Dantrolene inhibition of ryanodine receptor Ca\(^{2+}\) release channels: molecular mechanism and isoform selectivity

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Abbreviations used:
RYR, ryanodine receptor; SR, sarcoplasmic reticulum; MH, malignant hyperthermia; EC, excitation-contraction; CaM, calmodulin; AMPPCP, adenosine 5'-(β,γ-methylene)triphosphate; PIPES, 1,4-piperazinediethanesulfonic acid.
SUMMARY

As an inhibitor of Ca\(^{2+}\) release through ryanodine receptor (RYR) channels, the skeletal muscle relaxant dantrolene has proven to be both a valuable experimental probe of intracellular Ca\(^{2+}\) signaling, and a lifesaving treatment for the pharmacogenetic disorder malignant hyperthermia (MH). However, the molecular basis and specificity of dantrolene’s actions on RYR channels have remained in question. Here we utilize \[^{3}\text{H}]\text{ryanodine binding to further investigate dantrolene’s action on the three mammalian RYR isoforms. Inhibition of the pig skeletal muscle RYR1 by dantrolene (10 µM) was associated with a three-fold increase in the } K_d \text{ of } \[^{3}\text{H}]\text{ryanodine binding to sarcoplasmic reticulum (SR) vesicles, such that dantrolene effectively reversed the three-fold decrease in the } K_d \text{ for } \[^{3}\text{H}]\text{ryanodine binding resulting from the MH RYR1 Arg}^{615} \rightarrow \text{Cys mutation.} \text{ Dantrolene inhibition of the RYR1 was dependent on the presence of adenine nucleotide and calmodulin (CaM), and reflected a selective decrease in the apparent affinity of RYR1 activation sites for Ca}\(^{2+}\) relative to Mg\(^{2+}\). In contrast to the RYR1 isoform, the cardiac RYR2 isoform was unaffected by dantrolene, both in native cardiac SR vesicles and when heterologously expressed in HEK-293 cells. By comparison, the RYR3 isoform expressed in HEK-293 cells was significantly inhibited by dantrolene, and the extent of RYR3 inhibition was similar to that displayed by the RYR1 in native SR vesicles. Our results thus indicate that both the RYR1 and the RYR3—but not the RYR2—may be targets for dantrolene inhibition } \text{in vivo.} \text{ }

**Keywords:**
sarcolemmal reticulum; calcium release channel; excitation-contraction coupling; malignant hyperthermia
INTRODUCTION

Ryanodine receptors (RYRs) are intracellular Ca\(^{2+}\) channels specialized for the rapid and massive release of Ca\(^{2+}\), as is necessary for excitation-contraction (EC) coupling in striated muscle (1). Three different RYR isoforms have been identified in mammalian tissues: the RYR1, which is predominantly expressed in skeletal muscle; the RYR2, which is predominantly expressed in cardiac muscle; and the RYR3, which is expressed at comparatively low levels in a variety of tissues, including the brain. Because RYR channels play critical roles in the diverse physiologic/pathophysiologic cell processes that are controlled by Ca\(^{2+}\) release from intracellular stores (2), these channels represent potentially important pharmacologic targets for modulating cell regulation (3). However, the key functional properties that may distinguish the different RYR isoforms remain unclear (4,5), and few if any drugs are known to act as isoform-specific modulators of these channels (3).

To date, the muscle relaxant dantrolene remains the only drug targeting RYR channels that is used clinically (3,5). Early investigations indicated that dantrolene may act selectively on the physiologic mechanism responsible for activating Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) during skeletal muscle EC coupling (6,7,8). Accordingly, dantrolene (~10 µM) shifts the sensitivity of contractile activation to higher voltages and reduces skeletal muscle twitch force by more than half (7,8). Dantrolene inhibition of SR Ca\(^{2+}\) release in skeletal muscle has provided a lifesaving treatment for the pharmacogenetic disorder malignant hyperthermia (MH). Thus, the uncontrolled SR Ca\(^{2+}\) release, muscle contracture, and accelerated metabolism that threaten the MH-susceptible (MHS) patient exposed to volatile anesthetics during surgery are effectively suppressed upon treatment with dantrolene (9,10). Dantrolene also reverses the increased sensitivity of MHS muscle to activation by caffeine (11), which constitutes the basis of in vitro diagnostic tests of this syndrome (12,13). Dantrolene’s efficacy in the treatment of MH is in large part a function of this drug’s selective action on SR Ca\(^{2+}\) release in skeletal muscle, while exerting no comparable negative inotropic effect on the beating heart (6,10,14). Notably, the absence of major effects of dantrolene on SR Ca\(^{2+}\) release in the heart is consistent with the possibility that dantrolene may act selectively on the RYR1 but not the RYR2 channel isoform (15). At the same time, it is also clear that dantrolene’s effects on Ca\(^{2+}\) release from intracellular stores are not strictly limited to skeletal muscle, but extend to certain non-muscle cells, including central neurons (16-19). The basis of dantrolene’s effects in non-muscle cells remains unclear,
however, due to uncertainty regarding the precise molecular mechanism of dantrolene inhibition, and the selectivity of this mechanism for different Ca\textsuperscript{2+} release channel isoforms.

