LABORATORY INVESTIGATIONS

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Dantrolene Sodium Can Increase or Attenuate Activity of Skeletal Muscle Ryanodine Receptor Calcium Release Channel

Clinical Implications

Thomas E. Nelson, Ph.D., Marina Lin, M.S.,* Gisele Zapata-Sudo, M.D., Ph.D.,† Roberto Takashi Sudo, M.D., Ph.D.†*

Background: **Dantrolene sodium (DS) is a direct-acting skeletal muscle relaxant whose only known action is to block calcium release from intracellular storage sites. The exact site of action for DS is unknown, but its efficacy in treating and preventing anesthetic-induced malignant hyperthermia (MH) is well established.**

Methods: **Single ryanodine (Ry_1) receptor calcium release channels were incorporated into a planar lipid bilayer for electrophysiologic recording and for subsequent analysis of the channel's gating and conductance properties. The cellular effects of low DS concentrations were investigated by isometric contracture tension responses in biopsied MH human and dog muscle fascicles and in normal, single fibers from human vastus lateralis muscle.**

Results: **Two concentration-dependent DS effects on the isolated Ry_1 receptor were discovered, suggesting at least two different binding sites. At nanomolar concentrations, DS activated the channel by causing three- to fivefold increases in open-state probability and dwell times. At micromolar concentrations, DS first increased then reduced activity in the channels; with the dominant effect being reduced activity. A 20 nM concentration of DS produced significant contracture tension in human muscle from one MH subject and caused potentiation of twitch in muscle from another MH patient. Halothane contracture in MH dog muscle was followed by an additional increase in tension when treated with 20 nM DS. Other investigations on chemically skinned, human fibers showed that calcium loaded in the sarcoplasmic reticulum was partially released by nM DS.**

Conclusions: **The study results suggest that at least two binding sites for DS exist on the Ry_1 receptor calcium channel. A low-affinity (μM) site is associated with reduced channel gating and open-state dwell time and may relate to the established pharmacologic muscle relaxant effect of DS. The proposed high-affinity (nM) DS binding site activates the channel, producing Ca^{2+} release to the myoplasm, which, under environmentally adverse conditions, could damage genetically predisposed MH muscle. Such a phenomenon, if it occurs in DS-treated MH patients, could generate a recrudescence of the syndrome. (Key words: Complications: malignant hyperthermia. Ions: calcium channels. Muscle: skeletal. Pharmacology: dantrolene. Receptors: ryanodine.)**

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DANTROLENE sodium is a unique skeletal muscle relaxant acting distally to the myoneural junction to block calcium release from inside the muscle cell.[1] In human volunteers, the intravenous administration of 2.4 mg/kg dantrolene produces a blood concentration of about 10 μM and a 75% decrease in muscle twitch force.[2] The exact site(s) for dantrolene's action has not been determined, although indirect and conflicting evidence has been reported.[3–5] Studies using radiolabeled dantrolene have indicated that at least two binding sites exist in skeletal muscle sarcoplasmic reticulum membranes,[6–8] a high- (nM) and a low- (μM) affinity site. The high-affinity binding site was not evident in membranes from cardiac and smooth muscle.[7] Knowledge about dantrolene's specific site of action is important because it is the only drug efficacious for treating ma-

* Department of Anesthesia, The Bowman Gray School of Medicine of Wake Forest University.

† Department of Basic and Clinical Pharmacology, Universidade Federal do Rio de Janeiro.

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Address reprint requests to Dr. Nelson: Department of Anesthesia, The Bowman Gray School of Medicine of Wake Forest University, 300 South Hawthorne Road, Winston-Salem, North Carolina 27103.

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lignant hyperthermia (MH), and although it can block skeletal muscle contraction only by 75%, no similar effects on cardiac or smooth muscle contractility have been noted. The ryanodine receptor protein (Ry_1) is the major calcium release channel in skeletal muscle, and its mutational forms are linked to MH susceptibility in pigs[9] and in some human families.[10–13] The Ry_1 receptor remains a probable molecular target for dantrolene's action, even though previous studies produced conflicting results.[3–5]

An analysis of the Adverse Metabolic Response to Anesthesia database in North American MH Registry‡ showed that 32 of 232 (13.8%) MH cases recrudesced and that 30 of these 32 patients received dantrolene therapy. While testing a hypothesis that DS acts on the Ry_1 receptor protein, we discovered two effects of micromolar, therapeutic DS concentration on the channel's gating properties. The first observed effect was activation of the channel, which was followed in time by the second effect of inactivation. The activating effect of DS was shown to occur at concentrations 0.001 times the therapeutic, inactivating concentration. Subsequently, we showed that these low concentrations of DS can produce contracture in isolated normal and MHS skeletal muscle, providing one possible explanation for the recrudescence of MH among dantrolene-treated MH patients.

