Dantrolene Stabilizes Domain Interactions within the Ryanodine Receptor*

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Shigeki Kobayashi‡, Mark L. Bannister‡, Jaya P. Gangopadhyay‡, Tomoyo Hamada‡, Jerome Parness‡, and Noriaki Ikemoto‡

From the §Boston Biomedical Research Institute, Watertown, Massachusetts 02472, the §Departments of Anesthesia, Pharmacology, Pediatrics, and Physiology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854, and the ¶Department of Neurology, Harvard Medical School, Boston, Massachusetts 02115

Interdomain interactions between N-terminal and central domains serving as a “domain switch” are believed to be essential to the functional regulation of the skeletal muscle ryanodine receptor-1 Ca\(^{2+}\) channel. Mutational destabilization of the domain switch in malignant hyperthermia (MH), a genetic sensitivity to volatile anesthetics, causes functional instability of the channel. Dantrolene, a drug used to treat MH, binds to a region within this proposed domain switch. To explore its mechanism of action, the effect of dantrolene on MH-like channel activation by the synthetic domain peptide DP4 or anti-DP4 antibody was examined. A fluorescence probe, methylcoumarin acetate, was covalently attached to the domain switch using DP4 as a delivery vehicle. The magnitude of domain unzipping was determined from the accessibility of methylcoumarin acetate to a macromolecular fluorescence quencher. The Stern-Volmer quenching constant (K\(_{SV}\)) increased with the addition of DP4 or anti-DP4 antibody. This increase was reversed by dantrolene at both 37 and 22 °C and was unaffected by calmodulin. \(^{1}\)H\(^{-}\)ryanodine binding to the sarcoplasmic reticulum and activation of sarcoplasmic reticulum Ca\(^{2+}\) release, both measures of channel activation, were enhanced by DP4. These activities were inhibited by dantrolene at 37 °C, yet required the presence of calmodulin at 22 °C. These results suggest that the mechanism of action of dantrolene involves stabilization of domain-domain interactions within the domain switch, preventing domain unzipping-induced channel dysfunction. We suggest that temperature and calmodulin primarily affect the coupling between the domain switch and the downstream mechanism of regulation of Ca\(^{2+}\) channel opening rather than the domain switch itself.

Dantrolene (hydrated 1-(5-(4-nitrophenyl)-2-furanyl)methylene)amino)-2,4-imidazolidinedione sodium salt) is an intracellularly acting skeletal muscle relaxant used for the treatment of malignant hyperthermia (MH). MH is a potentially deadly, pharmacogenetically mediated, hypermetabolic response to volatile anesthetics that results from unregulated intramyoplasmic Ca\(^{2+}\) release (1). The drug is known to inhibit excitation-contraction coupling of skeletal muscle (2) by suppressing depolarization-induced sarcoplasmic reticulum (SR) Ca\(^{2+}\) release in normal and MH-susceptible skeletal muscle without affecting voltage sensor activation (3). In MH, the voltage dependence of contractile activation is shifted to lower voltages (4), whereas in the presence of clinical concentrations of dantrolene, i.e. 10 \(\mu\)M (5), the voltage dependence of contractile activation is shifted to higher voltages (6, 7). Normalization of the voltage dependence of contractile activation may therefore be one of the important components of the therapeutic action of dantrolene. Dantrolene also confers a normal Mg\(^{2+}\) sensitivity to MH-susceptible muscle fibers, which would otherwise show a considerably reduced sensitivity to the normal inhibitory action of myoplasmic Mg\(^{2+}\) on the SR Ca\(^{2+}\) release mechanism (8, 9). Conferring normal Mg\(^{2+}\) sensitivity to mutated ryanodine receptor (RyR1) may be another key component of dantrolene therapeuticus in MH.

Extensive studies have been carried out to examine the effect of dantrolene on the function of isolated skeletal muscle SR. It has been shown that both dantrolene and its equipotent, water-soluble analog azumolene suppress SR Ca\(^{2+}\) release induced by Ca\(^{2+}\) and various pharmacological agents (3, 10–12). Although dantrolene has been shown to suppress the ryanodine binding activity of the SR (13, 14), this finding is not universal (15). Dantrolene (1–5 \(\mu\)M) has been reported to have a biphasic effect on the open probability of RyR1 channels in lipid bilayers, first activating and then blocking at nanomolar concentrations (16), but others have not been able to see any effect of this drug on single channels (3). Importantly, dantrolene has been shown to at least partially restore the normal properties of RyR1 Ca\(^{2+}\) channels in SR isolated from MH-susceptible pigs (1, 17, 18). Thus, these studies together suggest that dantrolene interacts with the RyR to suppress the channel dysfunction that occurs with MH mutations.

As widely recognized, MH mutations are not randomly distributed along the RyR1 sequence. The vast majority of them are localized to two restricted regions, the N-terminal (Cys\(^{329–331}\)) and the central (Asp\(^{1122–1124}\)) domains, whereas a third, C-terminal region (Ile\(^{3916}\)-Gly\(^{4942}\)) contains fewer MH-sensitive sites than in the Banyu Fellowship Award in Cardiovascular Medicine (to S. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ”advertisement” in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

1 To whom correspondence should be addressed; Boston Biomedical Research Inst., 64 Grove St., Watertown, MA 02472. Tel.: 617-658-7774; Fax: 617-972-1761; E-mail: ikemoto@bbri.org.

