Cardiac Calcium Release Channel (Ryanodine Receptor 2) Regulation by Halogenated Anesthetics

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ABSTRACT

Background: Halogenated anesthetics activate cardiac ryanodine receptor 2–mediated sarcoplasmic reticulum Ca^{2+} release, leading to sarcoplasmic reticulum Ca^{2+} depletion, reduced cardiac function, and providing cell protection against ischemia-reperfusion injury. Anesthetic activation of ryanodine receptor 2 is poorly defined, leaving aspects of the protective mechanism uncertain.

Methods: Ryanodine receptor 2 from the sheep heart was incorporated into artificial lipid bilayers, and their gating properties were measured in response to five halogenated anesthetics.

Results: Each anesthetic rapidly and reversibly activated ryanodine receptor 2, but only from the cytoplasmic side. Relative activation levels were as follows: halothane (approximately 4-fold; n = 8), desflurane and enflurane (approximately 3-fold, n = 9), and isoflurane and sevoflurane (approximately 1.5-fold, n = 7, 10). Half-activating concentrations (K_a) were in the range 1.3 to 2.1 mM (1.4 to 2.6 minimum alveolar concentration [MAC]) with the exception of isoflurane (5.3 mM, 6.6 minimum alveolar concentration). Dantrolene (10 μM with 100 nM calmodulin) inhibited ryanodine receptor 2 by 40% but did not alter the K_a for halothane activation. Halothane potentiated luminal and cytoplasmic Ca^{2+} activation of ryanodine receptor 2 but had no effect on Mg^{2+} inhibition. Halothane activated ryanodine receptor 2 in the absence and presence (2 mM) of adenosine triphosphate (ATP). Adenosine, a competitive antagonist to ATP activation of ryanodine receptor 2, did not antagonize halothane activation in the absence of ATP.

Conclusions: At clinical concentrations (1 MAC), halothane desflurane and enflurane activated ryanodine receptor 2, whereas isoflurane and sevoflurane were ineffective. Dantrolene inhibition of ryanodine receptor 2 substantially negated the activating effects of anesthetics. Halothane acted independently of the adenine nucleotide–binding site on ryanodine receptor 2. The previously observed adenosine antagonism of halothane activation of sarcoplasmic reticulum Ca^{2+} release was due to competition between adenosine and ATP, rather than between halothane and ATP. (Anesthesiology 2017; 126:495-506)

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RYANODINE receptor 2 receptors (RyR2) are the Ca^{2+} release channel in cardiac sarcoplasmic reticulum (SR). RyR2 can be activated by Ca^{2+} binding to cytoplasmic or luminal sides of the channel[1–3] and can be inhibited by cytoplasmic and luminal Mg^{2+}, which acts as a competitive Ca^{2+} antagonist.[4,5] RyR2 also possesses cytoplasmic facing adenine nucleotide–binding sites[6] by which physiologic concentrations of adenosine triphosphate (ATP; 5 mM) strongly activates channel opening.[7]

Many ion transporters in cardiomyocytes are modulated by halogenated anesthetics such as halothane, isoflurane, enflurane, desflurane, and sevoflurane.[8] Such modulation includes activation of RyR2-mediated SR Ca^{2+} release and inhibition of SERCA2a-driven Ca^{2+} uptake into the SR, both leading to SR Ca^{2+} depletion and reducing cardiac function,[9] contributing to protecting against injury due to ischemia and reperfusion.[10] (During ischemia, there is Ca^{2+} overload of the SR because of RyR2 inhibition by acidosis, reduced [ATP], and

What We Already Know about This Topic

- The ryanodine receptor 2 (RyR2) is a critical component of muscle calcium release and potential determinant of cardiac function and cell protection from ischemia-reperfusion injury
- The volatile anesthetic activation of the RyR2 receptor remains poorly defined

What This Article Tells Us That Is New

- The author's incorporated ryanodine receptor 2 (RyR2) receptors in an *in vitro* lipid bilayer model to show differential effects of clinically relevant concentrations of volatile anesthetics on RyR2 receptor activation
- Halothane, desflurane, and enflurane produced RyR2 activation, while isoflurane and sevoflurane were ineffective in activating the RyR2 receptor
- The study adds important mechanistic insight into the potential protective functions of volatile anesthetics on the ryanodine receptor and the heart

Corresponding article on page 373.

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elevated [Mg^{2+}]. Upon reperfusion, there is a massive release of SR Ca^{2+} as RyR2 recovers from these ischemic conditions.[11])

Halothane has been proposed to act as a surrogate for ATP, activating RyR2 during periods of ischemia, thus ameliorating Ca^{2+} overloading of the SR.[12] This proposal was based on the findings that (1) halothane had a more pronounced effect on Ca^{2+} release when ATP levels were low and (2) adenosine appeared to antagonize halothane activation, supporting the notion that halothane and ATP act at a common adenine nucleotide–binding site on RyR2. However, a previous study using permeabilized rat ventricular myocytes found no competitive action between halothane and ATP on SR Ca^{2+} release.[13] Given the difficulty in precisely controlling the RyR2 environment in cell studies, unequivocal determination of the nature of these molecular interactions requires direct measurements of RyR2 activation in single-channel studies. Although single-channel measurements of RyR2 in artificial lipid bilayers find that clinically relevant concentrations of halothane cause substantial activation of RyR2,[14,15] there has been no examination of competition between halothane and adenine nucleotides in single channels. In this study, we use single-channel measurements to test the hypothesis that halothane and adenine nucleotides share a common activation site on RyR2.

Dantrolene is a well-known inhibitor of Ca^{2+} release in skeletal and cardiac muscles[16–20] that has been used for treatment for halothane-induced malignant hyperthermia in skeletal muscle and arrhythmias in cardiac muscle. Our recent finding that calmodulin is required for dantrolene inhibition of ryanodine receptor 1 (RyR1) and RyR2 explains why dantrolene had no effect on the activity of RyR2 in previous single-channel studies,[15,21–23] where calmodulin would have been absent (calmodulin readily dissociates from the ryanodine receptor (RyR) complex[24]). By adding exogenous calmodulin, we have for the first time employed single-channel recording to probe the mechanism for dantrolene inhibition of halothane-induced Ca^{2+} release by RyR2.

Overall, the mechanism of activation by halogenated anesthetics is poorly defined. Here, we make the first measurements of sevoflurane and desflurane activation of RyR2, the half-activating concentrations (K_a) for other anesthetics and how they alter RyR2 regulation by intracellular ATP, Ca^{2+}, and Mg^{2+}.