Methods and Materials

A heavy fraction of sarcoplasmic reticulum membranes (SR) was isolated from porcine longissimus dorsi according to methods previously described.[14] Muscle, biopsied from animals anesthetized with thiopental with their lungs mechanically ventilated with 100% O_2, was homogenized then centrifuged at varying speeds[14] to obtain a crude heavy SR membrane fraction. The membranes were finally suspended in 20 mM histidine (*p*H 6.8), 150 mM KCl, and 0.3 M trehalose and protease inhibitors and maintained at −75° C. The animals from which the muscle was obtained were phenotyped to MH by *in vitro* gracilis muscle contracture testing and by a halothane-succinylcholine MH challenge protocol.[15] Single-channel reconstitution experiments were as previously reported.[16] The heavy SR membrane fraction concentrated with Ry_1 receptor protein was incorporated into a phospholipid bilayer (palmitoyl - oleoyl - phosphatidylethanolamine:palmitoyl-oleoyl-phosphatidylcholine, 7:3) painted across a 250-μm diameter aperture separating two chambers. $CsCH_3SO_3$-equilibrated-agar Ag/AgCl electrodes were used to measure the Cs^+ current flowing from one chamber (cis) to the other (trans). The cis chamber represents cytoplasmic and the trans chamber intraluminal reference to the SR membrane. The SR membrane fusion into the bilayer occurred in the cis chamber, and each chamber maintained at 25°C contained 250 mM $CsCH_3SO_3$, 20 mM HEPES (*p*H 7.4), and 20 μM Ca^{2+}. A pulsed-voltage protocol was used for sampling single-channel data. From a holding membrane potential of 0 mV, a 50-mV (cis) polarization was applied for 200 ms at a frequency of 1.4 Hz. Recordings of 250 episodes, each 2 s in duration, filtered at 2,500 Hz, and sampled at a rate of 10 kHz, were obtained for control predantrolene values. After the control recordings, dantrolene was added to the cis chamber and mixed for 1 min. A 0.5-mM stock solution of dantrolene sodium in water was prepared immediately before adding to the cis chamber. After adding and mixing dantrolene in the cis chamber, channel records were obtained as described above for the controls except that recordings continued for times ranging from 27 min up to 1 h among the channels. Data acquisition software and hardware (pClamp, TL-1 Interface, Axon, Burlingame, CA) were computer-interfaced. Analysis software by TRANSIT 2.0 (Department of Molecular Physiology and Biophysics, Baylor College of Medicine) was used to obtain measurements of open-state probability (Po), current amplitude (picoamperes, pA), and the open and closed time constants. The time constants were derived by fitting logarithmically binned histograms of the open and closed dwell times using an automated maximum likelihood method. The software program TRANSIT also produced current integral values by measuring the area (current amplitude × time) under each opening. Summation of the Cs^+ current integral values during channel lifetimes provided data from which different activity states were identified. In the absence of dantrolene, the slope of the cumulative current integral *versus* time was relatively constant and identified as a control state. After adding dantrolene, activated (cumulative current integral slopes > control) or reduced activity (slope < control) states were identified. Consequently, for this study, three channel activity states were identified as control (±dantrolene), activated, and reduced activity. Among the channels recorded, five were tested in the presence of 1 nM and

‡ Allen G: Personal communication. 1995.

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four with 5 μM dantrolene. Nine variables were determined for each channel recording period represented by control, activated, and attenuated channel activity levels. Experimental design was repeated-measures with unbalanced subsampling. Contrasts were made to compare dantrolene concentration effect on the three different channel activity levels. Proc Mixed in SAS (SAS, Cary, NC) was used for a mixed model, repeated-measures analysis of variance to determine statistical significance ($\alpha = 0.05$) of dantrolene effects on the channel variables. Corrections for multiple comparisons, when necessary, were made with Bonferroni's method. Transformations were required for the cumulative current integral values (ranked within channel with respect to size) and for the dwell-time constants (log transformation to normalize τ value distributions).

The activating effects of low DS concentrations on channels from MHS (n = 5) and MHN (n = 6) pig muscle were compared by exposing each channel to DS concentrations increasing from 0.1 to 4.0 nM. Single-channel data acquisition and analysis was similar to that described above except that each record was 200 episodes, each 2 s duration, and only one 200-episodes control record was acquired before initiating the DS additions.

The effects of low DS concentrations on tension development in isolated human and dog skeletal muscle were tested as follows. Human vastus lateralis and dog gracilis muscle was obtained under general, MH-nontrigger anesthesia for diagnostic contracture testing by the North American MH Registry Protocol procedures.[17] Biopsied muscle fascicles were exposed to dantrolene in 37°C Krebs-Ringer solution within 6 h of surgery. Small, 2–3-mm fascicles of human muscle were tied to a wooden applicator stick and chemically skinned[18] in a relaxing solution containing (mM): 172 K-propionate, 2.5 Mg-acetate, 5 K_2ethyleneglycol-bis(beta aminoethylether) N,N,N′,N′-tetraacetate (EGTA), 10 imidazole propionate (*p*H = 7.0), and 2.5 Na_2K_2ATP. From this bundle of skinned muscle, a single fiber was dissected, transferred to a chamber filled with 650 μl of relaxing solution maintained at 25°C, and attached to a force transducer (Grass FT 03) for tension measurement. The force transducer signal was digitized and conditioned by a Digidata 1200 and a Cyberamp, respectively (Axon) and stored in a computer for subsequent analysis with pClamp6 software (Axon). Wash solution was identical to relaxing solution except the K-propionate was 185 mM and no K_2EGTA was present. Caffeine and DS solutions were prepared in the washing solution immediately before use. The pCa levels were based on appropriate mixture of CaEGTA:K_2EGTA calculated by the computer program described by Fabiato[19,20] using the constants provided by Orentlicher *et al.*[21] The protocol for tension measurements was as follows: (1) 1-min calcium (pCa = 6.6) loading into the sarcoplasmic reticulum; (2) two 20-s washes in washing solution; (3) 5-min exposure of fiber to 5 nM DS; and (4) exposure of fiber to a caffeine concentration. After step 3, steps 1 and 2 were repeated, and the next concentration of caffeine was added. Each fiber was tested with 20 mM caffeine at the beginning and end of each protocol. If the 20-mM caffeine response at the end of the protocol was <80% of the initial response, the fiber data were discarded. The human and animal tissues used in these experiments were obtained by protocols approved by our institutional review committee.

Results

Isolated Porcine RY_1 Channel Behavior

Although channel activity was measured from current produced by Cs^+ conductance, the channels are identified as Ry_1 receptor calcium channels by a characteristic unitary conductance of cesium (450 pS), inhibition by ruthenium red, activation by ATP, and transformation to a prolonged, suboptimal conducting state by ryanodine.[22] In a previous, preliminary study,[23] we observed activation followed by activity below control level when dantrolene was applied to channels at concentrations ranging from 5 to 25 μM. Speculating that a low-affinity binding site may represent the dantrolene effect that reduces activity of the Ry_1 receptor channel, we compared the effects of 1 and 5 μM dantrolene on a single Ry_1 receptor channel (fig. 1). Addition of 1 nM dantrolene was followed by a period of increased channel open-state probability that was never observed during control recordings (fig. 1). Overall, the open-state probability was increased from 0.02 to 0.07. Next, dantrolene concentration was increased from 1 to 5 μM, and after a brief period of increased activity, the channel became inactivated by this higher concentration of dantrolene (fig. 1) and was never again observed in an activated state. Among four channels exposed to 5 μM dantrolene, each was first activated, and this was followed by periods during which the channels had a marked reduction in activity. During the total recording time of 78.8 min for which these four channels were