2 The abbreviations used are: MH, malignant hyperthermia; SR, sarcoplasmic reticulum; RyR, ryanodine receptor; MCA, methylcoumarin acetate; CaM, calmodulin; SAED, sulfo-S-maleimydyl; 2-(7-azido-4-methylcyclohexylamino)-2′,4′-dinitrophenyl acid; AMP-PCP, adenosine 5′-(β,γ-methylene)triphosphate; MOPS, 3-(N-morpholino)propanesulfonic acid; BAPTA, 1,2-bis(2-aminophenox)-ethane-N,N′,N′,N′-tetraacetic acid.

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Molecular Mechanism of Dantrolene Action

Preparation of SR Vesicles—The SR microsomes were prepared from rabbit back paraspinal and hind leg skeletal muscles (Pel-Freez Biologicals, Rogers, AR) using the method of differential centrifugation described previously (35).

Domain Peptide and Site-specific Antibody—We used a domain peptide (DP4) corresponding to Leu2442–Cys2477 of RyR1 both as a channel-activating reagent and as a site-directed carrier to incorporate the fluorescence probe MCA into RyR1. The peptide was synthesized on an Applied Biosystems Model 431A synthesizer, purified by reversed-phase high-pressure liquid chromatography, and evaluated by mass spectrometry. To localize the DP4-binding site within the primary structure of RyR1, the site-specific anti-DP1 (Leu269–Cys309), anti-DP4 (Leu2442–Cys2477), and anti-DP3 (Asp324–Val351) polyclonal antibodies were used (31).

Site-specific MCA Labeling at the DP4-binding Site of RyR1—Site-specific fluorescence labeling of the DP4-binding sites on RyR1 in skeletal muscle SR was performed using the cleavable heterobifunctional cross-linking reagent sulfo-SS-maleimidyl 2-[4-azido-4-oxo]ethyl-methionyl-3-acetamidoethyl-1,3-dithiopropionate (SAED) (36).

Localization of the Site of MCA Attachment within the RyR—MCA-labeled RyR1 was purified from fluorescently labeled SR using heparin and hydroxylapatite affinity columns as described by Inui and Fleischer (37). MCA-labeled RyR was digested with trypsin (1:100, 1:10 trypsin/protein ratio) at 22 °C for 45 min, and the resultant tryptic fragments were separated for fluorescent analysis by SDS-PAGE on SDS-polyacrylamide gels. For Western blot analysis, tryptic fragments were transferred to Immobilon-P membranes (Millipore Corp, Bedford, MA) at 90 V in 10% methanol and 10 mM CAPS (pH 11.0) at 4 °C. The blots were developed with anti-DP1, anti-DP3, and anti-DP4 primary antibodies and peroxidase-conjugated secondary antibodies.

[H3]ryanodine Binding assay—Isolated skeletal muscle SR (0.5 mg/ml) that had been labeled with MCA was incubated with 10 nM [H3]ryanodine (68.4 Ci/ml; PerkinElmer Life Sciences) and the desired concentration of dantrolene or an equivalent volume of methanol vehicle (final concentration of 0.7%), and 2.0 μM MCA uptake was monitored in a Cary Eclipse spectrophotometer (Varian Inc.) using fluo-3 (excitation at 488 nm and emission at 525 nm) as a Ca2+ indicator. The [Ca2+]i was kept at 0.1 μM using BAPTA/calcium buffer (0.449 mM CaCl2, 1 mM BAPTA, and 20 mM MOPS [pH 7.2]). The effect of dantrolene on MCA uptake (1 μM) was determined under identical assay conditions. Assays were carried out in duplicate according to established protocols (38), and each datum point was obtained by averaging the duplicates.

Ca2+ Release assay—Isolated skeletal muscle SR (0.2 mg/ml) was incubated at 22 or 37 °C in a solution containing 0.15 M KCl, 0.1 μM CaCl2, 1 mM BAPTA/calciuem buffer, and either 10 μM dantrolene or an equivalent volume of methanol vehicle (final concentration of 0.7%). 2.0 μM fluo-3, and 20 mM MOPS (pH 7.2) in the presence or absence of MCA. Ca2+ uptake was initiated by the addition of 1 mM MgATP to the cuvette, and the time course of Ca2+ uptake was monitored in a Cary Eclipse spectrophotometer (Varian Inc.) using fluo-3 (excitation at 488 nm and emission at 525 nm) as a Ca2+ indicator (31). After Ca2+ uptake had reached a plateau, various concentrations of DP4 were added, and the resultant Ca2+ release was monitored. During the Ca2+ uptake/release reaction, the sample was continuously stirred, and the temperature was kept constant at either 22 or 37 °C.

