[^{3}H]Azidodantrolene: Synthesis and Use in Identification of a Putative Skeletal Muscle Dantrolene Binding Site in Sarcoplasmic Reticulum[◊]

Sanjay S. Palnitkar,[‖,⊥] Bin Bin,[§,⊥] Leslie S. Jimenez,[§] Hiromi Morimoto,[▽] Philip G. Williams,[▽,†] Kalanethee Paul-Pletzer,[‖] and Jerome Parness*[,‖,‡]

Departments of Anesthesia, Pharmacology, and Pediatrics, UMDNJ–Robert Wood Johnson Medical School, New Brunswick, New Jersey 08901, Department of Chemistry, Rutgers University, Piscataway, New Jersey 08855, National Tritium Labelling Facility and Physical Biosciences Division, E. O. Lawrence Berkeley National Laboratory, One Cyclotron Road, Berkeley, California 94720, and Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143

Received September 2, 1998

Dantrolene sodium is a medically important hydantoin derivative that interferes with release of Ca^{2+} from intracellular stores of skeletal muscle by an unknown mechanism. Identification of the molecular target of dantrolene would greatly aid in understanding both the mechanism of action of the drug and the dynamics of intracellular Ca^{2+} release in muscle. [^{3}H]-Azidodantrolene was designed and synthesized as a photoaffinity analogue in order to identify a putative dantrolene receptor in skeletal muscle. Introduction of 1 mole-atom of tritium into aldehyde **5b** was required during radioligand synthesis in order to ensure high enough specific activity for detection of photo-cross-linked proteins by fluorographic methods. This was accomplished by reduction of ester **3** with custom synthesized, 100% tritium-labeled lithium triethylborotritide, followed by oxidation to **5b** by manganese(IV) oxide. Compound **6b** was demonstrated to be ≥95% tritium-labeled at the imine position by NMR spectroscopy, and the specific radioactivity of [^{3}H]azidodantrolene sodium was empirically determined by HPLC and liquid scintillation counting to be 24.4 Ci/mmol, ~85% of theoretical maximum. [^{3}H]Azidodantrolene was found to be pharmacologically active in ligand–receptor binding studies with skeletal muscle sarcoplasmic reticulum membranes. Photo-cross-linking experiments analyzed by SDS–PAGE and tritium fluorography have identified a ~160-kDa specifically labeled protein as the putative, intracellular, skeletal muscle dantrolene receptor. This photolabeled protein comigrates with a protein in Western blots immunologically cross-reactive to a polyclonal anti-rabbit skeletal muscle ryanodine receptor antibody. Thus, the putative dantrolene receptor may be related to the skeletal muscle ryanodine receptor.

Introduction

Dantrolene (hydrated 1-(((5-(4-nitrophenyl)-2-furanyl)methylene)amino)-2,4-imidazolidinedione sodium salt) (Figure 1) is the only drug currently available for the therapy of a life-threatening, genetic sensitivity to volatile anesthetics and depolarizing skeletal muscle relaxants known as malignant hyperthermia (MH). Once MH is triggered, it results in massive, intracellular release of Ca^{2+} from stores in the sarcoplasmic reticulum (SR) of skeletal muscle.[1,2] Although the molecular mechanism by which dantrolene acts is not known, its therapeutic effects parallel an inhibition of anesthetic-triggered Ca^{2+} release.[3,4] Dantrolene is presumed to inhibit the function of the primary Ca^{2+} release channel of SR, also known as the ryanodine receptor. The

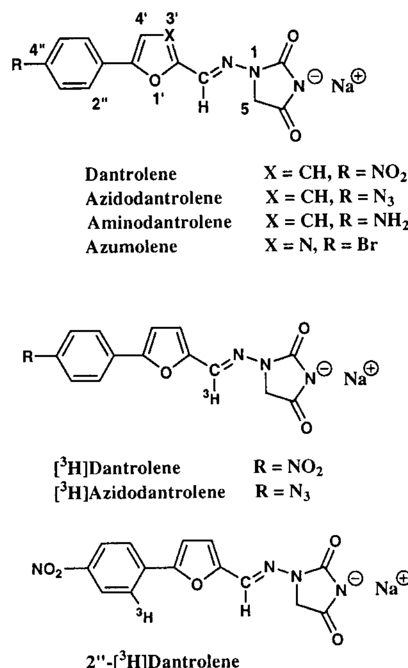


Figure 1. Dantrolene and its congeners.

◊ We dedicate this paper to the memory of Dr. Tom Schwan (deceased) of Proctor & Gamble, Norwich, NY, for providing us with many dantrolene congeners and synthetic intermediates and for his abiding interest in dantrolene chemistry and pharmacology.

* To whom correspondence should be addressed at: Department of Anesthesia, UMDNJ–Robert Wood Johnson Medical School, Clinical Academic Building/Suite 3100, 125 Paterson St, New Brunswick, NJ 08901. Tel: (732) 235-4824. E-mail: parness@umdnj.edu.

‖ Department of Anesthesia, UMDNJ–Robert Wood Johnson Medical School.

‡ Departments of Pharmacology and Pediatrics, UMDNJ–Robert Wood Johnson Medical School.

§ Rutgers University.

