Dantrolene requires Mg^{2+} to arrest malignant hyperthermia

Rocky H. Choi[a], Xaver Koenig[a], and Bradley S. Launikonis[a,1]

[a]School of Biomedical Sciences, The University of Queensland, Brisbane, QLD, 4072, Australia

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Malignant hyperthermia (MH) is a clinical syndrome of skeletal muscle that presents as a hypermetabolic response to volatile anesthetic gases, where susceptible persons may develop lethally high body temperatures. Genetic predisposition mainly arises from mutations on the skeletal muscle ryanodine receptor (RyR). Dantrolene is administered to alleviate MH symptoms, but its mechanism of action and its influence on the Ca^{2+} transients elicited by MH triggers are unknown. Here, we show that Ca^{2+} release in the absence of Mg^{2+} is unaffected by the presence of dantrolene but that dantrolene becomes increasingly effective as cytoplasmic-free $[Mg^{2+}]$ (free $[Mg^{2+}]_{cyto}$) passes mM levels. Furthermore, we found in human muscle susceptible to MH that dantrolene was ineffective at reducing halothane-induced repetitive Ca^{2+} waves in the presence of resting levels of free $[Mg^{2+}]_{cyto}$ (1 mM). However, an increase of free $[Mg^{2+}]_{cyto}$ to 1.5 mM could increase the period between Ca^{2+} waves. These results reconcile previous contradictory reports in muscle fibers and isolated RyRs, where Mg^{2+} is present or absent, respectively, and define the mechanism of action of dantrolene is to increase the Mg^{2+} affinity of the RyR (or "stabilize" the resting state of the channel) and suggest that the accumulation of the metabolite Mg^{2+} from MgATP hydrolysis is required to make dantrolene administration effective in arresting an MH episode.

malignant hyperthermia | dantrolene | ryanodine receptor | magnesium | skeletal muscle fiber

Ryanodine receptors (RyRs) are essential regulators of cytoplasmic Ca^{2+} in muscle, heart, and brain (1–3). Congenital or acquired mishandling of Ca^{2+} by RyRs is associated with organ dysfunction, myopathy, and the increased risk of sudden death (1, 4–7). Consequently, the search for drugs to modulate RyR function is an area of intense research (8–10). An example of a successful drug controlling the Ca^{2+} mishandling of the RyR is the muscle relaxant dantrolene (11). It is primarily used to treat malignant hyperthermia (MH), a life-threatening condition in which genetically predisposed individuals adversely react to the exposure of volatile anesthetics (5, 12). Since its approval, the drug has cut the mortality rate associated with MH from more than 80% to below 2% (11). Despite this success, the exact mechanism of how dantrolene antagonizes MH episodes and depresses overactive Ca^{2+} release during MH episodes remains unknown.

Susceptibility to MH most commonly arises from mutations in the RyR1 gene, the Ca^{2+} release channel of skeletal muscle. Mg^{2+} is present in the muscle at ∼1 mM and exerts an inhibitory action over the RyR1, which needs to be overcome for normal voltage-controlled Ca^{2+} release (13–16). RyR1 variants have a lowered affinity for Mg^{2+} (13, 17), making them more prone to opening and resulting in increased sensitivity to RyR agonist. The ensuing abnormal Ca^{2+} release in RyR variants (18–24) during an MH event leads to excessive heat production as the muscle attempts to clear the persistently high cytoplasmic Ca^{2+}.

Functional studies on muscle fibers suggested the RyR as the potential target for the action of dantrolene (25, 26), and respective binding sites were mapped to the protein (27–29). However, no action of dantrolene was found in most studies on single RyRs incorporated into lipid bilayers (26, 30, 31). A major difference between the experiments examining the effect of dantrolene in bilayers and intact fibers is the ionic conditions; the bilayer studies significantly diverge from physiological ionic conditions, in particular with the exclusion of Mg^{2+} from the cytoplasmic solution (see, e.g., ref. 13). This is necessary in bilayer studies to remove the resting inhibition on the RyR and induce a measureable opening probability in the isolated channel.

