Pharmacological distinction between dantrolene and ryanodine binding sites: evidence from normal and malignant hyperthermia-susceptible porcine skeletal muscle

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Dantrolene inhibits and ryanodine stimulates calcium release from skeletal-muscle sarcoplasmic reticulum (SR), the former by an unknown mechanism, and the latter by activating the ryanodine receptor (RyR), the primary Ca^{2+} -release channel of SR. Dantrolene is used to treat malignant hyperthermia (MH), a genetic predisposition to excessive intracellular Ca^{2+} release upon exposure to volatile anaesthetics. Porcine MH results from a point mutation in the SR RyR that alters the open probability of the channel, and is reflected in altered [^3H]ryanodine binding parameters. Specific binding sites for [^3H]dantrolene and [^3H]ryanodine co-distribute on SR that has been isolated by discontinuous sucrose gradient centrifugation. If the two drug-binding sites are functionally linked, [^3H]dantrolene binding might be affected both by pharmacological and by genetic modulators of the functional state of the RyR. Accordingly, we compared the characteristics of [^3H]dantrolene binding to porcine malignant-hyperthermia-susceptible and normal-skeletal-muscle

SR, and examined the effects of RyR modulators on [^3H]dantrolene binding to these membranes. Additionally, the feasibility of separating the SR binding sites for [^3H]dantrolene and [^3H]ryanodine was investigated. No significant differences in [^3H]dantrolene binding characteristics to SR membranes from the two muscle types were detected, and the B_{max} ratio for [^3H]dantrolene/[^3H]ryanodine was $1.4(\pm 0.1):1$ in both muscle types. [^3H]Dantrolene binding is unaffected by the RyR modulators caffeine, ryanodine, Ruthenium Red and calmodulin, and neither dantrolene nor azumolene have any effect on [^3H]ryanodine binding. Additionally, distinct peaks of [^3H]dantrolene and [^3H]ryanodine binding are detected in SR membranes fractionated by linear sucrose centrifugation, although no differences in protein patterns are detected by SDS/PAGE or Western-blot analysis. We suggest that the binding sites for these two drugs are pharmacologically distinct, and may exist on separate molecules.

INTRODUCTION

Malignant hyperthermia (MH) is a genetic disorder of skeletal muscle resulting in a pattern of muscle physiology resembling aberrant excitation–contraction coupling [1,2]. In susceptible individuals, the syndrome is triggered by exposure to volatile anaesthetics and/or depolarizing skeletal muscle relaxants, and is characterized by a sustained elevation of myoplasmic Ca^{2+} , probably resulting from a defect in the mechanism of sarcoplasmic reticulum (SR) Ca^{2+} release. This process leads to hypercontracture, hypermetabolism, hyperthermia and, if left untreated, death [3]. Dantrolene (1-([5-(*p*-nitrophenyl)furfurylidene]amino)hydantoin sodium) [4] is the only drug currently available for the effective treatment of MH, and is thought to act by inhibiting Ca^{2+} release from the SR [5,6]. However, the exact mechanism and site of action of this drug are not known, although recent evidence would suggest that it is at the level of the SR Ca^{2+} -release channel itself [7].

The SR Ca^{2+} -release channel, also known as the ryanodine receptor (RyR) for its ability to bind and be activated by the plant alkaloid ryanodine, is a member of a family of intracellular Ca^{2+} -release channels with genes expressed primarily in skeletal

muscle (RYR1), heart (RYR2) and brain (RYR3), although overlap in expression exists [8]. The functioning of this channel can be altered both by pharmacological modulation and through genetic modifications. For example, RyR channel activity is enhanced by caffeine, Ca^{2+} , ATP and nanomolar ryanodine, and is inhibited by Ruthenium Red, Mg^{2+} and micromolar ryanodine [9]. [^3H]Ryanodine binding studies demonstrate that the binding of this ligand to the RyR reflects the functional state of the Ca^{2+} channel, in that channel activators enhance [^3H]ryanodine binding, whereas channel inhibitors suppress binding [9]. Additionally, the point mutation in RYR1 resulting in the Arg⁶¹⁵ → Cys amino acid substitution linked to porcine MH [2,10] leads to an increased rate of SR Ca^{2+} efflux, and both an increased receptor affinity for [^3H]ryanodine and an altered Ca^{2+} dependence for [^3H]ryanodine binding [11–13].