▽ E. O. Lawrence Berkeley National Laboratory.

† University of California.

⊥ These authors contributed equally to this paper.

10.1021/jm9805079 CCC: $18.00 © 1999 American Chemical Society
Published on Web 05/14/1999

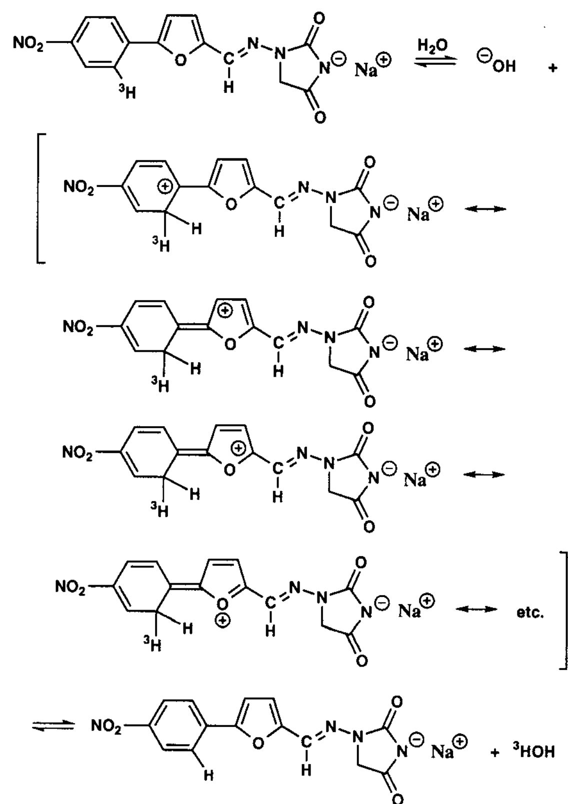
ryanodine receptor is a homotetrameric protein (monomer M_r = 565 kDa), whose channel function is known to be regulated by a number of proteins, enzymes, and small molecules, including the neutral plant alkaloid, ryanodine.[2,5-7] Deciphering whether dantrolene interacts directly with the channel or an accessory/regulatory protein has profound implications for how a skeletal muscle cell is able to regulate intracellular Ca^{2+} release in both normal and pathological states. The identification of a molecular target for dantrolene, therefore, acquires particular significance.

We have recently synthesized [2″-^{3}H]dantrolene and, using a drug binding assay, identified the presence of specific dantrolene binding sites in SR of porcine skeletal muscle.[8] Due to the reversibility of classical ligand−receptor interactions, drug binding studies allow only the demonstration that discrete drug binding sites exist. This technique is useful in identifying active fractions during receptor purification and molecular identification only in cases of high-affinity ligand−receptor interactions measurable with high signal-to-noise ratios. Dantrolene, however, possesses intermediate affinity for its receptor (K_d = 200−300 nM), and previous binding studies demonstrate a poor signal-to-noise ratio of ~0.2.[8] An experimental protocol designed to enhance the signal-to-noise ratio of the [^{3}H]dantrolene binding assay is therefore necessary to increase the likelihood of receptor identification.

A direct approach to the molecular identification of a drug receptor involves the synthesis and use of radioactively labeled, photoactive congeners in photoaffinity labeling experiments.[9-11] Specific photoaffinity labeling of a receptor−ligand binding site results when a photo- and pharmacologically active ligand bound to its receptor binding site is irradiated. The resultant photoactivated compound attacks chemically susceptible groups in its immediate vicinity by any of a variety of mechanisms, leading to the formation of a covalent bond(s) with an amino acid residue(s) in or near the ligand binding site. The specifically cross-linked, putative receptor is then identified using a detection system appropriate to the ligand label. Though many photoactivatable and radioactive groups are available for conjugation to a ligand, our search was limited by the relatively stringent requirements of structure−activity relationships for dantrolene.[12] Small changes in charge distribution and molecular configuration of dantrolene result in large decrements in biological activity. Our aim, therefore, was to use photoactivatable groups and radioactive atoms that would be expected to minimally perturb the chemical structure and pharmacological activity of this ligand.

After examination of structure−activity relationships described in the first report on dantrolene synthesis and biological activity,[12] we reasoned that replacing the strongly electron-withdrawing nitro group of dantrolene with the more weakly electron-withdrawing azido group would still result in a pharmacologically active, photoactivatable derivative, azidodantrolene (Figure 1). Moreover, examination of the structure of dantrolene reveals only two potential sites for a nonexchangeable tritium atom. Interestingly, our first synthesis of [^{3}H]dantrolene placed the tritium atom at the 2″ position on the phenyl ring because of the relative ease of its synthesis.[8] The resulting material (8.92 Ci/mmol, ChemSyn Laboratories, Lenexa, KS) was subject to substantial tritium exchange and underwent significant radiochemical decay over a period of 2 years (data not shown). Indeed, the tritium at this position is expected to undergo hydrogen/tritium exchange because of the adjacent electron-rich furan ring (Scheme 1). Conversely, hydrogen/tritium exchange at either the 3″ position (ortho to the electron-withdrawing nitro group) or the imine hydrogen would not be expected to occur. The relative ease of synthesis of the proposed imine-tritiated congener versus the 3″-tritiated derivative led us to concentrate on synthesizing the former. Preliminary experiments with dantrolene in basic deuterium oxide revealed no hydrogen−deuterium exchange at the imine position (data not shown).