Materials and Methods

Preparation of SR Vesicles

Animal tissues were obtained with approval from the Animal Care and Ethics Committee of the University of Newcastle (Newcastle, New South Wales, Australia; approval number A-2009-153). Sheep hearts were obtained from ewes anesthetized with 2% propofol intravenous (IV) followed by oxygen or isoflurane and euthanized by barbiturate overdose (pentobarbitone, 150 mg/kg IV) before the heart was removed. RyR2 was isolated from the heart muscle as described elsewhere.[25]

Single-Channel Measurements

RyRs from sheep heart were incorporated into artificial lipid bilayers that were formed from phosphatidylethanolamine and phosphatidylcholine (8:2 wt/wt) in n-decane (50 mg/ml). For single-channel recording, the *cis* (cytoplasmic) and *trans* (luminal) solutions contained 250 mM Cs^{+} (230 mM cesium methane sulfonate plus 20 mM cesium chloride). Before experiments, halogenated anesthetic concentration in the *cis* solutions was adjusted by adding liquid anesthetic and sealing the solutions in glass-teflon syringes. During recordings, these *cis* solutions were applied to the bilayer using a syringe pump (constructed in-house), providing continuous local perfusion *via* a tube placed in close proximity to the bilayer (fig. 1). Thus, switching between any of eight syringe pumps could produce solution exchange at the bilayer within 3 s. The composition of *trans* solution was altered by means of aliquot additions directly to the bath.[26]

Halogenated anesthetics were obtained from ICI Pharmaceuticals (Australia). Solutions were pH buffered using 10 mM TES (*N*-tris[hydroxymethyl] methyl-2-amino-ethanesulfonic acid, ICN Biomedicals [Australia]) and titrated to pH 7.4 using CsOH (ICN Biomedicals). A Ca^{2+} electrode (Radiometer) was used to determine the purity of Ca^{2+} buffers and Ca^{2+} stock solutions as well as free [Ca^{2+}] greater than 100 nM. Free Ca^{2+} was adjusted with $CaCl_2$ and buffered using (1) 4.5 mM BAPTA (1,2-bis(o-aminophenoxy)ethane- *N*,*N*,*N′*,*N′*-tetraacetic acid, obtained from Invitrogen [Australia]) for free [Ca^{2+}] less than 1 μM; (2) dibromo BAPTA (up to 2 mM) for free [Ca^{2+}] between 1 and 10 μM; or (3) sodium citrate (up to

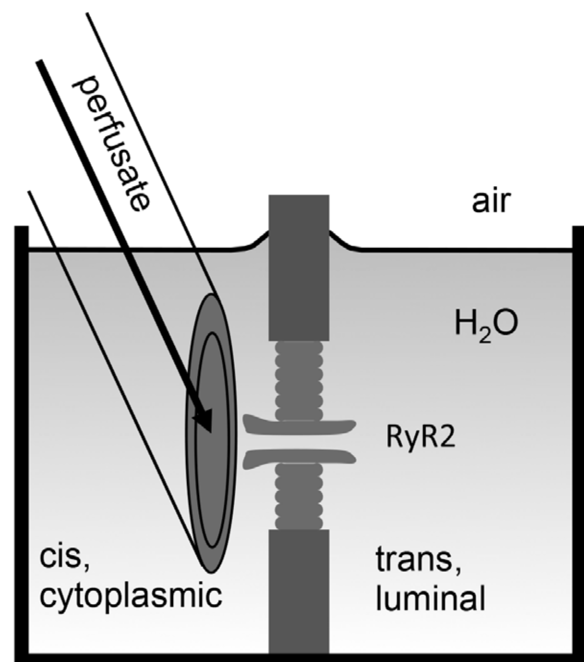

Fig. 1. Schematic arrangement of the lipid bilayer containing ryanodine receptor 2 (RyR2), cytoplasmic (*cis*) and luminal (*trans*) baths, and the solution perfusion tube.

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6 mM) for free [Ca^{2+}] between 10 and 50 μM. In solutions containing ATP (ATP chelates Ca^{2+} and Mg^{2+}), free levels of Mg^{2+} (added as $MgCl_2$) were calculated using estimates of ATP purity and effective Mg^{2+} binding constants that were determined previously under our experimental conditions.[27] Cesium salts were obtained from Sigma-Aldrich Chemical Company (Australia) and $CaCl_2$ and $MgCl_2$ from BDH Chemicals (USA).

Acquisition and Analysis of Ion Channel Recordings

An Axopatch 200B amplifier (Axon Instruments Pty, Ltd, USA) controlled the bilayer potential and recorded unitary currents. Electrical potential differences are expressed as cytoplasmic potential relative to luminal potential. The channel currents were recorded during the experiments using a 50-kHz sampling rate and 5-kHz low-pass filtering. Before analysis, the current signal was redigitized at 5 kHz and low-pass filtered at 1 kHz with a Gaussian digital filter. Single-channel open probability (P_o), mean open time (T_o), mean closed times (T_c), and channel opening rate ($k_o = 1/T_c$) were measured using a threshold discriminator at 50% of channel amplitude. These parameters were measured from single-channel records using Channel3 software (Nicholas W. Laver). Unless otherwise stated, measurements were performed with the *cis* solution voltage clamped at −40 mV.

Statistics

Data are presented as means ± SD or 95% CIs as indicated in figure legends. Individual readings of channel properties were derived from 30 to 100 s of RyR2 recording depending on experimental conditions as described previously.[25] Hill equations were fitted to the dose–response data by the method of least-squares, and errors on the Hill equation parameters were derived using the simplex method.[28] Hypothesis testing was carried out using either two-tailed Student's *t* test or ANOVA (6.0; Graphpad Software Inc., USA) as described in the text. In the case of figure 3, B and C, statistical analyses were programmed using SAS v9.4 (SAS Institute, USA). Opening rate (k_o) and opening duration (T_o) were regressed in separate models on dose, anesthetic, and a dose–anesthetic interaction using mixed-effect regression models, with a random effect for channel and fixed effect for anesthetic. A negative binomial distribution was utilized for the k_o outcome, and a γ distribution was utilized for the T_o outcome. Normal and Poisson distributions were initially trialed for the k_o outcome; however, nonnormality of the residuals in the former and overdispersion in the latter models led to the final choice of the negative binomial distribution. A normal distribution was originally considered for the T_o outcome; however, nonnormality of the residuals led to the final choice of a γ distribution instead.

The data analysis for figure 6 compared the effect of adenosine in the presence and absence of ATP. A two-way ANOVA with repeated measures was used for the no-halothane data; a mixed model was used for the with-halothane data to handle missing values in the repeated measures. Unpaired *t* tests for interactions from both models were then used to compare combinations of adenosine and ATP in the absence of halothane and in the presence of halothane. *Post hoc* pairwise comparisons were made if significant differences were detected. Recommended adjustments were made for multiple comparisons (*e.g.*, Holm–Sidak or Tukey–Kramer). In this study, it was not feasible to randomly assign RyR2 to different experimental conditions or to impose blinding methods. The number of experiments was chosen based on the previous experience of experimental scatter.

