results provide the first evidence that an inhalational anesthetic can modulate activation of $InsP_3R$ channels. Interestingly, isoflurane activates $InsP_3R$ channels with a biphasic dose–response with optimal concentrations approximately 0.4 to 1 mM, close to clinically used concentrations for general anesthesia. This activation of $InsP_3R$ channels by isoflurane showed a strong Ca^{2+} concentration dependence, with the optimum $[Ca^{2+}]_i$ of 2 μM, qualitatively similar to the $[Ca^{2+}]$ dependence of $InsP_3$-activated channel activity. The $InsP_3$ competitive antagonist, heparin, blocked the ability of isoflurane to activate $InsP_3Rs$. Whether isoflurane requires $InsP_3$ for it to activate the channel, or isoflurane activation is not only direct but also sensitive to heparin inhibition, is unclear. The former may be likely because isoflurane (0.4 mM) potentiated activation of the $InsP_3R$ by low concentrations of $InsP_3$, but failed to further enhance $InsP_3R$-3 activity at saturating $[InsP_3]$. Nevertheless, further studies are needed to investigate possible biochemical interactions between isoflurane and the $InsP_3R$. Together, these results establish the $InsP_3R$ as a novel target of isoflurane and perhaps other inhalational or intravenous general anesthetics.

Isoflurane Enhances Ca^{2+} Signals and Cell Apoptosis via *Activation of $InsP_3R$*

The biphasic effects of $[Ca^{2+}]_i$ on $InsP_3R$ activation play important roles on intracellular Ca^{2+} oscillations, waves, and spreading of global Ca^{2+} signals.[37] Our finding that isoflurane activates $InsP_3R$-3 channels raised the possibility that it could enhance $[Ca^{2+}]_i$ signals. Application of 400 μM isoflurane resulted in a transient increase in $[Ca^{2+}]_i$ in DT40 cells expressing $InsP_3R$-3 but not in DT40 cells lacking the channels. In addition, $[Ca^{2+}]_i$ signals generated by immunoglobulin M stimulation of DT40-R3 cells, both single-peaks and oscillations, were more prominent in the presence of isoflurane (fig. 5, F and G).

We previously showed in *in vitro* and *in vivo* models that exposure to isoflurane for prolonged durations significantly induced apoptosis that required activation of $InsP_3Rs$.[11,13] Our results here suggest that this is medicated at least in part by isoflurane activation of $InsP_3R$ channel gating. The effects of isoflurane on changes in $[Ca^{2+}]_i$ are in good agreement with the conclusions reached from the single-channel recordings of the effects of isoflurane on $InsP_3R$ activity observed in the current study. It has long been known that anesthetics including halothane activate the other major ER Ca^{2+}-release channels, the RyR channel complex,[38] and this is thought to be the basis for malignant hyperthermia.[39] Similar to $InsP_3Rs$, RyRs are expressed throughout the nervous system and play important roles in both normal cell functions[40] and in various neurodegenerative diseases.[2,41–43] Our previous study indicated a role of RyR activation in isoflurane-induced apoptosis in neuronal tissue cultures.[29] Both $InsP_3R$ and RyR contribute to regulation of intracellular Ca^{2+} homeostasis and may interact with each other through Ca^{2+}-induced Ca^{2+} release in a common pathway in normal neuronal function and neurodegeneration. Excessive Ca^{2+} release from the ER *via* these release channels could cause Ca^{2+} overload in mitochondria and depletion of ER Ca^{2+}, both of which can contribute to apoptosis.[6,34,44] In addition, mitochondrial Ca^{2+} overload causes cytochrome C release, activating caspase-3, which can cleave the $InsP_3R$, resulting in a constituent Ca^{2+} leak from the ER.[45] Thus, excessive or prolonged activation of $InsP_3R$ by isoflurane may set in motion a cascade of events resulting in apoptosis in different tissue culture cells including neurons[3,11,13,15–17] and in developing brains.[16] Our data suggest that the $InsP_3R$ may represent an ideal target for prevention of the harmful side effects of inhalational anesthetics. Inhibition of excessive activation of the $InsP_3R$ may ameliorate or prevent anesthesia-mediated neurodegeneration as demonstrated in animal models.[16] This is especially relevant in pediatric[46,47] and aged patients,[48] who seem to be the most vulnerable to the harmful side effects of anesthetics. Unfortunately, there is no good pharmacology for the $InsP_3R$. Although heparin is an $InsP_3R$ antagonist, its poor penetration across cell membranes limits its use for protection against anesthetic neurotoxicity in animals or patients. It should be noted that anesthetics have also been shown to be protective against various stresses, also by activation of $InsP_3Rs$ in different tissue culture models.[3,12,14,49,50] As demonstrated here (fig. 6), mild or moderate activation of $InsP_3R$ and moderate Ca^{2+} release from the ER by isoflurane provides cytoprotection, possibly *via* physiological Ca^{2+} uptake into mitochondria and stimulation of adenosine triphosphate production,[25] or by activation of AKT and microtubule-associated protein kinase/extracellular-signal-regulated kinases

