Essential Role of Calmodulin in RyR Inhibition by Dantrolene

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ABSTRACT

Dantrolene is the first line therapy of malignant hyperthermia. Animal studies suggest that dantrolene also protects against heart failure and arrhythmias caused by spontaneous Ca$^{2+}$ release. Although dantrolene inhibits Ca$^{2+}$ release from the sarcoplasmic reticulum of skeletal and cardiac muscle preparations, its mechanism of action has remained controversial, because dantrolene does not inhibit single ryanodine receptor (RyR) Ca$^{2+}$ release channels in lipid bilayers. Here we test the hypothesis that calmodulin (CaM), a physiologic RyR binding partner that is lost during incorporation into lipid bilayers, is required for dantrolene inhibition of RyR channels. In single channel recordings (100 nM cytoplasmic [Ca$^{2+}$] + 2 mM ATP), dantrolene caused inhibition of RyR1 (rabbit skeletal muscle) and RyR2 (sheep) with a maximal inhibition of $P_0$ ($E_{\text{max}}$) to 52 ± 4% of control only after adding physiologic [CaM] = 100 nM. Dantrolene inhibited RyR2 with an IC$_{50}$ of 0.16 ± 0.03 μM. Mutant N98S-CaM facilitated dantrolene inhibition with an IC$_{50}$ = 5.9 ± 0.3 nM. In mouse cardiomyocytes, dantrolene had no effect on cardiac Ca$^{2+}$ release in the absence of CaM, but reduced Ca$^{2+}$ wave frequency (IC$_{50}$ = 0.42 ± 0.18 μM, $E_{\text{max}}$ = 47 ± 4%) and amplitude (IC$_{50}$ = 0.19 ± 0.04 μM, $E_{\text{max}}$ = 66 ± 4%) in the presence of 100 nM CaM. We conclude that CaM is essential for dantrolene inhibition of RyR1 and RyR2. Its absence explains why dantrolene inhibition of single RyR channels has not been previously observed.

Introduction

Dantrolene is a well known inhibitor of Ca$^{2+}$ release in skeletal muscle (Hainaut and Desmedt, 1974) that has been used clinically as the treatment of malignant hyperthermia (MH). MH is a potentially fatal inherited disorder of skeletal muscle in which mutations in the proteins involved in excitation-contraction coupling (e.g., RyR1 and DHPR) (McCarthy et al., 1990; Monnier et al., 1997; Jung et al., 2012) cause uncontrolled sarcoplasmic reticulum (SR) calcium release and muscle contracture in the presence of volatile anesthetics. Notably, mutations in the cardiac ryanodine receptor (RyR) isofrom (RyR2) that correspond to the MH mutations in RyR1 cause catecholaminergic polymorphic ventricular tachycardia (Yano, 2005). Recent in vitro and animal studies suggest that dantrolene has antiarrhythmic effects in catecholaminergic polymorphic ventricular tachycardia and possibly also in heart failure (Jung et al., 2012; Kobayashi et al., 2009, 2010; Maxwell et al., 2012).

Dantrolene acts on skeletal and cardiac muscle by inhibiting Ca$^{2+}$ release from the SR (Hainaut and Desmedt, 1974; Kobayashi et al., 2005; Uchinoumi et al., 2010). Assays of Ca$^{2+}$ release in intact myocytes and cell homogenates containing SR vesicles (Fruen et al., 1997) suggest that dantrolene inhibits the SR Ca$^{2+}$ release channel with a half-inhibiting concentration (IC$_{50}$) of 0.3 μM (Kobayashi et al., 2009). Even though a dantrolene binding site has been identified in the DP1 regions in RyR1 and RyR2 (Parness and Paliptikar, 1995; Paul-Pletzer et al., 2002, 2005; Kobayashi et al., 2009), there has been only one direct observation of RyR inhibition by dantrolene in bilayer-based single channel recordings (Nelson et al., 1996). Studies since then find no effect of dantrolene in single channel recordings (Szentesi et al., 2001; Cherendichenko et al., 2008; Diaz-Sylvester et al., 2008; Wagner et al., 2014). Hence, it is not clear if dantrolene acts directly on the RyR or some other protein involved in excitation-contraction coupling such as the DHPR (Salata et al., 1983; Chou et al., 2014).

