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Halothane modulation of skeletal muscle ryanodine receptors: dependence on Ca^{2+} , Mg^{2+} and ATP

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Abstract

Malignant hyperthermia (MH) susceptibility is a genetic disorder of skeletal muscle associated with mutations in the ryanodine receptor isoform 1 (RyR1) of sarcoplasmic reticulum (SR). In MH-susceptible skeletal fibers, RyR1-mediated Ca^{2+} release is highly sensitive to activation by the volatile anesthetic halothane. Indeed, studies with isolated RyR1 channels (using simple Cs^+ solutions) found that halothane selectively affects mutated but not wild-type RyR1 function. However, studies in skeletal fibers indicate that halothane can also activate wild type RyR1-mediated Ca^{2+} release. We hypothesized that endogenous RyR1 agonists (ATP, luminal Ca^{2+}) may increase RyR1 sensitivity to halothane. Consequently, we studied how these agonists affect halothane action on rabbit skeletal RyR1 reconstituted into planar lipid bilayers. We found that cytosolic ATP is required for halothane-induced activation of the skeletal RyR1. Unlike RyR1, cardiac RyR2 (much less sensitive to ATP) responded to halothane even in the absence of this agonist. ATP-dependent halothane activation of RyR1 was enhanced by cytosolic Ca^{2+} (channel agonist) and counteracted by Mg^{2+} (channel inhibitor). Dantrolene, a muscle relaxant used to treat MH episodes, did not affect RyR1 or RyR2 basal activity and did not interfere with halothane-induced activation. Studies with skeletal SR microsomes confirmed that halothane-induced RyR1-mediated SR Ca^{2+} release is enhanced by high ATP/low Mg^{2+} in the cytosol and by increased SR Ca^{2+} load. Thus, physiological or pathological processes that induce changes in cellular levels of these modulators could affect RyR1 sensitivity to halothane in skeletal fibers, including the outcome of halothane-induced contracture tests used to diagnose MH susceptibility.

Keywords

volatile anesthetics; dantrolene; sarcoplasmic reticulum; excitation-contraction coupling; Malignant Hyperthermia

INTRODUCTION

During excitation contraction (EC) coupling in skeletal muscle, T-tubule membrane depolarization promotes activation of the ryanodine receptor channel isoform 1 (RyR1) located at the terminal cisternae of sarcoplasmic reticulum (SR) intracellular Ca^{2+} stores. This results in massive Ca^{2+} release to the cytosol, which activates the contractile system.

Several hundred mutations in RyR1 have been mapped and characterized. They were shown to cosegregate with phenotypes corresponding to several diseases including malignant

hyperthermia (MH) susceptibility, central core disease (CCD) and multi-minicore disease (reviewed in (2 and 33).

Although the primary target of the volatile anesthetic halothane is the central nervous system, it has been found that this anesthetic triggers MH episodes. It is apparent that, in MH-susceptible individuals, RyR1-mediated calcium release from skeletal SR is hypersensitive to halothane (2,33 and 46). Indeed, [³H]Ryanodine binding experiments (32) as well as channel recordings in planar bilayers (26) confirmed RyR1 abnormal behavior and halothane-induced activation of mutated but not wild-type RyR1. Consequently, halothane was chosen as an MH susceptibility test agent for both the North American caffeine halothane contracture test (CHCT) and the European in-vitro contracture test (IVCT) (1,27,39). On average, dissected muscle fiber bundles taken from MH-susceptible individuals develop contracture in response to low doses of halothane/caffeine. However, there is variability and the responses of MH-susceptible fibers have a degree of overlapping with those of normal fibers. Thus, a limitation of these tests is misdiagnosis due to their relatively low specificity (1,21,39).

Previous findings suggest that some factors that could change with altered metabolism, such as cytosolic Mg²⁺ levels and lumenal Ca²⁺ content, could make wild-type muscle fibers responsive to halothane (10). Indeed, halothane has been shown to largely stimulate RyR1-mediated Ca²⁺ release from SR vesicles isolated from wild-type rabbit skeletal muscle (30). It is important to mention that while these Ca²⁺ release studies were performed with high lumenal Ca²⁺ and in the presence of cytosolic modulators (Mg²⁺/ATP) (30), the bilayer experiments reporting lack of halothane modulation of wild type RyR1 were done using simple (Cs⁺ or K⁺) solutions (26). It has been reported that the nature of the current carrier as well as the presence or absence of Mg²⁺/ATP may affect the RyR1 behavior (6,9,13). Thus, we hypothesized that halothane modulation of RyR1 is highly sensitive to these environmental factors and that the discrepancies between reports on the action of halothane on wild-type RyR1 (26,30) may reflect differences in the channel environment. Therefore, we investigated how changes in cytosolic levels of ATP and Mg²⁺ as well as lumenal Ca²⁺ affect the action of halothane on wild-type RyR1 reconstituted in planar bilayers. We compared the effects of halothane on skeletal RyR1 to those on cardiac RyR2 since, unlike RyR1, RyR2 was reported to be responsive to halothane when isolated in simple bilayer solutions (4) as well as in myocardial cells (16,40). Since the muscle relaxant dantrolene is effective for the treatment of MH (19,42) and it may target RyR1 (14,25,38), we also tested if this agent interferes with the action of halothane on RyR channels. We found that halothane affects the behavior of both RyR isoforms. Still, the action of halothane on skeletal RyR1 depends on the levels of physiological cytosolic modulators (Ca²⁺, Mg²⁺ and ATP) as well as on lumenal Ca²⁺ content. Some of the results have been presented in preliminary form (8).

METHODS

Drugs and chemicals

CaCl₂ standard for Ca²⁺ calibration was from Word Precision Instruments Inc. (Sarasota, FL, USA). Phospholipids were obtained from Avanti (Alabaster, AL, USA). Dantrolene was from Calbiochem (San Diego, CA). All other drugs and chemicals were from Aldrich, Sigma, Fluka or were reagent grade. Dantrolene stock solutions (20 mM) were prepared by dissolving the drug in DMSO. Gas chromatography measurements (performed at Carbon Dynamics Inc., Springfield, IL) indicated that the dantrolene present in these stock solutions dissolved well up to ~30 μM in HEPES-TRIS. Halothane stock solutions were prepared fresh at the beginning of each experiment by vigorously mixing halothane and HEPES-TRIS (1:3 v/v) for 20-60 minutes. The mixture is allowed to rest on ice until the halothane-saturated HEPES-TRIS phase (~20 mM halothane, estimated using gas chromatography at Carbon Dynamics Inc.) separates

from the halothane phase. Halothane stock solutions were kept on ice in vials sealed with a silicon-teflon septum cap.

Sarcoplasmic reticulum vesicle preparation

Experimental work on animals was done in conformity to the guidelines laid out in the Guide for the Care and Use of Laboratory Animals.