Results

Concentration Dependencies of RyR2 Activation by Halogenated Anesthetics

RyR2 from sheep heart was incorporated into lipid bilayers, and their activity was measured in the presence of a 0.1 μM cytoplasmic Ca^{2+} (*i.e.*, typical of end diastole) and 0.1 mM luminal Ca^{2+}, with 2 mM cytoplasmic ATP (vehicle solution; fig. 2). Perfusion of the cytoplasmic bath with vehicle solution containing halothane at mM concentrations caused an increase RyR2 activation (fig. 2A) that was reversed on washout within the time taken for solution exchange at the bilayer (approximately 3 s). The degree of RyR2 activation (fig. 3A) depended on the anesthetic ($P < 0.0001$, one-way ANOVA) with *post hoc* multiple comparisons test (Holm–Sidak; $P < 0.05$), showing that halothane (figs. 2B and 3A), desflurane (fig. 2C), and isoflurane (fig. 2D) caused the largest activation followed by enflurane (fig. 2E) and sevoflurane (fig. 2F). The concentration dependencies of RyR2 activation by these halogenated anesthetics are shown in figure 3, and the parameters of the Hill fits to concentration dependencies of RyR2 open probability (P_o; fig. 3A) are shown in table 1. Most half-maximal activating concentration (K_a) showed no significant dependency on the type of anesthetic with values in the range of 1.3 to 2.1 mM. The exception was isoflurane (K_a = 5.3 mM), which had a significantly higher K_a than that for halothane (K_a = 1.5 mM; $P < 0.05$, one-way ANOVA with Tukey–Kramer multiple comparisons test). Using regression analysis (see Methods), halothane, enflurane, and desflurane activation could be resolved as increases in both channel opening rate (k_o, the reciprocal of mean closed duration, $P = 0.0005$, $P < 0.0001$, and $P = 0.08$, respectively) and mean open duration (T_o; figs. 3, B and C, respectively; $P < 0.0001$, $P < 0.0001$, and $P = 0.0006$, respectively).

Cytoplasmic Site of Anesthetic Action

We added halothane and enflurane to the luminal bath in bilayer experiments (5 or 50 mM by aliquot additions of liquid anesthetic) to see if they could activate RyR2 from that side of the membrane. The relative effects of these halogenated anesthetics on P_o are shown in figure 4 where it can

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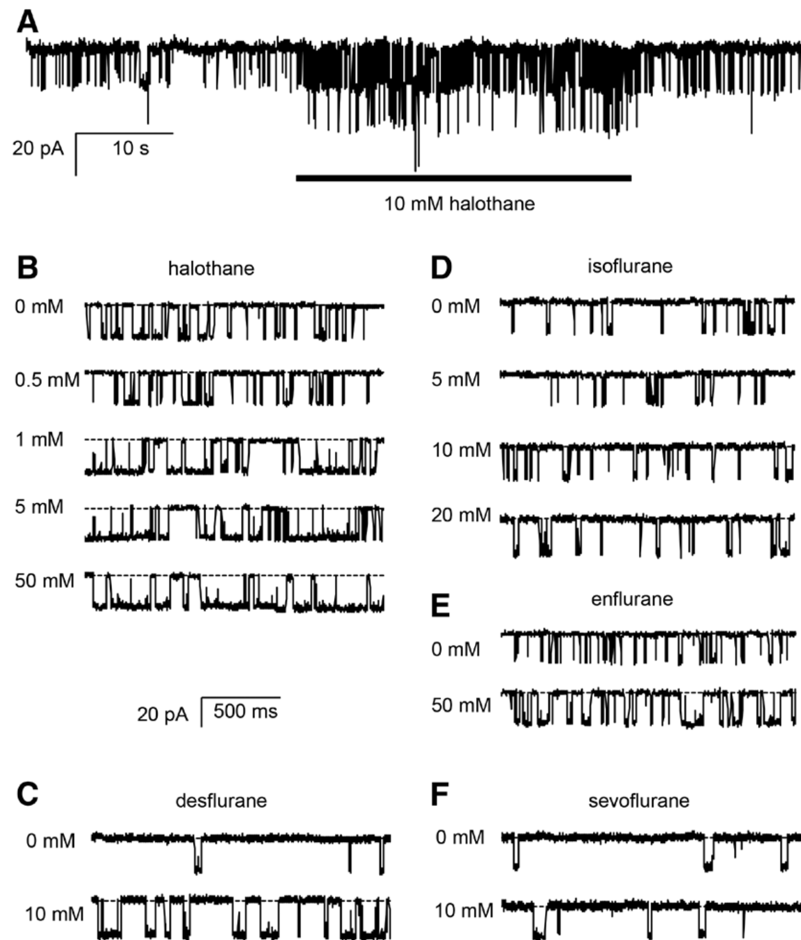

Fig. 2. Activation of ryanodine receptor 2 (RyR2) activity by the halogenated anesthetics. (*A*) Response of three RyR2 to application and washout of halothane. (*B–F*) RyR2 activity on an expanded timescale in the presence of various cytoplasmic concentrations of (*B*) halothane, (*C*) desflurane, (*D*) isoflurane, (*E*) enflurane, and (*F*) sevoflurane. The anesthetic concentrations in the cytoplasmic (*cis*) baths are shown at the edge of each trace. The cytoplasmic bath contained 2 mM adenosine triphosphate and 0.1 μM Ca^{2+} and the luminal bath contained 0.1 mM Ca^{2+}. Cytoplasmic and luminal baths also contained 230 mM $CsCH_3O_3S$ and 20 mM CsCl (pH 7.4). Recordings obtained at −40 mV show channel openings as downward current jumps from the baseline (*dashed lines*).

be seen that they produced no significant activation of RyR2 from the luminal bath.

Combined Effects of Halothane, ATP, and Adenosine

Halothane-induced activation of SR Ca^{2+} release in permeabilized ventricular myocytes has been found to require higher halothane concentrations in the presence of ATP.[12] We looked for similar phenomena in the activation of single RyR2 in bilayers and found that halothane was a potent activator of RyR2 in both the absence and presence of 2 mM ATP (fig. 5; one-way ANOVA, $P < 0.012$). We also found that the K_a for halothane activation was substantially higher (one-way ANOVA, $P < 0.001$) in the absence of ATP (table 1), which is the opposite of that expected from competition between ATP and halothane (see Discussion). In permeabilized ventricular myocytes, adenosine (5 mM) has been seen to antagonize halothane activation of SR Ca^{2+} release in the near-absence of ATP (0.05 mM).[12] Here, we used the same combinations of adenine nucleotide in bilayer experiments to investigate if halothane and adenosine competitively bind to a common site on RyR2 (fig. 6). In the absence of halothane, adenosine (5 mM) did not inhibit RyR2 activity in the absence of ATP but did so in the presence of ATP (two-way ANOVA with repeated measures, interaction $P = 0.0077$; see Methods). Adenosine caused a two-fold increase in RyR2 activation, whereas 0.05 mM ATP caused a 10-fold increase in RyR2 activity. However, in the presence of 0.05 mM ATP, addition of 5 mM adenosine inhibited channel activity and totally abolished the activating effect of ATP, in accord with these previous studies,[29,30] demonstrating competition between ATP and adenosine for the adenine nucleotide–binding site on RyR2. In the presence of halothane, ATP and adenosine had similar relative effects on RyR2 activity as in the absence of halothane, albeit with higher levels of activation; *i.e.*, adenosine did not inhibit RyR2 activity in the absence of ATP but did so in the