Calmodulin (CaM) is known to regulate the activity of RyR1 and RyR2 (Tripathy et al., 1995; Balshaw et al., 2001). CaM inhibits RyR2 directly by binding to residues 3583–3603 of each RyR2 subunit (Huang et al., 2013) with high affinity (K$_d$ 20–100 nM) (Guo et al., 2011). Similarly, CaM may either increase RyR1 activity at resting cytoplasmatic [Ca$^{2+}$] or decrease activity at higher [Ca$^{2+}$] (Tripathy et al., 1995). Fruen and colleagues (Fruen et al., 1997; Zhao et al., 2001)
found that dantrolene reduces the effect of RyR1 activators (but interestingly, not in RyR2) including CaM, suggesting that CaM might augment dantrolene inhibition of RyR1. During the process of RyR2 isolation from the heart and their incorporation into artificial lipid bilayers, the RyR macromolecular complex stays mostly intact (Marks et al., 2002), except for CaM, which is reported to dissociate from the RyR complex with a time constant of less than 1 minute (Guo et al., 2011). Hence, bilayer-based channel studies would generally have been made devoid of this important regulatory molecule in the RyR complex, whereas CaM is abundant in intact cell and cell homogenates. Therefore, we hypothesize that CaM is the missing protein and that its absence in bilayer experiments provides an explanation as to why dantrolene inhibition has not been observed in single channel RyR recording experiments. We test this hypothesis by examining the effects of dantrolene, in the absence and presence of CaM, on the gating of RyR1 and RyR2 Ca$^{2+}$ release channels incorporated into artificial lipid bilayers and on the frequency and amplitude of Ca$^{2+}$ waves in permeabilized cardiomycocytes.

### Materials and Methods

#### Chemicals.

SR vesicles containing RyR1 were isolated from rabbit skeletal muscle, and RyR2 were isolated from sheep hearts (Laver et al., 1995) and incorporated in artificial bilayer membranes composed of a lipid mixture of phosphatidylethanolamine and phosphatidylcholine (8:2 wt/wt, Avanti Polar Lipids, Alabaster, AL) in n-decane (50 mg/ml, ICN Biomedicals, Irvine, CA). Experimental solutions contained (in millimolar) 150 Cs$^+$ (130 CsCl, 20 CsCl). All solutions were pH buffered using N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (ICN Biomedicals) and titrated to pH 7.4 using CsOH (ICN Biomedicals). Cytoplasmic solutions were buffered to a redox potential of -232 mV with reduced glutathione disulfide (0.2 mM) and glutathione (GSH; 4 mM), and luminal solutions were buffered to a redox potential of ~180 mV with reduced glutathione disulfide (3 mM) and GSH (2 mM). A Ca$^{2+}$ electrode (Radiometer, Brea, CA) was used in our experiments to determine the purity of Ca$^{2+}$ buffers and Ca$^{2+}$ stock solutions as well as free Ca$^{2+}$ > 100 nM. The cesium salts were obtained from Sigma-Aldrich (St. Louis, MO). CaCl$_2$ was obtained from BDH Chemicals (WVR, Radnor, PA). Calmodulin was obtained from two sources, Sigma-Aldrich (prepared from bovine testes) and Enzo Life Sciences (Farmingdale, NY; prepared from pig brain). Dantrolene (powder) was obtained from Sigma. Dantrolene was prepared as stock solutions in dimethylsulfoxide, and calmodulin was prepared in milliQ (EMD Millipore, Billerica, MA). During experiments, the concentrations of calmodulin, dantrolene, and Ca$^{2+}$ in the cytoplasmic solution were altered by a local perfusion system (O’Neill et al., 2003), which allowed exposure of a single channel to multiple bathing conditions applied in any chosen sequence with an exchange time of ~3 seconds.

#### Data Acquisition and Analysis.

Experiments were carried out at room temperature (23 ± 2°C). Electric potentials are expressed using standard physiologic convention (i.e., cytoplasm relative to SR lumen at virtual ground). Control of the bilayer potential and recording of unitary currents were done using an Axopatch 200B amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA). The current signal was digitized at 5 kHz and low pass-filtered at 1 kHz. Single channel dwell-time histograms of open and closed time, open probability, and mean open and mean closed time, were measured using a threshold discriminator at 50% of channel amplitude (Channell3 software; N. W. Laver, nic@niclaver.com). Individual readings were derived from 45-120 seconds of RyR2 recording. Hill equations were fitted to the dose-response data by the method of least squares. Average data are given as mean ± S.E.M. The statistical significance of differences was tested using Student’s t test.