Skeletal and cardiac SR microsomes (six different preparations each) were used as a source of RyR1 and RyR2, respectively. Microsomes rich in RyR1 channels (terminal cisternae fraction, TC microsomes) were isolated from predominantly fast-twitch skeletal muscle (back and leg; adult New Zealand white rabbits), as previously described (34). Heavy sarcoplasmic reticulum membrane fractions from dog ventricle, rich in RyR2 channels, isolated using cellular subfractionation methods based on Chamberlain *et al.* (3), were kindly provided by Dr. S. Fleischer (Dept. Biological Sciences, Vanderbilt University). All preparations were kept in liquid nitrogen. Aliquots (15 μ l each) of membranes were prepared every month and stored at -80°C . For experiments, aliquots were quickly defrosted in water, kept on ice and used within 5 hours.

Ryanodine receptor channel recordings and data analysis

Reconstitution of RyR1 and RyR2 in planar lipid bilayers was done as previously described (5,6). Briefly, planar lipid bilayers were formed on 80 to 150 μm -diameter circular holes in teflon septa, separating two 1.3 ml compartments. The *trans* compartment was filled with a HEPES- Ca^{2+} solution (HEPES 250 mM, $\text{Ca}(\text{OH})_2$ 53 mM; pH 7.4) and subsequently clamped at 0 mV by an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). The *cis* compartment (ground) was filled with HEPES-Tris solution (HEPES 250 mM, Tris 120 mM, pH 7.4). Bilayers of a 5:4:1 mixture of bovine brain phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine (45-50 mg/ml in decane) were painted onto the holes of the teflon septa from the *cis* side. To promote vesicle fusion, 500-1000 mM CsCl and 1 mM CaCl_2 were added to the *cis* solution. Cardiac or skeletal SR microsomes (5-15 μg) were then added to the *cis* solution. After RyR currents (or Cl^- currents >100 pA at 0 mV) were observed, the *cis* chamber was perfused for 5 min at 4 ml/min with HEPES-TRIS solution. A mixture of BAPTA and dibromoBAPTA was used to buffer free $[\text{Ca}^{2+}]$ on the cytosolic surface of the channel ($[\text{Ca}^{2+}]_{\text{cyt}}$), (5). Free $[\text{Mg}^{2+}]$ in mixtures of Mg^{2+} and ATP was estimated using Winmaxc2.5 by Chris Patton, Stanford University (available for free download at <http://www.stanford.edu/~cpatton/maxc.html>). Unless otherwise stated, “presence of Mg^{2+} /ATP” means total Mg^{2+} = 4 mM; total ATP = 5 mM (free $[\text{Mg}^{2+}]$ = 0.25 mM).

Cardiac and skeletal RyR channels were identified by their unique characteristics of slope conductance (from 80 to 100 pS) and reversal potential (+40 to +50 mV; *trans-cis*). Channel modulation by agonists, blockers and conductance modifiers was also assessed as previously described (5). RyR channel currents are depicted as positive (upward deflections) in figures and reflect cation flux from *trans* (luminal) to *cis* (cytosolic) compartments.

Recordings of RyR activity (4 to 8 min in duration) were carried out at 0 mV, filtered through a low-pass Bessel filter at 1-10 kHz, digitized at 20-100 kHz with a 12-bit analog to digital converter and stored on DVD for computer analysis, using the pClamp9 software (Axon Instruments). For data analysis, idealized traces were created. Open probabilities were determined by half amplitude threshold analysis of single-channel recordings as previously described (5). In multichannel experiments, we estimated the global open probability (nP_o). In the figures, we show the P_o (for single channels) or nP_o/x (for multiple channels), with x representing the maximal number of current levels observed.

When indicated, 1-50 μL of halothane stock solution were added to the *cis* chamber (final halothane levels ranging from 10 μM to 0.5 mM). During the time of the experiments (20-30 minutes), the loss of halothane from the *cis* chamber was less than 10% (estimated using gas chromatography at Carbon Dynamics Inc.).

Measurements of Ca^{2+} uptake/leak by SR microsomes

Ca^{2+} uptake by SR microsomes was measured with a spectrophotometer (Cory 50, Varian, Walnut Creek, CA) using the Ca^{2+} -sensitive dye antipyrilazo III (APIII). SR membrane vesicles (50 $\mu\text{g}/\text{ml}$) were added to 1 ml phosphate buffer containing (in mM): 100 KH_2PO_4 , MgCl_2 1-7 (free $\text{Mg}^{2+} = 0.2$ -2), 1-4 ATP and 0.2 APIII; pH 7.0. Ca^{2+} uptake was initiated by the addition of 30 μM Ca^{2+} to the medium and measured as changes in absorbance of APIII between 710 nm and 790 nm. In some experiments, ruthenium red (10 μM) was used to block the Ca^{2+} leak from SR. In SR vesicles preloaded with Ca^{2+} , we also studied the effects of halothane on the rate of Ca^{2+} leak after addition of cyclopiazonic acid (20 μM), which inhibits the SR calcium pump (SERCA). The Ca^{2+} efflux from the vesicles is mainly via the ryanodine receptors as indicated by its block by ruthenium red (10 μM).

Statistical Analysis

Data are presented as means $\pm$ S.E.M. of *n* measurements. Statistical comparisons between groups were performed with Student's *t* test of paired differences. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Halothane affects skeletal ryanodine receptors (RyR1) activity in the presence of cytosolic $\text{Mg}^{2+}/\text{ATP}$

Figure 1 shows changes in open probability (P_o) of wild-type RyR1 channels in response to halothane added to the cytosolic surface of the channel. Bilayer experiments were performed using 50 mM luminal Ca^{2+} as the charge carrier. The channel openings are observed as discrete (~ 4 pA) upward deflections of the current. The cytosolic free Ca^{2+} concentration was kept at 2 μM . As previously reported (5), in the absence of $\text{Mg}^{2+}/\text{ATP}$ skeletal RyR1 channels have low or moderate activity. Under these conditions, cumulative increase of halothane levels (0.12 to 0.5 mM) in the cytosolic bathing solution had no significant effect on RyR1 activity (Figs. 1A and 1C, open triangles). However, in the presence of $\text{Mg}^{2+}/\text{ATP}$ (4 mM/5 mM; free $\text{Mg}^{2+} = 0.25$ mM), halothane induced a marked increase in RyR1 activity (Fig. 1B). P_o increased from 0.13 ± 0.09 (control) to 0.38 ± 0.11 with 0.125 mM halothane, to 0.48 ± 0.14 with 0.25 mM halothane and to 0.62 ± 0.12 with 0.5 mM halothane (Fig. 1C, filled triangles).