Porcine MH, like human MH, is characterized by an exaggerated, volatile, anaesthetic-induced release of Ca^{2+} into the myoplasm that results in symptoms of the syndrome. Unlike porcine MH, the inheritability of human MH susceptibility is genetically pleiotropic, as only half the cases examined contain a mutation in the RYR1 gene [2,14]. Yet patients and all strains of malignant hyperthermia susceptible (MHS) pigs who develop the

Abbreviations used: HSR, heavy sarcoplasmic reticulum; LSR, light sarcoplasmic reticulum; MH, malignant hyperthermia; MHS, malignant hyperthermia susceptible; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

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syndrome can be successfully treated with dantrolene, indicating that the site of action of dantrolene may be a common regulatory site in the pathway of SR Ca^{2+} release.

Studies in normal and MHS pigs have suggested that there is little difference between the two in the effects and concentration dependence of intravenous dantrolene on various parameters of skeletal muscle relaxation [15,16]. In contrast, dantrolene is reported to differentially affect mean channel open time of the RyR from normal and MHS tissue in lipid bilayer studies, leading the authors to suggest that dantrolene acts directly on the RyR [7]. The last implies that, like ryanodine binding, dantrolene binding is also functionally linked to the activity state of the RyR/ Ca^{2+} -release channel. We would predict therefore that the characteristics of the dantrolene binding site may differ between normal and MH tissue, and that solution conditions or drugs that regulate RyR function should also affect [^3H]dantrolene binding. We therefore tested whether differences in [^3H]dantrolene binding could be distinguished in SR derived from the two muscle types, and whether RyR modulators have any effect on [^3H]dantrolene binding. We now present evidence that [^3H]dantrolene and [^3H]ryanodine binding sites in isolated SR are present on separable, overlapping membrane subsets in linear sucrose gradients, and are pharmacologically distinct and non-interacting. These data imply a mechanism of action of dantrolene that is independent of the ryanodine binding site.

MATERIALS AND METHODS

Materials

Dantrolene sodium, $3\frac{1}{2}\text{H}_2\text{O}$, azumolene sodium, $2\text{H}_2\text{O}$ and amino-dantrolene sodium were graciously given by Proctor & Gamble, Norwich, NY, U.S.A. [^3H]Dantrolene was custom synthesized by ChemSyn (Lenexa, KS, U.S.A.; 8.92 Ci/mmol), or Vitrox (Placentia, CA, U.S.A.; 10.0 Ci/mmol). [^3H]Ryanodine (65.0 Ci/mmol) and the dihydropyridine antagonist [^3H]PN200-110 (78.0 Ci/mmol) were purchased from NEN and Amersham respectively. Purified cow brain calmodulin was given by Dr. Donald J. Wolff, U.M.D.N.J.-Robert Wood Johnson Medical School, Piscataway, NJ, U.S.A. PMSF, benzamidine, leupeptin and pepstatin A were purchased from Boehringer-Mannheim. CytoScint-ES and hyamine hydroxide were purchased from ICN, and glass-fibre filters (GF/C) from Whatman. Common laboratory chemicals were purchased from Sigma.

Preparation of skeletal-muscle membrane fractions

MHS and normal pigs were from closed herds maintained by the Department of Animal Science, University of Minnesota. The herds have been genotyped on the basis of the C1843T mutation (R615C) in the RYR1 gene [2]. Fast-twitch skeletal muscle was dissected and immediately frozen in liquid N_2 and maintained at -72°C until use. Membrane fractions corresponding to transverse tubules, light SR (LSR) and heavy SR (HSR) were prepared using discontinuous sucrose-density-gradient centrifugation [11,17], essentially as described previously [18]. Membrane fractions were stored at -80°C and thawed just before use. These fractions have been characterized for their ability to bind [^3H]ryanodine, [^3H]dantrolene and [^3H]PN200-110, and have maintained binding activity for up to 2 years [18–20].