Spectroscopic Monitoring of Domain Unzipping—To make a macro-molecular collisional quencher, QSYTM 7 carboxylic acid (2.5 mM) was conjugated with 0.5 mM bovine serum albumin by incubation in 20 mM HEPES (pH 7.5) at 22 °C for 30 min in the dark. Unreacted QSYTM 7 carboxylic acid was removed by Sephadex G-50 gel filtration. Fluorescence quenching by the conjugate was performed by measuring the steady-state fluorescence of SR labeled with MCA (excitation at 348 nm and emission at 445 nm; Cary Eclipse spectrophotometer). Fluorescently labeled SR (0.2 mg/ml) was incubated at either 22 or 37 °C in a solution containing 0.15 M KCl, 1 mM AMP-PCP, 0.1 μM CaCl2 (BAPTA/calcium buffer), and either various concentrations of dantrolene or an enhancement of [H3]ryanodine binding and activation of SR Ca2+ release, both measures of Ca2+ channel activation, it required calmodulin to do so at 22 °C. These results suggest that inhibition of RyR1 domain unzipping plays a role in the therapeutic action of dantrolene. The present data also suggest that the temperature and CaM dependence of dantrolene activity on RyR1 is due to their effects on the mechanism by which the domain switch is coupled to Ca2+ channel function.

EXPERIMENTAL PROCEDURES

Mutations (19–24). The pathophysiological consequences of MH mutations are hyperactivation and/or hypersensitization of RyR1 Ca2+ channel activity to stimulating conditions (both pharmacological and voltage-dependent) (1, 18). In contrast, most mutations conferring susceptibility to central core disease, a rare myopathy showing a different phenotype (19, 25, 26), are located in the C-terminal putative channel pore region (Val3916–Ala41942) (19, 26). The Ca2+ release properties of expressed RyR1 channels containing randomly selected MH mutations from the N-terminal and central domains have been shown to display similar properties of hyperactivation and hypersensitization (27). To explain these results, we have proposed a model of channel regulation that involves interdomain interactions between the N-terminal and central domains of RyR1 serving as a “domain switch” for Ca2+ channel regulation (28–31). In the resting state, the N-terminal and central domains make close contact at several as yet undetermined subdomains. The conformational constraints imparted by the “zipped” configuration of these two domains stabilize and maintain the closed state of the Ca2+ channel. Stimulation of the RyR via excitation-contraction coupling weakens these critical interdomain contacts (unzipping of the domain switch), thereby lowering the energy barrier for Ca2+ channel opening. Partial unzipping or weakening of the domain switch may also occur secondary to MH mutations in either the N-terminal or central domain. As a result of this, MH-susceptible RyR1 channels are hypersensitive to agonist stimuli.

Recently, Parness and co-workers (32) localized a dantrolene-binding site within the primary structure of RyR1 using [3H]azidodantrolene, a pharmacologically active photoaffinity analog of dantrolene. [3H]Azidodantrolene specifically photolabels the N-terminal 1400-amino acid fragment of RyR1 cleaved by an endogenous, SR membrane-bound calpain (33) and is localized within sequence Leu590–Cys609 based on the following evidence. 1) Of several synthetic RyR1 domain peptides examined, [3H]azidodantrolene specifically photolabels only peptides containing the Leu590–Cys609 sequence (named DP1 for domain peptide-1), and 2) an anti-RyR1 monoclonal antibody recognizing DP1 inhibits [3H]azidodantrolene photolabeling of RyR1 (34). The dantrolene-binding site is therefore located within the domain comprising the N-terminal portion of our proposed domain switch. Since dantrolene seems to inhibit Ca2+ release, these findings have led us to suggest that dantrolene may act by reinforcing interactions between the N-terminal and central portions of the RyR1 domain switch that favor a zipped conformation. Here, we present new evidence suggesting that this is indeed at least a portion of the mechanism of action of dantrolene.

In this study, we produced MH-like conditions of RyR1 (i.e. hyperactivation and hypersensitization of Ca2+ channels) in SR isolated from normal skeletal muscle by adding DP4, a synthetic domain peptide corresponding to amino acids 2442–2477 of RyR1, or anti-DP4 antibody, which, as we have recently shown, induces spectroscopic changes consistent with domain unzipping (29, 31). The magnitude of domain unzipping was determined from the accessibility of a fluorescence probe, methylcoumarin acetate (MCA), attached to the N-terminal domain, to a macromolecular fluorescence quencher (bovine serum albumin-conjugated QSY). The Stern-Volmer quenching constant (Ksv), a measure of domain unzipping, increased with the addition of either DP4 or anti-DP4 antibody. Here, we show that the addition of dantrolene reversed the increase in Ksv that was produced by DP4 or anti-DP4 antibody with an IC50 of 0.3 μM. This blocking effect of domain unzipping by dantrolene was present at both 37 and 22 °C and was independent of calmodulin (CaM). Although, at 37 °C, dantrolene alone inhibited DP4 release of RyR1 Ca2+ channel activity, both measures of Ca2+ channel activation, it required calmodulin to do so at 22 °C. These results suggest that inhibition of RyR1 domain unzipping plays a role in the therapeutic action of dantrolene. The present data also suggest that the temperature and CaM dependence of dantrolene activity on RyR1 is due to their effects on the mechanism by which the domain switch is coupled to Ca2+ channel function.
equivalent volume of methanol vehicle (final concentration of 0.7%) and 20 mM MOPS (pH 7.2). Various concentrations of DP4 or 20 μg/ml anti-DP4 antibody was used to induce domain unzipping, and DP4-mut, in which one mutation was made to mimic R2458C MH mutation, was used as a negative control. The effect of dantrolene on domain unzipping was investigated by determining the effect of this drug on RyR1 MCA fluorescence in the presence or absence of 1 μM CaM. The data were analyzed using the Stern-Volmer equation:

\[ F_0/F = 1 + KQ \]

where \( F \) and \( F_0 \) are fluorescence intensities in the presence and absence of added quencher, respectively; \( K \) is the quenching constant, a measure of the accessibility of the protein-bound probe to the quencher; and \([Q]\) is the concentration of the quencher (QSY) (29, 31).