In a previous report, we demonstrated that dantrolene acts directly on the RYR1 to reduce the extent of channel activation by calmodulin (CaM), and thereby decrease the Ca\textsuperscript{2+} sensitivity of channel activation (15). Here we further define the mechanism and isoform selectivity of dantrolene as an inhibitor of RYR channels.

**EXPERIMENTAL PROCEDURES**

MHS pigs homozygous for the RYR1 Arg\textsuperscript{615}→Cys mutation were obtained from the University of Minnesota Experimental Farm; normal control animals were obtained from commercial suppliers. Skeletal muscle SR vesicles were prepared from longissimus dorsi muscle of MHS and normal pigs, and cardiac SR vesicles were prepared from porcine ventricular tissue, as described previously (20,21). All isolation buffers contained a mixture of protease inhibitors (100 nM aprotinin, 1 µM leupeptin, 1 µM pepstatin, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride). [\textsuperscript{3}H]Ryanodine was purchased from NEN Life Science Products (Boston, MA), and non-radioactive ryanodine was purchased from Calbiochem (La Jolla, CA). Dantrolene, porcine brain CaM, and AMPPCP (a nonhydrolyzable ATP analog) were from Sigma. Azumolene (a water soluble analog of dantrolene) was manufactured by Proctor & Gamble (Norwich, NY) and provided by Dr. Esther Gallant (University of Minnesota). Dantrolene stock solutions (1 mM) were prepared fresh every 3-5 days in 50% methanol (0.5% methanol final concentration) and stored in the dark at room temperature.

Cloning and sequencing of the full-length cDNA encoding the mouse cardiac RYR2 (22) and the rabbit uterus RYR3 (23) have been described previously. RYR clones were transiently expressed in HEK-293 cells following transfection by Ca\textsuperscript{2+} phosphate precipitation. HEK-293 whole-cell lysates were prepared as described (24) in buffer containing 137 mM NaCl, 25 mM Tris/HEPES (pH 7.4), 1% CHAPS, and 0.5% phosphatidyl choline. [\textsuperscript{3}H]Ryanodine binding to SR vesicles (2 mg/ml) or HEK-293 cell lysates (1.4-1.9 mg/ml) was determined following ~10 min preincubations in 37\textdegree C media containing 120 mM K-propionate, 10 mM PIPES (pH 7.0), 2 mM Na\textsubscript{2}AMPPCP, 1 µM CaM, ± 10 µM dantrolene or methanol vehicle. Following addition of
[^3]H]ryanodine (20 nM), samples were incubated 90 min at 37°C, then collected on Whatman GFC filters with 3X 3 ml washes with ice-cold 100 mM KCl. RYR expression levels in HEK-293 cells were quantitated via determinations of the maximal[^3]H]ryanodine binding capacity of cell lysates in media containing 600 mM KCl, 10 mM Na2ATP, and 100 µM Ca2+ (0.01 ± 0.009 pmol/mg for mock-transfected controls; 0.85 ± 0.06 pmol/mg for RYR2-expressing cells; 0.68 ± 0.5 pmol/mg for RYR3-expressing cells). Data were corrected for nonspecific binding determined in the presence of 100-fold excess nonradioactive ryanodine. Concentrations of ionized Ca2+ were obtained using calcium acetate-EGTA buffers (25).

RESULTS

**Dantrolene alters the **K_d** of[^3]H]ryanodine binding to the MHS and normal RYR1** — Previous results from our laboratory demonstrated that dantrolene inhibition of skeletal muscle SR vesicle Ca2+ release was associated with reduced levels of[^3]H]ryanodine binding to the isolated RYR1 (15), and thus indicated that dantrolene may act directly at the RYR1 to inhibit channel activation. Other laboratories, however, have proposed that dantrolene’s effects on SR Ca2+ release may be mediated via its binding to non-RYR proteins (26,27), and have questioned whether the magnitude of dantrolene’s action on[^3]H]ryanodine binding in vitro may adequately explain this drug’s clinical effects. The current experiments therefore sought to further define the mechanism and selectivity of dantrolene’s action on[^3]H]ryanodine binding, and to determine if this mechanism might reasonably account for the in vivo actions of dantrolene in reversing MH. The experiments shown in Fig. 1 compared the effects of dantrolene and the pig MHS mutation on the **K_d** and **B_max** of[^3]H]ryanodine binding to skeletal muscle SR vesicles. The concentration of dantrolene in these experiments was 10 µM, a concentration which approximates therapeutic drug levels in vivo (28), and at which the inhibition of SR vesicle[^3]H]ryanodine binding by dantrolene was maximal (15). In the absence of dantrolene, the **K_d** for[^3]H]ryanodine binding to MHS SR vesicles was approximately one-third of that for normal SR vesicles (Table I), consistent with the magnitude of the MHS mutation’s effect on[^3]H]ryanodine binding affinity documented previously (20). In comparison, the **K_d** for[^3]H]ryanodine binding to both MHS and normal SR vesicles was increased ~3-fold in the presence of dantrolene. Neither
the MHS mutation nor dantrolene significantly altered the $B_{\text{max}}$ of SR vesicle $[^3\text{H}]$ryanodine binding. Consequently, $[^3\text{H}]$ryanodine binding to MHS SR vesicles in the presence of dantrolene was essentially equivalent to that of normal SR vesicle $[^3\text{H}]$ryanodine binding in the absence of dantrolene over the range of ryanodine concentrations examined (Fig. 1). These results thus demonstrate that dantrolene inhibition of the RYR1 was associated with a reduced affinity of the channel for $[^3\text{H}]$ryanodine, and further indicate that the magnitude of dantrolene’s effect on $[^3\text{H}]$ryanodine binding was comparable to that of the MHS Arg$^{615}$$\rightarrow$Cys mutation.