Scheme 1. Proposed Mechanism of Hydrogen−Tritium Exchange at the 2″ Position in [^{3}H]Dantrolene

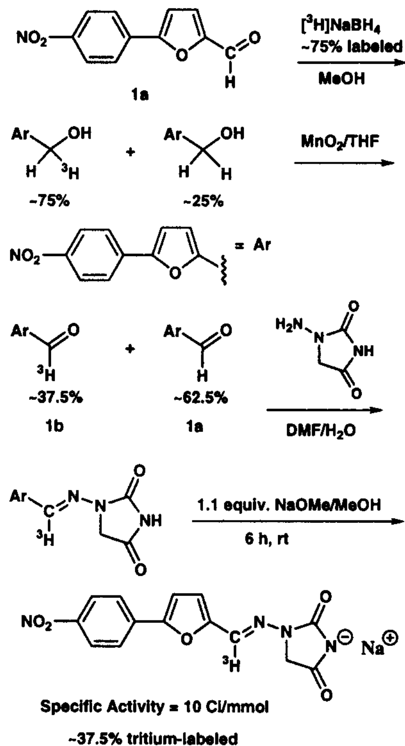


This manuscript reports an improved synthesis of [^{3}H]-dantrolene (Scheme 2), the successful synthesis and tritiation (Scheme 3) of the photoaffinity analogue of dantrolene, azidodantrolene (Figure 1), and the identification of a putative molecular target of the drug.

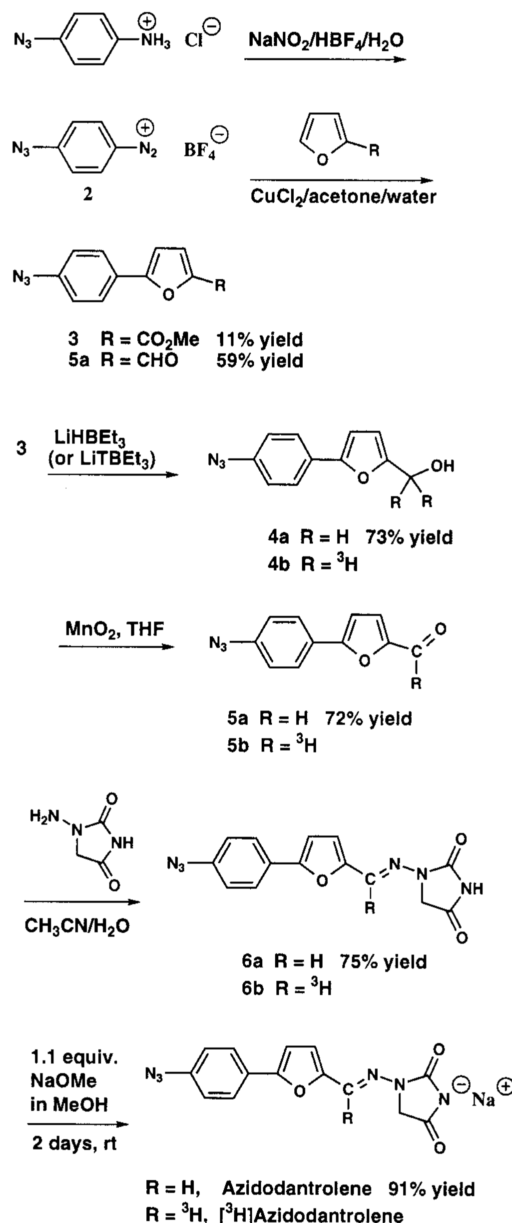
Results and Discussion

Chemistry. 1. Improved Synthesis of [^{3}H]Dantrolene. The nonexchangeable imine position of dantrolene was tritiated by reacting a tritide-reducing agent with the formyl group of **1a**, an intermediate in the synthesis of dantrolene, and reoxidation to the tritiated analogue **1b**. This adds only two reaction steps to the overall synthetic scheme of unlabeled dantrolene. Dantrolene was tritiated (Vitrax, Inc., Placentia, CA) by reduction of the aldehyde **1a** with commercially available sodium borotritide (~75% tritium labeled), followed by reoxidation with manganese(IV) oxide to the tritiated

Scheme 2. Synthesis of [^{3}H]Dantrolene



Scheme 3. Synthesis of Azidodantrolene



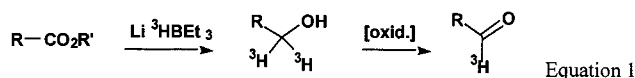
aldehyde **1b** (Scheme 2). Theoretically, using the above source of tritium, reduction of the formyl group with tritride to its corresponding alcohol, and reoxidation to the tritiated formyl group should introduce at best only 0.375 mole-atom of tritium. In fact, condensation with 1-aminohydantoin followed by deprotonation with sodium methoxide resulted in [^{3}H]dantrolene with a specific activity of 10 Ci/mmol. This is ~93% of the theoretical value if isotope effects are ignored, since the specific activity of 1 mole-atom of tritium is 28.76 Ci/mmol.[13]