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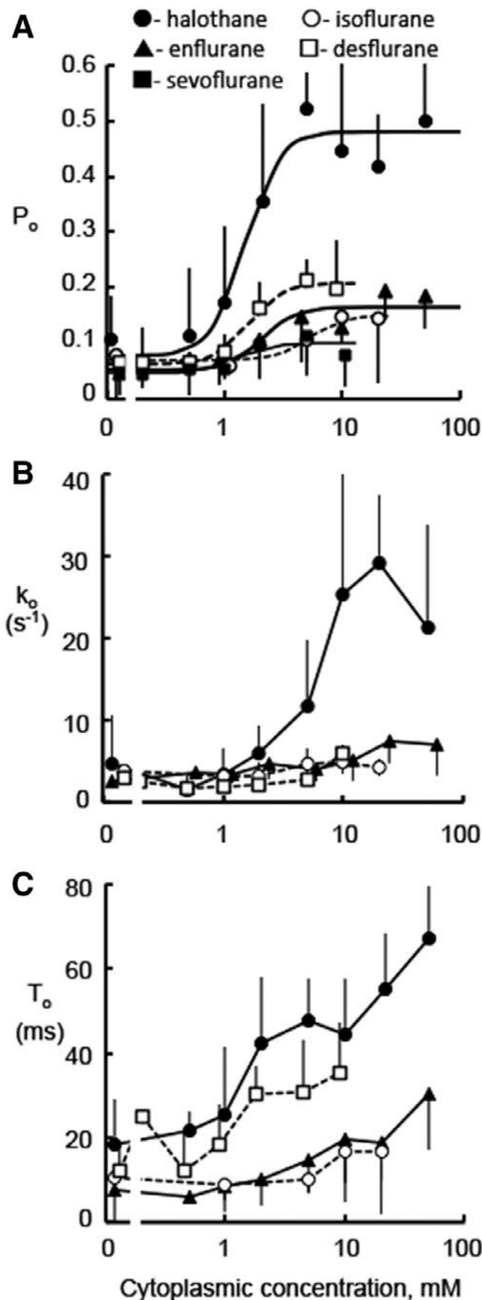

Fig. 3. Concentration dependencies of anesthetic activation of ryanodine receptor 2 (RyR2) open probability (*A*), opening rate, k_o (*B*), and mean open time, T_o (*C*). Experimental conditions and composition of vehicle solution are given in figure 2. (*B* and *C*) Sevoflurane data are omitted for clarity. Data show the mean ± SD. *Curves* show fits of Hill equation to the P_o data using the criterion of least-squares. Fit parameters and numbers of channels are given in table 1.

presence of ATP (two-way ANOVA with repeated measures, interaction $P = 0.0226$). These data indicate that adenosine is not a competitive antagonist for halothane, but rather it is a competitive antagonist for ATP. Therefore, the data in figures 5 and 6 do not reveal any competition between halothane and adenine nucleotides on RyR2 activity.

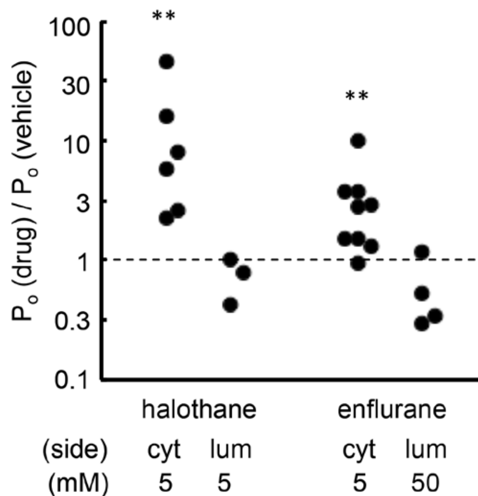

Fig. 4. The halogenated anesthetics have a cytoplasmic site of action. Relative activation of ryanodine receptor 2 (RyR2) by halothane or enflurane in the cytoplasmic (cyt) or luminal (lum) solutions. Experimental conditions and composition of vehicle solution are given in figure 2. *Significant difference from 1 (two-tailed, Student's *t* test, $^{**}P < 0.01$).

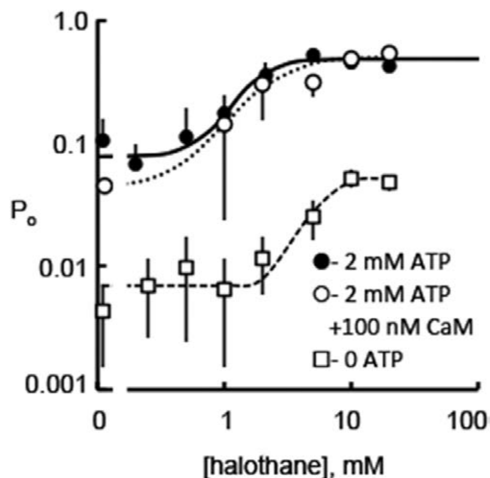

Fig. 5. Halothane activation of ryanodine receptor 2 (RyR2) and its dependencies on cytoplasmic adenosine triphosphate (ATP) and calmodulin (CaM). Concentration dependence of halothane activation of RyR2 open probability (P_o) in the presence and absence of ATP and in the presence of CaM. Experimental conditions and composition of vehicle solution are given in figure 2. Data show the mean ± SD. The *curves* show Hill fits to the data. Fit parameters and numbers of experiments are given in table 1.