RESULTS

Dantrolene Inhibits an Abnormal Unzipping of the Domain Switch—To investigate the effect of dantrolene on the mode of interdomain interaction, we utilized the MCA fluorescence quenching technique we developed to determine the extent of unzipping of the RyR1 domain switch (see "Experimental Procedures") (29, 31). As shown previously (29), DP4 mediates the specific MCA labeling of the N-terminal 1400-amino acid segment of RyR1. The precise location of DP4-mediated MCA labeling of RyR1 within the N-terminal 1400-amino acid region has not been determined, and we do not yet know whether it occurs in the N-terminal MH domain (Cys35–Arg614). To better define where MCA is incorporated, we used DP4 conjugated with the heterobifunctional cross-linker SAED, as a site-directing carrier, as described previously (29). The SAED-DP4 conjugate was photo-cross-linked to its binding site, and the DP4 moiety was removed under reducing conditions. MCA-labeled RyR1 was purified and subjected to tryptic digestion. The process of degradation of the fluorescently labeled polypeptide chain was then followed by SDS-PAGE. As shown in Fig. 1, partial digestion of fluorescently labeled RyR1 with a low concentration of trypsin (100:1 (w/w) RyR protein/trypsin ratio) resulted in the appearance of two major fluorescently labeled fragments with approximate molecular masses of 155 and 140 kDa. Digestion with a higher concentration of trypsin (10:1 RyR protein/trypsin ratio) resulted in the appearance of several fluorescently labeled fragments. As shown in the Western blot of Fig. 1, the 155- and 140-kDa MCA-labeled bands stained with both anti-DP1 (Leu590–Cys609) and anti-DP3 (Asp324–Val351) antibodies, whereas the 51-kDa MCA-labeled region is included in the N-terminal MH domain encompassed by Cys35–Arg614, these results indicate that DP4-mediated MCA labeling has taken place within the presumed N-terminal domain portion of the domain switch. These results also suggest that DP4, hence Leu2442–Pro2477 of the central domain of RyR1, binds to the N-terminal domain, as predicted from our domain switch hypothesis.
The domain switch hypothesis predicts that, if the MCA probe attached to the N-terminal domain is buried in the zipped configuration, it will be relatively inaccessible to a macromolecular fluorescence quencher, bovine serum albumin-conjugated QSY. Unzipping should, however, render the MCA probe more accessible to the quencher. DP4 has been previously shown to enhance [3H]ryanodine binding (28), to induce Ca\(^{2+}\) release from the SR (29), to induce contraction in skinned muscle fiber at an inhibitory Mg\(^{2+}\) concentration (41), to increase the frequency of Ca\(^{2+}\) sparks in saponin-permeabilized fibers (42), and to increase the open probability of single channels (42). Thus, DP4 mimics MH-like hyperactivation and hypersensitization of the RyR1 Ca\(^{2+}\) channel in the otherwise wild-type system. Furthermore, a mutation in DP4 (DP4-mut) that mimics the R2458C MH mutation results in virtually complete loss of the activities of wild-type DP4 (28, 29, 41, 42). This is consistent with the idea that MH mutations weaken domain-domain interactions within RyR that are mimicked by synthetic domain peptide-domain (RyR) interaction. Thus, we used DP4 herein to mimic MH-like channel dysfunction and to investigate the effects of dantrolene on these functions.

We first determined the fluorescence intensity of bound MCA as a function of increasing concentrations of the quencher in the absence or presence of DP4 at 22°C. Fig. 2A shows the Stern-Volmer plot of fluorescence quenching of MCA attached to the N-terminal domain in the presence of various concentrations of DP4. The slope of the plot, which is equal to the Stern-Volmer quenching constant \(K_Q\), is a measure of the degree of domain unzipping. As shown in Fig. 2B, the \(K_Q/K_Q\) value (where \(K_Q\) is the quenching constant in the presence of DP4 or DP4-mut, and \(K_Q\) is the quenching constant in their absence) was used to assess the extent of domain unzipping. \(K_Q/K_Q\) increased with increasing concentrations of DP4 and leveled off at \(\sim 100 \text{µM}\) peptide. Significantly, the DP4 concentration dependence of the increase in \(K_Q/K_Q\) correlates well with the DP4 concentration dependence of activation of RyR Ca\(^{2+}\) channels (cf. Fig. 5). As shown in Fig. 2B, the single MH mutation in DP4-mut has made the peptide incapable of enhancing fluorescence quenching, presumably due to mutation-induced loss of affinity for the N-terminal domain. DP4-induced domain unzipping provides a simple and versatile model for the study of pathogenic and therapeutic mechanisms of MH.