[^3H]Dantrolene binding assay

Specific [^3H]dantrolene binding to HSR was determined by a rapid filtration assay, exactly as described previously [18]. Specifically bound [^3H]dantrolene was typically 20–30% of the

total binding activity. Non-specific binding was determined in the presence of $150\text{ }\mu\text{M}$ azumolene. Data was analysed by using non-linear regression (InPlot 4.0, GraphPad Inc.). The K_i values for unlabelled congeners of [^3H]dantrolene were calculated using the Cheng–Prusoff correction, which relates the tracer ligand concentration and the concentration of inhibitor at which 50% ligand binding is inhibited [21]. Protein was determined according to Bradford [22]. Statistical analysis of the data was done by using StatMost (Version 2.5, DataMost, Inc.). Graphic data are presented as means $\pm$ S.E.M. Free Ca^{2+} concentrations were determined using the freeware program Maxchelator version 6.63 [23].

[^3H]Ryanodine binding assay

The binding of [^3H]ryanodine to membrane fractions was determined using a rapid filtration assay according to the method of Valdivia et al. [19]. Briefly, HSR membranes were incubated with appropriate concentrations of [^3H]ryanodine in a buffer containing 20 mM Tris/HCl (pH 8.5), 0.15 M NaCl and $50\text{ }\mu\text{M}$ CaCl_2 , at 37°C for 90 min. Samples were rapidly filtered through Whatman GF/C filters using a Brandel cell harvester, and washed with ice-cold buffer ($2 \times 5\text{ ml}$). The filters were counted for radioactivity in CytoScint-ES. Non-specific binding was measured in the presence of $10\text{ }\mu\text{M}$ ryanodine.

Fractionation of [^3H]dantrolene and [^3H]ryanodine SR binding sites

Porcine skeletal-muscle HSR was isolated as described above. After thawing, membranes were further dispersed in [^3H]dantrolene binding buffer using two manual strokes of a Teflon/glass Dounce homogenizer. SR membranes ($500\text{ }\mu\text{l}$, 6 mg/ml) were layered on to linear 20–40% (w/v) sucrose gradients and centrifuged at $100\,000\text{ g}$ for 18 h at 4°C . The gradients were then fractionated into $500\text{ }\mu\text{l}$ fractions and the specific binding of [^3H]dantrolene (250 nM) and [^3H]ryanodine (5 nM) was determined in each fraction. Total and non-specific drug binding was determined in duplicate aliquots from each fraction as described above. Final sucrose concentrations did not exceed 75 mM, and had no effect on [^3H]dantrolene binding (see the Results section).

Protein electrophoresis and immunoblot analysis

Constituent proteins of SR membranes separated by linear sucrose-density-gradient centrifugation were resolved by electrophoresis on SDS/7.5% (w/v)-polyacrylamide gels according to the method of Laemmli [24], and either stained with silver [25], or transferred to PVDF membranes (Immobilon-P membranes, Millipore) for subsequent Western-blot analysis. Proteins in sample buffer were kept at room temperature for 1 h before sample loading, as boiling caused precipitation of protein including the RyR. For immunoblot analysis, proteins were transferred from gels using a semi-dry blotting apparatus (Idea Scientific, Minneapolis, MN, U.S.A.) at 24 V for 1 h. Blotted PVDF membranes were then incubated with 5% (w/v) non-fat dry milk in TBS buffer (20 mM Tris/HCl/150 mM NaCl, pH 7.5) for 1 h at room temperature to block non-specific antibody binding. Specific antibody binding was obtained by incubating blots with appropriate primary antibody in TBS buffer containing 1% non-fat dry milk, either for 2 h at room temperature, or overnight at 4°C . Blots were then incubated with appropriate secondary antibody (Sigma) conjugated to alkaline phosphatase for 2 h at room temperature, and visualized after colour development with Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl

phosphate (Sigma). The primary antibodies to skeletal muscle RyR, dihydropyridine receptor (α_1 subunit), calsequestrin and $\text{Ca}^{2+}/\text{Mg}^{2+}$ ATPase were generously given by Dr. Kevin P. Campbell.