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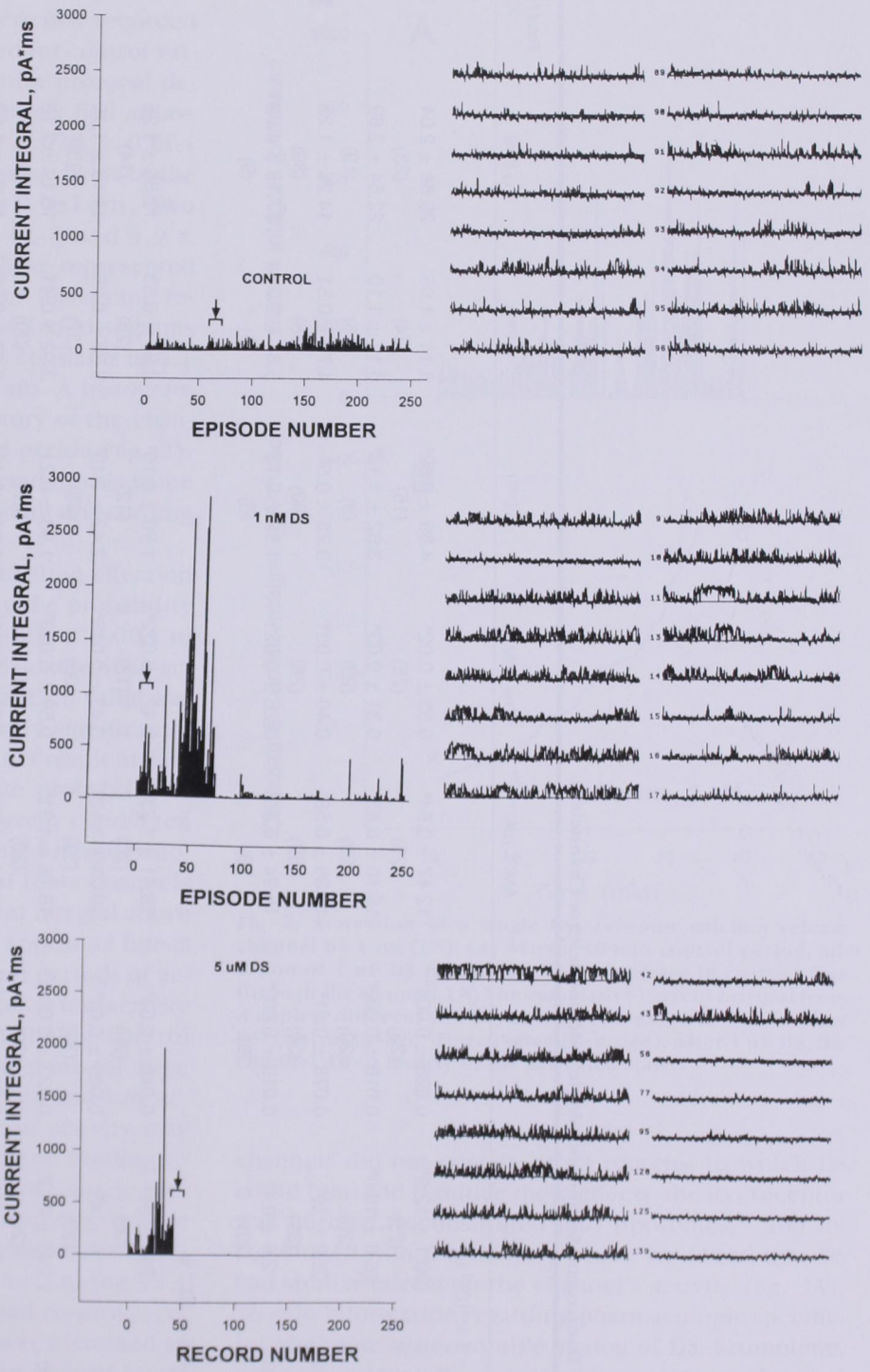

Fig. 1. The alteration of single calcium release channel gating by dantrolene sodium (DS). (*Top*) The left figure is a record of the current integral across 250 episodes, each 2 s long. The right figure shows single channel raw data with upward spikes representing open states from the closed, baseline state. (*Middle*) After adding 1 nM DS, the channel has increased activity as illustrated by increased current integral period (left) and by increased open-state probability (right). (*Bottom*) After adding 5 μM DS, the channel changes from an activated to an inactivated state. Bracket with arrow above represents that portion of the record from which the raw data for channel gating was obtained.

exposed to 5 μM dantrolene, 37.7 ± 4% of this time represented control level activity, 30.6 ± 3% of time the channel was in active state, and 31.7 ± 1.2% of time the channels were attenuated. The average values for the cumulative current integral, Po, open- and closed-state dwell times and time constants, and current amplitude were calculated for each of these states before and after 5 μM dantrolene (table 1). The five chan-

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Table 1. Effects of Dantrolene Sodium on Single Calcium Release Channels

	CCI	Po	AMPL (pA)	τ_{01} (ms)	τ_{02} (ms)	τ_{c1} (ms)	τ_{c2} (ms)	Total No. of Channel Openings
Dantrolene sodium = 1×10^{-9} M								
Control	90.1 ± 12.9[a] (25)	0.028 ± 0.004[a] (25)	12.47 ± 0.64[a] (25)	0.33 ± 0.02[a] (25)	4.89 ± 0.82[a] (16)	8.14 ± 1.05 (24)	20.68 ± 2.04 (23)	76,488 (24)
Dantrolene control	55.6 ± 10.0[a] (20)	0.016 ± 0.002[a] (20)	15.10 ± 0.63[a] (20)	0.31 ± 0.03[a] (20)	3.62 ± 1.13[a] (9)	6.12 ± 1.10 (15)	22.54 ± 2.69 (19)	30,740
Dantrolene activated	252 ± 29[b] (39)	0.078 ± 0.01[b] (39)	16.36 ± 0.56[b] (39)	0.40 ± 0.03[b] (28)	3.73 ± 0.5[a] (30)	4.79 ± 0.51 (38)	14.26 ± 1.28 (39)	146,280 (41)
Dantrolene attenuated	2.6 ± 0.4[c] (9)	0.010 ± 0.002[a] (9)	16.24 ± 0.80[a] (9)	0.28 ± 0.02[a]	1.98 ± 0.13[a] (6)	5.81 ± 2.21 (6)	27.18 ± 4.02 (9)	5,995
Dantrolene sodium = 5×10^{-6} M								
Control	252 ± 61[a] (20)	0.049 ± 0.01[a] (20)	19.41 ± 1.23[a] (20)	0.42 ± 0.022[a] (18)	1.96 ± 0.15[a] (12)	3.91 ± 0.53 (20)	12.55 ± 1.25 (14)	57,947
Dantrolene control	376 ± 43[a] (33)	0.089 ± 0.01[a,b] (33)	20.03 ± 1.12[b] (33)	0.43 ± 0.016[a] (30)	2.42 ± 0.19[a] (23)	13.32 ± 0.39 (37)	14.10 ± 1.10 (23)	57,790
Dantrolene activated	1,245 ± 210[b] (20)	0.223 ± 0.034[c] (20)	18.16 ± 1.91[a] (20)	0.59 ± 0.033[b] (11)	1.98 ± 0.21[a] (21)	3.02 ± 0.41 (30)	16.19 ± 2.18 (13)	58,840
Dantrolene attenuated	80 ± 16[c] (32)	0.024 ± 0.005[a,c] (32)	15.74 ± 1.00[c] (32)	0.26 ± 0.021[c] (25)	1.94 ± 0.19[a] (20)	3.62 ± 0.36 (16)	17.87 ± 1.93 (20)	31,948