Cytosolic Ca^{2+} modulates RyR1 sensitivity to halothane in the presence of $\text{Mg}^{2+}/\text{ATP}$

As previously reported (5,13), RyR1 channels are moderately sensitive to activation by increasing cytosolic Ca^{2+} levels, with peak activation at ~ 100 μM , regardless of the presence/absence of $\text{Mg}^{2+}/\text{ATP}$ (Fig. 2A, filled circles and Fig. 2B, filled circles). In the absence of $\text{Mg}^{2+}/\text{ATP}$ halothane had no effect on channel activity (Fig. 2A). In the presence of $\text{Mg}^{2+}/\text{ATP}$, halothane-induced activation of RyR1 was observed when cytosolic free Ca^{2+} levels were higher than 2 μM (Fig. 2B). Our results suggest that RyR1-sensitivity to halothane not only depends on the presence of $\text{Mg}^{2+}/\text{ATP}$ but also on the cytosolic Ca^{2+} levels.

Halothane-induced RyR1 activation is affected by cytosolic free $[\text{Mg}^{2+}]$

Cytosolic Mg^{2+} is a well known inhibitor of RyRs (13). As shown in Fig. 3A, increasing cytosolic free Mg^{2+} resulted in a decrease in RyR1 activity. The average P_o dropped from 0.29 ± 0.11 at 0.25 mM free Mg^{2+} to 0.07 ± 0.05 at 1 mM free Mg^{2+} . Figure 3B shows representative

multiple-channel recordings of RyR1 after addition of halothane. With 1 mM cytosolic free Mg^{2+} , RyR1 activity was not significantly affected by halothane. However, at lower free Mg^{2+} levels, halothane induced a significant increase in RyR1 activity from 0.12 ± 0.06 to 0.32 ± 0.07 and from 0.29 ± 0.11 to 0.5 ± 0.07 at 0.5 mM and 0.25 mM cytosolic Mg^{2+} , respectively (Fig. 3C).

Dantrolene does not affect halothane-induced RyR1 activation

It is known that dantrolene inhibits excitation-contraction coupling in skeletal muscle but its mechanism of action is still not well defined (19). Some studies suggested that dantrolene directly inhibits RyR1 while others found no effect (14,25,38). Since dantrolene prevents halothane-induced contraction (19,25), we tested possible interactions between these drugs.

As shown in Fig. 4, halothane-induced activation of RyR1 was not affected by addition of 20 μM dantrolene. Under control conditions (2 μM Ca^{2+} , presence of Mg^{2+}/ATP), RyR1 had low or moderate activity ($P_o = 0.05 \pm 0.02$). Halothane (0.5 mM) increased RyR1 activity to $P_o = 0.33 \pm 0.05$. Subsequent addition of 20 μM dantrolene did not change RyR1 activity ($P_o = 0.36 \pm 0.09$, $n=8$). Dantrolene, at its limit of solubility ($\sim 33 \mu M$), was also found without effects on the activity of halothane-activated RyR1 ($P_o = 0.32 \pm 0.07$; $n=8$).

In the absence of halothane, dantrolene (20 μM) was also without effect on the activity of RyR1 activated by 100 μM Ca^{2+} ($P_o = 0.48 \pm 0.09$ and 0.46 ± 0.08 before and after addition of dantrolene, respectively; $n=6$).

Halothane increases activity of cardiac ryanodine receptors (RyR2) in the absence of Mg^{2+}/ATP

Single channel experiments were performed using 50 mM luminal Ca^{2+} as the current carrier and in the absence of Mg^{2+}/ATP . Figure 5 shows examples of multiple channel recordings before (Fig. 5A) and after (Fig. 5B) addition of 0.5 mM halothane to the cytosolic chamber. Halothane (0.5 mM) increased cardiac ryanodine receptor activity at resting (100 nM) cytosolic free Ca^{2+} levels (Fig. 5(a1) versus fig 5(b1)) as well as at activating (1 μM) cytosolic free Ca^{2+} levels (Fig. 5(a2) versus fig 5(b2)). The average open probabilities (P_o) are shown in Fig. 5C. We also found that halothane was an effective agonist of cardiac RyR2 in the presence of Mg^{2+}/ATP (results not shown).

As observed with RyR1, dantrolene did not affect halothane-induced RyR2 activation. Under control conditions (100 nM Ca^{2+}), P_o was 0.06 ± 0.03 . RyR activity increased upon addition of halothane ($P_o = 0.56 \pm 0.11$) but remained unchanged after subsequent addition of dantrolene ($P_o = 0.52 \pm 0.12$ and 0.49 ± 0.11 with 20 and $\sim 33 \mu M$ dantrolene, respectively; $n=8$). In the absence of halothane, dantrolene (20 μM) was also without effect on the activity of RyR2 activated by 5 μM Ca^{2+} ($P_o = 0.39 \pm 0.07$ and 0.40 ± 0.07 before and after addition of dantrolene, respectively; $n=6$).

Halothane affects SR Ca^{2+} uptake and leak in skeletal muscle SR microsomes

The rate of Ca^{2+} uptake by isolated skeletal SR microsomes is a very sensitive macroscopic measurement to test modulation of both RyR1 and sarcoplasmic reticulum Ca^{2+} ATP-ase (SERCA) activity. This method, which evaluates populations of RyR1s, was used here to confirm our results of halothane action in planar bilayers. Figure 6A shows the time course of Ca^{2+} uptake into SR microsomes in response to Ca^{2+} spikes under control conditions as well as for SR microsomes incubated with halothane and halothane + ruthenium red. After Ca^{2+} is added, there is rapid uptake. The net Ca^{2+} uptake equals the SR Ca^{2+} influx (mediated by SERCA) minus the Ca^{2+} leak rate from the SR vesicles (via RyRs). In the experiments conducted in the presence of ruthenium red (10 μM), where the RyR-mediated Ca^{2+} leak was

fully inhibited, the time courses of Ca^{2+} uptake were not affected by halothane (Fig. 6B, open *versus* filled triangles and Fig. 6C, striped *versus* dotted bars; $n=4$ paired experiments). This suggests that halothane does not affect SERCA-mediated Ca^{2+} uptake.

In the absence of ruthenium red, addition of halothane decreased the rate of Ca^{2+} uptake. This is expected since halothane-induced RyR1 activation would increase the Ca^{2+} leak rate, while the Ca^{2+} influx remains constant. This effect of halothane was more evident at lower concentrations of Mg^{2+} (Fig. 6B, open circles; $n=4$). Furthermore, the halothane-induced reduction in Ca^{2+} uptake relative to the control condition was larger when the SR Ca^{2+} content was higher (Fig. 6C, compare black *versus* white bars; $n=4$ paired experiments).

A more direct estimation of macroscopic RyR1 activity can be obtained by loading the skeletal muscle SR microsomes with Ca^{2+} and measuring its release (or leak) after addition of cyclopiazonic acid (an inhibitor of SERCA). Figure 7A shows the time course of Ca^{2+} release from SR microsomes after addition of CPZ. Notice the large increase in Ca^{2+} leak from the SR microsomes incubated with halothane. Contrarily, in the presence of ruthenium red, no Ca^{2+} leak was observed under control conditions or after incubation with halothane. This indicates that in our conditions Ca^{2+} leak was entirely mediated by RyRs. As shown in Fig. 7B, the effect of halothane on SR Ca^{2+} leak was highly dependent on ATP and Mg^{2+} concentrations ($n = 5$ paired experiments). Maximal halothane-induced activation of Ca^{2+} release was observed with 4 mM ATP (Fig. 7B, open circles) at 0.25 mM free Mg^{2+} .