**Effect of dantrolene on RYR1 sensitivity to caffeine, Mg$^{2+}$, and Sr$^{2+}$—**Previous results demonstrated that dantrolene decreased the sensitivity of the isolated RYR1 to activation by Ca$^{2+}$ (15), and thereby suggested that a reduced affinity of Ca$^{2+}$ binding to RYR1 activation sites may constitute the basis of dantrolene inhibition of Ca$^{2+}$ release in skeletal muscle. To further examine this possibility, we determined the effect of dantrolene on the RYR1’s sensitivity to other effectors known to modulate RYR activation by Ca$^{2+}$. Caffeine, for example, is known to activate by increasing the Ca$^{2+}$ sensitivity of RYR channels (29). Furthermore, a reduced threshold for caffeine activation is characteristic of MHS RYR1 mutations at diverse sites within the primary sequence of this channel protein (12). Accordingly, Fig. 2 indicates that the threshold for caffeine activation of $[^3\text{H}]$ryanodine binding to MHS SR was decreased relative to that of normal SR, and the EC$_{50}$ for caffeine activation of MHS SR was reduced by approximately one-half (Table II). Conversely, dantrolene (10 µM) shifted the caffeine threshold for activation of $[^3\text{H}]$ryanodine binding to higher caffeine concentrations, and increased the EC$_{50}$ for caffeine activation of both MHS and normal SR vesicles by $\geq$ 2-fold. Dantrolene thus reduced the apparent caffeine sensitivity of the RYR1, and opposed the effect of the MHS mutation on caffeine activation of the channel.

Mg$^{2+}$ is an endogenous inhibitor of RYR channels that acts by competing with Ca$^{2+}$ at channel activation sites (30,31). Consequently, the physiologic significance of any changes in RYR1 Ca$^{2+}$ sensitivity in the presence of dantrolene will depend on whether dantrolene also affects corresponding changes in the Mg$^{2+}$ sensitivity of the channel. To better understand how dantrolene may influence the selectivity of RYR1 activation sites for Ca$^{2+}$ relative to Mg$^{2+}$, we determined the Mg$^{2+}$ sensitivity of $[^3\text{H}]$ryanodine binding in media containing 100 nM Ca$^{2+}$ (Fig. 3A). In the absence of dantrolene, $[^3\text{H}]$ryanodine binding to MHS SR vesicles was slightly less
sensitive to inhibition by MgCl$_2$ than was normal SR (IC$_{50}$ for MHS SR increased 23%, $p = 0.03$; Table II). Dantrolene, however, did not significantly alter the IC$_{50}$ for MgCl$_2$ inhibition of $[^{3}H]$ryanodine binding to either MHS or normal SR vesicles.

Whereas Mg$^{2+}$ competitively blocks RYR1 activation by Ca$^{2+}$, Sr$^{2+}$ ions may replace Ca$^{2+}$ in activating RYR channels (31). Accordingly, results in Fig. 3B indicate that SR vesicle $[^{3}H]$ryanodine binding was activated by Sr$^{2+}$, and that the EC$_{50}$ for activation was significantly decreased for MHS SR vesicles (Table II). Furthermore, dantrolene significantly increased the EC$_{50}$ for Sr$^{2+}$. These results are thus in agreement with dantrolene’s effect on the Ca$^{2+}$ sensitivity of the RYR1 (15), but are in contrast to the lack of any effect of dantrolene on RYR1 sensitivity to Mg$^{2+}$ (Fig. 3B). Together, our results therefore support the possibility that dantrolene inhibits SR Ca$^{2+}$ release in situ by reducing the sensitivity of the RYR1 to activation by Ca$^{2+}$, and further indicate that this mechanism may operate independently of any effect of dantrolene on the Mg$^{2+}$ sensitivity of the channel.