RESULTS

To determine whether MH susceptibility altered the subcellular distribution of [^3H]dantrolene binding, drug binding was determined for the four major subfractions of porcine skeletal-muscle membranes purified by discontinuous sucrose-density-gradient centrifugation. [^3H]Dantrolene binding to MHS skeletal muscle membranes followed the pattern seen with normal skeletal muscle membranes [18], with the greatest number of sites present in the HSR and only half as many sites detected in the LSR fraction. As reported previously [18], no specific [^3H]dantrolene binding sites were detected in membrane fractions enriched in either sarcolemma or transverse tubules, and specific

Figure 1 Characterization of [^3H]dantrolene binding to HSR membranes from MHS porcine skeletal muscle

(A) The concentration dependence of equilibrium binding of [^3H]dantrolene to HSR was determined by rapid filtration, as described in the Materials and methods section. Non-specific binding was determined in the presence of $150\ \mu\text{M}$ azumolene. Points are means $\pm$ S.E.M. of triplicate determinations ($n = 3$). (B) Inhibition of [^3H]dantrolene binding to HSR membranes. MHS porcine skeletal muscle HSR membranes were incubated with [^3H]dantrolene ($200\ \text{nM}$) and increasing concentrations of unlabelled dantrolene, azumolene or aminodantrolene. Binding was determined in triplicate as in (A), and expressed as means $\pm$ S.E.M. Stock solutions of drugs were made in ethanol ($n = 3$).

Figure 2 Effect of osmolarity on [^3H]dantrolene binding

HSR membranes were incubated with [^3H]dantrolene ($200\ \text{nM}$) and increasing concentrations of solute ($\pm 150\ \mu\text{M}$ azumolene). Experiments with increasing salt concentrations were performed with SR from normal and MHS muscle, and those using sucrose, with SR from normal muscle only. Specific [^3H]dantrolene binding was determined using rapid filtration as described in the Materials and methods section. Points are means $\pm$ S.E.M. ($n = 2$).

[^3H]ryanodine binding to the membrane fractions paralleled the distribution of [^3H]dantrolene binding sites in HSR and LSR. Moreover, no significant differences in the absolute number of binding sites for the two drugs in HSR and LSR from normal or MHS muscle were noted (results not shown).

We characterized the concentration dependence and the equilibrium binding parameters (K_d and B_{max}) of [^3H]dantrolene binding to MHS HSR isolated from porcine fast-twitch skeletal muscle using the rapid filtration assay described above. The specific binding curve, best characterized by a rectangular hyperbola, indicated a single class of [^3H]dantrolene binding sites, with calculated K_d and B_{max} values of $365 \pm 50\ \text{nM}$ and $15.1 \pm 3.2\ \text{pmol/mg}$ of membrane protein respectively (Figure 1A). Specifically bound [^3H]dantrolene could be displaced by unlabelled dantrolene and azumolene, but not by the physiologically inactive congener aminodantrolene (Figure 1B). The apparent K_i value for either dantrolene or azumolene inhibition of [^3H]dantrolene binding was approx. $600\ \text{nM}$. The ratio of maximal calculated dantrolene/ryanodine binding sites in MHS SR is $1.4(\pm 0.1):1$ ($n = 4$). These values are not significantly different from earlier data obtained with HSR from normal porcine skeletal muscle [18].

[^3H]Dantrolene binding to its binding site on MHS HSR was further characterized with respect to pH, ionic strength and bivalent cation concentration. The pH sensitivity of [^3H]dantrolene binding to MHS HSR is essentially indistinguishable from that of normal HSR reported previously (results not shown) [18]. Furthermore, univalent salt concentrations greater than $100\ \text{mM}$ inhibit [^3H]dantrolene binding to HSR isolated from both muscle types, using either sodium or potassium as the univalent cation, or chloride or acetate as the corresponding anion (Figure 2). To distinguish ionic from osmolar effects, [^3H]dantrolene binding was measured as a function of increasing sucrose concentration and compared with the results for sodium acetate and KCl. The inhibition profiles for all three solutes are essentially identical (Figure 2). No differences in [^3H]dantrolene binding to HSR from either muscle type as a function of increasing Ca^{2+} concentrations were observed (results

Table 1 Effect of calcium-channel modulators on [3 H]dantrolene and [3 H]ryanodine binding

Dantrolene binding to HSR from both normal and MHS pig skeletal muscle was determined in the presence of various calcium-channel modulators and 200 nM [3 H]dantrolene, as described in the Materials and methods section. Ryanodine binding to HSR was determined in the presence of ryanodine, dantrolene or azumolene in the presence of 5 nM [3 H]ryanodine, as described in the Materials and methods section ($n = 3$). *Values are significantly different from those for control, as calculated by Student's t test ($P < 0.05$).