We hypothesized that the lack of dantrolene action on single RyRs was due to the fact that the experiments were carried out either in the absence of or in low free $[Mg^{2+}]_{cyto}$ (26, 30, 31). In contrast, the intact fiber experiments, where dantrolene inhibited the RyR, maintained the endogenous level of free $[Mg^{2+}]_{cyto}$ (26). Using mechanically skinned fibers, where the cytoplasmic environment can be rapidly manipulated (14, 15), we tested the effectiveness of dantrolene across the relevant range of free $[Mg^{2+}]_{cyto}$. Thus, we used skinned fibers to mimic the ionic conditions of both bilayer experiments and intact fiber experiments in the same preparation. Additionally, because the skinned fiber is bathed in, effectively, an infinite volume of internal solution, any metabolites generated during experiments diffuse from the preparation in less than a second so that the ionic conditions set in the bath are maintained inside the fiber. Using this approach, we identify that dantrolene shifts the affinity of the RyR for Mg^{2+} (alternatively termed "stabilizing the closed state of the channel"), and therefore, the drug requires the presence of Mg^{2+} to properly close the channel. Furthermore, we imaged Ca^{2+} transients in the presence of halothane (volatile anesthetic) in human MH-susceptible muscle and observed that the metabolite Mg^{2+} is required to supplement the resting free $[Mg^{2+}]_{cyto}$ for dantrolene to depress halothane-induced overactive Ca^{2+} release.

Significance

The nature of overactive Ca^{2+} release in malignant hyperthermia (MH) and the mechanism of action of the drug dantrolene that arrests MH events are poorly understood. Here, we show that dantrolene stops overactive Ca^{2+} release by increasing the affinity of the ryanodine receptor (RyR) to Mg^{2+}. In particular, Ca^{2+} waves induced by MH triggers in human muscle are not affected by dantrolene unless Mg^{2+} increases above resting levels, a condition met by the increase in MgATP hydrolysis during an MH episode. We suggest that only the combination of dantrolene and increased Mg^{2+} can depress overactive Ca^{2+} release and the resulting excessive heat production to arrest MH.

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[1]To whom correspondence should be addressed. Email: b.launikonis@uq.edu.au.

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Results

To simulate cytoplasmic ionic conditions during Ca^{2+} release occurring in bilayer experiments (zero or low free $[Mg^{2+}]_{cyto}$) (26, 30, 31), we directly released Ca^{2+} through the RyRs of skinned fibers by lowering free $[Mg^{2+}]_{cyto}$ (14, 15, 32) (note that our notation of $[Mg^{2+}]_{cyto}$ always indicates the free $[Mg^{2+}]_{cyto}$, which has been calculated taking into the account the cytoplasmic buffers, which include ATP, creatine phosphate, and EGTA; see *Methods*). The lowering of free $[Mg^{2+}]_{cyto}$ removes the resting inhibition on the RyRs and allows Ca^{2+} release from the sarcoplasmic reticulum (SR), as reported by the Ca^{2+}-dependent fluorescence of rhod-2 in the internal bathing solution (24, 32). This form of Ca^{2+} release was not inhibited upon the introduction of 50 μM dantrolene (Fig. 1). Profiles of the spatially averaged fluorescence showed that the amplitude and full duration at half magnitude (FDHM) were not affected by the presence of dantrolene (Fig. 1). The addition of exogenous calmodulin (100 nM) did not affect this result (Fig. S1).

Next, we wished to test the possibility that the inhibitory action of dantrolene on RyR Ca^{2+} release required the presence of physiologically relevant free $[Mg^{2+}]_{cyto}$, consistent with the suppression of Ca^{2+} release by dantrolene in intact and skinned fiber preparations (21, 26). To release Ca^{2+} in the presence of mM levels of free $[Mg^{2+}]_{cyto}$, we used electrical field stimulation at 1 Hz and recorded Ca^{2+} release by confocal line-scanning of Ca^{2+}-dependent rhod-2 fluorescence in rat skinned fibers (33). The activation of the t-system voltage-sensor via action potential stimulation causes the temporary removal of Mg^{2+} inhibition exerted on the RyR to allow rapid release of Ca^{2+} through the transition of the RyR from the closed to the open state (14, 15). Note that the rate of Ca^{2+} release evoked by action potential in skinned fibers is not distinguishable from that occurring in intact fibers (33, 34).