CCI = cumulative current integral (pA · ms); Po = open state probability; AMPL = average current amplitude, picoamperes; τ_{01} and τ_{02} = open state time constants, ms; τ_{c1} and τ_{c2} = closed state time constants, ms.

Values are mean ± SE with number of observations in parentheses. Values in a column with different letter superscripts are significantly different ($P < 0.05$).

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nels treated with 1 nM dantrolene were first recorded over a 10-min period that was analyzed for control values. After activation by the voltage pulse protocol described above (methods), these channels had an average open-state probability (Po) of 0.028 ± 0.004 and an amplitude of 12.5 ± 0.64 pA and dwelt in the open state for an average of 0.37 ± 0.031 ms. Two open time constants of 0.33 ± 0.02 (τ_{O1}) and 4.9 ± 0.82 (τ_{O2}) ms were obtained, and these represented 95.4% and 4.6% of the total dwell time histogram, respectively. The same procedure applied to histograms of the closed times provided two time constants of 8.1 ± 1.05 (τ_{C1}) and 20.7 ± 2.04 (τ_{C2}) ms. A histogram of the current integral provides a history of the channel's conductance over the measured period (fig. 2). During the control period of measurement, this value averaged 90.1 ± 12.9 pA · ms over the total recording time of 10 min.

Dantrolene (1 nM) produced an activating effect on the Ry_1 receptor channel by increasing the probability of opening almost threefold from 0.028 ± 0.004 to 0.078 ± 0.01 ($P < 0.01$). During this dantrolene-activated state, the open-state time constant τ_{O1} value was increased (table 1) and was statistically significantly different from the control values. The net result of dantrolene-induced increase in open-state probability is an increase in the total amount of current conducted by the channel over time. As represented in the histogram of the current integral for one of these channels (fig. 2), dantrolene increases the current integral above the control level, and these changes appear as bursts of increased activity occurring between periods of activity at the control, predantrolene level. A few activity states were identified as having activity below control levels. Because the current integral histograms for these channels did not display periods of high and low activity before dantrolene, this change in activity may represent on and off times for dantrolene binding to apparent high- and/or low-affinity sites of a single protein molecule. During the 73.6 min the channels were exposed to 1 nM dantrolene, the activated-state dwell times totaled 56.9 ± 9% of the total time. During 30.4 ± 7% of the total time, the channels had control level activity, and 12.7 ± 7% of the time was identified as reduced activity ranging from less than control levels to a completely closed channel. Some of the averaged channel variables in these different states are statistically described for the channels treated with 1 nM dantrolene (table 1). To rule out the possibility that the crude SR membranes used to incorporate Ry_1 receptor

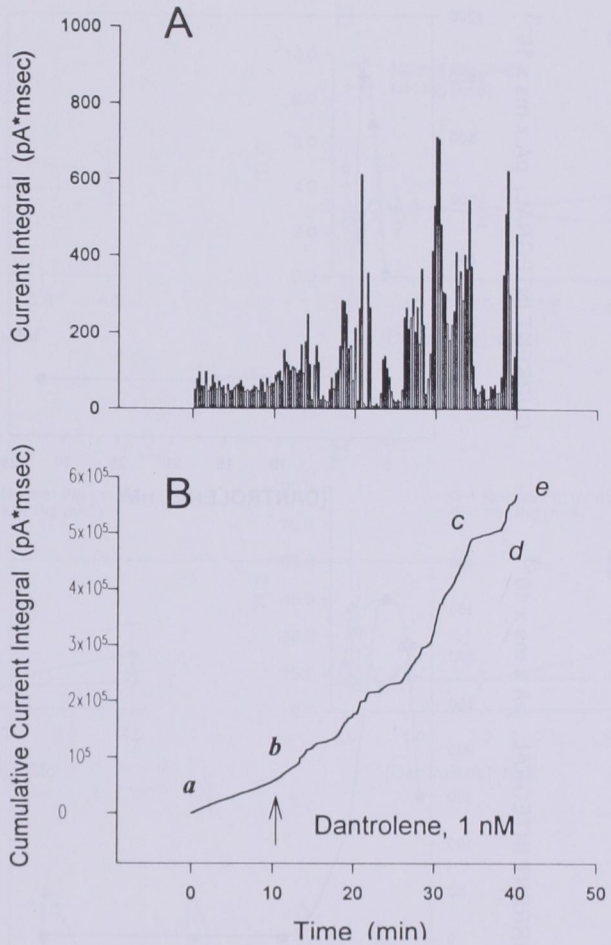

Fig. 2. Activation of a single Ry_1 receptor calcium release channel by 1 nM (DS). (*A*) After a 10-min control period, addition of 1 nM DS produced a large increase in current flux through the channel. (*B*) The cumulative current integral from *A* depicts different activity states (see text) before and after DS: control (a–b, c–d); activated (b–c, d–e). After 1 nM DS, the channel dwelt mostly in the activated state.

channels did not contain other proteins to which DS could bind and produce these effects, the Ry_1 receptor was purified, reconstituted into liposomes,[24] and incorporated into the bilayer, where we found that DS had similar effects on the channel's activity (fig. 3A). To gain information regarding pharmacologic specificity, the more water-soluble analog of DS, azumolene, was exposed to an Ry_1 receptor channel. As we observed for DS, azumolene at nanomolar concentration activated the channel, whereas micromolar concentrations markedly reduced channel activity (fig. 3B).