Modulator	[3 H]Dantrolene binding (pmol/mg of protein)		[3 H]Ryanodine binding (pmol/mg of protein)	
	Normal	MHS	Normal	MHS
Control (0)	5.8 $\pm$ 0.6	5.5 $\pm$ 0.8	4.10 $\pm$ 0.09	3.9 $\pm$ 0.1
Dantrolene (10 μ M)	0	0	4.13 $\pm$ 0.12	3.94 $\pm$ 0.11
Azumolene (25 μ M)	0	0	4.09 $\pm$ 0.10	3.92 $\pm$ 0.08
Ryanodine (1 nM–100 μ M)	5.8 $\pm$ 0.6	5.6 $\pm$ 0.8	0	0
Caffeine (1 mM)	5.6 $\pm$ 0.8	5.4 $\pm$ 0.9	4.71 $\pm$ 0.10	4.81 $\pm$ 0.13*
(10 mM)	5.8 $\pm$ 0.6	5.4 $\pm$ 0.7	5.45 $\pm$ 0.09*	5.61 $\pm$ 0.08*
Ruthenium Red (1 μ M)	5.8 $\pm$ 0.7	5.5 $\pm$ 0.6	0.03 $\pm$ 0.09*	0.18 $\pm$ 0.11*
(100 μ M)	5.9 $\pm$ 0.8	5.4 $\pm$ 0.7	0	0

Table 2 Effect of calcium/calmodulin on [3 H]dantrolene binding

HSR membranes from normal skeletal muscle were incubated with [3 H]dantrolene (200 nM) and the indicated concentrations of Ca^{2+} and/or calmodulin ($\pm 150 \mu\text{M}$ azumolene). Specific [3 H]dantrolene binding was determined using rapid filtration as described in the Materials and methods section ($n = 3$).

Parameter	[3 H]Dantrolene bound (pmol/mg)
Control	5.5 $\pm$ 0.7
1 μM Ca^{2+}	5.4 $\pm$ 0.9
5 μM Ca^{2+}	5.0 $\pm$ 1.0
10 nM calmodulin	5.5 $\pm$ 0.7
100 nM calmodulin	5.1 $\pm$ 0.8
500 nM calmodulin	5.1 $\pm$ 0.9
10 nM calmodulin + 1 μM Ca^{2+}	5.9 $\pm$ 0.7
100 nM calmodulin + 1 μM Ca^{2+}	5.9 $\pm$ 0.6
500 nM calmodulin + 5 μM Ca^{2+}	5.2 $\pm$ 0.8

not shown). Additionally, the Mg^{2+} dependence of [3 H]dantrolene binding was similar for both muscle types, with 20 mM Mg^{2+} inhibiting binding by 40–50% (results not shown).

The effects of various RyR modulators on both [3 H]dantrolene and [3 H]ryanodine binding to HSR derived from either normal or MHS skeletal muscle were determined (Table 1). The results demonstrate that ryanodine, at concentrations ranging from 1 nM to 100 μM , neither inhibits nor enhances [3 H]dantrolene binding from both muscle types. Consistent with previous reports [9,26], millimolar concentrations of caffeine enhanced [3 H]ryanodine binding to HSR vesicles (33–40% increase at 10 mM), but had no effect on [3 H]dantrolene binding. Ruthenium Red, an RyR channel antagonist that completely inhibits [3 H]ryanodine binding (Table 1) [9,27,28], also had no effect on specific [3 H]dantrolene binding to HSR. In the converse experiment, neither dantrolene (25 μM) nor azumolene (100 μM) inhibited [3 H]ryanodine binding to the RyR, either at pH 8.5, the optimum pH *in vitro* pH for [3 H]ryanodine binding to the RyR [29], or pH 7.4, the physiological pH at which [3 H]dantrolene binding is maximal [18]. Calmodulin has been shown to bind to the RyR [30,31], and have a calcium-dependent biphasic effect on

Figure 3 (A) [3 H]Dantrolene and [3 H]ryanodine binding to SR membranes fractionated on linear sucrose gradients and (B) SDS/PAGE and Western-blot analysis of peak [3 H]dantrolene and [3 H]ryanodine binding fractions from linear sucrose gradients