Fig. 2*A*, *Left* shows examples of Ca^{2+} release in the presence of 0.4, 1, and 3 mM free $[Mg^{2+}]_{cyto}$. The increase of free $[Mg^{2+}]_{cyto}$ from 0.4 to 1 mM did not affect the shape or amplitude of the Ca^{2+} transients. However, increasing free $[Mg^{2+}]_{cyto}$ to 3 mM significantly reduced the amplitude of the Ca^{2+} transient. Field stimulation failed to elicit Ca^{2+} transients in the presence of 10 mM free $[Mg^{2+}]_{cyto}$. This effect of increasing free $[Mg^{2+}]_{cyto}$ suppressing Ca^{2+} release is consistent with the results of others (14, 15) and provides further verification of the free $[Mg^{2+}]_{cyto}$ present in the internal bathing solutions.

Ca^{2+} transients in the presence of 50 μM dantrolene are shown in Fig. 2*A*, *Right*. In the presence of 0.4 mM free $[Mg^{2+}]_{cyto}$, 50 μM dantrolene did not affect the amplitude of the Ca^{2+} transient (Fig. 2*A*, *Top*). However, when the free $[Mg^{2+}]_{cyto}$ was increased from 0.4 to 1 and 3 mM, 50 μM dantrolene reduced the Ca^{2+} transient amplitude (Fig. 2*A*, *Middle* and *Bottom*). We also tested the effect of exogenous calmodulin (100 nM) at each free $[Mg^{2+}]_{cyto}$ with dantrolene present on Ca^{2+} release evoked by action potential stimulation. No change in Ca^{2+} release amplitude was observed (Fig. 2*B*, red data points).

The effect of dantrolene on suppressing electrically evoked Ca^{2+} transients across free $[Mg^{2+}]_{cyto}$ of 0.4–3 mM is summarized in Fig. 2*B*. This figure shows the inhibitory effect of free $[Mg^{2+}]_{cyto}$ on suppressing RyR Ca^{2+} release (14, 15) is shifted to the left by dantrolene, and Fig. 2*C* shows that the % inhibition of 50 μM dantrolene on Ca^{2+} release increased with increasing free $[Mg^{2+}]_{cyto}$. These results indicate that mM levels of free $[Mg^{2+}]_{cyto}$ are required for dantrolene to affect RyR Ca^{2+} release. We also determined the concentration response of dantrolene on electrically evoked Ca^{2+} transients in the presence of 3 mM free $[Mg^{2+}]_{cyto}$, where the inhibitory effect of dantrolene was greatest. A Hill curve fitted these data (Fig. 2*D*).

Next, we wished to determine the action of dantrolene at various free $[Mg^{2+}]_{cyto}$ against halothane-induced Ca^{2+} release in MHS human muscle. We used skinned fibers isolated from needle biopsies obtained from unrelated subjects. As it was possible to isolate many fibers from each biopsy, a significant number of repeat measures, with appropriate controls, could be conducted on each biopsy (35). The collection of repeat measures from individual biopsies allowed for assessment of the subject's sensitivity to halothane and dantrolene. Subject A carried RyR1 variant in exon 36, pArg1976Cys. The sensitivity of these mutant fibers to relatively low concentrations of RyR agonists