Dantrolene inhibition is dependent on adenine nucleotide—Ohta and coworkers originally noted that dantrolene’s effect on skinned muscle fibers were more pronounced when media contained ATP (32); and more recently, Palnitkar and coworkers reported that adenine nucleotides enhanced the binding of $[^{3}H]$dantrolene to SR vesicles (33). To better define the role of adenine nucleotides in the mechanism of RYR1 inhibition by dantrolene, we examined the effect of a non-hydrolysable ATP analog (AMPPCP) on the inhibition of skeletal muscle SR vesicle $[^{3}H]$ryanodine binding by dantrolene (Fig. 4). Initial experiments compared the CaM-dependent activation of MHS SR vesicle $[^{3}H]$ryanodine binding in the presence and absence of AMPPCP. Results in Fig. 4A show that SR vesicle $[^{3}H]$ryanodine binding was dependent on both AMPPCP and CaM. Thus, in the presence of AMPPCP (2 mM), dantrolene decreased by one-third the extent of CaM-activated $[^{3}H]$ryanodine binding to MHS SR vesicles. In contrast, dantrolene did not significantly inhibit $[^{3}H]$ryanodine binding when AMPPCP was omitted from the binding media (Fig. 4A). This loss of dantrolene inhibition was confirmed in determinations of $K_d$ and $B_{max}$ values for $[^{3}H]$ryanodine binding in the AMPPCP-free media (Fig. 4C; Table I). A comparison of the dose dependence of dantrolene inhibition at different [AMPPCP] also indicated that RYR1 inhibition was strictly dependent on adenine nucleotide (Fig. 4C); however, neither the extent nor the concentration dependence of dantrolene inhibition was altered when
the concentration of nucleotide was increased from 1 to 4 mM (IC$_{50}$ for dantrolene = 129 ± 48 nM versus 112 ± 32 nM, respectively).

Effect of dantrolene on the RYR2 and RYR3 isoforms—Possible effects of dantrolene on the $K_d$ of [$^3$H]ryanodine binding to cardiac SR vesicles were investigated in media that contained either 100 nM or 300 nM Ca$^{2+}$ (Fig. 5). In 100 nM Ca$^{2+}$-containing media, neither $K_d$ nor $B_{max}$ determinations (Table I) were significantly affected by dantrolene (10 µM), whereas in the same media dantrolene increased the $K_d$ of [$^3$H]ryanodine binding to skeletal muscle SR vesicles 3-fold (Fig. 1). When Ca$^{2+}$ was increased to 300 nM, the affinity of [$^3$H]ryanodine binding to cardiac SR vesicles was increased (~7-fold); however, dantrolene again had no effect on either $K_d$ or $B_{max}$ determinations. These results thus demonstrate that, in comparison to the RYR1, the RYR2 isoform in cardiac SR vesicles was insensitive to clinical concentrations of dantrolene.

To further investigate the isoform selectivity of dantrolene as an inhibitor of RYR channels, we also examined [$^3$H]ryanodine binding to recombinant RYRs heterologously expressed in HEK-293 cells (22). HEK-293 cells provide a valuable system for the functional expression of the different RYR isoforms, as any endogenous RYRs in these cells are expressed only at very low levels (34). Accordingly, Fig. 6A shows that the [$^3$H]ryanodine binding activity of lysates prepared from mock-transfected HEK-293 cells was low (5 fmol/mg protein), and was not significantly affected by dantrolene (10 µM). [$^3$H]Ryanodine binding to RYR2-transfected HEK-293 cell lysates was increased ~20-fold relative to mock-transfected controls. Moreover, [$^3$H]ryanodine binding to the recombinant RYR2 in these cells was unaffected by dantrolene (Fig. 6A), consistent with the results obtained using cardiac SR vesicles (Fig 5). In comparison, [$^3$H]ryanodine binding to lysates prepared from RYR3-expressing cells was significantly inhibited by dantrolene ($p < 0.001$). Furthermore, the magnitude of dantrolene’s effect on the recombinant RYR3 (60% of control [$^3$H]ryanodine binding in the presence of dantrolene) was comparable to dantrolene’s effect on the RYR1 in native SR vesicles (Fig. 1 and data not shown). These results therefore demonstrate that a cell’s sensitivity to dantrolene may be determined by the RYR isoform(s) expressed, and identify the RYR3 isoform as a target for dantrolene action.

Dantrolene inhibition of the recombinant RYR3 isoform was further characterized to investigate whether a similar mechanism may explain dantrolene’s effects on the RYR1 and
RYR3 isoforms. The Ca$^{2+}$ dependence of $[^3]$Hryanodine binding to HEK-cell lysates containing heterologously expressed RYR3 (Fig. 6B) indicated that dantrolene inhibition of RYR3 was most pronounced at ~100 nM Ca$^{2+}$. In the presence of dantrolene (10 µM), the EC$_{50}$ for Ca$^{2+}$ activation of $[^3]$Hryanodine binding was increased 2.3-fold (from 97.8 ± 37 nM to 229 ± 48 nM). Although this increase in the Ca$^{2+}$ EC$_{50}$ for RYR3 did not reach statistical significance ($p = 0.07$), the magnitude of the effect was comparable to that previously documented for dantrolene inhibition of the RYR1 (~2.5-fold increase in Ca$^{2+}$ EC$_{50}$ for RYR1 in the presence of 10 µM dantrolene, reference 15). Furthermore, results in Fig. 6C indicated that $[^3]$Hryanodine binding to RYR3 was also inhibited by azumolene (10 µM), a dantrolene analog known to inhibit the RYR1 isoform (33,15). Finally, as for the RYR1 (15), RYR3 inhibition by dantrolene was also abolished when the temperature in the $[^3]$Hryanodine binding media was reduced to 20°C, or when AMPPCP was omitted from the binding media (Fig. 6C). These results thus indicate that dantrolene inhibition of the RYR3 isoform exhibited properties similar to those previously demonstrated for the dantrolene inhibition of the RYR1 isoform in SR vesicles (15; Fig. 4).