(A) HSR membranes were fractionated on a 20–40% linear sucrose density gradient using ultracentrifugation at 100 000 g , and specific drug binding to each 500 μl fraction was determined as described in the Materials and methods section. The exact sucrose concentration in each fraction was determined by refractive index ($n = 3$). (B) Protein (5 μg each) from the peak [3 H]dantrolene (35% sucrose) and [3 H]ryanodine (37.5% sucrose) binding fractions were loaded on to 7.5% polyacrylamide gels, electrophoresed, and stained with silver, as described in the Materials and methods section. Alternatively, gel-resolved proteins were electroblotted on to PVDF membranes, immunostained with antibodies to RyR1, dihydropyridine receptor (DHPR), calsequestrin (CS) and $\text{Ca}^{2+}/\text{Mg}^{2+}$ ATPase, and visualized as described in the Materials and methods section. Silver-stained protein patterns are on the outside lanes and immunoblots are in the centre. Molecular masses (in kDa) are indicated on the right.

RyR Ca^{2+} release and [3 H]ryanodine binding in both normal and MHS tissue [30,32]. Yet it has virtually no effect on [3 H]dantrolene binding at any Ca^{2+} concentration measured (Table 2).

Unable to demonstrate any modification of [3 H]dantrolene binding by RyR modulators, we questioned whether or not [3 H]dantrolene and [3 H]ryanodine binding sites on SR membranes could be physically distinguished. Accordingly, HSR vesicles were further fractionated on a linear 20–40% sucrose gradient, and specific drug binding was determined (Figure 3A). There appear to be two distinct but overlapping populations of drug binding HSR vesicle fractions, with the population of membranes that preferentially binds [3 H]dantrolene possessing a lower average buoyant density than the fraction that preferentially binds [3 H]ryanodine. In order to determine whether these separable peaks of drug binding would reflect observable

differences in membrane protein pattern, equal amounts of protein (5 μ g) from each 500 μ l fraction were electrophoresed on reducing SDS/7.5%-polyacrylamide gels and silver-stained, as described in the Materials and methods section. The membrane protein profiles of the peak [3 H]ryanodine and [3 H]dantrolene binding fractions shown in Figure 3(B) are identical with each other and with those all across the gradient (results not shown). Moreover, immunoblot analysis of key skeletal-muscle-specific proteins (RyR1, dihydropyridine receptor, calsequestrin and $\text{Ca}^{2+}/\text{Mg}^{2+}$ ATPase) also did not manifest any discernible differences between the two peaks (Figure 3B).

DISCUSSION

Many studies comparing normal and MHS porcine skeletal muscle have revealed that the SR from the latter is much more sensitive to a variety of Ca^{2+} -release triggers, including Ca^{2+} [13,33], caffeine [34], volatile anaesthetics, pH [33], polylysine and chemical depolarization [35] and ryanodine [36]. The increased sensitivity of SR Ca^{2+} release to ryanodine in MHS muscle is reflected by an increased affinity of [3 H]ryanodine for its binding site on the RyR [11,37,38], and an enhanced sensitivity of this binding to Ca^{2+} [37]. Indeed, to date, all RyR modulators that have been examined have been shown to affect [3 H]ryanodine binding. Thus the characteristics of [3 H]ryanodine binding to SR appear to reflect the functional state of the RyR channel activity and can therefore be used as a probe of such activity [26,38].

Earlier reports of the effects of intravenous dantrolene in both normal and MHS pigs on *in vivo* muscle relaxation and myoplasmic Ca^{2+} levels suggest little significant difference in drug susceptibility between the two strains of animals [15,16]. In contrast, recent studies noted both a biphasic action of dantrolene on porcine RyR channel activity in lipid bilayers, and a statistically significant difference in the effect of the drug on normal and MHS channel open-state probability [7]. This suggests differences in the efficacy of dantrolene on MHS and normal RyR channels. Therefore if dantrolene's site of action is the RyR itself, and since dantrolene and ryanodine have reciprocal effects on SR Ca^{2+} release, these two drugs might reciprocally affect the binding of the other to the RyR. Indeed, the pig MH RyR defect, which concomitantly altered the characteristics of [3 H]ryanodine binding [11,12], might similarly alter [3 H]dantrolene binding characteristics, including alterations in Ca^{2+} sensitivity.