We extended the above observations to investigate the effect of various concentrations of dantrolene on DP4-induced domain unzipping, again using the fluorescence quenching technique (Fig. 3). As shown in Fig. 3A, 1 µM dantrolene reduced \(K_Q\) from the level induced by 100 µM DP4 to near control levels. Fig. 3B shows the concentration dependence of the dantrolene-induced decrease in the degree of fluorescence quenching \((K_Q/K_Q)\). The major effect of dantrolene was seen in the concentration range of 0.1–1.0 µM, with half-maximal inhibition at 0.3 µM, agreeing well with the reported \(K_D\) of \(\sim 0.275 \text{µM}\) for [3H)dantrolene binding to the SR (43). The maximal inhibitory effect (\(\sim 73%\) of the control) was attained well below 10 µM, the serum concentration generally achieved during clinical treatment. Fig. 3C shows the effect of increasing concentrations of DP4 on \(K_Q/K_Q\) in the presence of 10 µM dantrolene. The drug suppressed fluorescence quenching at all concentrations of DP4 tested. These results are consistent with the view that dantrolene reverses DP4-induced domain unzipping and restabilizes the zipped configuration of the domain switch.

To confirm that the observed dantrolene inhibition represents an effect on the functionality of the domain switch rather than a localized specific binding effect (e.g., competition with DP4 for its mating domain), we used anti-DP4 polyclonal antibody as another agent to induce domain unzipping. Like DP4, anti-DP4 antibody induces domain unzipping and also produces MH-like hyperactivation and hypersensitization effects (31) by binding to the central domain portion of the domain switch. Anti-DP4 antibody (20 µg/ml) was added to MCA-labeled SR in the absence or presence of increasing concentrations of dantrolene, and the magnitude of macromolecular fluorescence quenching was determined (Fig. 4). Here, too, dantrolene inhibited anti-DP4 antibody-induced enhancement of macromolecular fluorescence quenching. The concentration dependence of dantrolene inhibition of anti-DP4 antibody-induced fluorescence quenching parallels that of its inhibition of DP4-induced activity, i.e. it occurred in the concentration range of 0.1–1.0 µM, with half-maximal inhibition at 0.3 µM. In this case, however, the extent of inhibition was significantly greater than when “domain unzipping” was produced by DP4. In all the experiments above, the addition of 10 µM dantrolene in the absence of DP4 (Fig. 3B) or anti-DP4 antibody (Fig. 4) produced no significant effect on \(K_Q/K_Q\).
According to the literature, higher temperature (37 °C) (cf. Refs. 7, 12, 13, and 45) and calmodulin (14) are required for significant inhibition of RyR1 Ca\textsuperscript{2+}/H\textsubscript{11001} channels by dantrolene. We investigated the effect of 10 \textmu M dantrolene on domain unzipping (\(K_{Q}/K_{Q}\)) induced by 100 \textmu M DP4 at both 22 and 37 °C. As shown in Table I, raising the temperature from 22 to 37 °C produced only a statistically insignificant increase in the extent of dantrolene inhibition of domain unzipping from 73.7 ± 10.9 to 80.9 ± 7.4%. The addition of 1 \textmu M CaM at 22 °C also produced virtually no change in the extent of dantrolene inhibition of DP4-induced domain unzipping.

Inhibition of Skeletal Muscle SR Ryanodine Binding and \(\text{Ca}^{2+}\) Release by Dantrolene—Ryanodine binds to RyRs only when the channel is in the open state, and the degree of ryanodine binding is widely used as a biochemical measure of the degree of channel activation (44). Fig. 5 shows the concentration-dependent effects of DP4 or DP4-mut on \([^{3}\text{H}]\)ryanodine binding at 22 °C. In these experiments, MCA-labeled SR was used to correlate the physiological properties of the \(\text{Ca}^{2+}\) channel with the fluorescence quenching data. MCA-labeled and -unlabeled SR preparations showed essentially the same responses to DP4, dantrolene, and the other effectors (data not shown). Fig. 5 demonstrates that DP4 enhanced \([^{3}\text{H}]\)ryanodine binding to the SR in a concentration-dependent manner. Comparison of Figs. 3C and 5 reveals that the enhancement of \([^{3}\text{H}]\)ryanodine binding and fluorescence quenching occurred in approximately the same concentration range of DP4. This indicates that unzipping of the domain switch and activation of \(\text{Ca}^{2+}\) channels are well coordinated. Surprisingly, 10 \textmu M dantrolene caused little inhibition of \([^{3}\text{H}]\)ryanodine binding at 22 °C (Table II). At this temperature, dantrolene inhibition, if any, was not more than 10% of the control at all concentrations of DP4 tested (data not shown). CaM alone (1 \textmu M) increased the specific activity of \([^{3}\text{H}]\)ryanodine binding to the SR at 22 °C, but dantrolene was now able to significantly suppress radioligand binding (−35%) (Table II). When we carried out the same type of experiments at 37 °C, dantrolene produced equivalently significant inhibition of \([^{3}\text{H}]\)ryanodine binding (−59%) in the presence or absence of CaM, in agreement with previous reports (7, 12, 13, 45). These results suggest that higher temperature and CaM have similar effects on the extent of dantrolene inhibition of DP4-induced activation of RyR \(\text{Ca}^{2+}\) channels. These effects of temperature and CaM on the extent of 10 \textmu M dantrolene. The drug suppressed fluorescence quenching at all concentrations of DP4. Each datum point represents the mean ± S.D. obtained from at least four different experiments.
the fast and slow phases of release, respectively; and \(k_1\) and \(k_2\) are the rate constants of the fast and slow phases, respectively. The calculated values of the initial rate of Ca\(^{2+}\) release ((dy/dt)\(_{t=0}\) = \(a k_1 + b k_2\)) and the amount of Ca\(^{2+}\) release (a + b) are listed in Table III. At 22 °C and in the absence of CaM, dantrolene produced no appreciable effect on the initial rate of Ca\(^{2+}\) release, although there was a small but appreciable inhibition of the slow phase of Ca\(^{2+}\) release (Fig. 6A and Table III). The addition of 1 μM CaM resulted in a significant increase in the initial rate of Ca\(^{2+}\) release. Under these conditions, 10 μM dantrolene produced a significant reduction in the initial rate (~ 41%) and an appreciable reduction in the total amount of Ca\(^{2+}\) released, as well. Interestingly, raising the experimental temperature to 37 °C produced an effect similar in magnitude to that produced by CaM at 22 °C. Thus, at 37 °C, dantrolene produced a significant reduction in both the initial rate and the amount of Ca\(^{2+}\) release even in the absence of CaM. These results are again consistent with the view that CaM and temperature seem to affect the dantrolene-blocking signal from the domain switch to the Ca\(^{2+}\) channel pore domain.