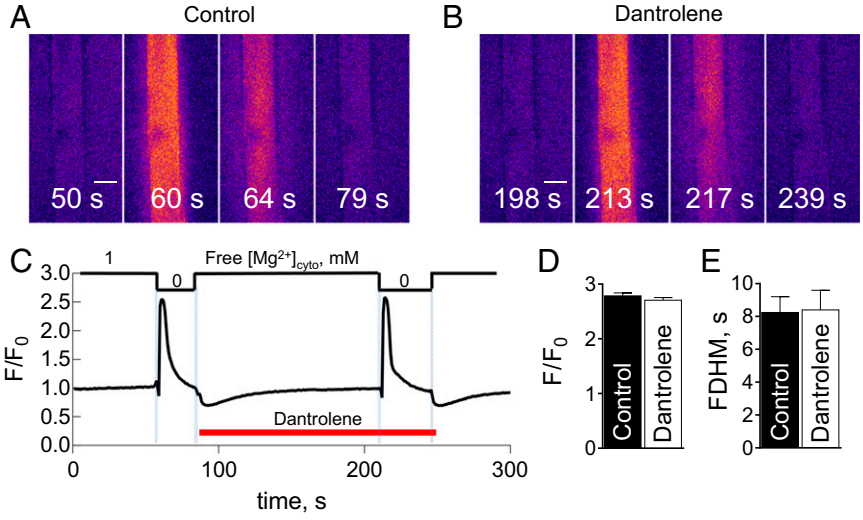

Fig. 1. Ca^{2+} transients evoked by the removal of Mg^{2+} are not inhibited by dantrolene. Selected images of cytoplasmic rhod-2 fluorescence during continuous *xyt* recordings (at 0.8 s·frame^{-1}) in rat skinned fibers acquired while applying Ca^{2+} release inducing 0 Mg^{2+} solution either in the absence (*A*) or presence of dantrolene (*B*) and 1 mM EGTA and 300 nM Ca^{2+} (see Table S1). (Scale bar: 50 μm.) (*C*) Spatially averaged values of normalized fluorescence intensity (F/F_0) versus elapsed time from the full experiment that is represented in *A* and *B*. Applied free $[Mg^{2+}]_{cyto}$ is given at the top, and exchange of solutions is indicated by pale blue vertical bars. A 2-min time interval was allowed for after each low Mg^{2+} transient to recover SR Ca^{2+}. The applied free $[Ca^{2+}]_{cyto}$ was kept constant throughout the recording. The presence of 50 μM dantrolene is indicated by the horizontal bar. Mean normalized peak amplitude values (F/F_0) (*D*) and FDHM (*E*) in the absence and presence of dantrolene ($n = 5$ fibers). A paired Student's *t* test revealed no significant difference ($P > 0.05$) in both *D* and *E*.