Although [3 H]ryanodine binding to SR isolated from MHS skeletal muscle is reportedly increased more than twice over that of SR isolated from normal tissue in the presence of 1–10 μM Ca^{2+} [37], we detected no significant differences in [3 H]dantrolene binding parameters between normal and MHS skeletal-muscle membranes. We observed no differences in the sensitivity of [3 H]dantrolene binding to inhibition by unlabelled azumolene or solution conditions (changes in pH, ionic strength, Ca^{2+} and Mg^{2+} concentrations). Our results also demonstrate that ryanodine does not inhibit [3 H]dantrolene binding to HSR isolated from either normal or MHS muscle. Consistent with earlier results [39], neither dantrolene nor azumolene has any effect on the binding of [3 H]ryanodine to HSR, despite a three-orders-of-magnitude molar excess of unlabelled ligand. Additionally, the RyR modulators, caffeine, Ruthenium Red and calmodulin all have no effect on [3 H]dantrolene binding, and the mutation in the RyR of MHS muscle does not alter [3 H]dantrolene binding to SR, even under conditions known to modulate both the open/closed state of the RyR channel and [3 H]ryanodine binding [26,40]. The data suggest therefore that, although dantrolene and ryanodine have reciprocal effects on

Ca^{2+} fluxes from the SR, their binding sites are pharmacologically distinct and non-interacting. If dantrolene is affecting RyR-dependent channel activity, it may do so by mechanisms that do not involve the high-affinity ryanodine binding site. Such regulation could theoretically occur whether the dantrolene binding site exists on the RyR or on an associated regulatory molecule. Alternatively, dantrolene could be affecting a cryptic, non-ryanodine-sensitive Ca^{2+} channel in SR. Although no such channel has been definitively identified in skeletal muscle, Ca^{2+} -induced Ca^{2+} release has been shown to occur in myotubes from RyR1 knockout mice [41,42], indicating a non-RyR1-dependent pathway for SR Ca^{2+} efflux. Moreover, a dantrolene-sensitive non-ryanodine-sensitive Ca^{2+} pool has recently been described in cultured cerebellar granule neurons [43].

The data reported here, along with earlier structure-activity studies [4,44], demonstrate that both the hydrophobic and the polar characters of dantrolene and azumolene are important in determining drug-binding interactions at their receptor sites. This is illustrated by the fact that both the binding and the physiological activities of dantrolene are significantly attenuated by a reduction of the electron-withdrawing nitro group to the neutral amino group [4,18]. The physiological activity of azumolene is similarly inhibited by the replacement of the highly electronegative bromine atom with a hydrogen atom [44]. Furthermore, and consistent with earlier results [18], [3 H]dantrolene binding is inhibited both by high ionic strength and by sucrose (Figure 2). These data indicate that both electrostatic and hydrophobic interactions contribute to the binding activity of this drug. Since no significant differences in [3 H]dantrolene binding to normal and MHS SR membranes were detected, membrane lipid-content differences, previously noted to exist between the two muscle types [45,46], do not seem to play a role in determining the binding characteristics of this drug. Irrespective of which molecule in the SR is ultimately proven to contain the dantrolene binding site, the above is consistent with a potential transmembrane region or hydrophobic pocket site of action for dantrolene and azumolene.

We have also been able to partially separate ryanodine and dantrolene binding sites *in vitro* on subsets of SR membranes of non-identical buoyant densities on linear sucrose gradients. Analysis of the peak [3 H]dantrolene and [3 H]ryanodine binding fractions by denaturing PAGE and immunostaining did not reveal any obvious differences in the distribution of key skeletal-muscle-specific proteins involved in excitation-contraction coupling. This observation has a number of potential interpretations. First, dantrolene and ryanodine may bind to separate, interacting molecules that partition into different membrane populations, one of which may be an unrecognized SR Ca^{2+} channel. Secondly, two forms of the RyR may exist on subsets of SR membranes in porcine skeletal muscle, and these bind dantrolene and ryanodine with differing affinities and/or degrees. Thirdly, a putative dantrolene receptor may interact with only one of the forms of the RyR found in SR. To date, there is no direct support for any of these explanations, but there is convincing evidence both for the presence of many potential RyR regulatory proteins in the SR-T-tubule junction and for multiple RyR isoforms in skeletal muscle (see [9,47] for reviews).

In summary, the data reported herein indicate that a [3 H]dantrolene binding site exists in skeletal muscle SR that is pharmacologically independent of the ryanodine binding site and unaffected by the genetic mutation in the RyR that results in porcine MH. Moreover, the two binding sites can be partially separated into different microsomal subfractions. Further studies aimed at the molecular identification of the dantrolene binding site are in progress.

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