Combined Effects of Halothane, Calmodulin, and Dantrolene

Although calmodulin associates with RyR2 in the cell, it is labile and can readily dissociate when RyR2 is isolated and incorporated into artificial lipid bilayers.[24,31] To investigate the effect of halothane on the RyR2/calmodulin complex, we added 100 nM calmodulin in the cytoplasmic solution (fig. 5). In the absence of halothane, calmodulin caused a moderate (approximately 50%) reduction in the open

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Table 1. Parameter Values for the Hill Equation from Least-Squares Fits to RyR2 Activation by Cytoplasmic Application of Halogenated Anesthetics in figure 3A

ATP CaM	X	P_{max}	P_{min}	P_{max}/P_{min}	K_a, mM	K_a, MAC	H_a	Activation at 1 MAC,* %	n
+ –	Sevoflurane	0.10 ± 0.08	0.05 ± 0.03	1.4 ± 0.3	1.3 ± 1.3	2.6	3‡	4	10
+ –	Isoflurane	0.15 ± 0.06	0.07 ± 0.04	1.6 ± 0.1†	5.3 ± 3.1	6.6	3‡	1	7
+ –	Desflurane	0.21 ± 0.03	0.06 ± 0.01	2.7 ± 0.3†	1.6 ± 0.3	1.7	3 ± 2	42	10
+ –	Enflurane	0.16 ± 0.08	0.06 ± 0.03	3.2 ± 0.3†	2.1 ± 1.6	1.4	3‡	45	9
+ –	Halothane	0.48 ± 0.05	0.08 ± 0.03	4.2 ± 2.6†	1.5 ± 0.3	1.7	3 ± 2	120	8
+ +	Halothane	0.43 ± 0.05	0.04 ± 0.04	10 ± 3†	1.4 ± 0.3	1.5	4 ± 2	160	8
– –	Halothane	0.052 ± 0.005	0.007 ± 0.002	13 ± 4†	5.2 ± 0.6b	5.8	4 ± 2	1	5

In the leftmost column, + symbols indicate the presence of cytoplasmic ATP (2 mM) and calmodulin (100 nM). The Hill equation is

$$P_o = \frac{(P_{max} - P_{min})([X]/K_a)^{H_a}}{\left(1 + ([X]/K_a)^{H_a}\right)} + P_{min}$$

P_{max}/P_{min} were derived from values paired for each experiment. The K_a values are also given as multiples of minimum alveolar concentration (MAC at one atmosphere ambient pressure [%, vol/vol] that produces immobility in 50% of animals subjected to a noxious stimulus) values. In rats at 37°C, these values are (%, vol/vol, mM), respectively, 1.04, 0.9 for halothane, 1.52, 0.8 for isoflurane, and 2.17, 1.5 for enflurane.[35,51] Similar values are found in humans where we obtained (%, vol/vol, mM) 2, 0.5 for sevoflurane and 6, 0.95 for desflurane.[52]

*Significantly different to 1 (P < 0.05). †RyR2 activation of P_o at anesthetic concentrations of 1 MAC relative to that in the absence of anesthetic, calculated from fitted curves. ‡H_a was not adjusted during fitting.

ATP = adenosine triphosphate; CaM = calmodulin; n = the number of channels; X = anesthetic agent.

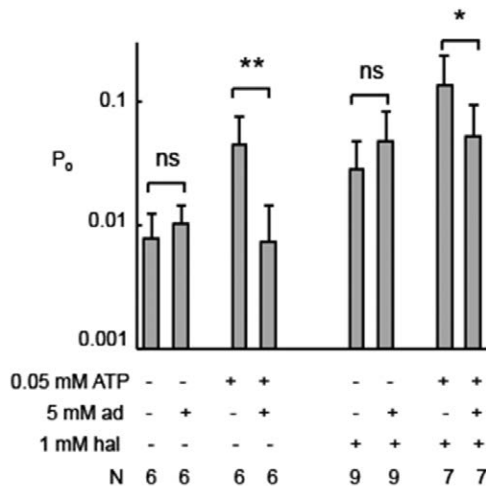

Fig. 6. Adenine nucleotide regulation of ryanodine receptor 2 (RyR2) in the presence and absence of halothane. RyR2 open probability in vehicle solutions containing adenosine triphosphate (ATP), adenosine (ad), and halothane (hal) as indicated by present (+) and absent (–). Experimental conditions and composition of vehicle solution are given in figure 2 except that the cytoplasmic solution contained 1 μM Ca^{2+}. Data show the mean ± 95% CIs. N = numbers of channels; ns = nonsignificant. ns P > 0.05; *P < 0.05; **P < 0.01, determined by two-way ANOVA on repeated measures of adenosine inhibition.

probability of RyR2 in accord with previous studies.[32,33] Addition of halothane caused RyR2 activation with the same K_a and P_{max} as seen in the absence of calmodulin (table 1). In the presence of calmodulin, dantrolene (10 μM in the cytoplasmic solution) caused an approximately 40% reduction in RyR2 open probability (fig. 7). There was no significant difference in dantrolene inhibition in the absence of halothane, in the presence of halothane concentrations that produce half activation (2 mM), or at full halothane activation of RyR2 (10 mM; fig. 7B).

Effects of Halothane on RyR2 Regulation by Ca^{2+} and Mg^{2+}

The effect of halothane (10 mM) on the activation of RyR2 by cytoplasmic Ca^{2+} (using 0.1 mM luminal Ca^{2+}) is shown in figure 8, and the parameters of the Hill fits to the Ca^{2+}-dependencies of P_o in figure 8A are given in table 2. Halothane reduced the K_a for RyR2 Ca^{2+} activation from 4.3 to 1.6 μM and caused a four-fold increase in P_o at nM cytoplasmic Ca^{2+} (table 2; P_{min}). These changes in activity were associated with significant increases in both mean opening rates (Student's t test, $P = 6 \times 10^{-4}$) and open time ($P = 1.5 \times 10^{-6}$; fig. 8, B and C).

In the presence of 0.1 μM cytoplasmic Ca^{2+}, luminal Ca^{2+} is a moderate activator of RyR2 (fig. 9). At –40 mV bilayer potential (lumen at ground) and in the absence of halothane, RyR P_o had a bell-shaped response to luminal Ca^{2+} with half-activation at 53 μM and half-inhibition at 0.8 mM (table 2). The activating phase is associated with increases in both mean open time and opening rate, whereas the inhibiting phase was due only to a decrease in mean open time (fig. 9, B and C). A +40 mV bilayer potential, which opposes the flow of Ca^{2+} from lumen to cytoplasm, nearly abolished luminal Ca^{2+} activation at submicromolar concentrations (fig. 9, D–F). The presence of 10 mM halothane had no significant effect in the absence of luminal Ca^{2+}, but it did increase peak luminal Ca^{2+} activation by nearly 10-fold (P_{max} in table 2) along with a three-fold decrease in K_a. Again, these effects were due to increases in mean open time and opening rate.