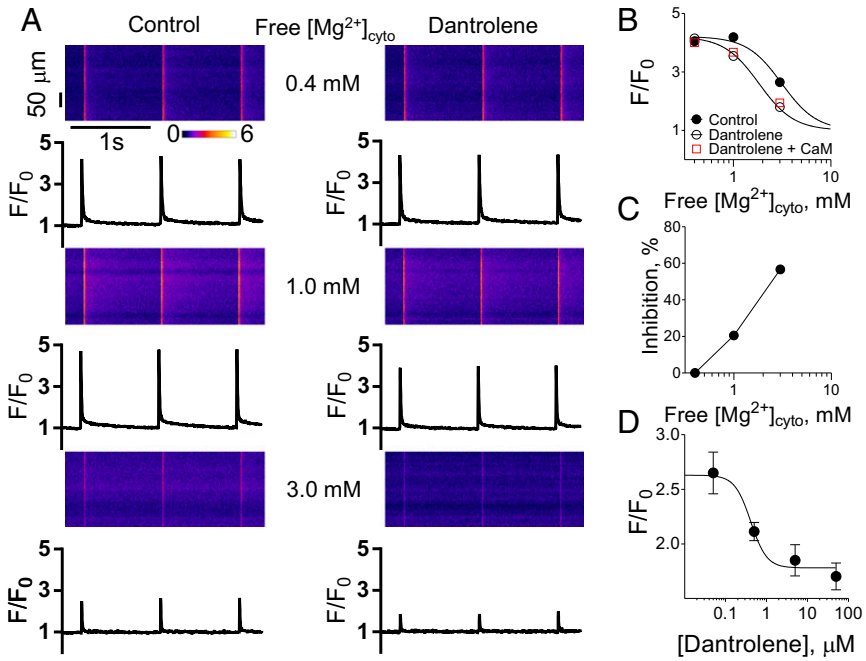

Fig. 2. Inhibition of electrically evoked Ca^{2+} transients by dantrolene is dependent on the free $[Mg^{2+}]_{cyto}$. (*A*) Original recordings in rat skinned fibers as obtained by confocal line scans parallel to the fiber long axis (*Top*), with corresponding line-averaged and normalized rhod-2 fluorescence signals (F/F_0, *Bottom*). Cytosolic Ca^{2+} transients were elicited by electrical field stimulation at 1 Hz in the absence (*Left*) or presence (*Right*) of 50 μM dantrolene at free $[Mg^{2+}]_{cyto}$ of 0.4 (*Top*), 1 (*Middle*), or 3 mM (*Bottom*). All solutions contained 1 mM EGTA and 100 nM free Ca^{2+} (see Table S1). Note that rhod-2 fluorescence was recorded at 2 ms·line^{-1}. (*B*) Effect of dantrolene on Ca^{2+} transient peak amplitudes for the $[Mg^{2+}]_{cyto}$ as shown in *A*. Best fit of the data to a Hill equation in the absence and presence of 50 μM dantrolene yielded IC_{50} values of 2.95 ± 1.42 and 1.73 ± 1.63 mM, respectively. Curves are significantly different from each other (Extrasum of square *F* test). The red data points represent the Ca^{2+} transient amplitudes in the presence of 100 nM exogenous calmodulin (CaM) in the presence of dantrolene. Note that these points are not different from the responses in the presence of dantrolene with only the endogenous calmodulin. (*C*) % inhibition of Ca^{2+} transient by dantrolene at indicated $[Mg^{2+}]_{cyto}$. (*D*) Concentration-dependent inhibition of electrically evoked Ca^{2+} transients by dantrolene at 3 mM free $[Mg^{2+}]_{cyto}$. Best fit Hill curve had an IC_{50} value of 0.41 ± 2.61 μM. All data are derived from 5 to 18 fibers and presented as mean ± SEM.

(1 mM halothane and 3 mM caffeine; Fig. 3 and Fig. S2) was consistent with MHS status, as reported previously from a calibration of Ca^{2+} release in human skinned fibers and the in vitro contracture test (20). Subject B had previously submitted to MH diagnosis at The Royal Melbourne Hospital, Melbourne, Australia. A diagnosis of MHS (halothane) was returned following the in vitro contracture test (with responses of 0.07 g at 2 mM caffeine and 0.26 g at 2% halothane) (36).

Fig. 3*A* shows an isolated fiber initially exposed to nominally zero free $[Mg^{2+}]_{cyto}$ to directly stimulate Ca^{2+} release through the RyR from the biopsy of subject A. Following reloading the SR with Ca^{2+} (2 min in 100 nM Ca^{2+}), exposure to 1 mM halothane in the presence of 1 mM free $[Mg^{2+}]_{cyto}$ induced repetitive Ca^{2+} waves (Fig. 3*A*). Incrementally increasing free $[Mg^{2+}]_{cyto}$ from 1 up to 3 mM had a progressive slowing effect on Ca^{2+} wave frequency in the presence of 1 mM halothane, which provided some variance between the subjects (Fig. 3*B* and Fig. S3) that is fully expected (36). Note that free $[Ca^{2+}]_{cyto}$ was held at 100 nM throughout these experiments. Clinically relevant plasma levels of dantrolene [1.6 μg/mL (5 μM)] (37) and raised concentrations of dantrolene [16 μg/mL (50 μM)] were then tested on the frequency of halothane-induced Ca^{2+} waves in fibers from both subjects. Note that dantrolene was introduced in the internal bathing solution ∼15 s before the administration of halothane. The addition of the relatively high level of dantrolene (50 μM) had no effect on the halothane-induced Ca^{2+} waves in the presence of the resting level of free $[Mg^{2+}]_{cyto}$ (1 mM) in fibers from subject A (Fig. 3*C*) or subject B (Fig. 3*D*). At 5 μM dantrolene, Ca^{2+} wave frequency was slowed when free $[Mg^{2+}]_{cyto}$ was equal to or greater than 1.5 mM (Fig. 3*D*). Consistent with this, 50 μM dantrolene caused a significant impediment to halothane-induced Ca^{2+} waves when free $[Mg^{2+}]_{cyto}$ was 2 mM or greater (Fig. 3*C*).