The lack of effects observed for dantrolene in bilayers was confirmed using the loading/release assay. The rate of Ca^{2+} release induced by 0.5 mM halothane was not affected by dantrolene (20 μM) (0.72 ± 0.01 *versus* $0.68 \pm 0.02 \text{ min}^{-1}$, 0.5 mM halothane *versus* 0.5 mM halothane + 20 μM dantrolene, respectively, $n=4$ paired observations). In the absence of halothane, dantrolene was also without effect on the rate of caffeine-induced Ca^{2+} release from SR microsomes (0.71 ± 0.02 *versus* $0.68 \pm 0.04 \text{ min}^{-1}$, caffeine *versus* caffeine + 20 μM dantrolene, respectively, $n=4$ paired observations). To rule out the possibility that the concentration of halothane (0.5 mM) is too high to be counteracted by dantrolene, we tested if the presence of dantrolene (2 μM or 20 μM) affects the Ca^{2+} leak induced by a lower concentration of halothane (0.1 mM). Again, we found no effect of dantrolene on the rate of halothane-induced Ca^{2+} release (0.66 ± 0.02 *versus* $0.71 \pm 0.01 \text{ min}^{-1}$, 0.1 mM halothane *versus* 0.1 mM halothane + 2 μM dantrolene, respectively, $n=3$ paired observations and 0.65 ± 0.02 *versus* $0.64 \pm 0.03 \text{ min}^{-1}$, 0.1 mM halothane *versus* 0.1 mM halothane + 20 μM dantrolene, respectively, $n=3$ paired observations). Not shown, the loading rate of unstimulated SR vesicles (i.e. in the absence of caffeine or halothane) was not affected by 2 or 20 μM dantrolene suggesting that this agent does not affect SERCA activity.

Thus, the results obtained with skeletal muscle SR microsomes are in agreement with the observations using RyR1 reconstituted in lipid bilayers. These results confirm that halothane action is synergized by high ATP/low Mg^{2+} in the SR bathing solution as well as by increasing SR luminal Ca^{2+} load. In addition, the SR Ca^{2+} release studies also indicate that dantrolene may not directly affect RyR1 in native SR microsomes.

DISCUSSION

We found that halothane is a direct activator of wild-type skeletal RyR1 and cardiac RyR2 channels. The action of halothane on skeletal muscle RyR1 was profoundly affected by the levels of Ca^{2+} , Mg^{2+} and ATP bathing the cytosolic surface of the channel as well as on the luminal SR Ca^{2+} load. Unlike RyR1, halothane-induced activation of cardiac RyR2 did not require the presence of ATP or high cytosolic Ca^{2+} . We also found that dantrolene did not

change the basal level of gating of both RyR isoforms and did not interfere with their activation by halothane.

Our results indicate that halothane-sensitivity of RyR-mediated Ca^{2+} release in skeletal fibers, but not in cardiac myocytes, would heavily depend on the cellular levels of endogenous modulators (Mg^{2+} , ATP, Ca^{2+}). They also suggest that dantrolene inhibition of RyR-mediated Ca^{2+} release reported in cells may result from an “indirect” action on RyRs.

Endogenous agonists affect halothane-induced activation of RyR1

It has been shown that halothane stimulates contraction and SR Ca^{2+} release from normal skeletal muscle (24,30). Here, we demonstrate that halothane induces an increase in the activity of skeletal RyR1 channels taken from wild-type rabbit skeletal muscle. In a previous report, halothane has been shown to be without effect on skeletal RyR1 taken from wild-type animals reconstituted in lipid planar bilayers (26). This apparent discrepancy, however, can be explained by the differences in experimental conditions. Indeed, we found that the halothane efficacy as an RyR1 activator was highly dependent on the presence of certain cellular cytosolic modulators (i.e., Ca^{2+} , Mg^{2+} and ATP) as well as on SR Ca^{2+} load. Early reports were carried out using simple cytosolic solutions, which did not contain Mg^{2+} /ATP and low Ca^{2+} (10-100 μM) in the luminal solutions (26). We also found no effects of halothane under similar conditions.

In all the conditions tested, cytosolic ATP was required to obtain halothane-induced increase in RyR1 activity. Calcium was also a limiting component, since no effect of halothane was observed at cytosolic resting Ca^{2+} levels (i.e., when the channels are mainly closed). Any condition that favors an increase in RyR1 activity (decrease in $[\text{Mg}^{2+}]_{\text{cyt}}$ or increase in $[\text{Ca}^{2+}]_{\text{cyt}}$) made the channels more susceptible to activation by halothane. These results are consistent with a previous report, where 1 mM halothane induced SR Ca^{2+} release in normal skeletal muscle skinned fibers only at low (0.1-0.4 mM) Mg^{2+} (11).

Possible mechanism of action of halothane on RyR1 activity

The present channel studies indicate that the RyR1 must be, at least minimally, active to be sensitive to halothane since no effect of halothane was observed when the channels were closed (i.e., at resting $[\text{Ca}^{2+}]_{\text{cyt}}$, high $[\text{Mg}^{2+}]_{\text{cyt}}$ or in the absence of ATP). Accordingly, our SR Ca^{2+} loading/release assays suggest that the action of halothane requires a moderate level of Ca^{2+} leak, as induced by high luminal Ca^{2+} levels as well as high ATP / low Mg^{2+} in the cytosol.

It is known that RyR1 is an ATP-gated channel (6,13,36). ATP binding greatly increases the activity of skeletal RyR1, including those channels that display low open probabilities ($P_o \sim 0.01$) in the presence of maximal activating cytosolic Ca^{2+} levels (6). On the other hand, Mg^{2+} is an inhibitor of RyR1 activity. Mg^{2+} interferes with Ca^{2+} binding to cytosolic high affinity (micromolar) divalent cation binding sites which activate the channel (9,13). In addition, Mg^{2+} binds to low affinity (millimolar) inhibitory sites (9,13). Thus, in the conditions that favored halothane action, the high affinity (activating) binding sites in RyR1 were partially occupied by Ca^{2+} . Furthermore, high levels of ATP contributed to maintain RyR1 in a high-activity mode.

The halothane-counteracting action of Mg^{2+} can be explained by decreased occupancy by Ca^{2+} of high-affinity (activating) sites, increased occupancy by Mg^{2+} of low-affinity (inhibitory) sites and formation of Mg-ATP complexes (decrease in free ATP levels). All these actions will greatly decrease RyR1 activity (6,13).

A previous report showed that halothane induces little or no Ca^{2+} release from SR membrane vesicles if the Ca^{2+} content in the SR vesicles is below a threshold level suggesting that luminal Ca^{2+} fundamentally affects the action of halothane (48). Likewise, we found that halothane action was dependent on luminal Ca^{2+} levels. High luminal Ca^{2+} increases RyR1 open probability and sensitivity to ATP (13,35) and appears to decrease binding of divalent ions to cytosolic low affinity sites (15).