It is well known that Mg^{2+} in either the cytoplasmic or luminal solutions inhibit RyRs as shown in figure 10.[34] Hill fit parameters for cytoplasmic and luminal Mg^{2+} inhibition are

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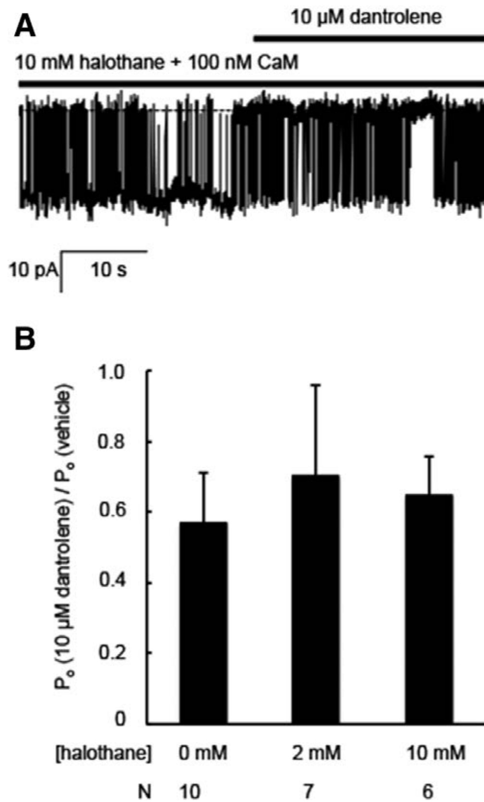

Fig. 7. Dantrolene inhibition of ryanodine receptor 2 (RyR2) in the presence of halothane. (*A*) The experimental conditions are as indicated in figure 2 except that the cytoplasmic (*cis*) bath also contains 100 nM calmodulin (CaM) and 10 mM halothane. Channel openings as downward current jumps from the baseline (*dashed line*). (*B*) Relative inhibition of RyR2 by 10 µM dantrolene in the cytoplasmic bath. Data show the mean ± 95% CIs and numbers of channels, N, are indicated.

shown in table 3. The addition of 10 mM halothane had no significant effect on either of these Mg^{2+} inhibition phenomena.

Discussion

RyR2 Sensitivity to Anesthetics

This study shows that halogenated anesthetics directly and reversibly activate cardiac RyR2 with halothane being the strongest activator, in agreement with previous single-channel studies of RyR2.[14,15] We also (1) demonstrate that the reversibility of activation is rapid and occurs within the time of solution exchange (fig. 2A); (2) demonstrate that luminal application of these anesthetics does not significantly activate RyR2 (fig. 4), (3) make the first direct measurements of the K_a's for RyR2 activation by halogenated anesthetics (fig. 3; table 1); and (4) determine the effects of the clinically used anesthetics, sevoflurane and desflurane on RyR2. K_a values for anesthetics were in the range of 1.3 to 2.1 mM (1.4 to 2.6 minimum alveolar concentration [MAC]) with the exception of isoflurane (5.3 mM, 6.6 MAC). The effect of anesthetics at clinically used concentrations (1 MAC) was calculated from theoretical Hill fits to their dose–responses in figure 3. Under

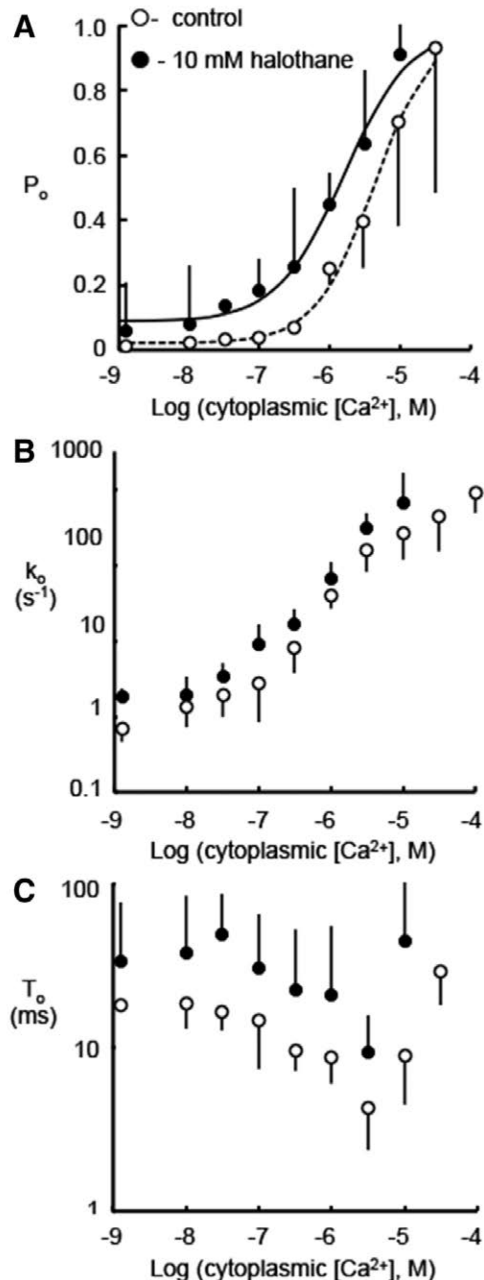

Fig. 8. The effects of cytoplasmic 10 mM halothane on cytoplasmic Ca^{2+} activation of ryanodine receptor 2 (RyR2). Data show the action of halothane on RyR2 open probability (P_o; *A*), opening rate (k_o; *B*), and mean open duration (T_o; *C*). The experimental conditions are the same as described in figure 2. Data points show the mean ± SD. Curves show Hill fits to the data. The fit parameters and numbers of experiments are listed in table 2.

these conditions, RyR2 activity increases by 120% with halothane, approximately 50% with enflurane and desflurane and is not significantly increased by isoflurane and sevoflurane, consistent with the effects of anesthetics on activation of SR Ca^{2+} release in cardiac muscle.[35,36] Thus, we show that halothane is the most active of the anesthetics on RyR2 activity.

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Table 2. Parameter Values for the Hill Equation from Least-Squares Fits to Cytoplasmic and Luminal Ca^{2+} Regulation of P_o in figures 8A and 9A

Treatment	P_{max}	P_{min}	K_a, µM	H_a	K_i, mM	H_i	n
Cytoplasmic Ca^{2+}							
Control	1.0 ± 0.10	0.02 ± 0.02	4.3 ± 1.0	1.0 ± 0.4	—	—	10
10 mM Hal	1.0 ± 0.16	0.08 ± 0.02*	1.6 ± 0.7*	0.9 ± 0.5	—	—	13
Luminal Ca^{2+}							
Control	0.04 ± 0.004	(5 ± 5) × 10⁻⁴	53 ± 15	2‡	0.8 ± 0.1	2‡	14
10 mM Hal	0.37 ± 0.05†	(2.2 ± 0.6) × 10⁻³*	13 ± 7*	2‡	0.6 ± 0.2	2‡	12

The Hill equation for Ca^{2+} activation is

$$P_o = \frac{(P_{max} - P_{min})\left(\left[Ca^{2+}\right]/K_a\right)^{H_a}}{\left(1+\left(\left[Ca^{2+}\right]/K_a\right)^{H_a}\right)} + P_{min} P_o = \frac{P_{max}\left(\left[Ca^{2+}\right]/K_a\right)^{H_a}}{\left(1+\left(\left[Ca^{2+}\right]/K_a\right)^{H_a}\right)} + P_{min}.$$

The Hill equation for Ca^{2+} inhibition is

$$P_o = \frac{P_{max}}{\left(1+\left(\left[Ca^{2+}\right]/K_i\right)^{H_i}\right)}.$$

Significant difference to control values ($P < 0.05$, †$P < 0.01$). ‡H was not adjusted during fitting.
Hal = halothane; n = number of channels.