Discussion

Our results indicate that dantrolene increases the affinity of the RyR for Mg^{2+}, to increase the ability of free $[Mg^{2+}]_{cyto}$ to suppress Ca^{2+} release through the RyR (Figs. 1 and 2) (18–20, 22, 23). Critically, endogenous levels of resting free $[Mg^{2+}]_{cyto}$ were found to be insufficient to effect the repetitive releases of Ca^{2+} in human MHS muscle in the presence of halothane, and it required an increase to at least 1.5 mM free $[Mg^{2+}]_{cyto}$ for dantrolene to antagonize RyR activity (Fig. 3), conditions that likely arise inside a muscle fiber during an MH episode.

The apparent contradictory results in regard to the inhibitory effect of dantrolene on the RyR previously obtained with intact muscle fibers (26) and with isolated RyRs incorporated into bilayers (26, 30, 31) can now be reconciled. The key difference between the experimental approaches was the presence of mM levels of free $[Mg^{2+}]_{cyto}$ (Figs. 1 and 2). Zero or very low Mg^{2+} in bilayer experiments and 1 mM Mg^{2+} in intact fibers is consistent with the presence and absence of the effect of dantrolene on Ca^{2+} flux through RyRs, respectively (Figs. 1 and 2). We have been able to directly assess the function of dantrolene across this broad range of free $[Mg^{2+}]_{cyto}$ by using skinned fibers, where we could rapidly and accurately manipulate the ionic environment of the cytoplasm in conjunction with assessing RyR activity by directly imaging the Ca^{2+}-dependent fluorescence that arises from RyR-mediated Ca^{2+} transients (14, 15, 24).

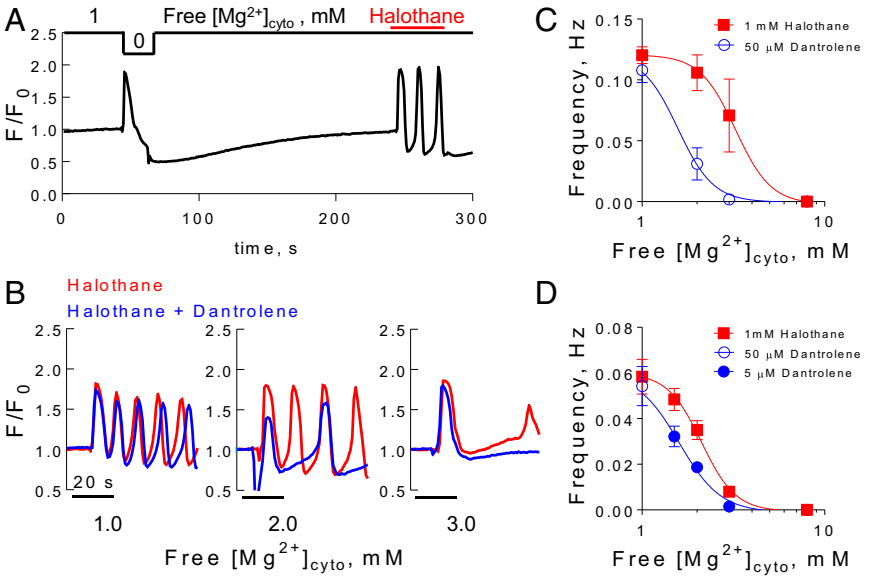

Fig. 3. Halothane-induced Ca^{2+} waves in human MHS fibers require increases in free $[Mg^{2+}]_{cyto}$ to be affected by dantrolene. (*A*) Spatially averaged cytoplasmic rhod-2 fluorescence in a fiber from subject A during changes in the internal bathing solution from one containing 1 mM free $[Mg^{2+}]_{cyto}$ to 0 Mg^{2+} followed by reintroduction of 1 mM free $[Mg^{2+}]_{cyto}$ and subsequent exposure to 1 mM halothane. Note that halothane induced repetitive Ca^{2+} waves in the presence of 1 mM free $[Mg^{2+}]_{cyto}$ and that data were acquired at 0.8 s·frame^{-1}. $[Ca^{2+}]$ was 100 nM in all solutions. (*B*) Examples of MHS fibers from subject A exposed to 1 mM halothane and 1, 2, and 3 mM free $[Mg^{2+}]_{cyto}$ in the presence or absence of 50 μM dantrolene. Frequency of halothane-induced Ca^{2+} waves in the presence of 1–3 mM free $[Mg^{2+}]_{cyto}$ and the presence and absence of dantrolene in fibers isolated from the biopsies of subject A (*C*) and subject B (*D*). Data in *C* and *D* were fit with a Hill equation. IC_{50} values amounted to 3.2 ± 1.05 and 1.6 ± 1.06 mM (*C*) and 2.1 ± 1.05 and 1.6 ± 1.06 mM (*D*) in the presence of halothane and halothane plus dantrolene, respectively. Curves in the presence and absence of 5 (*D*) and 50 μM dantrolene (*C*) were significantly different from each other (extra sum of squares *F* test). All data points are derived from four to five fibers and presented as mean ± SEM.