The apparent high-affinity DS binding site was characterized for its DS concentration dependency, which

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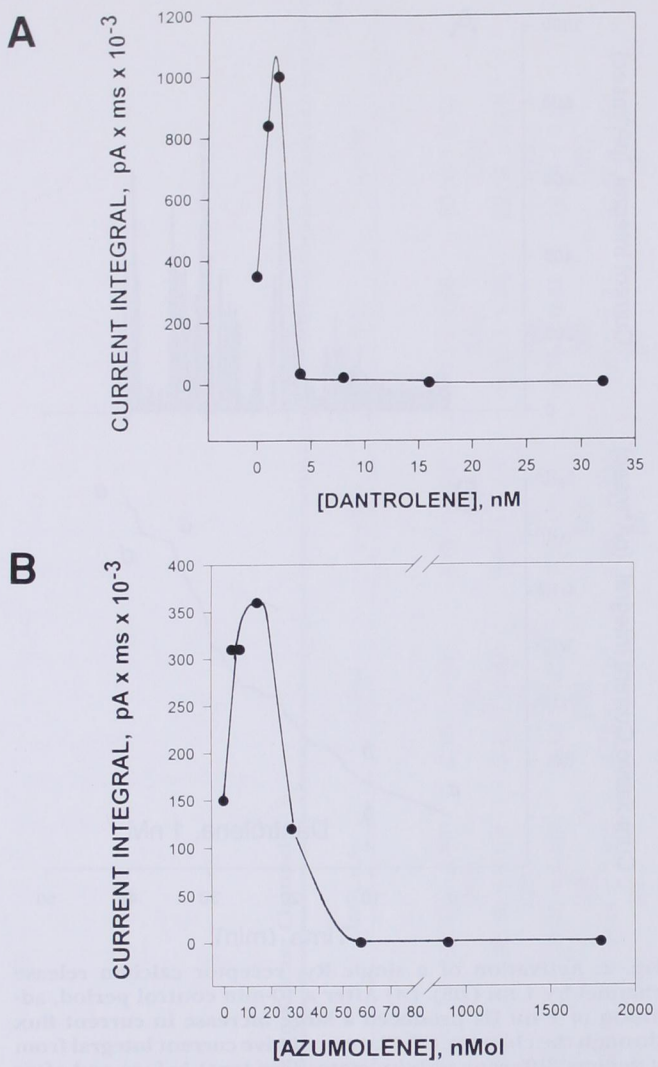

Fig. 3. (*A*) Effect of dantrolene sodium (DS) on the current flux through a single, purified Ry_1 receptor calcium release channel. After purification of Ry_1 receptor and reconstitution into liposomes, the channel was incorporated into a bilayer for single channel recording. At 2 nM DS, the channel is activated 2.8-fold above control, pre-DS levels. Higher DS concentrations reduced current through the channel to levels below the control (*B*). The effect of azumolene, a more hydrophilic analog of dantrolene sodium, on the activity of a single skeletal muscle Ry_1 receptor calcium release channel.

was compared between MHS and MHN channels from pig skeletal muscle. The open-state probability of MHN channels increased biphasically, with DS concentrations increasing from 0.1 to 4.0 nM (fig. 4). The first phase appeared to be saturated at about 0.5 nM, and the second phase at 2.0 nM (fig. 4). The Po was increased twofold at 0.5 nM and sixfold at 2.0 nM DS (fig. 4). Channels from MHS muscle had 1.8 ± 0.39-fold increase in Po at 0.5 nM, but from 1 to 4 nM DS, no further increase in Po was observed. The open-state dwell time histograms were logarithmically binned and fitted for time constants, for which two were found. The shorter time constant, τ_{O1} averaged 0.26 ± 0.02 ms and 0.48 ± 0.17 ms for MHN and MHS channels, respectively, and these dwell time constants represented 95.9 ± 0.02 and 95.8 ± 0.025% of the total histograms, respectively. The τ_{O1} values increased with increasing DS concentrations with a biphasic response (fig. 5) similar to that observed for DS effects on Po. However, the τ_{O1} value was increased by 1.5-fold compared to a sixfold increase for Po. Similar to the DS concentration dependency for Po, the biphasic τ_{O1} responses showed saturation at 0.5 and 1.0 nM DS (fig. 5A). As previously reported,[22] the τ_{O1} value for MHS channels is greater than for MHN channels. The DS concentrations (0.1–4 nM) had no statistically significant effect on τ_{O1} (fig. 5A). The longer open time constants, τ_{O2}, were not statistically significantly affected by DS application to MHN or MHS channels (fig. 5B). Two closed-state time constants were fitted for MHS and MHN channels and DS, 0.1–4 nM, had no effect on these values (fig. 6).

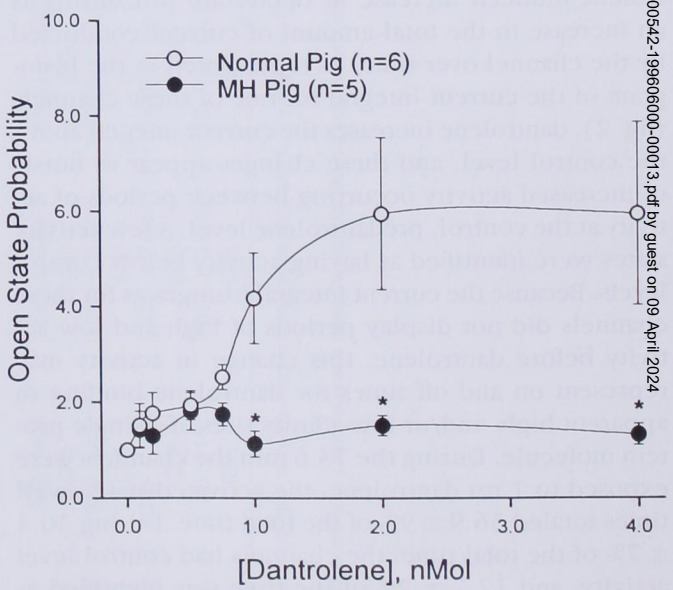

Fig. 4. A comparison of dantrolene sodium (DS) effects on open-state probability of malignant hyperthermia (MH) mutant and wild-type channels from pig skeletal muscle. In wild-type channels, DS increased Po above the normalized (1.0), control value. A biphasic, [DS]-dependent response was evident in the wild-type channels. In MH mutant channels, only the first phase of DS activation was observed. *Statistically significant difference between wild-type and mutant channels.