**DISCUSSION**

Dantrolene acts as an intracellular skeletal muscle relaxant and is the primary therapeutic agent used for the treatment of MH (46). A considerable amount of information accumulated in the literature suggests that, at micromolar concentrations, dantrolene blocks some step(s) of activation of RyR1 Ca\(^{2+}\) channels, but the detailed mechanism of its pharmacological action remains obscure. The main aim of this study was to gain a deeper insight into the molecular mechanism of the pharmacological action of dantrolene.

We proposed the domain switch model to explain both the mechanism of channel activation under normal conditions and the mechanism of pathogenesis of channel dysfunction. The model assumes that the mode of interaction between the N-terminal and central domains of the RyR (the two major regions harboring many of the reported MH mutations) is involved in Ca\(^{2+}\) channel regulation and also in the pathogenesis of MH. In the resting state, the two domains make close contact at several subdomains forming the zipped configuration (i.e. the "off" configuration of the on/off switch), and this configuration stabilizes the closed state of the Ca\(^{2+}\) channel. Stimulation of the RyR by T tubule depolarization or chemical agonists causes unzipping of the domain switch (the "on" configuration of the switch), leading to Ca\(^{2+}\) channel opening. Mutations in conformationally active portions of either region of the domain switch weaken the inter-domain interactions, causing partial unzipping of the switch. As a result, MH-susceptible RyR1 channels are more readily opened in response to the lack of effect of these factors on the extent of dantrolene inhibition of fluorescence quenching.

**Our data above (Fig. 2B) demonstrate that DP4-mut had virtually no effect on fluorescence quenching. This suggests that MH mutations in DP4 destroy the ability of this sequence to bind to its mating domain on the N-terminal region of RyR1. Accordingly, there should be no channel activation by DP4-mut, as was indeed demonstrated by the inability of this mutated peptide to enhance \(^{3}H\)ryanodine binding at 22 °C (Fig. 5) and at 37 °C (data not shown). Taken together, our results suggest (a) that domain unzipping is a prerequisite for DP4-induced channel opening, (b) that dantrolene exerts its inhibitory action directly upon the domain switch, and (c) that the inhibition signal elicited in the domain switch is transmitted to the Ca\(^{2+}\) channel in a temperature- and CaM-dependent manner.**

We next investigated the effect of temperature and CaM on the ability of dantrolene to inhibit DP4-induced SR Ca\(^{2+}\) release, as measured by the time- and Ca\(^{2+}\)-dependent changes in fluo-3 fluorescence spectrophotometry (Fig. 6). These Ca\(^{2+}\) release experiments were carried out with an MCA-unlabeled SR preparation because MCA labeling did not produce appreciable changes in the RyR properties in preliminary experiments (data not shown). Approximately 20 s after Ca\(^{2+}\) uptake was initiated by the addition of MgATP, 100 μM DP4 was added to induce Ca\(^{2+}\) release, and the effect of 10 μM dantrolene on the Ca\(^{2+}\) release time course in the absence and presence of 1 μM CaM was analyzed. The Ca\(^{2+}\) release time courses (shown in Fig. 6) were fitted to a double exponential equation: \(y = y_0 + a(1 - e^{-kt}) + b(1 - e^{-kt})\), where \(a\) and \(b\) are the magnitude of

| Table I | Dantrolene inhibition of DP4-induced macromolecular fluorescence quenching of MCA-labeled RyR1: effects of temperature and CaM |
|---|---|
| | 22 °C | 37 °C |
| 100 μM DP4 | 1.76 ± 0.09 (7) | 1.82 ± 0.03 (5) |
| 100 μM DP4 + 10 μM dantrolene | 1.20 ± 0.07 (7) | 1.16 ± 0.06 (5) |
| Inhibition (%) | 74.3 ± 9.7 (7) | 80.9 ± 7.4 (5) |
| 100 μM DP4 + 1 μM CaM | 1.80 ± 0.09 (6) | ND |
| 100 μM DP4 + 1 μM CaM + 10 μM dantrolene | 1.21 ± 0.07 (6) | ND |
| Inhibition (%) | 74.3 ± 8.7 (6) | ND |

\(a p < 0.05 \text{ versus } 100 \mu\text{M DP4.} \)

\(b p < 0.05 \text{ versus } 100 \mu\text{M DP4 + 1 \muM CaM.} \)