2. Nonradioactive Azidodantrolene. The synthesis of azidodantrolene followed a route analogous to that for dantrolene itself.[12] It began with the coupling of the aryldiazonium tetrafluoroborate **2** with 2-furaldehyde using cupric chloride as the catalyst (Scheme 3). A modification[14,15] of the procedure reported by Snyder et al.[12] raised the yield of aldehyde **5a** from 14% to 59%. Condensation of **5a** with aminohydantoin hydrochloride in 2:3 acetonitrile:water occurred readily to give a 75% yield of **6a**. Reaction of **6a** with 1.1 equiv of sodium methoxide in methanol gave the corresponding sodium salt in a 91% yield. Unreacted **6a** was cleanly separated from the sodium salt by removing methanol in vacuo and then washing the resulting residue with absolute ethanol. Only the sodium salt is soluble in ethanol; therefore, it can be isolated in a pure form by concentrating the ethanol filtrate under reduced pressure.

3. [^{3}H]Azidodantrolene. A photoaffinity label with the highest possible specific activity was desired to aid in the identification of a putative dantrolene receptor, a low abundance cellular protein. To synthesize [^{3}H]-azidodantrolene with the highest possible specific activity, we devised a reaction scheme in which 1 mole-atom of tritium would be inserted into the imine position (Scheme 3). The reduction of ester **3** (Scheme 3) with custom synthesized 100%-labeled lithium triethylborotritide (Super Tritide)[16] was proposed to give the completely labeled alcohol **4b**, which could then be reoxidized as above to yield the corresponding aldehyde **5b**. In theory, this should allow the introduction of 1 mole-atom of tritium into the imine position.

To accomplish this goal, 5-(4-azidophenyl)-2-furoate (**3**) was a necessary intermediate. The synthesis of **3** was carried out using the same reaction conditions as for **5a** except that methyl 2-furoate was substituted for 2-furaldehyde (Scheme 3). The yield in this reaction was substantially lower, a mere 11%. Reduction of **3** with lithium triethylborohydride cleanly reduced the methyl ester in the presence of the azido group, resulting in a 73% yield of **4a**. Oxidation with manganese(IV) oxide in tetrahydrofuran gave a 72% yield of **5a**. The synthesis of [^{3}H]azidodantrolene was performed as outlined in Scheme 3.[16] Reduction of **3** with lithium triethylborotritide gave an approximately 2:3 ratio of **3**:**4b** as judged by the ^{1}H NMR spectra. However, oxidation of the alcohol **4b** (as a mixture with **3**) with manganese(IV)

oxide was considerably slower than the reaction rate observed for the unlabeled alcohol **4a**. A significant isotope effect is to be expected, since a carbon–tritium bond must be broken during the course of the reaction. In the future, an alternative method such as tetrapropylperruthenate (TPAP)/4-methylmorpholine *N*-oxide (NMO) oxidation[17] may be more useful since this method has been previously used successfully in the rapid oxidation of deuterated alcohols.[18] In the present situation, the unreacted alcohol **4b** was carried forward as a mixture with **5b,** since only **5b** will undergo condensation with 1-aminohydantoin to give **6b**. Deprotonation with sodium methoxide followed by HPLC purification gave [^{3}H]azidodantrolene. Theoretically, this method should result in 100% tritium labeling. A specific activity of only 24.4 ± 4 Ci/mmol was determined by HPLC for this sample of [^{3}H]azidodantrolene. However, the ^{1}H and ^{3}H NMR spectra of the [^{3}H]-azidodantrolene showed no sign of hydrogen in the imine position (Figure 2). Although NMR spectroscopy is a relatively insensitive technique, it can be concluded that the imine position is ≥95% tritium-labeled. Both [^{3}H]dantrolene and [^{3}H]azidodantrolene, prepared as described above, have shown no sign of hydrogen/tritium exchange during a period of over 1 year. Reduction of an ester with lithium triethylborotritide followed by oxidation appears to be a good general method for synthesizing nonexchangeable, ~100% tritium-labeled aldehydes (eq 1).



Pharmacological Activity. 1. Nonradioactive Azidodantrolene. The pharmacological utility of unlabeled azidodantrolene was evaluated using a modification of the [^{3}H]dantrolene binding inhibition assay reported previously (see Experimental Section).[8] Preliminary experiments revealed that [^{3}H]dantrolene binding in a buffer containing 500 μM–3 mM β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP), a physiologically nonhydrolyzable analogue of adenosine triphosphate (ATP), is enhanced 3–4-fold over our earlier results, and the signal-to-noise ratio increased from 0.2 to 0.6 (data not shown). The K_d for [^{3}H]dantrolene binding in this buffer system is ~210 nM (data not shown) and does not differ significantly from that obtained in the absence of AMP-PCP (~275 nM).[8] Therefore, AMP-PCP was included in all binding experiments reported herein.