Taken together, our results suggest that halothane binding *per se* is not enough to activate quiescent RyR1 channels but appears to stabilize the open-state of partially activated channels. To be effective, halothane requires the interaction of the RyR1 channel with physiological modulators. Thus, studies on the modulation of isolated RyR1 channels by halothane-like agents should be conducted in the presence of these modulators to get a better comparison with the effects of those agents on RyR1-mediated calcium release in the cellular environment.

Pathophysiological implications - Malignant hyperthermia

Our main observation is that the action of halothane (and possibly halothane-like anesthetics) on skeletal RyR1-mediated Ca^{2+} release would heavily depend on the cellular levels of endogenous agonists. These results may have importance to explain the variability in the contracture tests that are utilized in the diagnosis of MH susceptibility. Although the RyR1 mutations *per se* could be the main cause for the hypersensitivity to halothane observed in MH susceptible individuals, it is also plausible that, in some cases, changes in the channel environment might synergize the action of halothane. In this regard, increased resting cytosolic Ca^{2+} levels have been found in cells carrying some MH mutations (17,22,28,41,42). This increase in cytosolic Ca^{2+} correlated with higher resting activity of mutated RyR1 channels as detected using [^3H]ryanodine binding (42,43). Increased RyR1 resting activity would make the channels more susceptible to halothane effects. Notice, also, that increased resting RyR1 activity could also result from diminished negative control by dihydropyridine receptor (DHPR) L-type calcium channels (31,47).

It is known that RyR1 carrying MH mutations are less sensitive to Mg^{2+} inhibition (29,37). Decreased Mg^{2+} sensitivity would result in higher RyR1 basal activity and thus, higher sensitivity to halothane. Increased RyR1 basal activity would also be found with decreased cytosolic Mg^{2+} levels, again increasing RyR1 sensitivity to halothane. In this regard, MH susceptibility has been associated with increased insulin levels (7), which are known to decrease intracellular Mg^{2+} in muscle (20).

It is known that ATP levels could largely change in skeletal fibers depending on fiber type and cellular metabolism (18). This would greatly affect the activity of ATP-gated RyR1 and consequently, change its sensitivity to halothane-induced activation. If a decrease in ATP levels occurs during manipulation of skeletal fibers under *in vitro* conditions (1,27,39), the basal activity of RyR1 would decrease and thus the response to halothane could be significantly attenuated, originating false negatives in MH-susceptibility tests.

RyR1 channels are modulated by luminal Ca^{2+} levels (35). Accordingly, we show that halothane-induced SR Ca^{2+} release is enhanced when the SR Ca^{2+} content is increased. It has been reported that the response to the agonist caffeine of skeletal fibers from MH-susceptible individuals is less sensitive to decreasing luminal Ca^{2+} (10). Moreover, MH-susceptible pigs display halothane-induced Ca^{2+} release at much lower levels of Ca^{2+} content than normal pigs (48). Therefore, we would expect an increased response to halothane when comparing MH-susceptible to normal muscle with equivalent SR Ca^{2+} load. Notice that increases in SR Ca^{2+} load could also enhance halothane-induced RyR1 activation.

In summary, the increased sensitivity to halothane observed in MH-susceptible skeletal fibers could be due to mutations in the RyR1 that increase its binding affinity for volatile anesthetics, or mutations that change RyR1 sensitivity to cytosolic modulators (Ca^{2+} , ATP, Mg^{2+}) or to SR Ca^{2+} load. Our results also suggest that environmental changes in levels of these modulators (either induced by pharmacological agents or due to pathologies) could make the wild type RyR1 channels more susceptible to halothane.

Modulation of cardiac RyR2 activity mediated by halothane

Halothane has been reported to increase Ca^{2+} release in isolated myocytes (40). Halothane was also found to be effective on chemically skinned myocardium, where its action on ruthenium red-sensitive Ca^{2+} release did not require the presence of nucleotides or relatively high $[\text{Ca}^{2+}]$ (16). Accordingly, we show that halothane has a robust activating effect on RyR2 regardless of the presence/absence of Mg^{2+} /ATP. Previous studies also found that halothane is able to activate cardiac RyR2 with Cs^{+} as current carrier, i.e., with low levels of luminal Ca^{2+} (4). Thus, our studies and previous reports consistently show that halothane-induced activation of cardiac RyR2 is much less sensitive to the channel environment than halothane-induced activation of skeletal RyR1 isoform.

Lack of effect of dantrolene on halothane-induced activation of RyRs

The muscle relaxant dantrolene has been shown to prevent halothane effects both in MH-susceptible individuals and experimental animal models (19,25,42). Dantrolene has also been found to improve intracellular Ca^{2+} handling in heart (23). It has been well established that the skeletal RyR1 and cardiac RyR2 are direct targets of dantrolene, as revealed by biochemical photoaffinity labeling experiments (49,50). Dantrolene binding appears to stabilize interactions between domains in the RyR1 molecule believed to be important for the functional regulation of the channel (51). However, the results shown in the present work indicate that dantrolene action may not be related to direct inhibition of RyR channels.

Our results in cardiac RyR2 are in agreement with previous reports (14). On the other hand, previous studies on the effect of dantrolene on skeletal muscle RyR1 lead to inconsistent results. Early reports suggested that nanomolar levels of dantrolene activate skeletal RyR1 while micromolar levels inhibit the channels (25). Differently, $[\text{H}^3]$ -ryanodine binding studies only found partial inhibitory effects with micromolar levels of dantrolene (12,14). Partial inhibition by dantrolene of tyrobutyrate-induced SR Ca^{2+} release was also found (38). In that report, as well as in a recent communication (52), no effect of dantrolene was observed on RyR1 function studied in bilayers (38), which is in agreement with the present work.

The cause of the discrepancies among studies of dantrolene action on RyR1 channels is unclear. Possible candidates could include differences in the experimental conditions (e.g., Mg^{2+} /ATP, luminal and cytosolic Ca^{2+}), which may affect dantrolene efficacy. The method utilized for the isolation of SR microsomes may also introduce variability in the results. In skeletal fibers, L-type Ca^{2+} channels (DHPR) in t-tubular membranes are a key component of excitation-contraction coupling as they modulate RyR1 through physical interactions (13). In “triad-like” SR preparations but not in “terminal cisternae-like” preparations used here (34), those t-tubular structures containing DHPR remain associated and may modulate RyR1 in SR membranes. Thus, the action of dantrolene on RyR1-mediated Ca^{2+} release observed in cells (38,42) could reflect changes in these DHPR-RyR1 interactions. Indeed, dantrolene was found to inhibit the binding of dihydropyridine analogs to DHPR more effectively than the binding of $[\text{H}^3]$ ryanodine to RyR1 (12). Dantrolene was also found to inhibit DHPR Ca^{2+} currents (38). In addition, azumolene (a dantrolene analog) decreased the frequency but not the shape (amplitude, duration and width) of local events of RyR1-mediated Ca^{2+} release (Ca^{2+} sparks), indicating that the gating of RyR1 channels during the events was not affected by dantrolene

derivatives (44). Similar decrease in Ca^{2+} spark frequency is found with the DHPR antagonist nifedipine, which appears to stabilize physical interactions between DHPR and RyR1 (47).