**dantrolene inhibition of RyR Ca\(^{2+}\) channels are in sharp contrast to the lack of effect of these factors on the extent of dantrolene inhibition of fluorescence quenching.**

**Fig. 5.** DP4-induced activation of \(^{3}H\)ryanodine binding versus DP4 or DP4-mut concentration at 22 °C. The data were fitted to the equation \(y = aK^x/(1 + K^x)\), where \(y\) is the amount of ryanodine bound at \(x\) concentration of DP4, \(a\) is the maximal binding, and \(K\) is the association constant. Each datum point represents the mean ± S.D. of at least four experiments carried out in duplicate.
Dantrolene inhibition of DP4-induced enhancement of $[^{3}H]$ryanodine binding activity: effects of temperature and CaM

$[^{3}H]$Ryanodine binding to skeletal muscle SR vesicles was determined in the absence or presence of 10 μM dantrolene at 22 or 37 °C as described under “Experimental Procedures.” The same experiments were carried out in the presence of 1 μM CaM. Each datum point represents the mean ± S.D. The number of experiments is shown in parentheses. A paired t test was employed to determine the statistical significance of the data (p value).

![Table II](#)

**Table II**

|                      | 22 °C | 37 °C |
|----------------------|------|------|
| **[3]H**ryanodine bound pmol/L |      |      |
| 100 μM DP4           | 0.76 ± 0.07 (12) | 0.64 ± 0.18 (6) |
| 100 μM DP4 + 10 μM dantrolene | 0.72 ± 0.04 (10) | 0.26 ± 0.06 (6)$^a$ |
| Inhibition (%)        | 5.5 ± 18.1 (10) | 59.1 ± 9.3 (6) |
| 100 μM DP4 + 1 μM CaM | 1.95 ± 0.46 (6)$^a$ | 1.85 ± 0.26 (6)$^a$ |
| 100 μM DP4 + 1 μM CaM + 10 μM dantrolene | 1.28 ± 0.16 (6)$^b$ | 0.75 ± 0.11 (6)$^b$ |
| Inhibition (%)        | 34.5 ± 13.2 (6) | 59.2 ± 6.2 (6) |

$^a$ p < 0.05 versus 100 μM DP4.

$^b$ p < 0.05 versus 100 μM DP4 + 1 μM CaM.

**Molecular Mechanism of Dantrolene Action**

The initial rate ($ak_1 + bk_2$) of Ca$^{2+}$ release was calculated by fitting a double exponential function ($y = y_0 + a(1 - e^{-kt}) + b(1 - e^{-kt'})$, where $a$ and $b$ are the magnitude of the fast and slow phases of release, respectively; and $k_1$ and $k_2$ are the rate constants of the fast and slow phases, respectively) to the first 20 s of the time course of fluo-3 fluorescence change after the addition of DP4. Each datum point represents the mean ± S.D. The number of experiments is shown in parentheses. A paired t test was employed to determine the statistical significance of the data (p value). ND, not determined.

|                      | 22 °C | 37 °C |
|----------------------|------|------|
| Initial rate ($ak_1 + bk_2$) nmol/mgs |      |      |
| 100 μM DP4           | 7.86 ± 1.75 (5) | 6.79 ± 0.47 (5) |
| 100 μM DP4 + 10 μM dantrolene | 8.53 ± 1.03 (4) | 3.35 ± 0.97 (6)$^a$ |
| Inhibition (%)        | −8.5 ± 12.9 (4) | 50.7 ± 14.3 (6) |
| 100 μM DP4 + 1 μM CaM | 12.02 ± 3.11 (7)$^a$ | ND |
| 100 μM DP4 + 1 μM CaM + 10 μM dantrolene | 7.07 ± 1.11 (6)$^b$ | ND |
| Inhibition (%)        | 41.2 ± 8.8 (6) | ND |

$^a$ p < 0.05 versus 100 μM DP4.

$^b$ p < 0.05 versus 100 μM DP4 + 1 μM CaM.

**FIG. 6. Dantrolene inhibits DP4-induced Ca$^{2+}$ release with a temperature and CaM dependence.** A, time course of DP4-induced Ca$^{2+}$ release in the absence of dantrolene (black trace) and in the presence of 10 μM dantrolene (gray trace) at 22 °C. After the Ca$^{2+}$ uptake initiated by the addition of 1 mM MgATP had reached a plateau, various concentrations of DP4 were added, and the resultant Ca$^{2+}$ release was monitored by fluo-3 fluorescence spectrophotometry. B, the same experiment as described for A, except that 1 μM CaM was present during the course of the Ca$^{2+}$ release experiment. C, the same experiment as described for A, except that it was carried out at 37 °C. Each Ca$^{2+}$ release trace presented here was obtained by signal averaging a total of 35–75 traces originating from four to seven experiments.