Oo et al. (38) previously reported that calmodulin is an important factor in the action of dantrolene in muscle by examining the activity of RyRs in isolated bilayers. However, the effect of dantrolene on RyRs in bilayers in the presence of calmodulin was only to lower open probability (P_o) to levels that would be equivalent to that with excessively leaky RyRs in a muscle fiber. That is, if channel P_o is 1.0 during normal Ca^{2+} release in a muscle fiber, a Ca^{2+} flux close to 200 mM·s^{-1}, and the normal resting RyR Ca^{2+} leak is about 5 nM·s^{-1} (34, 39) (which hence equates to a P_o of ~2.5×10^{-8}), then the P_o of 0.009 reported by Oo et al. under conditions of dantrolene and calmodulin still corresponds to a very large leak flux through the RyRs of about 0.18 mM•s^{-1}. Those results with isolated RyRs, however, were found under ionic conditions far from those pertaining in vivo, particularly in the absence of Mg^{2+}. Importantly, it has been shown that in skinned fibers calmodulin remains bound to the RyR (40). Thus, both free $[Mg^{2+}]_{cyto}$ and calmodulin are present at normal physiological levels in our experiments (40, 41). By manipulating free $[Mg^{2+}]_{cyto}$, we could clearly show that mM levels of free $[Mg^{2+}]_{cyto}$ are required for the inhibitory action of dantrolene on action potential-induced or halothane-induced Ca^{2+} release (Figs. 2 and 3). We further demonstrated that in the absence of free $[Mg^{2+}]_{cyto}$, dantrolene fails to significantly slow the release of Ca^{2+} through the RyR in the presence of endogenous or exogenous calmodulin (Figs. 1 and 2 and Fig. S1).

Dantrolene, even at a high concentrations (50 μM), did not affect halothane-induced Ca^{2+} release in the presence of resting levels of free $[Mg^{2+}]_{cyto}$ (Fig. 3). Ca^{2+} waves induced by the action of halothane could only be suppressed by dantrolene when the free $[Mg^{2+}]_{cyto}$ was increased above its normal resting levels (Fig. 3). Preadministration of dantrolene before halothane did not affect this requirement for raised free $[Mg^{2+}]_{cyto}$ (Fig. 3). These results suggest that in a clinical setting, even if dantrolene was preadministered to an MHS person undergoing a general anesthesia, free $[Mg^{2+}]_{cyto}$ would still need to rise above its normal resting level of 1 mM for dantrolene to be effective. A rise in muscle metabolites must precede any clinical symptoms of MH such as increasing body temperature and other changes, as they are a result of MgATP hydrolysis, which includes the defining heat production of the condition. Evidence for the rise of free $[Mg^{2+}]_{cyto}$ during extensive muscle use is best documented during metabolic fatigue, which can be used as a model of metabolic changes inside the muscle during overactive Ca^{2+} release under an MH trigger. It has been shown in human type II fibers that ATP is depleted in the order of seconds during maximal activity (42). Similarly, we can expect that muscle contractures in an MH episode lead to major [ATP] decline. This decline of [ATP] significantly retards its capacity as the major cytoplasmic buffer of Mg^{2+}, causing a parallel rise in free $[Mg^{2+}]_{cyto}$. Indeed, increases in free $[Mg^{2+}]_{cyto}$ to levels in the order of 1.5–3 mM are reached during metabolic fatigue (43–45).