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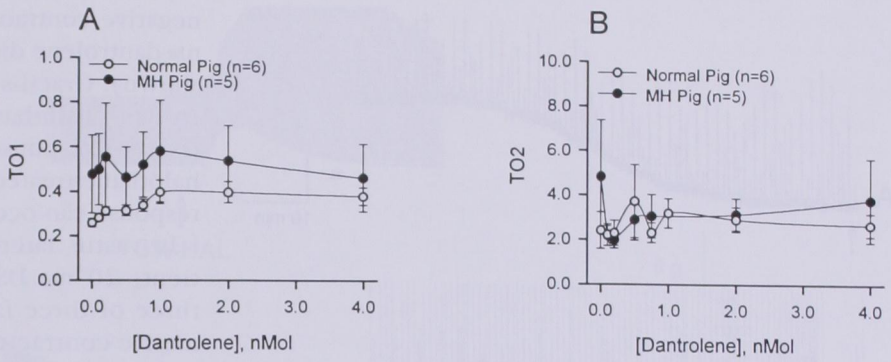

Fig. 5. Open-state dwell time constants for malignant hyperthermia (MH) mutant and wild-type channels with varying concentrations of dantrolene sodium. (*A*) Short dwell time constant τ_{O1} is greater in MH mutant channel and dantrolene sodium (DS) produced greater increase in τ_{O1} of wild-type channels. (*B*) Longer open time constant, τ_{O2} was not different between MH mutant and wild-type channels, and DS effect is not significant.

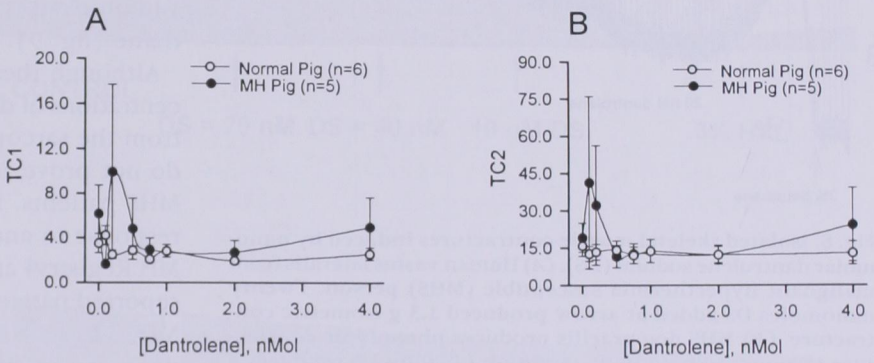

Fig. 6. Closed-state dwell time constants for malignant hyperthermia (MH) mutant and wild-type channels with varying concentrations of dantrolene sodium. Short (*A*) and long (*B*) closed-state dwell times were not altered by dantrolene sodium and did not differ between MH mutant and wild-type channels.

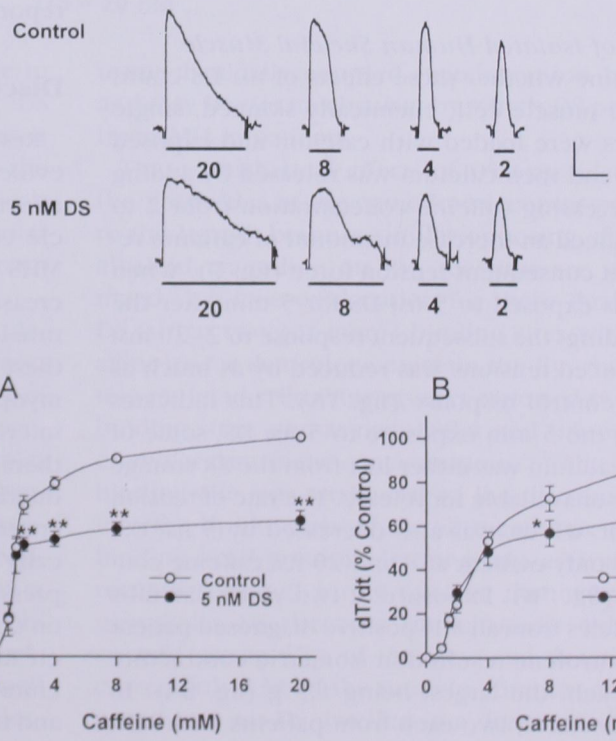

Fig. 7. Effect of nanomolar dantrolene sodium on caffeine-induced tensions in human single skinned fiber. Isometric tension recorded from human skeletal single skinned fiber. Both records were obtained from same fiber. Control: sarcoplasmic reticulum was loaded during 1 min pCa 6.6 solution. After two washes (in washing solution), tension was challenged by caffeine. To investigate the effect of low concentration of dantrolene sodium (DS) on Ca^{2+} sequestration by SR, fibers were pretreated by 5 nM of DS during 5 min, and then control protocol was repeated. Bar = exposure time for each concentration of caffeine tested. Calibrations: vertical, 15 mg; horizontal, 40 s. Pooled data (N = 10, mean ± SE) of: (*A*) peak tension and (*B*) dT/dt induced by incremental concentration of caffeine using protocol described above. Ordinate of *A* and *B* represent, respectively, percent of maximal tension and percent of dT/dt maximal induced by 20 mM of caffeine. $^{*}P < 0.05$. $^{**}P < 0.01$.