#### Effect of Dantrolene on RyR Dwell Times.

To gain more insight into the mechanism of dantrolene inhibition, we compiled dwell-time histograms of channel open and closed events of sheep RyR2 at four cytoplasmic [Ca$^{2+}$], ranging from 0.1 μM (end diastolic) to 100 μM (systolic) (Fig. 2, A and B; Supplemental Fig. 1). Histograms are displayed using the log-bin method of Sigworth and Sine (1987), where individual
exponential components appear as peaks centered on their time constant value. In the absence of dantrolene, open and closed dwell times in 1 μM cytoplasmic Ca2+ exhibited peaked distributions that were fitted by two exponential components (see Supplemental Table 1). Addition of dantrolene (10 μM) shifted the peak of the open distributions to shorter times and closed distribution to longer times. Dantrolene had a similar effect in 0.1 μM cytoplasmic Ca2+ but had no effect at 100 μM cytoplasmic Ca2+ (Supplemental Fig. 1). It was not possible to resolve significant differences in the parameters of the exponential fits except for the slow time-constant of the closed distributions that were fitted by two exponential components appearing at higher [Ca2+]m (see Supplemental Table 1). Addition of dantrolene (10 μM) diminished and there was no significant inhibition of RyR2 mean open and closed durations (Fig. 2C). In 0.1 μM cytoplasmic Ca2+, dantrolene reduced RyR2 Po via a decrease in mean channel open duration and an increase in mean closed duration. At 1 μM cytoplasmic Ca2+, the effect of dantrolene was diminished and there was no significant inhibition occurring at higher [Ca2+]m. The effect of dantrolene was to shift the Ca2+–activation response of RyR2 to higher [Ca2+]m.

**Effect of Dantrolene on Ca2+ Waves in Mouse Cardiomyocytes.** The amplitude and frequency of spontaneous Ca2+ waves, two parameters that have been implicated as independent predictors of arrhythmogenicity (Galimberti and Knollmann, 2011), were measured in mouse ventricular cardiomyocytes. Examples of the effect of 30-minute exposure to dantrolene (3, 10, 50 μM) on Ca2+ waves recorded in the presence or absence of CaM are presented in Fig. 3A. Dantrolene reduced Ca2+ wave amplitude and frequency in the presence of CaM but had no effect in the absence of CaM (Fig. 3A). This finding is consistent with the single channel experiments with the concentration dependence of these effects (Fig. 3, B and C) exhibiting remarkably similar IC50 and Emax values to that measured in the single channel experiments (Fig. 1).

**Dantrolene Inhibition Can Be Mediated by CaM Mutants.** Because both dantrolene and CaM are RyR2 inhibitors, we investigated the possibility that dantrolene acts by amplifying CaM inhibitory action on RyR2. To test this possibility we measured dantrolene inhibition of sheep RyR2 in the presence of 10 nM (C; mean ± S.E.M., n = 3 to 4) and 100 nM CaM (D; mean ± S.E.M., n = 7 to 20). The luminal [Ca2+]c is 0.1 mM and cytoplasmic [Ca2+]m is 100 nM. The solid curve shows the Hill fit to the data using the equation: where IC50 = 0.16 ± 0.03 μM, H = 1.3 ± 0.3, and Emax = 52 ± 4%. The dashed curve uses the same parameter values, except Emax = 80 ± 5%.

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We also investigated dantrolene inhibition in the presence of N98S-CaM that is a CaM mutant that has no inhibitory effect on RyR2 in the absence of dantrolene (Fig. 4C, ○). The advantage of this CaM mutant is that we can examine the effect of varying its concentration on facilitating dantrolene inhibition without the confounding effect of CaM inhibition. In the absence of CaM, dantrolene (10 μM) had no effect on RyR Po. Figure 4C shows that addition of only 6 nM N98S-CaM was sufficient to facilitate significant dantrolene inhibition of RyR2. The N98S-CaM facilitation of dantrolene inhibition had a sigmoidal dependence on log-concentration with an IC50 of 5.9 ± 0.3 nM, a Hill coefficient of 5 ± 2.6, and an Emax of 53 ± 4%.

Discussion

Our study presents the first demonstration of dantrolene inhibition of mammalian RyR1 and RyR2 from recordings of single RyR and permeabilized cardiomyocytes. The finding that a physiologic concentration of CaM is required for dantrolene inhibition of these RyRs provides an answer to the long-standing question of why dantrolene, an inhibitor of SR Ca2+ release, had no effect on the activity of mammalian RyR1 and RyR2 in previous single channel studies (Szentesi et al., 2001; Diaz-Sylvester et al., 2008; Wagner et al., 2014). Because CaM readily dissociates from the RyR complex (Guo et al., 2011), CaM would have been absent during those experiments. IC50 for CaM facilitation of dantrolene inhibition appears to be ~10 nM for wt-CaM (Fig. 1E) and 5.9 nM for N98S-CaM (Fig. 4C). These values are ~2-fold lower than the binding affinities for these CaMs on RyR2 (Guo et al., 2011; Hwang et al., 2014).