Therefore, it is possible that the action of dantrolene on skeletal muscle and heart, including therapeutic properties, would reside in its interaction with target molecules other than RyRs. As indicated, a possible candidate would be the DHPR. However, various alternative dantrolene targets have been suggested in other studies, including the inhibition of RyR1-coupled store-operated calcium entry (45).

Concluding remarks

The present work shows that the response of RyR1 channels to the volatile anesthetic halothane is highly sensitive to the levels of cytosolic modulators (Ca^{2+} , ATP, Mg^{2+}) as well as to the SR Ca^{2+} load. Consequently, these factors represent important variables to be controlled when studying the action of halothane-like anesthetics at the cellular level. In particular, tests used to determine MH susceptibility, should take into consideration that variations in the intracellular levels of endogenous RyR1 modulators induced by an abnormal metabolic status of the patients or by changes in the cell environment during the manipulation of the samples, would affect the sensitivity of the skeletal fibers to halothane, inducing variability in the outcome of the tests.

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REFERENCES

1. Allen GC, Larach MG, Kunselman AR, The North American Malignant Hyperthermia Registry of MHAUS. The sensitivity and specificity of the caffeine-halothane contracture test. A report from the North American Malignant Hyperthermia Registry. *Anesthesiology* 1998;88:579–588. [PubMed: 9523799]
2. Avila G. Intracellular Ca^{2+} dynamics in malignant hyperthermia and central core disease: established concepts, new cellular mechanisms involved. *Cell Calcium* 2005;37:121–127. [PubMed: 15589992]
3. Chamberlain BK, Levitsky DO, Fleischer S. Isolation and characterization of canine cardiac sarcoplasmic reticulum with improved Ca^{2+} transport properties. *J Biol Chem* 1983;258:6602–6609. [PubMed: 6304048]
4. Connelly TJ, Coronado R. Activation of the Ca^{2+} release channel of cardiac sarcoplasmic reticulum by volatile anesthetics. *Anesthesiology* 1994;81:459–469. [PubMed: 8053596]
5. Copello JA, Barg S, Onoue H, Fleischer S. Heterogeneity of Ca^{2+} gating of skeletal muscle ryanodine receptor (RyR-1) compared with cardiac RyR-2. *Biophys J* 1997;73:141–156. [PubMed: 9199779]
6. Copello JA, Barg S, Sonnleitner A, Porta M, Diaz-Sylvester PL, Fill M, Schindler H, Fleischer S. Mg^{2+} block of cardiac and skeletal muscle ryanodine receptors. Effects of Ca^{2+} , ATP and caffeine. *J Memb Biol* 2002;187:51–64.
7. Denborough MA, Warne GL, Moulds RF, Martin FI. Insulin secretion in malignant hyperpyrexia. *Br Med J* 1974;3:493–495. [PubMed: 4413915]
8. Diaz-Sylvester PL, Porta M, Fill M, Copello JA. Halothane action on skeletal muscle ryanodine receptor (RyR1) channels. *Biophys J* 2005;88(Part 2 Suppl S):485A–486A.
9. Diaz-Sylvester PL, Porta M, Nani A, Escobar A, Fill M, Copello JA. Modulation of cardiac ryanodine receptors (RyR2) by divalent cations $\text{M}(2+)$. *Biophys J* 2002;82(Part 2):361.

10. Duke AM, Hopkins PM, Steele DS. Effects of Mg^{2+} and SR luminal Ca^{2+} on caffeine-induced Ca^{2+} release in skeletal muscle from humans susceptible to malignant hyperthermia. *J Physiol* 2002;544:85–95. [PubMed: 12356882]
11. Duke AM, Hopkins PM, Steele DS. Mg^{2+} dependence of halothane-induced Ca^{2+} release from the sarcoplasmic reticulum in rat skeletal muscle. *J Physiol* 2003;551:447–454. [PubMed: 12909676]
12. el-Hayek R, Parness J, Valdivia HH, Coronado R, Hogan K. Dantrolene and azumolene inhibit [3H] PN200-110 binding to porcine skeletal muscle dihydropyridine receptor. *Biochem Biophys Res Commun* 1992;187:894–900. [PubMed: 1326958]
13. Fill M, Copello JA. Ryanodine receptor calcium release channels. *Physiol Rev* 2002;82:893–922. [PubMed: 12270947]
14. Fruen BR, Mickelson JR, Louis CF. Dantrolene inhibition of sarcoplasmic reticulum Ca^{2+} release by direct and specific action at skeletal muscle ryanodine receptors. *J Biol Chem* 1997;272:26965–26971. [PubMed: 9341133]
15. Györke I, Györke S. Regulation of the cardiac ryanodine receptor channel by luminal Ca^{2+} involves luminal Ca^{2+} sensing sites. *Biophys J* 1998;75:2801–2810. [PubMed: 9826602]
16. Herland JS, Julian FJ, Stephenson DG. Halothane increases Ca^{2+} efflux via Ca^{2+} channels of sarcoplasmic reticulum in chemically skinned rat myocardium. *J Physiol* 1990;426:1–18. [PubMed: 1700102]
17. Iaizzo PA, Klein W, Lehmann-Horn F. Fura-2 detected myoplasmic calcium and its correlation with contracture force in skeletal muscle from normal and malignant hyperthermia susceptible pigs. *Pflugers Arch* 1988;411:648–653. [PubMed: 3412868]
18. Karatzaferi C, de Haan A, Ferguson RA, van Mechelen W, Sargeant AJ. Phosphocreatine and ATP content in human single muscle fibres before and after maximum dynamic exercise. *Pflugers Arch* 2001;442:467–474. [PubMed: 11484780]
19. Krause T, Gerbershagen MU, Fiege M, Weissborn R, Wappler F. Dantrolene—a review of its pharmacology, therapeutic use and new developments. *Anaesthesia* 2004;59:364–373. [PubMed: 15023108]
20. Lares MJ, Monteiro CP, Bicho M. Role of cellular magnesium in health and human disease. *Front Biosci* 2004;9:262–276. [PubMed: 14766364]
21. Litman RS, Rosenberg H. Malignant hyperthermia: update on susceptibility testing. *JAMA* 2005;293:2918–2924. [PubMed: 15956637]
22. Lopez JR, Alamo LA, Jones DE, Papp L, Allen PD, Gergely J, Sreter FA. [Ca^{2+}]i in muscles of malignant hyperthermia susceptible pigs determined in vivo with Ca^{2+} selective microelectrodes. *Muscle Nerve* 1986;9:85–86. [PubMed: 3951486]
23. Min JY, Meissner A, Feng X, Wang J, Malek S, Wang JF, Simon R, Morgan JP. Dantrolene: effects on abnormal intracellular Ca^{2+} handling and inotropy in postinfarcted rat myocardium. *Eur J Pharmacol* 2003;471:41–47. [PubMed: 12809951]
24. Nelson TE, Denborough MA. Studies on normal human skeletal muscle in relation to the pathopharmacology of malignant hyperpyrexia. *Clin Exp Pharmacol Physiol* 1977;4:315–322. [PubMed: 891045]
25. Nelson TE, Lin M, Zapata-Sudo G, Sudo RT. Dantrolene sodium can increase or attenuate activity of skeletal muscle ryanodine receptor calcium release channel. Clinical implications. *Anesthesiology* 1996;84:1368–1379. [PubMed: 8669678]
26. Nelson TE. Halothane effects on human malignant hyperthermia skeletal muscle single calcium-release channels in planar lipid bilayers. *Anesthesiology* 1992;76:588–595. [PubMed: 1312798]
27. Ording H, Brancadoro V, Cozzolino S, Ellis FR, Glauber V, Gonano EF, Halsall PJ, Hartung E, Heffron JJ, Heytens L, Kozak-Ribbens G, Kress H, Krivosic-Horber R, Lehmann-Horn F, Mortier W, Nivoche Y, Ranklev-Twetman E, Sigurdsson S, Snoeck M, Stieglitz P, Tegazzin V, Urwyler A, Wappler F, The European Malignant Hyperthermia Group. In vitro contracture test for diagnosis of malignant hyperthermia following the protocol of the European MH Group: results of testing patients surviving fulminant MH and unrelated low-risk subjects. *Acta Anaesthesiol Scand* 1997;41:955–966. [PubMed: 9311391]
28. Otsu K, Nishida K, Kimura Y, Kuzuya T, Hori M, Kamada T, Tada M. The point mutation Arg⁶¹⁵→Cys in the Ca^{2+} release channel of skeletal sarcoplasmic reticulum is responsible for