In the experiment shown, [^{3}H]dantrolene, 250 nM, was incubated with SR membranes and increasing concentrations of azidodantrolene in the dark, for 1 h at 37 °C. After filtration and washing of assay aliquots, the glass fiber filters were processed and assayed for radioactivity by liquid scintillation counting. The results, shown in Figure 3, reveal that azidodantrolene specifically inhibits [^{3}H]dantrolene binding to SR membranes in a concentration-dependent manner (K_i ~ 1.5 μM). Analysis of binding inhibition (Inplot 4.0) reveals that the data are best fit by a curve mathematically consistent with ligand competition for binding at a single site. These results confirm that azidodantrolene competes with [^{3}H]dantrolene at the same ligand binding site.

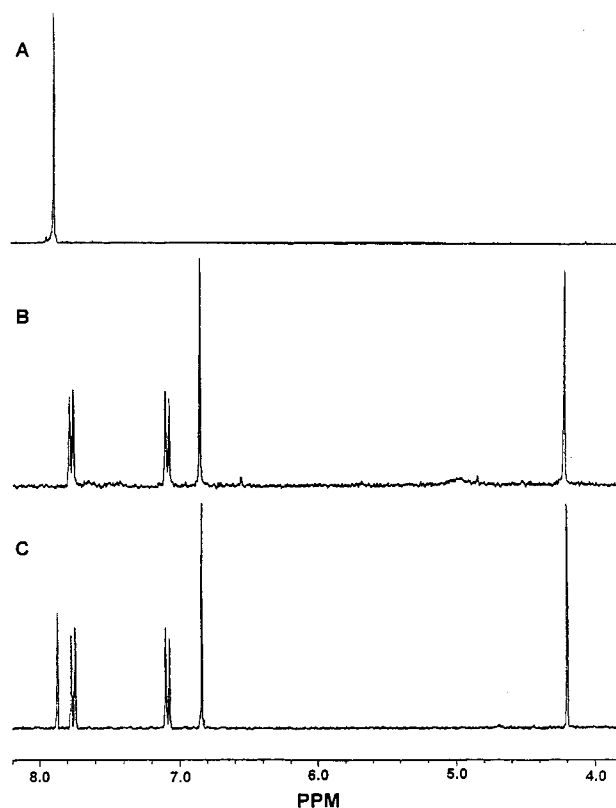


Figure 2. Determination of the specific activity of **6b** by ^{1}H and ^{3}H NMR spectroscopy: (A) ^{3}H NMR spectrum of **6b** in THF-d_8, (B) ^{1}H NMR spectrum of **6b** in THF-d_8, (C) ^{1}H NMR spectrum of **6a** in THF-d_8.

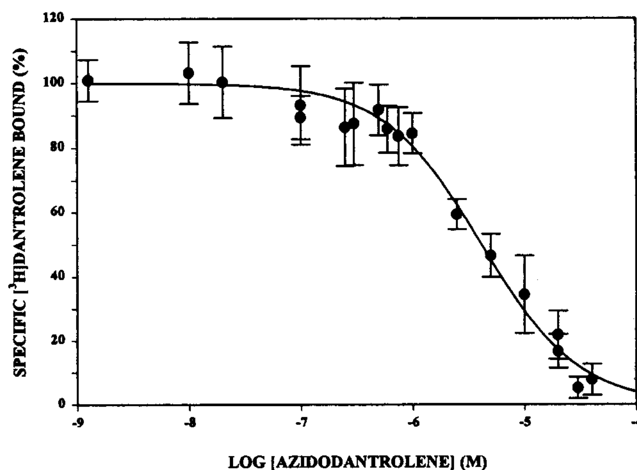


Figure 3. Inhibition of [^{3}H]dantrolene binding to SR by azidodantrolene. SR membranes were incubated with [^{3}H]-dantrolene in the presence of increasing concentrations of azidodantrolene as explained in the Experimental Section. Azidodantrolene dose-dependently inhibited the specific binding of [^{3}H]dantrolene to SR membranes.

As expected, azidodantrolene is extremely sensitive to ambient and UV light. When irradiated at 366 nm (2-cm distance, 20 °C), azidodantrolene undergoes complete photolysis within 5 s, as determined by scanning UV spectrophotometry (200–700 nm, λ_{max} = 360 nm). Indeed, even under ambient light, degradation is evident within 2 min (data not shown).

2. [^{3}H]Azidodantrolene. The specific binding activity of [^{3}H]azidodantrolene was determined using an inhibition binding assay reciprocal to the one described

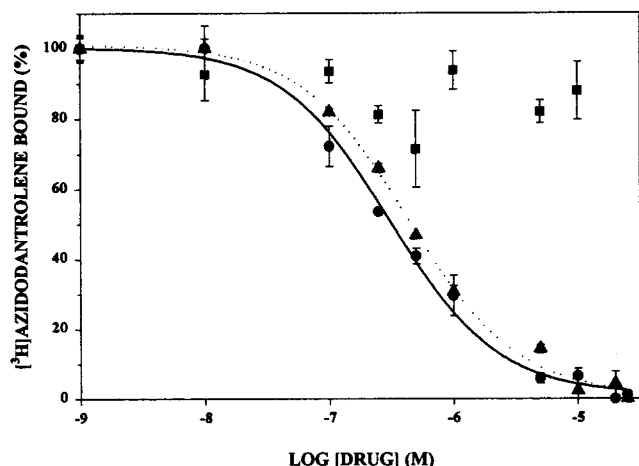


Figure 4. Inhibition of [3H]azidodantrolene binding to SR by dantrolene and its congeners. SR membranes were incubated in the presence of 200 nM [3H]azidodantrolene and increasing concentrations of dantrolene (▲), azumolene (●), or aminodantrolene (■). Both dantrolene and azumolene were equipotent in their inhibitory activity, whereas aminodantrolene was inactive.