Halothane is rarely used anymore, and the modern anesthetics tested had very little effect in inducing RyR2 opening.

Our previous study[37] has shown that in the presence of calmodulin, dantrolene inhibits RyR2 by up to 45% with a concentration for half-maximal inhibition of 160 nM. Therefore, at clinical concentrations used here (approximately 10 µM[38]), dantrolene exerts its maximal inhibitory action. Here, we show that dantrolene produces similar relative inhibition (approximately 40%) regardless of the halothane concentration, indicating that dantrolene does not alter the K_a for halothane activation. This degree of inhibition is sufficient to completely negate the activating effects of sevoflurane, isoflurane, desflurane, and enflurane. It also negates halothane activation at 1 MAC and substantially alleviates its activation at 2 MAC.

Our results indicate that halothane activation of RyR2 is different from that seen in the skeletal isoform, RyR1. RyR2 is much less sensitive to halogenated anesthetics than RyR1; the K_a's for anesthetic activation of RyR2 given in table 1 are an order of magnitude higher than those for RyR1 (K_a approximately 0.1 mM[39,40] and substantially higher than seen in SR Ca^{2+} release through drosophila RyR (K_a approximately 0.6 mM[41]). Also, halothane activation of RyR1 required the presence of ATP and high concentrations of Ca^{2+} in the cytoplasmic solutions,[15] whereas here and in a previous study,[14] halothane activation of RyR2 does not have this requirement for ATP. However, a common feature of halothane activation of RyR1 and RyR2 is that in both isoforms, halothane strongly potentiates the activating effect of luminal Ca^{2+}.[23]

Mechanisms of Halothane Potentiation of Ca^{2+} Activation of RyR2

There is now substantial evidence that Ca^{2+} binding at luminal or cytoplasmic facing sites on the RyR2 protein complex can trigger channel openings.[1–3] Maximally activating concentrations of halothane increased RyR2 sensitivity to activation by cytoplasmic Ca^{2+} indicating that halothane can potentiate the cytoplasmic facing Ca^{2+} activation mechanism of RyR2.

The largest effect of halothane was its potentiation of luminal Ca^{2+} activation of RyR2. Previous studies have found that luminal Ca^{2+} can activate RyR2 by binding to luminal facing sites on the RyR2 molecule and by permeating the open RyR2 pore and binding to cytoplasmic facing sites to sustain channel openings (*i.e.*, Ca^{2+} feed-through).[1–3] Our analysis of the voltage-dependence of RyR2 mean open time and opening rates indicates that halothane potentiates luminal Ca^{2+} activation by a direct action on both the luminal Ca^{2+} sites and cytoplasmic Ca^{2+} sites. Evidence for the former is that halothane decreases RyR2 closed times (fig. 9, B and E; $k_o = 1/T_c$), during periods where there can be no Ca^{2+} feed-through. Evidence for the latter is that halothane substantially increases RyR2 mean open durations, but only when the bilayer potential difference favors the luminal-to-cytoplasm flow of Ca^{2+} (−40 mV; fig. 9, C and F). Therefore, we envisage that halothane-induced changes in the cytoplasmic Ca^{2+} activation mechanism would account for the increased effect of cytoplasmic Ca^{2+} on mean open durations and opening rates seen in figure 8, B and C together with most of the increased effect of luminal Ca^{2+} on mean open durations in figure 9C.

Interaction of Halothane and Adenine Nucleotide Activation Mechanisms

The literature is currently divided on whether halothane and ATP share a common activating site on RyR2.[12,13] A key finding in the study by Yang *et al.*,[12] supporting the idea that halothane and adenine nucleotides act at a common site was that adenosine was able to reduce halothane activation

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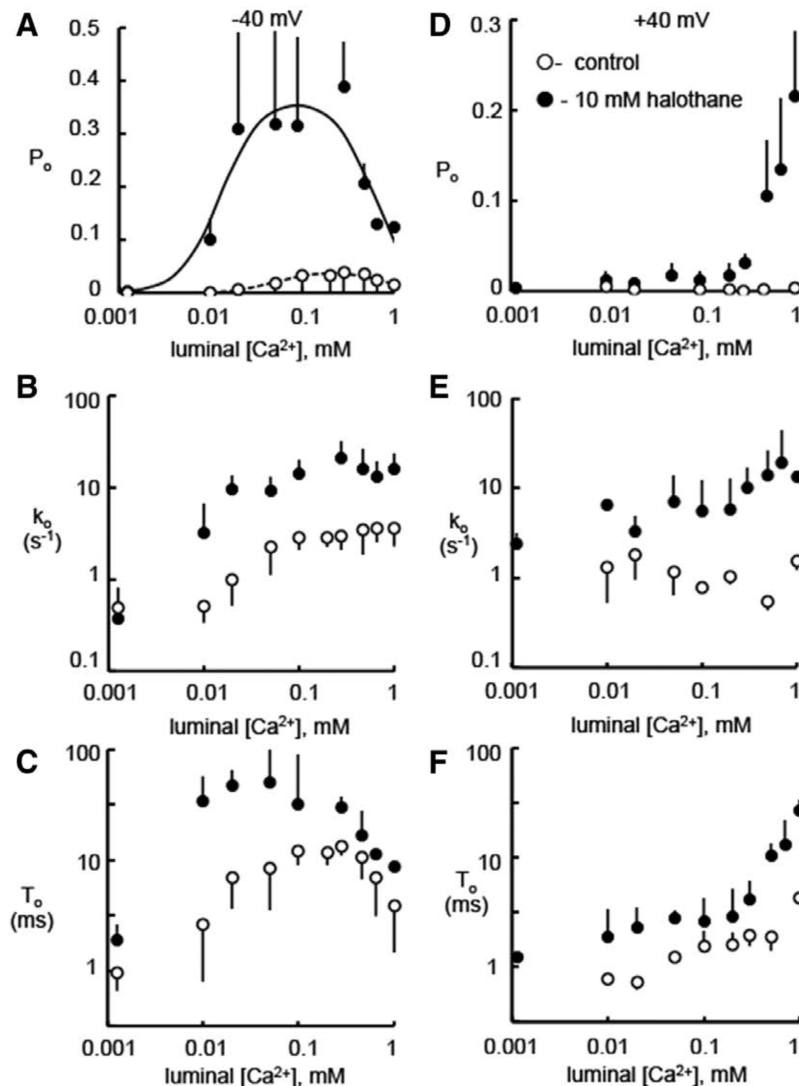

Fig. 9. The effects of cytoplasmic 10 mM halothane on luminal Ca^{2+} activation of ryanodine receptor 2 (RyR2) at bilayer potentials of −40 and +40 mV. Data show the action of halothane on RyR2 open probability (P_o; *A* and *D*), opening rate (k_o; *B* and *E*), and mean open duration (T_o; *C* and *F*). The experimental conditions are the same as described in figure 2. Data points show the mean ± SD. The curve in *A* shows a Hill fit to the data. The parameters and numbers of channels are listed in table 2.