**DISCUSSION**

Despite dantrolene’s importance both in the treatment of MH, and as a pharmacologic probe of Ca$^{2+}$ release from intracellular stores, the molecular basis and specificity of dantrolene’s actions on RYR channels have remained uncertain. In this study, we have used $[^3]$Hryanodine binding to further characterize the mechanism by which dantrolene may selectively inhibit the RYR1 but not the RYR2 channel isoform, and to determine whether the RYR3 isoform may also be a target for dantrolene action.

*Dantrolene opposes increased RYR1 activity resulting from the MHS Arg$^{615}$→Cys mutation*—Although dantrolene is only a partial inhibitor of the isolated RYR1, the current results clearly demonstrate that the magnitude of dantrolene’s effect on the functional activity of this channel is comparable to the effect of the MHS RYR1 Arg$^{615}$→Cys mutation. Thus, dantrolene effectively opposed the ~3-fold increase in the affinity of SR vesicle $[^3]$Hryanodine binding resulting from the MHS mutation (Fig. 1). Dantrolene similarly opposed the ~2-fold increase in RYR1 caffeine
sensitivity resulting from the MHS mutation (Fig. 2). Increased RYR1 caffeine sensitivity is diagnostic for human MHS mutations at diverse sites in the primary sequence of the channel protein (12), and this result is therefore consistent with dantrolene’s general efficacy in the treatment of this genetically heterogeneous disorder. The basis of the increased caffeine sensitivity of MHS channels is uncertain (29), but is unlikely to reflect actual effects of the various mutations on the affinity of caffeine binding to the RYR1 (35). Rather, increased caffeine sensitivity may indicate that MHS mutations, like caffeine, act by reducing the threshold for RYR1 activation by Ca$^{2+}$ (29). In this view, the observed 2- to 3-fold increase in the caffeine EC$_{50}$ in the presence of dantrolene is consistent with the 2- to 3-fold shifts in the sensitivity of MHS and normal channels to both Ca$^{2+}$ (15) and Sr$^{2+}$ (Fig. 3B) in the presence of dantrolene.

Dantrolene alters selectivity of RYR1 activation sites for Ca$^{2+}$ relative to Mg$^{2+}$—Physiologic Mg$^{2+}$ concentrations (~1 mM) may fully block RYR1 activation by Ca$^{2+}$ (30,36) and this suggests that dynamic changes in this channel’s affinity for either Ca$^{2+}$ or Mg$^{2+}$ may be a requisite step for channel activation in situ (29,36). According to the model of Lamb and Stephenson, for example, RYR1 activation during EC coupling is dependent on a decrease in the Mg$^{2+}$ affinity of the channel, mediated via the coupling of RYRs to transverse tubule voltage sensors (37). It is also possible, however, that the Ca$^{2+}$ affinity of RYR1 activation sites might be modulated independently of major changes in the channel’s sensitivity to [Mg$^{2+}$]. In light of dantrolene’s effects both on EC coupling in intact muscle (7,8) and on the Ca$^{2+}$ sensitivity of the isolated RYR1, we examined the potential role of altered Mg$^{2+}$ sensitivity in the mechanism of dantrolene inhibition. Our results (Fig. 3) indicate that dantrolene inhibition of SR vesicle [$^3$H]ryanodine binding was not associated with an altered IC$_{50}$ for Mg$^{2+}$ (Table II). Because this inhibition reflects the competitive binding of Mg$^{2+}$ to RYR1 Ca$^{2+}$ sites (30,31), our results indicate that dantrolene’s effect on the apparent Ca$^{2+}$ affinity of RYR1 activation sites (15) is not associated with corresponding changes in the affinity of these same sites for Mg$^{2+}$. Similarly, Murayama and coworkers (38) recently concluded that the effect of caffeine on the Ca$^{2+}$ affinity of RYR activation sites was also not associated with changes in the affinity of Mg$^{2+}$ binding to these sites. Thus, in intact muscle, both dantrolene and caffeine may modulate the Ca$^{2+}$ sensitivity of the RYR1 via mechanisms that operate independently of any changes in the channel’s affinity for Mg$^{2+}$. 
Isoform-specific action of dantrolene on RYR channels—Accumulating evidence now supports a model in which dantrolene’s effects on SR Ca\(^{2+}\) release in skeletal muscle may be explained by the direct binding of dantrolene to the RYR1 channel protein, without invoking putative non-RYR dantrolene receptors (26). Accordingly, purified, solubilized preparations of the RYR1 channel protein retain sensitivity to dantrolene (15), and crosslinking experiments have identified the RYR1 as the major SR protein labeled with a photoaffinity dantrolene analogue (33). Nonetheless, the explanation for the dantrolene insensitivity of EC coupling in cardiac muscle has remained uncertain. For example, cardiac insensitivity to dantrolene might potentially be explained by a difference in the dantrolene binding properties of the RYR2 isoform itself, or by some cardiac-specific modification of the RYR2 protein, or by other differences in the molecular machinery that controls SR Ca\(^{2+}\) release in cardiac as compared to skeletal muscle. We therefore investigated possible effects of dantrolene on the RYR2 isoform heterologously expressed in a non-muscle cell (22). Our results (Fig. 6A) show that the RYR2 expressed in HEK-293 cells remained insensitive to dantrolene. In comparison, the RYR3 isoform expressed in the same cell type was significantly inhibited by dantrolene. These results indicate that the RYR2 itself is intrinsically insensitive to dantrolene, and thus suggest that this isoform may lack a high-affinity dantrolene site that is present in both the RYR1 and the RYR3 isoforms. We conclude that the absence of major effects of dantrolene on SR Ca\(^{2+}\) release in the heart is likely a simple function of the predominant expression of the RYR2 channel isoform in cardiac muscle.