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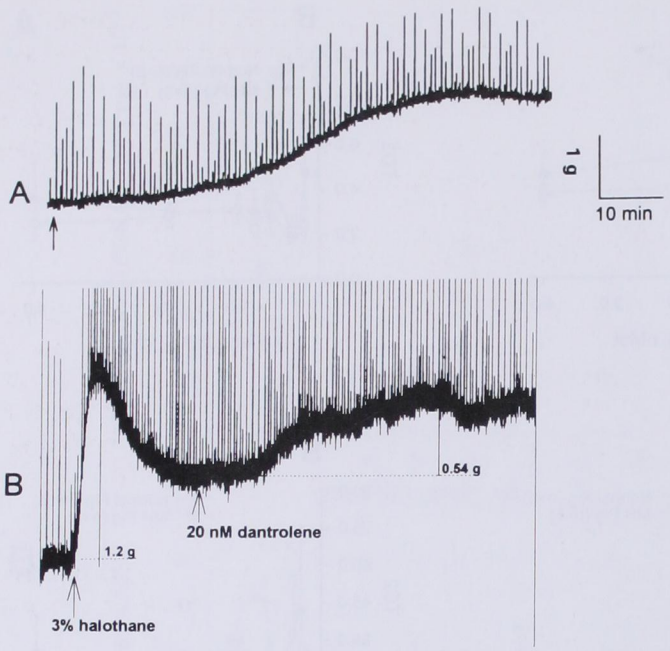

Fig. 8. Isolated skeletal muscle contractures induced by nanomolar dantrolene sodium (DS). (*A*) Human vastus lateralis from malignant hyperthermia susceptible (MHS) person. Twenty nanomoles DS added at arrow produced 1.3 g isometric contracture. (*B*) MHS dog gracilis produces phenotypic contracture response to halothane. Addition of 20 nM DS produces a 0.54-g contracture above the steady-state tension produced by halothane.

Response of Isolated Human Skeletal Muscle

To determine whether these effects of nM DS could occur in the muscle cell, chemically skinned, single human fibers were loaded with calcium and exposed to 5 nM DS, and then calcium was released by adding caffeine. Increasing caffeine concentration from 2 to 20 mM produced an increase in amount of calcium released and in consequent tension force (fig. 7). When the fiber was exposed to 5 nM DS for 5 min after the calcium loading, the subsequent response to 2–20 mM caffeine-induced tensions was reduced by as much as 40% of the control response (fig. 7A). This indicates that, during the 5 min exposure to 5 nM DS, some of the loaded calcium was either lost from the SR storage or was made unavailable for release. The rate of tension development, dT/dt, was also decreased by 5 nM DS, but this was only evident at 8 and 20 nM caffeine concentrations (fig. 7B). Exposure of two vastus lateralis muscle fascicles from an MH-positive diagnosed patient to 20 nM dantrolene resulted in isometric contracture tension in each, the largest being 1.3 g (fig. 8A). In four other fascicles, two each from patients with MH-negative contracture test results, 1-h exposure to 20 nM dantrolene did not produce a contracture (data not shown). Gracilis fascicles from MH dogs treated *in vitro* with halothane produced the MH-phenotypic contracture response, and when DS is applied to these halothane-treated fascicles, an additional contracture response can occur (fig. 8B).

In vastus lateralis fascicles from another MHS patient, 20 nM DS potentiated the twitch tension in three of three fascicles tested without evoking isometric contracture tension (fig. 9). Increasing DS to 40 nM produced no further effect, but an increase to 10 μM resulted in a 72% reduction in twitch tension and blockade of the contracture response to 3% halothane (fig. 9).

Although these *in vitro* studies show that low concentrations of dantrolene can cause a release of calcium from the sarcoplasmic reticulum stores, such findings do not prove *in vivo* dantrolene effects in normal or MHS patients. Data extracted from adverse metabolic response to anesthesia reports to the North American MH Registry‡ are interesting in this regard. Among 232 reported patients with clinical episodes suspicious for MH, 32 (13.8%) of these were reported to have recrudescence of the syndrome. Of these 32 patients, 30 had received an average initial DS dose of 120 mg. Among the 122 patients treated with DS, 24.6% were reported to have recrudesced.

Discussion

Results obtained from these investigations provide evidence at the cellular and molecular levels for two effects of DS on the regulation of $[Ca^{2+}]$ in skeletal muscle cells. In biopsied skeletal muscle fascicles from MHS humans, nanomolar concentrations of DS can increase twitch tension and produce isometric contracture in these genetically predisposed cells. Presumably, these tension increases are a consequence of increased myoplasmic $[Ca^{2+}]$ that is produced by dantrolene. At micromolar concentration, which corresponds to the therapeutic blood concentration, DS produces a reduction in twitch tension and blocks the MH muscle's contracture response to halothane. Thus, the genetically predisposed MH muscle cell is capable of expressing both of the DS concentration-dependent effects on contractility. Using chemically skinned single-muscle fibers, the muscle cell function is simplified to calcium uptake and release by the sarcoplasmic reticulum and to the generation of tension by the contractile ele-

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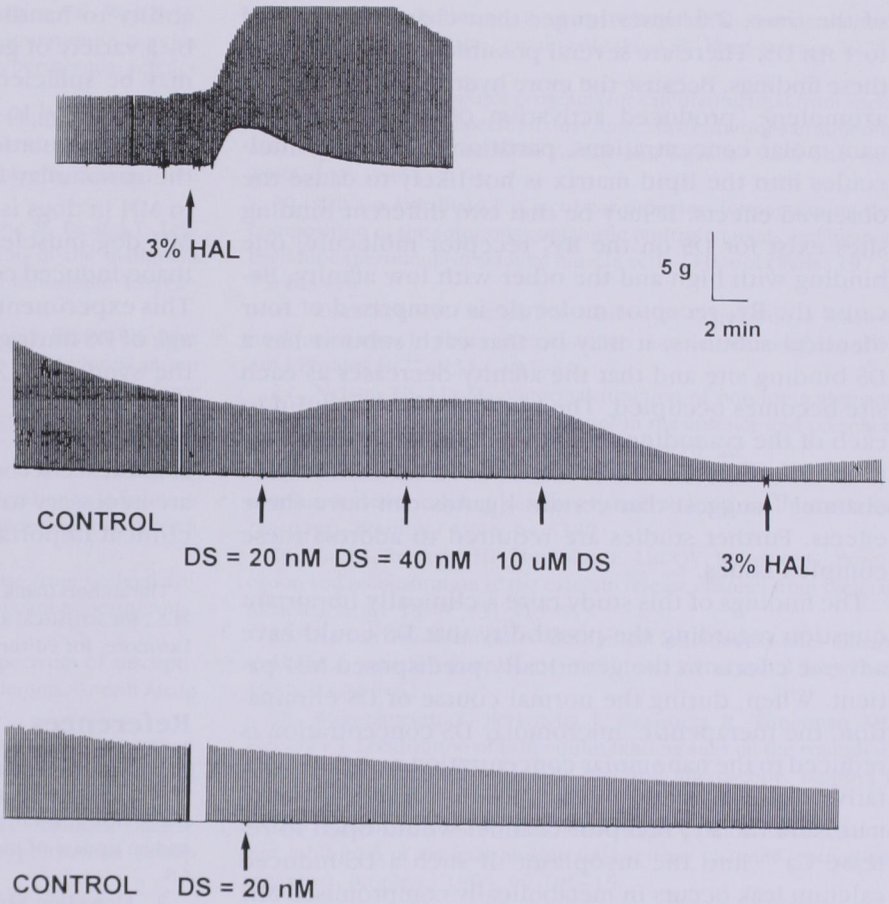