- hypersensitivity to caffeine and halothane in malignant hyperthermia. *J Biol Chem* 1994;269:9413–9415. [PubMed: 7511586]
29. Owen VJ, Taske NL, Lamb GD. Reduced Mg^{2+} inhibition of Ca^{2+} release in muscle fibers of pigs susceptible to malignant hyperthermia. *Am J Physiol* 1997;272:C203–C211. [PubMed: 9038826]
 30. Palade P. Drug-induced Ca^{2+} release from isolated sarcoplasmic reticulum. II. Releases involving a Ca^{2+} -induced Ca^{2+} release channel. *J Biol Chem* 1987;262:6142–6148. [PubMed: 2437115]
 31. Renganathan M, Messi ML, Delbono O. Dihydropyridine receptor-ryanodine receptor uncoupling in aged skeletal muscle. *J Membr Biol* 1997;157:247–253. [PubMed: 9178612]
 32. Richter M, Schleithoff L, Deufel T, Lehmann-Horn F, Herrmann-Frank A. Functional characterization of a distinct ryanodine receptor mutation in human malignant hyperthermia-susceptible muscle. *J Biol Chem* 1997;272:5256–5260. [PubMed: 9030597]
 33. Robinson R, Carpenter D, Shaw MA, Halsall J, Hopkins P. Mutations in RYR1 in malignant hyperthermia and central core disease. *Hum Mutat* 2006;27:977–989. [PubMed: 16917943]
 34. Saito A, Seiler S, Chu A, Fleischer S. Preparation and morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. *J Cell Biol* 1984;99:875–885. [PubMed: 6147356]
 35. Sitsapesan R, Williams AJ. The gating of the sheep skeletal sarcoplasmic reticulum Ca^{2+} -release channel is regulated by luminal Ca^{2+} . *J Membr Biol* 1995;146:133–144. [PubMed: 7473684]
 36. Smith JS, Coronado R, Meissner G. Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum. Activation by Ca^{2+} and ATP and modulation by Mg^{2+} . *J Gen Physiol* 1986;88:573–588. [PubMed: 2431098]
 37. Steele DS, Duke AM. Defective Mg^{2+} regulation of RyR1 as a causal factor in malignant hyperthermia. *Arch Biochem Biophys* 2007;458:57–64. [PubMed: 16620769]
 38. Szentesi P, Collet C, Sarkozi S, Szegedi C, Jona I, Jacquemond V, Kovacs L, Csernoch L. Effects of dantrolene on steps of excitation-contraction coupling in mammalian skeletal muscle fibers. *J Gen Physiol* 2001;118:355–375. [PubMed: 11585849]
 39. von Breunig F, Wappler F, Hagel C, von Richthofen V, Fiege M, Weissborn R, Stavrou D, Schulte am Esch J. Histomorphologic examination of skeletal muscle preparations does not differentiate between malignant hyperthermia-susceptible and -normal patients. *Anesthesiology* 2004;100:789–794. [PubMed: 15087612]
 40. Wheeler DM, Rice RT, Hansford RG, Lakatta EG. The effect of halothane on the free intracellular calcium concentration of isolated rat heart cells. *Anesthesiology* 1988;69:578–583. [PubMed: 3177918]
 41. Yang T, Esteve E, Pessah IN, Molinski TF, Allen PD, Lopez JR. Elevated resting $[Ca^{2+}]_i$ in myotubes expressing malignant hyperthermia RyR1 cDNAs is partially restored by modulation of passive calcium leak from the SR. *Am J Physiol Cell Physiol* 2007;292:C1591–C1598. [PubMed: 17182726]
 42. Yang T, Riehl J, Esteve E, Matthaei KI, Goth S, Allen PD, Pessah IN, Lopez JR. Pharmacologic and functional characterization of malignant hyperthermia in the R163C RyR1 knock-in mouse. *Anesthesiology* 2006;105:1064–1075. [PubMed: 17065911]
 43. Yang T, Ta TA, Pessah IN, Allen PD. Functional defects in six ryanodine receptor isoform-1 (RyR1) mutations associated with malignant hyperthermia and their impact on skeletal excitation-contraction coupling. *J Biol Chem* 2003;278:25722–25730. [PubMed: 12732639]
 44. Zhang Y, Rodney GG, Schneider MF. Effects of azumolene on Ca^{2+} sparks in skeletal muscle fibers. *J Pharmacol Exp Ther* 2005;314:94–102. [PubMed: 15831441]
 45. Zhao X, Weisleder N, Han X, Pan Z, Parness J, Brotto M, Ma J. Azumolene inhibits a component of store-operated calcium entry coupled to the skeletal muscle ryanodine receptor. *J Biol Chem* 2006;281:33477–33486. [PubMed: 16945924]
 46. Zhou H, Jungbluth H, Sewry CA, Feng L, Bertini E, Bushby K, Straub V, Roper H, Rose MR, Brockington M, Kinali M, Manzur A, Robb S, Appleton R, Messina S, D'Amico A, Quinlivan R, Swash M, Muller CR, Brown S, Treves S, Muntoni F. Molecular mechanisms and phenotypic variation in RYR1-related congenital myopathies. *Brain* 2007;130:2024–2036. [PubMed: 17483490]
 47. Zhou J, Yi J, Royer L, Launikonis BS, Gonzalez A, Garcia J, Rios E. A probable role of dihydropyridine receptors in repression of Ca^{2+} sparks demonstrated in cultured mammalian muscle. *Am J Physiol Cell Physiol* 2006;290:C539–C553. [PubMed: 16148029]