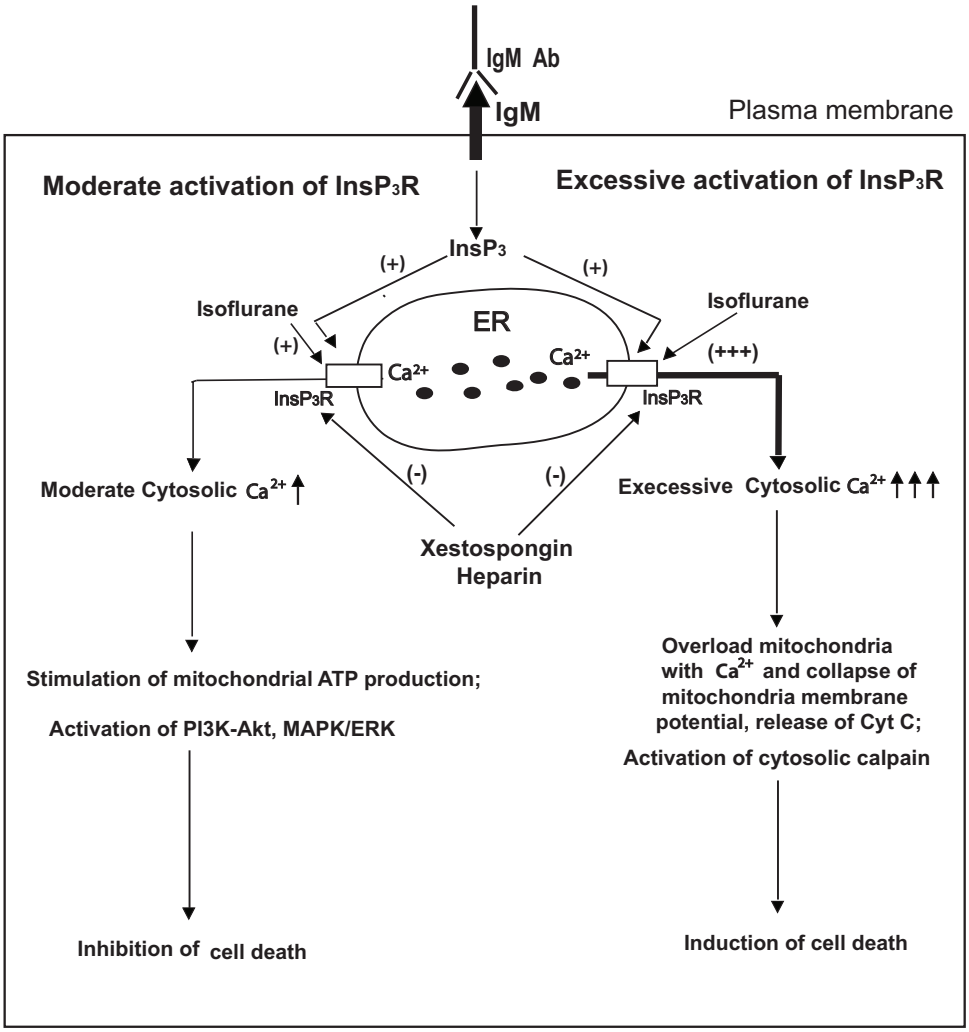

Fig. 6. Modulation of inositol trisphosphate ($InsP_3$) receptor ($InsP_3R$) activity by isoflurane and the effects on cell survival. Isoflurane can activate $InsP_3R$ or potentiate the activation of $InsP_3R$ by endogenous agonist $InsP_3$ generated by various extracellular stimulation including antibody for immunoglobulin M (IgM Ab). These effects can be inhibited by $InsP_3R$ antagonists xestospongin and heparin. Moderate activation of $InsP_3R$ by isoflurane at low concentration for short duration causes moderate Ca^{2+} release from the endoplasmic reticulum (ER) and increase of cytosolic Ca^{2+}, which in turn, inhibit cell death by inducing endogenous cytoprotective mechanisms (*left side*), such as activation of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-protein kinase B (AKT) and microtubule-associated protein kinase (MAPK)/extracellular-signal-regulated kinases (ERK) pathways. Excessive activation of $InsP_3R$ by isoflurane at high concentration for prolonged duration causes excessive Ca^{2+} release from the ER and abnormal increase of cytosolic Ca^{2+}, resulting in induction of cell death *via* apoptosis directly (*right side*), through rerelease of cytochrome C (Cyc C) from mitochondria into cytosolic space. ATP = adenosine triphosphate.

cytoprotective pathways.[12,14,50] Nevertheless, it is prudent to minimize the use of general anesthetics as much as possible, so that their beneficial effects can be used and the harmful effects be minimized.

In summary, our results indicate that the commonly used inhalational anesthetic isoflurane modulates gating of the $InsP_3R$ Ca^{2+}-release channel, enhancing ER Ca^{2+} release and contributing to isoflurane-mediated apoptosis. These results suggest that $InsP_3Rs$ are molecular targets of general anesthetics and that these receptors may provide the basis for some pharmacologic effects of general anesthetics and therapeutic interventions for anesthesia-mediated cell death by apoptosis.

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Competing Interests

The authors declare no competing interests.

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