Fig. 9. The effect of dantrolene sodium (DS) on normal and malignant hyperthermia-susceptible (MHS), biopsied human skeletal muscle contractility. (*A*) MHS muscle twitch potentiation and isometric contracture produced by 3% halothane. (*B*) MHS muscle exposed to 20 nM DS has potentiation of twitch, and increasing DS to 40 nM was without effect. At 10 μM DS, the twitch tension was reduced 72%, and when exposed to 3% halothane, no contracture occurred. (*C*) Normal human skeletal muscle exposed to 20 nM DS had neither twitch potentation nor contracture produced.

ments. In this experimental model, we were able to demonstrate that nanomolar concentrations of DS caused a release of calcium from the SR, whereas it had no effect on the amount of calcium loaded in the presence of MgATP. This finding suggests that the calcium release channel could be opened by nanomolar concentrations of DS, allowing calcium to flow down its concentration gradient into the myoplasm.

Experiments on single Ry_1 receptor protein molecules incorporated into a lipid bilayer provided direct evidence that DS could alter the gating properties of this calcium release channel. At the nanomolar concentration range, DS produces a marked increases in the open-state probability and dwell time of the channel, a finding that could explain how low concentrations of DS alter contractility in the intact muscle cell. When the calcium release channel is exposed to DS at micromolar concentration, an opposite effect is observed, *i.e.*, the channel open-state probability is markedly reduced. This finding is consistent with the well established pharmacologic effect of DS to reduce electromechanically coupled twitch tension by 70–80% and may be the mechanism by which DS prevents and treats MH in patients.

These antithetical effects of DS may relate more to the properties of the ryanodine receptor protein than to the ligand, because similar opposing effects of the alkaloid ryanodine on Ry_1 receptor are well recognized.[25–27] One explanation for these dual effects of DS is that two (or more) binding sites with different affinities for dantrolene exist on the Ry_1 receptor protein molecule. Previous studies reported two different binding sites, one at nanomolar and the other at micromolar dantrolene concentrations.[6–8] The low-affinity binding site was attributed to the distribution of DS into the lipid matrix of the SR membrane rather than binding to SR protein.[7] In our study, activation of Ry_1 receptor channel gating was the dominant effect for nanomolar DS, because the channels exhibited increased gating 57% of the time and deactivation occurred only 13% of the time. In contrast, channels exposed to 5 μM DS dwelled in a deactivated state 32%

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of the time; 2.5 times longer than channels exposed to 1 nM DS. There are several possible explanations for these findings. Because the more hydrophilic DS analog, azumolene, produced activation of Ry_1 receptor at nanomolar concentrations, partitioning of these molecules into the lipid matrix is not likely to cause the observed effects. It may be that two different binding sites exist for DS on the Ry_1 receptor molecule; one binding with high and the other with low affinity. Because the Ry_1 receptor molecule is comprised of four identical subunits, it may be that each subunit has a DS binding site and that the affinity decreases as each site becomes occupied. The binding of calmodulin to each of the ryanodine receptor subunits[26] and the antithetical effects of calmodulin on the calcium release channel[27] suggest that certain ligands can have these effects. Further studies are required to address these complex issues.

The findings of this study raise a clinically important question regarding the possibility that DS could have adverse effects in the genetically predisposed MH patient. When, during the normal course of DS elimination, the therapeutic, micromolar DS concentration is reduced to the nanomolar concentration range, the putative high affinity DS binding site effect would dominate, and the Ry_1 receptor channel would open to release Ca^{2+} into the myoplasm. If such a DS-induced calcium leak occurs in metabolically compromised MH muscle cells, myoplasmic $[Ca^{2+}]$ may increase to levels that reproduce the MH syndrome. In metabolically uncompromised cells, such a calcium leak may be controlled without serious consequences. The high incidence of MH syndrome recrudescence among dantrolene-treated MH patients suggests that our findings may have clinical relevance and represent a clinical paradox.

The genetic predisposition to MH in some human families is linked to one of several different mutations in Ry_1 receptor,[10–13] presenting another confounding possibility that some Ry_1 receptor mutations are adversely susceptible to the nanomolar DS effects and others are not. In this regard, it is interesting that nanomolar DS produced a greater increase in opening of the wild-type channel from pig muscle than it did from the MH mutant channels. Because only 5% humans demonstrate the pig defect, and the Ry_1 receptor may be involved in only about 50% of the mutations that demonstrate phenotypic MH, the increased opening caused by dantrolene in normal Ry_1 receptors may be of considerable significance. In muscles in which the ability to handle Ca^{2+} appropriately is compromised by a variety of genetic defects, the effect of dantrolene may be sufficient to induce hypermetabolism, which may proceed to an MH episode. It may be that the Ry_1 receptor mutation in pigs results in an attenuation of the nanomolar DS effects. The mutation predisposing to MH in dogs is not the pig MH mutation.§ In isolated MH dog muscle, addition of nanomolar DS to a halothane-induced contracture produced more contracture. This experiment suggests that inappropriately low dosage of DS during an MH clinical crisis could exacerbate the syndrome. Although our investigation unequivocally identifies an effect of low DS to increase myoplasmic $[Ca^{2+}]$, it does not prove that this can cause recrudescence of the MH syndrome. Further studies are necessary to determine whether our findings are of clinical importance.

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