above for unlabeled azidodantrolene. SR membranes were incubated in the dark with 200 nM [3H]azidodantrolene and increasing concentrations of dantrolene, azumolene, and the physiologically inactive congener, aminodantrolene, as described (see Experimental Section). After incubation, the samples were harvested via rapid filtration through glass fiber filters, and radioactivity was determined by liquid scintillation counting. The results, shown in Figure 4, demonstrate that both dantrolene and azumolene are equipotent in their specific competition of [3H]azidodantrolene binding (K_i ~175 nM), while aminodantrolene is inactive. Once again, nonlinear regression analysis of the inhibition binding data is consistent with a single-site model of ligand competition, and the data agree with the known pharmacological activity of these congeners. Data from experiments involving inhibition of [3H]azidodantrolene binding by unlabeled azidodantrolene were uninterpretable due to scatter of data at total ligand concentrations ≥ 1.0 μM. This phenomenon may be due to dark reactions of this azido compound with membrane lipids (see below).

Photoincorporation of [3H]azidodantrolene into SR membrane proteins was carried out as described in the Experimental Section. Briefly, SR membranes were incubated with 250 nM [3H]azidodantrolene in buffer containing AMP-PCP in the dark for 1 h at 37 °C, in the absence or presence of dantrolene (20 μM), azumolene (100 μM), or atropine (100 μM), and then immediately irradiated at 366 nm, as described. The irradiated proteins were resolved by SDS−PAGE and blotted onto PVDF membranes, and the tritium-labeled proteins were detected by fluorography. As depicted in the fluorogram (Figure 5A), there is only one specifically labeled protein band at M_r ~ 160 kDa. Both dantrolene and azumolene inhibit the specific photoincorporation of [3H]azidodantrolene into this ~160-kDa protein, while the chemically unrelated compound, atropine, has no effect. This cross-linking is a result of photoactivation of [3H]azidodantrolene, as it does not occur in the absence of sample irradiation, even at radioligand concentrations as high as 2 μM (data not shown). It proved impossible to use aminodantrolene as a negative control in this experiment, as an irradiated [3H]azidodantrolene/aminodantrolene mixture in the absence of membrane protein became trapped on the glass fiber filters, presumably due to photochemical interaction between one of the four known reactive species of the aryl azide probe[11] and aminodantrolene.

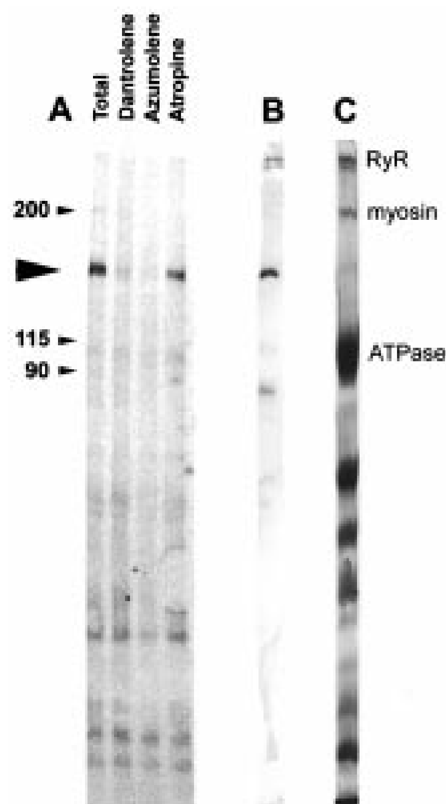


Figure 5. (A) Fluorographic detection of [3H]azidodantrolene cross-linked SR proteins. Fluograph of SDS−PAGE resolved proteins cross-linked with [3H]azidodantrolene in the presence or absence of dantrolene (20 μM), azumolene (100 μM), or atropine (100 μM) reveals specific photoincorporation into a single protein band denoted by the arrowhead (M_r ~ 160 kDa). (B) Western blot of SR proteins photo-cross-linked with [3H]-azidodantrolene. The same PVDF membrane used to generate the fluorograph in panel A was probed with a polyclonal antibody to rabbit RyR and visualized with a secondary antibody coupled to alkaline phosphatase, as described in the Experimental Section. Note the reactivity of both the RyR doublet and the 160-kDa band. (C) Coomassie blue staining of SR proteins separated by 7.5% SDS−PAGE. The RyR, myosin, and Ca^{2+}-ATPase are labeled for comparison.

As can be seen from Figure 5A, no specific [3H]-azidodantrolene cross-linking to the ryanodine receptor monomers resolved in our SDS−PAGE system was detected in any of these experiments (n = 15). In an attempt to determine if the cross-linked band could be related to the RyR, the proteins from this gel were electroblotted onto a PVDF membrane, and the membrane was probed with a polyclonal antibody to the RyR, as described in the Experimental Section. As can be seen from Figure 5C, both the RyR doublet at 565 and 410 kDa and the ~160-kDa band react with the antibody. No antibody reaction occurred with secondary antibody alone (data not shown).