[3H]ryanodine binding assays have demonstrated a reduction of CaM activation of purified pig RyR1 by dantrolene (Frueh et al., 1997). However, that finding was contradicted by a single channel study (Cherednichenko et al., 2008) that, using similar experimental conditions (100 nM cytoplasmic Ca2+ and 35°C), reported no inhibition by dantrolene (20 μM) of purified rabbit RyR1 channels in bilayers in the presence of exogenous FKBP12 and CaM. Together with the findings reported here, these results suggest that the inhibitory effect of dantrolene on RyR not only requires CaM but also other RyR-associated proteins that are present in native preparations but presumably absent in some purified RyR preparations.

The maximum RyR2 inhibition (Emax = 52%) and IC50 (0.16 ± 0.03 μM; Fig. 1D) are in close agreement with dantrolene inhibition of Ca2+ wave frequency and amplitude in saponin permeabilized cardiomyocytes (Fig. 3) and inhibition of Ca2+ release in SR vesicles from failing dog heart [IC50 = 0.3 ± 0.07 μM (Kobayashi et al., 2009)] and activity of purified RyR1 in [3H]ryanodine binding assays [0.15 ± 0.02 μM (Frueh et al., 1997)]. The dantrolene IC50 reported here coincides with the binding affinity of dantrolene to skeletal muscle SR vesicles [0.277 ± 0.025 μM (Parness and Palnitkar, 1995)] and its IC50 (0.3 ± 0.11 μM) for inhibiting the unzipping of the central and
N-terminal domains of RyR2 (Kobayashi et al., 2009). The potency of dantrolene in our study is also consistent with the inhibitory action of 1 \( \mu \)M dantrolene on Ca\(^{2+}\) spark frequency in isoproterenol-stimulated cardiomyocytes from R2474S knock-in mice (Kobayashi et al., 2010). However, the therapeutic actions of dantrolene in skeletal and cardiac muscle occur at much higher concentrations than required for inhibition of Ca\(^{2+}\) release from the SR. For example, 20 \( \mu \)M or more dantrolene was required to prevent exercise-induced cardiac arrhythmias in R2474S knock-in mice (Kobayashi et al., 2010), increase survival after ventricular fibrillation (Zamiri et al., 2014), and prevent anesthetic induced MH in skeletal muscle (Podranski et al., 2005). This has lead others to consider alternative therapeutic mechanisms for dantrolene such as modulating store-operated Ca\(^{2+}\) entry (Cherednichenko et al., 2008) or by acting as an antioxidant (Buyukokuroglu et al., 2001) or regulating antioxidant enzymes (Buyukokuroglu et al., 2002; Ucuncu et al., 2005).