The insensitivity of the cardiac RYR2 to dantrolene is associated with other notable differences in the regulation of this channel isoform. Thus, in comparison to both the RYR1 and the RYR3 isoforms, the RYR2 isoform is less responsive to activation by adenine nucleotide (21,24) and CaM (39,23). Recently, we reported that CaM together with adenine nucleotide activates the RYR1 by increasing the Ca\(^{2+}\) sensitivity of the channel (39). Conversely, dantrolene inhibits the RYR1 by reducing Ca\(^{2+}\) sensitivity, via a mechanism that is dependent on both adenine nucleotide and CaM. Thus, we postulate that dantrolene’s selective action on the RYR1 and RYR3 may in effect oppose the nucleotide- and CaM-dependent activation of these channel isoforms.
Dantrolene effects in non-muscle cells—The identification of the RYR3 as a target for dantrolene suggests that this more broadly-expressed channel isoform may potentially underlie dantrolene effects in various non-muscle tissues and cell types. In this regard, effects of dantrolene on Ca\textsuperscript{2+} signaling in central neurons are of particular interest. For example, dantrolene has been shown to inhibit elevations of neuronal Ca\textsuperscript{2+} evoked by NMDA, glutamate, or potassium depolarization (16). Moreover, dantrolene may protect central neurons from disruptions in Ca\textsuperscript{2+} homeostasis resulting from ischemic injury (16,17), epileptic seizure (18), or exposure to amyloid β–peptide (19). CNS effects of dantrolene are also suggested by reports that subjects treated with the drug may experience dizziness, blurred vision, and fatigue (40). However, resolving the molecular targets that underlie dantrolene’s effects on central neurons is difficult, as all three RYR isoforms are expressed in the brain, and multiple isoforms may be present within a single cell type (41,42,43). Yet notably, the predominant RYR in the brain as a whole is RYR2 (41,42), whereas our results indicate that RYR1 and RYR3—but not RYR2—may be targets for dantrolene. In light of our results, it may now be of interest to define the specific RYR isoforms may be responsible for dantrolene’s various effects on Ca\textsuperscript{2+} signaling in different neuronal cells. Regardless, it is clear that understanding the molecular basis of dantrolene’s effects on intracellular Ca\textsuperscript{2+} release channels may have implications that extend beyond skeletal muscle and MH, to diverse cell types and disease states.

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REFERENCES


FIGURE LEGENDS

Fig. 1. Scatchard analysis of \(^{3}\)Hryanodine binding to MHS and normal skeletal muscle SR vesicles in the presence or absence of dantrolene. Binding of \(^{3}\)Hryanodine (10-500 nM) to skeletal muscle SR vesicles was determined in the presence (filled symbols) or absence (open symbols) of 10 µM dantrolene in media containing 120 mM K-propionate, 10 mM PIPES (pH 7.0), 2 mM Na2AMPPCP, 1 µM CaM, and 100 nM Ca\(^{2+}\). Inset shows fits of the data to single rectangular hyperbola used for determinations of \(K_{d}\) and \(B_{max}\) (Table I). Data are means ± SE from five independent experiments comparing four MHS (squares) and four normal (circles) skeletal muscle SR vesicle preparations.

Fig. 2. Effect of dantrolene on the activation of skeletal muscle SR vesicle \(^{3}\)Hryanodine binding by caffeine. \(^{3}\)HRyanodine binding in the absence or presence of 10 µM dantrolene was determined in media containing 120 mM K-propionate, 10 mM PIPES (pH 7.0), 2 mM AMPPCP, 1.7 mM MgCl\(_2\), 1 µM CaM, and 100 nM Ca\(^{2+}\). Inset shows the data normalized to the maximal activation determined in the presence of 48 mM caffeine. Solid lines in figure and inset represent fits to the Hill equation. Data are means ± SE from four experiments comparing four MHS and three normal SR vesicle preparations.