In summary, we have shown that (*i*) dantrolene acts by increasing the affinity of the RyR for Mg^{2+}, (*ii*) repetitive Ca^{2+} waves generated by halothane can occur even with free $[Mg^{2+}]_{cyto}$ increased above resting levels, and (*iii*) dantrolene requires the metabolite Mg^{2+} to adequately close the RyR and inhibit overactive Ca^{2+} release. Overall these results suggest that dantrolene is a poor antagonist of RyR activity but one that is good enough to arrest MH, where the susceptibility to this condition arises from only a relatively minor decrease in RyR affinity for the endogenous inhibitory stabilizer Mg^{2+} (16, 17).

Methods

All experimental methods using rodents were approved by the Animal Ethics Committee at The University of Queensland, and the use of human muscle was approved by The University of Queensland Human Ethics Committee. Subjects signed informed consent forms before their involvement in this study. Wistar rats (University of Queensland Biological Resources, Brisbane) were killed by cervical dislocation, and the *extensor digitorum longus* (EDL) muscles were rapidly excised. Muscles were placed in a Petri dish under paraffin oil above a layer of Sylgard. Muscle biopsies were collected under local anesthesia (Xylocaine, 10 mg·mL^{-1}) from the midportion of the Vastus

Lateralis muscle, using a 6-mm Bergstrom biopsy needle modified for manual suction. Biopsy was taken from a 30-y-old female with a RyR1 variant (subject A) and a 45-y-old male (subject B). Muscle tissue collected from the biopsy needle was blotted on filter paper (Whatman no. 1) to remove blood and extracellular fluid. With both rat and human muscle, segments of individual fibers were isolated and mechanically skinned to completely remove the surface membrane. Skinned fibers were transferred to a custom-built experimental chamber with a coverslip bottom, where they were bathed in an "internal solution," containing (in mM) K^+, 126; Na^+, 36; free Mg^{2+}, 1 (total Mg, 8.5); MgATP, 7 (total ATP, 8); Ca^{2+}, 0.0001; rhod-2, 0.01; creatine phosphate, 10; EGTA, 1; and HDTA, 49 with pH adjusted to 7.1 ± 0.1 with KOH. To load Ca^{2+} into the SR, Ca^{2+} was increased to 300 nM (in rat skinned fiber experiments), and to release Ca^{2+}, a nominally Mg^{2+}-free solution was used (24, 32). In human muscle fiber experiments, Ca^{2+} was kept at 100 nM in all solutions. Free Mg^{2+} was lowered and raised to 0.4 and 10 mM for experiments involving electrical stimulation of skinned fibers without changing total [ATP]. Solution composition was calculated using MaxChelator software. BTS was added to all solutions to inhibit contraction. Dantrolene was added to internal solutions from a 10-mM stock dissolved in DMSO (38). At the highest [dantrolene] used (nominally 50 μM), it was possible that dantrolene partially precipitated upon dilution in aqueous solution, reducing its concentration to a value not lower than 20 μM (37). However, we did not observe any precipitate under the conditions we used to prepare dantrolene and consider the concentration of dantrolene to be close to the calculated value. Halothane was administered to fibers during imaging from a syringe to minimize exposure to air and reduce evaporation. In experiments using halothane to release Ca^{2+} from human muscle fibers, [EGTA] was lowered from 1 to 0.1 mM so that transients could be observed. Calmodulin derived from bovine testes was used in experiments. Table S1 provides the full details of the internal solutions composition used in this study. All chemicals were from Sigma, unless otherwise stated.

The experimental chamber containing a skinned fiber was placed above a water immersion objective (40×, N.A. 0.9) of the confocal laser scanning system (FV1000, Olympus). During low Mg^{2+}-induced Ca^{2+} release, the fiber was imaged in *xyt* mode at 0.8 s·frame^{-1} (Figs. 1 and 3). Images were analyzed to determine fluorescence amplitude and FDHM as described previously (24). For fibers exposed to electrical stimulation, field pulses at 1 Hz were applied across platinum electrodes parallel to the long axis of the fiber as previously described (33). Imaging for electrical stimulation experiments was in *xt* mode, at a rate of 2 ms·line^{-1} (Fig. 2). All imaging was performed at 21–24 °C. Statistical analysis and nonlinear curve fitting was performed with GraphPad Prism. IC_{50} values were derived from fits to Hill equations. All data are given as mean ± SEM.

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