**TABLE III**

Inhibition of SR Ca$^{2+}$ release by dantrolene: effects of temperature and CaM

The initial rate ($ak_1 + bk_2$) of Ca$^{2+}$ release was calculated by fitting a double exponential function ($y = y_0 + a(1 - e^{-kt}) + b(1 - e^{-kt'})$, where $a$ and $b$ are the magnitude of the fast and slow phases of release, respectively; and $k_1$ and $k_2$ are the rate constants of the fast and slow phases, respectively) to the first 20 s of the time course of fluo-3 fluorescence change after the addition of DP4. Each datum point represents the mean ± S.D. The number of experiments is shown in parentheses. A paired t test was employed to determine the statistical significance of the data (p value). ND, not determined.

|                      | 22 °C | 37 °C |
|----------------------|------|------|
| Initial rate ($ak_1 + bk_2$) nmol/mgs |      |      |
| 100 μM DP4           | 7.86 ± 1.75 (5) | 6.79 ± 0.47 (5) |
| 100 μM DP4 + 10 μM dantrolene | 8.53 ± 1.03 (4) | 3.35 ± 0.97 (6)$^a$ |
| Inhibition (%)        | −8.5 ± 12.9 (4) | 50.7 ± 14.3 (6) |
| 100 μM DP4 + 1 μM CaM | 12.02 ± 3.11 (7)$^a$ | ND |
| 100 μM DP4 + 1 μM CaM + 10 μM dantrolene | 7.07 ± 1.11 (6)$^b$ | ND |
| Inhibition (%)        | 41.2 ± 8.8 (6) | ND |

$^a$ p < 0.05 versus 100 μM DP4.

$^b$ p < 0.05 versus 100 μM DP4 + 1 μM CaM.

Putative critical domains of the RyR (domain peptides) and antibodies raised against these domain peptides, DP4 enhances ryanodine binding (28), induces Ca$^{2+}$ release from the SR (29), induces contraction in skinned muscle fibers at an inhibitory Mg$^{2+}$ concentration (41), increases the sensitivity to caffeine (41), increases the frequency of Ca$^{2+}$ sparks in saponin-permeabilized fibers (42), and increases the open probability of single channels (42). Similarly, anti-DP4 polyclonal antibody, directed against the Leu$^{2442}$–Pro$^{2477}$ region (a portion of the central domain) of RyR1, produces hyperactivation of Ca$^{2+}$ channels and hypersensitization of Ca$^{2+}$ channels to agonists (31).

The most important aspect of this study is the finding that dantrolene inhibits the DP4-induced increase in $K_Q$, i.e., dantrolene stabilizes a zipped configuration of the domain switch even in the presence of domain-unzipping agents such as DP4 and anti-DP4 antibody. Since domain unzipping produced by DP4 or anti-DP4 antibody seems to simulate the channel dysfunction caused by MH mutations, the present findings suggest that the molecular mechanism of therapeutic action of dantrolene in MH is to stabilize a zipped configuration of the
domain switch, despite the tendency of the mutation to destabilize the domain switch. As a result, the channel hyperactivity and hypersensitivity caused by MH mutations are suppressed by dantrolene. Since the dantrolene-binding site is located in the Leu509-Cys609 region of the proposed RyR1 domain switch (37), interdomain stabilization by dantrolene must be produced by direct binding of the drug to a region of the domain switch.

In agreement with previous reports (7, 12, 13, 45, 46), we found that the extent of dantrolene inhibition of DP4-induced RyR1 channel activation, as determined by both [3H]ryanodine binding and SR Ca2+ release, varies with the temperature, i.e. significant inhibition was observed at 37 °C, with only negligible inhibition at 22 °C. An interesting new finding in this study is that dantrolene inhibition of DP4-induced domain unzipping is independent of temperature. Thus, it seems that the requirement for physiological temperatures for dantrolene activity is not due to a temperature dependence for the stabilization of a zipped configuration of the domain switch. Rather, the temperature requirement is for the downstream mechanism(s) of actual channel opening. According to our working hypothesis, the on/off signal elicited in the domain switch is transmitted to the putative channel gating/pore domain by mediation of some intraprotein coupling mechanism that requires higher temperatures for activation or inhibition.

According to an earlier report (13), the addition of CaM is required to observe dantrolene inhibition of RyR1 channel function. We addressed the question of the possible role of CaM in modulating dantrolene activity in this study. We detected no further effect of CaM on dantrolene inhibition of ryanodine binding or Ca2+ release at 37 °C, where there was already significant inhibition by dantrolene. However, at 22 °C, where there was very little dantrolene intrinsic inhibition, the extent of dantrolene inhibition of both ryanodine binding and Ca2+ release was considerably increased in the presence of CaM. At 22 °C, however, CaM had no effect on the extent of dantrolene inhibition of domain unzipping. These results suggest that, like raising the temperature from 22 to 37 °C, CaM may lower the conformational energy barrier for the proposed coupling between the domain switch and the channel gating/pore domain and enhance blocking signals elicited in the domain switch to more effectively transmit them to the functional Ca2+ channel.

These results further suggest that the CaM-binding site on RyR1 is an intervening domain that mediates inhibitory signals from the domain switch to the channel gating/pore domain. Clearly, more work is required to elucidate the detailed molecular mechanism of pharmacological and therapeutic actions of dantrolene.

In summary, we speculate that dantrolene binding to the Leu509-Cys609 region of the N-terminal portion of the domain switch produces a local conformational change that results in reinforcement of the interdomain interactions between itself and its mating subdomain, likely located within the central domain portion of the domain switch. This stabilizes a zipped (i.e. closed) configuration. Thus, further characterization of the domain-domain interactions involving the dantrolene-binding region will be significant for further resolution of the molecular mechanism of dantrolene action on RyR1 in health and disease, as well as the mechanism of intraprotein control of channel opening.

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