Fig. 3. Effect of dantrolene on RYR1 sensitivity to Mg\(^{2+}\) and Sr\(^{2+}\). \(^{3}\)HRyanodine binding in the absence or presence of 10 µM dantrolene was determined in media containing 120 mM K-propionate, 10 mM PIPES (pH 7.0), 2 mM AMPPCP, and 1 µM CaM. The Mg\(^{2+}\) dependence of \(^{3}\)Hryanodine binding (A) was determined in the presence of 100 nM Ca\(^{2+}\); the Sr\(^{2+}\) dependence of \(^{3}\)Hryanodine binding (B) was determined in the presence of < 10 nM Ca\(^{2+}\). Insets at right show normalized data to emphasize effects of the MH mutation and dantrolene on RYR1 sensitivity to the divalent cations. Data are means ± SE from four experiments comparing four MHS and three normal skeletal muscle SR vesicle preparations.
Fig. 4. Effect of AMPPCP on the inhibition of skeletal muscle SR vesicle $[^3\text{H}]$ryanodine binding by dantrolene (10 μM).  A, CaM activation of SR vesicle $[^3\text{H}]$ryanodine binding determined in the absence (squares) or presence (circles) of 2 mM AMPPCP. Media also contained 120 mM K-propionate, 10 mM PIPES (pH 7.0), 100 nM Ca$^{2+}$, and 10 μM dantrolene, as indicated.  B, Ryanodine dependence of skeletal SR vesicle $[^3\text{H}]$ryanodine binding in the presence (filled symbols) or absence (open symbols) of 10 μM dantrolene, was determined as in Fig. 1, except that media lacked AMPPCP.  C, Dose dependence of dantrolene inhibition of $[^3\text{H}]$ryanodine binding at different concentrations of AMPPCP (1 μM CaM, 100 nM Ca$^{2+}$ throughout).  Data are means ± SE from three different MHS skeletal muscle SR vesicle preparations.

Fig. 5. Effect of dantrolene on cardiac SR vesicle $[^3\text{H}]$ryanodine binding. Binding of $[^3\text{H}]$ryanodine (10-500 nM) to cardiac SR vesicles in the presence or absence of 10 μM dantrolene was determined as in Fig. 1, in media containing either 100 nM Ca$^{2+}$ or 300 nM Ca$^{2+}$, as indicated.  Inset shows fits of the data to single rectangular hyperbola for determinations of $K_d$ and $B_{\text{max}}$ (Table I).  Data are means ± SE from four cardiac SR vesicle preparations.

Fig. 6. Effect of dantrolene on $[^3\text{H}]$ryanodine binding to the RYR2 and RYR3 isoforms in heterologously expressed in HEK-293 cells.  A, Comparison of $[^3\text{H}]$ryanodine binding to lysates prepared from HEK-293 cells mock-transfected with pCDNA vector, with binding to lysates from cells transiently expressing either RYR2 or RYR3.  $[^3\text{H}]$Ryanodine binding was determined in the presence or absence of dantrolene (10 μM) as described under “Experimental Procedures”.  B, Ca$^{2+}$ dependence of $[^3\text{H}]$ryanodine binding to the expressed RYR3 in the presence ( ) or absence ( ) of 10 μM dantrolene. Solid lines represent fits to the Hill equation (Ca$^{2+}$ EC$_{50}$ values given in the text).  C, Dantrolene inhibition of RYR3: effect of dantrolene analog, temperature, and adenine nucleotide. $[^3\text{H}]$Ryanodine binding was determined as in panels A and B, except that azumolene (10 μM) was substituted for dantrolene, or the temperature of the binding media was reduced to 20°C, or the binding media lacked AMPPCP, as indicated.  Means ± SE from 3-9 independent experiments.  Asterisks indicate significant differences from control binding in the absence of drug ($p < 0.05$, students $t$-test).
Table I. *Effect of dantrolene on SR vesicle[^H]ryanodine binding*

[^H]Ryanodine binding was determined as described under “Experimental Procedures” in the absence or presence of 10 µM dantrolene. Media contained 120 mM K-propionate, 10 mM PIPES (pH 7.0), 2 mM Na₂AMPPCP, 1 µM CaM, and 100 nM Ca²⁺, except as otherwise indicated at left in the table. *K*ₐ and *B*ₘₐₓ determinations are based on fits of the data presented in Figs. 1, 4B, and 5 to a single rectangular hyperbola.  aSignificantly different from control value in the absence of dantrolene (*p* < 0.05, student’s *t*-test).  bSignificantly different from corresponding value for normal SR.

<table>
<thead>
<tr>
<th></th>
<th>Kₐ (nM)</th>
<th>Bₘₐₓ (pmol/mg)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal SR</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>112 ± 8.5</td>
<td>11.9 ± 0.3</td>
<td>4</td>
</tr>
<tr>
<td>Dantrolene</td>
<td>303 ± 27</td>
<td>11.4 ± 0.5</td>
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<tr>
<td><strong>MHS SR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>32 ± 3.3</td>
<td>10.9 ± 0.3</td>
<td>4</td>
</tr>
<tr>
<td>Dantrolene</td>
<td>93 ± 6.3</td>
<td>10.4 ± 0.3</td>
<td>4</td>
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<tr>
<td><strong>MHS SR - AMPPCP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>419 ± 151</td>
<td>10.0 ± 2.1</td>
<td>3</td>
</tr>
<tr>
<td>Dantrolene</td>
<td>317 ± 61</td>
<td>8.8 ± 0.9</td>
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<tr>
<td><strong>Cardiac SR</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>1020 ± 920</td>
<td>3.1 ± 2.1</td>
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<tr>
<td>Dantrolene</td>
<td>640 ± 430</td>
<td>2.1 ± 0.4</td>
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<tr>
<td><strong>Cardiac SR + 300 nM Ca²⁺</strong></td>
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<tr>
<td>Control</td>
<td>141 ± 18</td>
<td>3.0 ± 0.2</td>
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<tr>
<td>Dantrolene</td>
<td>172 ± 45</td>
<td>3.0 ± 0.3</td>
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</tbody>
</table>
Table II. Effect of dantrolene on RYRI sensitivity to caffeine, Mg$^{2+}$, and Sr$^{2+}$