48. Ohnishi ST, Taylor S, Gronert GA. Calcium-induced Ca^{2+} release from sarcoplasmic reticulum of pigs susceptible to malignant hyperthermia. The effects of halothane and dantrolene. *FEBS Lett* 1983;161:103–107. [PubMed: 6884519]
49. Paul-Pletzer K, Palnitkar SS, Jimenez LS, Morimoto H, Parness J. The skeletal muscle ryanodine receptor identified as a molecular target of [^3H]azidodantrolene by photoaffinity labeling. *Biochemistry* 2001;40:531–542. [PubMed: 11148048]
50. Paul-Pletzer K, Yamamoto T, Ikemoto N, Jimenez LS, Morimoto H, Williams PG, Ma J, Parness J. Probing a putative dantrolene-binding site on the cardiac ryanodine receptor. *Biochem J* 2005;387:905–909. [PubMed: 15656791]
51. Kobayashi S, Bannister ML, Gangopadhyay JP, Hamada T, Parness J, Ikemoto N. Dantrolene stabilizes domain interactions within the ryanodine receptor. *J Biol Chem* 2005;280:6580–6587. [PubMed: 15611117]
52. Cherednichenko G, Ward CW, Feng W, Cabrales E, Michaelson L, Samso M, López JR, Allen PD, Pessah IN. Enhanced excitation-coupled calcium entry in myotubes expressing malignant hyperthermia mutation R163C is attenuated by dantrolene. *Mol Pharmacol* 2008;73:1203–1212. [PubMed: 18171728]

Figure 1. Effects of cumulative doses of halothane on skeletal muscle ryanodine receptors (RyR1) (A) Recordings of a group of RyR1 channels exposed to cumulative increases in halothane added to the cytosolic chamber. (B) Recordings of the same group of channels exposed to halothane in the presence of free $Mg^{2+} = 0.25$ mM / total ATP = 5 mM. Openings are shown as discrete upward deflections. The total number of channels was determined under conditions that maximally activate the RyR2, before the addition of halothane. (C) Mean open probability (P_o) $\pm$ SEM of RyR1 channels as a function of halothane concentration in the absence/presence of Mg^{2+} /ATP (open/filled triangles, respectively). * $p < 0.05$ compared to absence of halothane (n=6 paired experiments).

Figure 2. Effects of cytosolic Ca^{2+} levels on halothane-induced RyR1 activation

Mean open probability (P_o) $\pm$ SEM of RyR1 channels as a function of cytosolic free Ca^{2+} levels before (filled circles) and after (open circles) addition of 0.5 mM halothane to the *cis* chamber. Experiments were performed in the absence (A) as well as in the presence (B) of free Mg^{2+} = 0.25 mM / total ATP = 5 mM. * $p < 0.05$ compared to absence of halothane (n=6 paired experiments).

Figure 3. Effect of cytosolic free Mg^{2+} levels on halothane-induced RyR activation

(A) Recordings of a group of RyR1 channels subjected to changes in cytosolic Mg^{2+} levels. (B) Recordings of the same group of channels exposed to 0.5 mM halothane and subjected to the same changes in cytosolic Mg^{2+} levels. (C) Mean open probability (P_o) $\pm$ SEM of RyR1 channels as a function of cytosolic free Mg^{2+} levels before (filled circles) and after (open circles) addition of 0.5 mM halothane. * $p < 0.05$ compared to absence of halothane ($n=7$ paired experiments).

Figure 4. Effect of addition of dantrolene to halothane-activated RyR1

(A) Single channel recording of an RyR1 under control conditions (free $\text{Ca}^{2+} = 2\mu\text{M}$; free $\text{Mg}^{2+} = 0.25\text{ mM}$ / total ATP = 5 mM) and after subsequent addition of halothane (0.5 mM) and dantrolene (20 μM). (B) Mean open probability (P_o) $\pm$ SEM of RyR1 channels under control conditions and after addition of halothane (0.5 mM) and dantrolene (20 μM). * $p < 0.05$ compared to absence of halothane ($n=8$ paired experiments).

Figure 5. Halothane effect on cardiac ryanodine receptors (RyR2) activity

Representative recordings of RyR2 activity before (A) and after (B) addition of 0.5 mM halothane at cytosolic resting (100 nM; a1 and b1) and activating (1 μM ; a2 and b2) Ca^{2+} levels. (C) Mean open probability (P_o) $\pm$ SEM of RyR2 channels under control conditions (filled bars) and after addition of halothane (0.5 mM; open bars). Channel activity was measured at 100 nM and 1 μM cytosolic free Ca^{2+} . * $p < 0.05$ compared to absence of halothane ($n=6$ paired experiments).

Figure 6. Effects of halothane on Ca^{2+} loading into skeletal SR microsomes

(A) The loading of TC microsomes was initiated by $20 \mu\text{M}$ Ca^{2+} pulses. Each Ca^{2+} pulse produced a rapid spike followed by a slower active Ca^{2+} uptake as shown for control conditions and after incubation with halothane and halothane plus ruthenium red. (B) Loading rates as a function of free Mg^{2+} levels under control conditions (filled circles) and upon addition of 0.5 mM halothane (open circles). Triangles represent experiments performed in the presence of ruthenium red ($10 \mu\text{M}$). (C) Comparison of loading rates into SR microsomes with two different luminal Ca^{2+} levels, under control conditions (black bars) or incubated with 0.5 mM halothane (white bars). The same experiment was performed in the presence of ruthenium red (striped and dotted bars). In all cases values are means $\pm$ SE of $n = 4$ observations.

Figure 7. Mg^{2+} and ATP modify the effect of halothane on the rate of Ca^{2+} leak from skeletal SR microsomes

(A) Ca^{2+} leak from TC microsomes was induced by blocking the SERCA pump with $20 \mu\text{M}$ cyclopiazonic acid. Measurements were performed under control conditions (shaded lines) and upon addition of 0.5 mM halothane (black lines). In both cases Ca^{2+} leak was prevented by $10 \mu\text{M}$ ruthenium red, indicating that it is mainly mediated by RyR1 channels. (B) Ca^{2+} leak rate as a function of free cytosolic $[\text{Mg}^{2+}]$ in SR microsomes under control conditions (filled symbols) and upon incubation with 0.5 mM halothane (open symbols). Measurements were done in the presence of 1 mM total ATP (triangles) and 4 mM ATP (circles). In all cases, values are means $\pm$ SE of $n = 5$ observations.