of SR Ca^{2+} release in the near-absence of ATP (0.05 mM). However, Herland *et al.*[13] found that halothane could activate SR Ca^{2+} release regardless of the ATP concentration (0 to 11 mM), indicating that the mechanisms of activation were independent. Our experiments on the actions of ATP and adenosine on halothane activation of RyR2 reconcile these conflicting studies. We found that there was marked halothane activation of RyR2 in both the absence and presence of ATP (2 mM; fig. 6) in agreement with Ca^{2+} release in permeabilized cardiomyocytes[13] and with a previous single RyR2 channel study.[15] We also found that in the complete absence of ATP, halothane activation was slightly increased by adenosine, rather than inhibited as seen in the study of Yang *et al.*[12] However, in that study, adenine effects were actually measured in the presence of low levels of ATP (0.05 mM) because some ATP was required for loading of the SR by SERCA pump. We found that in the presence of 0.05 mM ATP, adenosine did reduce RyR2 activity in the presence of halothane, an effect also seen in the absence of halothane. Thus, our data are consistent with the findings of Yang *et al.*,[12] even though we found no evidence of competition between halothane and adenosine nucleotide for RyR2 activation. We conclude that the inhibitory action of adenosine in the presence of both halothane and low concentrations of ATP is due to competition between adenosine and ATP for the nucleotide-binding site on the RyR2, rather than between adenosine and halothane. It is interesting to note that Yang *et al.*,[12] reported that halothane had a larger effect on SR Ca^{2+} release at lower [ATP], an effect that could be explained by a common binding site. However, our finding that halothane sensitizes RyR2 to luminal Ca^{2+} (fig. 9), similar to that reported for increasing [ATP],[42] it increases

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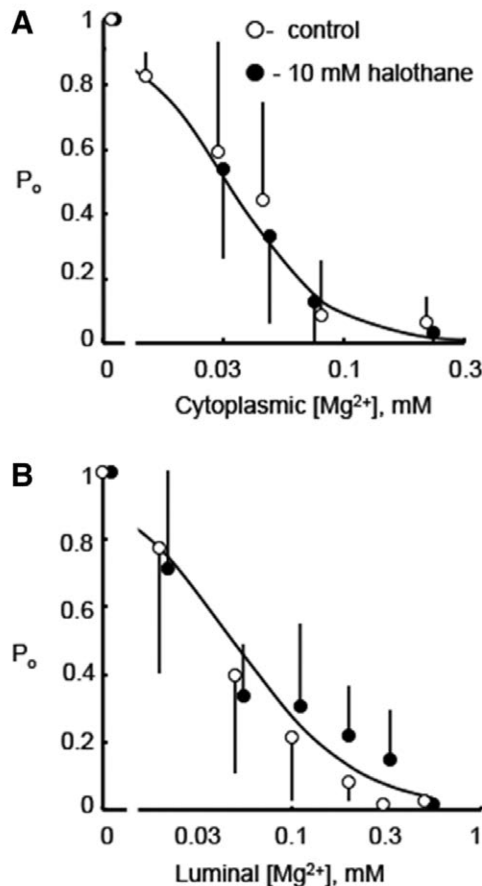

Fig. 10. Halothane (10 mM) does not affect ryanodine receptor 2 (RyR2) sensitivity to Mg^{2+} inhibition. RyR2 inhibition by cytoplasmic (*A*) and luminal Mg^{2+} (*B*) as measured by its relative effect on open probability (P_o). The experimental conditions are the same as described in figure 2. Halothane was added to the cytoplasmic solution. Data points show the mean ± SD. Curves show Hill fits to the 10 mM halothane data. The parameters and numbers of channels are listed in table 3.

Table 3. Hill Equation Parameters from Least-Squares Fits to Cytoplasmic and Luminal Mg^{2+} Inhibition of P_o in figure 10

Treatment	K_i, µM	H_i	n
Cytoplasmic Mg^{2+}			
Control	33 ± 5	1.7 ± 1.0	12
1 mM Hal	33 ± 7	2.0 ± 2	6
Luminal Mg^{2+}			
Control	41 ± 6	1.5 ± 0.8	4
1 mM Hal	49 ± 10	1.3 ± 0.8	9

The Hill equation for Mg^{2+} inhibition is

$$P_o = \frac{P_{max}}{\left(1+\left(\left[Mg^{2+}\right]/K_i\right)^{H_i}\right)}.$$

Hal = halothane; n = the number of channels.

an alternative explanation for the finding by Yang *et al.*[12] Increasing [ATP][43] or [halothane] decreases the SR luminal [Ca^{2+}] "set point" for RyR2 activation where the progressive rise in luminal [Ca^{2+}] *via* SERCA initiates propagating SR Ca^{2+} waves. The minimum attainable SR Ca^{2+} load and the basal luminal Ca^{2+} activation of RyR2 ultimately limit the degree to which this "setpoint" can be lowered. This limitation will produce experimental outcomes that mimic ligand competition at a limited number of binding sites.

Negative Inotropy and Cardioprotection by Volatile Anesthetics

Inhibition of the sarcolemmal L-type Ca^{2+} channel, Na/Ca exchanger,[44] and store-operated Ca^{2+} entry[45] by volatile anesthetics will inevitably lead to decreased Ca^{2+} loading by cardiomyocytes,[46] which is likely account for their sustained negative inotropic actions.[47] Negative inotropy would contribute to cardioprotective properties of anesthetics by depressing cardiac activity and reducing oxygen demand of the myocardium. As previously shown,[47] halothane, isoflurane, and sevoflurane have similar sustained negative inotropic actions on rat ventricular myocytes, which are largely independent of Ca^{2+} transport by the SR. This is consistent with our data, showing that these three anesthetics have very different actions on the SR Ca^{2+} release channel in spite of their similar inotropic effects.

Our data suggest an additional cardioprotective action of anesthetics whereby they would act as a surrogate agonist for the SR Ca^{2+} release channels in place of ATP when ATP is depleted during ischemia, thus preventing deactivation of RyR2 activity and Ca^{2+} overloading of the SR and the consequent transient aberrant release of SR Ca^{2+} during reperfusion. However, since sevoflurane and isoflurane do not activate RyR2 at clinical concentrations, this mechanism is likely to play a less significant role in their cardioprotective actions than their effects on sarcolemmal transporters or on cell survival signaling pathways in the cytoplasm and mitochondria.[48,49] It is interesting to note that activating RyR2 should potentially lead to anesthetic-induced cardiac arrhythmias.[50] However, no such concerns exist for these anesthetics. This might be because of compensatory effects of anesthetics on sarcolemmal transporters, a potentially important avenue for future studies.

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Competing Interests

The authors declare no competing interests.

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