[$^3$H]Ryanodine binding to skeletal muscle SR vesicles was determined as described under “Experimental Procedures” in the absence or presence of 10 µM dantrolene. Values for EC$_{50}$, IC$_{50}$, and Hill coefficients ($n_H$) are based on fits of the data presented in Figs. 2 and 3 to the Hill equation. $^a$Significantly different from control value in the absence of dantrolene ($p < 0.05$, student’s t-test). $^b$Significantly different from corresponding value for normal SR.

<table>
<thead>
<tr>
<th></th>
<th>Normal SR</th>
<th></th>
<th>MHS SR</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$EC_{50}$ caffeine (mM)</td>
<td>$n_H$</td>
<td>$EC_{50}$ caffeine (mM)</td>
<td>$n_H$</td>
</tr>
<tr>
<td>Control</td>
<td>8.0 ± 0.4</td>
<td>1.7 ± 0.1</td>
<td>3.8 ± 0.2$^b$</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Dantrolene</td>
<td>24.6 ± 2.6$^a$</td>
<td>1.2 ± 0.1</td>
<td>7.7 ± 0.6$^{a,b}$</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>$IC_{50}$ MgCl$_2$ (µM)</td>
<td>$n_H$</td>
<td>$IC_{50}$ MgCl$_2$ (µM)</td>
<td>$n_H$</td>
</tr>
<tr>
<td>Control</td>
<td>309 ± 10</td>
<td>1.1 ± 0.1</td>
<td>381 ± 22$^b$</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Dantrolene</td>
<td>304 ± 44</td>
<td>1.0 ± 0.1</td>
<td>337 ± 22</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>$EC_{50}$ SrCl$_2$ (µM)</td>
<td>$n_H$</td>
<td>$EC_{50}$ SrCl$_2$ (µM)</td>
<td>$n_H$</td>
</tr>
<tr>
<td>Control</td>
<td>1.2 ± 0.04</td>
<td>1.3 ± 0.1</td>
<td>0.42 ± 0.02$^b$</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Dantrolene</td>
<td>2.5 ± 0.16$^a$</td>
<td>1.1 ± 0.1</td>
<td>0.95 ± 0.09$^{a,b}$</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>
FIGURE 1
FIGURE 2

[Graph showing the relationship between caffeine concentration (mM) and [H]ryanodine binding (pmol/mg). The graph compares MHS SR, MHS + dantrolene, Normal SR, and Normal + dantrolene conditions.}

[Normalized binding graph on the right side of the figure.]

Legend:
- □ MHS SR
- ■ MHS + dantrolene
- ○ Normal SR
- ● Normal + dantrolene
FIGURE 3

A

\[ ^\text{3H} \text{Ryanodine Bound} \text{(pmol/mg)} \]

\[ \text{MgCl}_2 \text{ (mM)} \]

- MHS SR
- MHS + dantrolene
- Normal SR
- Normal + dantrolene

B

\[ ^\text{3H} \text{Ryanodine Bound} \text{(pmol/mg)} \]

\[ \text{SrCl}_2 \text{ (\mu M)} \]

- MHS SR
- MHS + dantrolene
- Normal SR
- Normal + dantrolene
FIGURE 4

A

\[ {}^3\text{H} \text{Ryanodine Bound (pmol/mg)} \]

\[ \text{CaM (nM)} \]

+ AMPPCP - Dan
+ AMPPCP + Dan
- AMPPCP + Dan

B

\[ {}^3\text{H} \text{Ryanodine Bound (pmol/mg)} \]

\[ \text{Ryanodine (nM)} \]

- AMPPCP

- Dan
+ Dan

C

\[ {}^3\text{H} \text{Ryanodine Bound (pmol/mg)} \]

\[ \text{Dantrolene (µM)} \]

+4 mM AMPPCP
+1 mM AMPPCP
- AMPPCP
FIGURE 5

[Graph showing the binding of [3H]Ryanodine with different concentrations of Ca^2+ and the effect of Dan treatment.]
Dantrolene inhibition of ryanodine receptor Ca\textsuperscript{2+} release channels: molecular mechanism and isoform selectivity
Fangyi Zhao, Pin Li, S.R. Wayne Chen, Charles F. Louis and Bradley R. Fruen

J. Biol. Chem. published online February 5, 2001

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