The efficiency of [3H]azidodantrolene incorporation into the 160-kDa band was determined. [3H]Azidodan-

trolene was incubated with SR protein in the presence or absence of ~100-fold molar excess of unlabeled dantrolene or azumolene for 90 min at 37 °C in the dark, as described in the Experimental Section. The reaction mixtures were split into two, one portion was filtered in the dark, and specific radioligand binding to SR protein was determined. The second portion was exposed to UV light, the SR membrane vesicles were sedimented at 14 000 rpm for 10 min in a microfuge, and the pellet was fractionated by SDS−PAGE (7.5%), as described. The proteins in the gels were then electroblotted onto PVDF membranes, and the specifically labeled 160-kDa protein was identified by Western blots (see above) and fluorography. This band was then cut out of the membrane and specific radioactivity determined by liquid scintillation counting. The resultant specific photoincorporation of radioactivity was normalized to starting protein and then divided by the specific radioactivity in the binding assay, yielding a specific incorporation of ~1.5%.

An accretion curve for [^{3}H]azidodantrolene binding to SR could not be performed since the scatter of radioactive signal among replicates was high at radioligand concentrations above 500 nM, precluding us from determining binding saturation. The scatter of radioactive counts may be attributable to nonspecific dark-phase reactions of [^{3}H]azidodantrolene with the sarcoplasmic reticulum membrane lipids, since, as described above, no dark-phase reactivity toward proteins is noted. Given the hydrophobic nature of this compound, such a result is not surprising.

Evidence for the molecular site of action of dantrolene has been circumstantial and contradictory. We had suggested that the binding sites for [^{3}H]dantrolene and [^{3}H]ryanodine might lie on separate molecular targets. This proposal was based on our ability to separate overlapping peaks of binding for the two drugs in porcine skeletal muscle SR membranes fractionated by linear sucrose gradient centrifugation and by lack of any pharmacological interaction between dantrolene and ryanodine in our binding studies.[21] On the other hand, recent reports present evidence suggesting that dantrolene acts directly at the level of the ryanodine receptor in skeletal muscle. Nelson and colleagues demonstrated a biphasic effect of dantrolene on partially purified ryanodine receptor channel activity in lipid bilayers and concluded that the action of dantrolene was directly on the channel itself. [19] A similar purification strategy was employed by Fruen et al. who reported the inhibitory actions of dantrolene on both [^{3}H]ryanodine binding to and Ca^{2+} release from SR membrane vesicles.[20] These authors also concluded that dantrolene acts directly and specifically on the ryanodine receptor itself. In an attempt to resolve the controversy, we synthesized a pharmacologically active, photoreactive, radiolabeled dantrolene analogue, [^{3}H]azidodantrolene, and directly identified the skeletal muscle dantrolene binding protein by specific photo-cross-linking.

The data above demonstrate that [^{3}H]azidodantrolene, under conditions designed to minimize nonspecific receptor−ligand interactions,[10] specifically labels one protein band that migrates at ~160 kDa in our SDS−PAGE system. Under these conditions, the radioligand does not specifically cross-link to the intact ryanodine receptor (565 kDa) or myosin (200 kDa), the two major high-molecular-weight proteins in our SR preparations. Western blot analysis of this ~160-kDa protein band using a polyclonal antibody raised against the intact RyR monomer reveals immunologic cross-reactivity. Though the definitive identity of this ~160-kDa protein is unknown at this time, our findings suggest a number of possibilities. The first and most likely possibility is that the dantrolene binding site is on a proteolytic cleavage fragment of the RyR. Indeed, a ~150−160-kDa rabbit skeletal muscle RyR fragment is among a number of fragments of different molecular weights known to be cleaved from the intact monomer by both exogenously added calpain and a specific, SR membrane-bound, n-calpain.[24−26] Moreover, the endogenous n-calpain of SR has been shown to cleave rabbit skeletal muscle RyR to primarily 375- and 150-kDa fragments.[25] Given the species differences between our muscle source (pig) and those of previous studies (rabbit), our 160-kDa fragment could easily be equivalent to the previously reported 150-kDa fragment. Second, the dantrolene binding site may be on a splice variant of the RyR, thereby accounting for the immunologic reactivity with the polyclonal anti-RyR antibody. Experimental support for this suggestion is to be found in reports of alternative splicing of both murine RyR1 and human RyR3 ryanodine receptors, though none of these splice variants have been shown to give rise to a ~160-kDa protein.[27,28] Third, dantrolene may specifically interact with a hitherto unidentified, epitopically cross-reactive, ~160-kDa protein of presumed RyR regulatory function. Unidentified proteins epitopically cross-reactive with anti-RyR antibodies have been demonstrated in osteoclasts and thymomas.[29,30] Finally, we cannot exclude the possibility of there being more than one protein migrating at 160 kDa in our gel system, one of which cross-reacts with the polyclonal, anti-RyR antibody.

In summary, we have synthesized [^{3}H]azidodantrolene and, for the first time, demonstrated the presence of a specific dantrolene binding protein with immunologic cross-reactivity to the RyR in skeletal muscle SR membranes. Attempts to purify and characterize this dantrolene binding protein are ongoing in our laboratory.