Our finding that dantrolene inhibition is seen only at cytoplasmic [Ca\(^{2+}\)] \( \leq 1 \) \( \mu \)M (Fig. 2C) is consistent with previous findings that dantrolene (1 \( \mu \)M) inhibits the frequency of Ca\(^{2+}\) sparks (and hence SR leak) yet does not inhibit the amplitude of Ca\(^{2+}\) transients (Maxwell et al., 2012; Zamiri et al., 2014). Thus dantrolene is a diastolic inhibitor of Ca\(^{2+}\) release in failing heart, which has the beneficial actions of increasing diastolic Ca\(^{2+}\) loading of the SR (Maxwell et al., 2012) and reducing diastolic SR Ca\(^{2+}\) leak after ventricular fibrillation (Zamiri et al., 2014). Because dantrolene is not an effective RyR inhibitor at high cytoplasmic [Ca\(^{2+}\)], it is not surprising that other dantrolene mechanisms may be more important for suppressing Ca\(^{2+}\) release during skeletal muscle twitches (Flewelling et al., 1983) or suppressing MH episodes. Single channel recordings of dantrolene inhibition provide a unique opportunity to probe the mechanism of dantrolene inhibition. RyR2 dwell-time distributions (Fig. 2, A and B) indicate that dantrolene decreases the duration of channel openings and increases the duration of closures, characteristics typical of an allosteric inhibitor rather than a channel blocker like the local anesthetics that cause distinct blocking events in single channel recordings that introduce new exponential components in closed time distributions (Tinker and Williams, 1993; Xu et al., 1993; Tashima et al., 2002). Like CaM, dantrolene inhibits RyR2 by destabilizing their open state and stabilizing their closed state. By using a CaM mutation that causes CaM to activate RyR2, we show that dantrolene does not merely increase the efficacy of CaM, but is an inhibitor in its own right (Fig. 4). The RyR has a homotetrameric structure that includes four dantrolene binding sites and at least four CaM binding sites (Fruen et al., 1997). Such a value indicates that the binding of only one dantrolene molecule is sufficient to cause inhibition of RyR2 activity. Interestingly, the dose-response of N98S-CaM facilitation of dantrolene inhibition (Fig. 4C) had a much higher Hill coefficient, consistent with a requirement for multiple CaM molecules on RyR2. The mechanism by which CaM facilitates dantrolene inhibition remains unclear. It is unlikely that dantrolene acts by binding to a site on CaM, because that would not explain the different Hill coefficients for the dantrolene and N98S-CaM dose responses. Also, given the redox buffering of our experimental solutions (4 mM GSH in bilayer experiments and 10 mM GSH in myocyte experiments) it is unlikely that the reducing
properties of dantrolene underlie its inhibition. However, one possibility is that CaM puts the RyR into a conformation that gives dantrolene access to its binding site on the RyR. Two studies have demonstrated that dantrolene has restricted access to its binding site that is regulated by RyR conformation and on the presence of RyR ligands such as Ca$^{2+}$ and ATP (Paul-Pletzer et al., 2001, 2005). An alternative possibility is that CaM is a part of the signaling pathway that transduces dantrolene binding into RyR inhibition. Several studies present evidence that dantrolene modulates interdomain interactions in RyR1 (Kobayashi et al., 2005) and RyR2 (Kobayashi et al., 2009; Uchinoumi et al., 2010; Suetomi et al., 2011; Maxwell et al., 2012) between the N-terminal (1–619 aa), central (2000–2500 aa), and C-terminal domains (3900–end). Our data are consistent with both these possibilities.

In conclusion, we show that CaM binding to the RyR is required to produce dantrolene inhibition in both RyR1 and RyR2. It is likely that other, as yet undefined, factors play a similar role in facilitating dantrolene inhibition.

Acknowledgments

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Author Contributions

Participated in research design: Oo, Imitiaz, Knollmann, Laver.

Conducted experiments: Oo, Gomez-Hurtado, Walwe.

Contributed to new reagents or analytic tools: Knollmann, Laver.

Performed data analysis: Oo, Gomez-Hurtado, Knollmann, Laver.

Wrote or contributed to the writing of the manuscript: Oo, Gomez-Hurtado, van Helden, Knollmann, Laver.

References


Fig. 4. Dantrolene inhibition of RyR in the presence of wt-CaM and mutant CaM. (A) Relative effect of wt-CaM (100 nM) on the open probability of RyR1 and RyR2 and N54I-CaM on RyR2. (B) Relative effect of 10 μM dantrolene (dan) on RyR2 P, in the presence of wt- and mutant-CaM. Mean values are indicated by the horizontal bars and S.E.M. by the vertical bars. P values indicate significant differences between wt- and mutant-CaM. (C) Facilitation of dantrolene (10 μM) inhibition by N98S-CaM on wt-RyR2. Asterisks indicate significantly different than 100% (*P < 0.05; **P < 0.01). The solid curve shows the fit of the Hill equation (see legend to Fig. 1) to the data.


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Molecular Pharmacology
Supplementary figure 1. Effect of dantrolene on open and closed dwell-times of RyR2. (A,C,E) Open and (B,D,F) closed dwell-time histograms compiled using the log-bin method of Sigworth and Sine (1987). Histograms are averages of three experiments obtained in cytoplasmic [Ca\(^{2+}\)] (indicated by pCa in each panel) in the absence (O) or presence (●) of 10 µM dantrolene. The constants of exponential constants fits to these dwell-time histograms are given in Supplementary Table 1. Figure 1C and D that the data from Figure 2A and B are re-plotted here for comparison purposes.
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Table 1. Parameter values for multi exponential fits to RyR2 dwell-time histograms ($H(t)$). The conditions give the cytoplasmic [Ca$^{2+}$] in units of pCa in the absence and presence of 10 µM dantrolene. T1 and T2 are the exponential time constants and A1 and A2 give fraction of dwell times in each exponential where $A1 + A2 = 100\%$. Asterisks indicate significant difference to absence of dantrolene in paired t-test (* $p<0.05$). The equation is: $H(t) = A1 \cdot T1 \cdot exp(-t/T1) + A2 \cdot T2 \cdot exp(-t/T2)$

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