Experimental Section

General. Elemental analyses were processed by Quantitative Technologies Inc., Whitehouse, NJ. Mass spectra were processed by the Center for Advanced Food Technology, Rutgers University, New Brunswick, NJ. Acetonitrile was distilled from calcium hydride. Tetrahydrofuran was freshly distilled from sodium and benzophenone. Triethylborane, tetramethylethylenediamine (TMEDA), and anhydrous methanol were purchased from Aldrich Chemical Co. Melting points were determined on a Thomas-Hoover melting point apparatus and were not corrected. Proton magnetic resonance spectra were recorded on a Varian Gemini 200-MHz or JEOL GSX 400-MHz NMR spectrometer. Tritium labeling was conducted at the National Tritium Labelling Facility, E. O. Lawrence Berkeley National Laboratory, Berkeley, CA.[22] Lithium triethylborotritide was prepared according to Andres et al.[16] Proton and tritium NMR spectroscopy experiments were carried out on an IBM Instruments, Inc. AF-300 spectrometer (^{3}H at 320 MHz, ^{1}H at 300 MHz) using a ^{3}H/^{1}H 5-mm dual probe. All potentially light-sensitive chemicals were handled under red light. Materials used in the assay of biological

activity of azidodantrolene were purchased from Sigma Chemical Co., St. Louis, MI, or ICN Pharmaceuticals Inc., Costa Mesa, CA, except for [^{3}H]dantrolene (10.0 Ci/mmol), which was custom synthesized by Vitrax, Placentia, CA. EtOAc, ethyl acetate; PE, petroleum ether, bp 35−60 °C.

Methyl 5-(4-Azidophenyl)-2-furoate (3). To a solution of 400 mg (2.34 mmol) of 4-azidoaniline hydrochloride in 1.5 mL of water was added 860 mg (4.69 mmol) of fluoroboric acid (48 wt % in water) dropwise. The reaction mixture was cooled in an ice bath, and a solution of 170 mg (2.46 mmol) of $NaNO_2$ in 0.5 mL of water (cooled in an ice bath) was added dropwise. The reaction mixture was stirred at 2−5 °C for 40 min; some white precipitate formed. A solution of 886 mg (7.03 mmol) of methyl 2-furoate in 2 mL of acetone was added, followed by the addition of 27 mg of $CuCl_2$ in 0.3 mL of water. The reaction mixture was allowed to warm to room temperature and stirred in the dark for 2 days. The solvents was removed in vacuo; the residue was diluted with CH_2Cl_2, washed with water twice, 1 N $NaHCO_3$ once, and brine once, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was dried with an oil pump overnight and was then chromatographed on silica using PE:EtOAc (4:1) as eluent. A total of 65 mg (11%) of a brown solid was obtained. Mp: 67−69 °C. ^{1}H NMR ($CDCl_3$): δ 7.70 (d, J = 8.7 Hz, 2H), 7.24 (d, J = 3.8 Hz, 1H), 7.07 (d, J = 8.7 Hz, 2H), 6.70 (d, J = 3.8 Hz, 1H), 3.92 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 159.2, 156.8, 143.6, 140.6, 126.4, 120.1, 119.5, 106.7, 51.92. MS (EI): *m/e* 243 (M^+), 215 ($M^+ - N_2$). Anal. ($C_{12}H_9N_3O_3$) C, H, N.

5-(4-Azidophenyl)-2-furanmethanol (4a). To a solution of 9.8 mg (0.04 mmol) of **3** in 1 mL of THF at 0 °C was added 101 μL (0.10 mmol) of 1 M $LiBEt_3H$ in THF solution. After the addition, the reaction mixture was stirred at 0 °C for 30 min and was then quenched with several drops of saturated NH_4Cl in MeOH solution. The solvents were removed in vacuo; the residue was dissolved in ethyl ether, washed with water twice and brine once, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was then chromatographed on silica using 4:1 PE/EtOAc as eluent. A total of 6.3 mg (73%) of a pale solid was obtained. Mp: 86−88 °C. ^{1}H NMR ($CDCl_3$): δ 7.64 (d, J = 8.5 Hz, 2H), 7.03 (d, J = 8.5 Hz, 2H), 6.55 (d, J = 3.4 Hz, 1H), 6.37 (d, J = 3.4 Hz, 1H), 4.66 (d, J = 4.7 Hz, 2H), 1.85 (broad s, 1H). ^{13}C NMR ($CDCl_3$): δ 153.6, 153.3, 139.0, 127.6, 125.3, 119.3, 110.1, 105.6, 57.6; MS (EI): *m/e* 215 (M^+), 187 ($M^+ - N_2$). Anal. ($C_{11}H_9N_3O_2$) C, H, N.

5-(4-Azidophenyl)-2-furaldehyde (5a). Procedure A: A total of 300 mg (1.76 mmol) of 4-azidoaniline hydrochloride was dissolved in 2 mL of water, 650 mg of 48% fluoroboric acid was added, and the mixture was cooled in an ice bath. A solution of 128 mg (1.85 mmol) of $NaNO_2$ in 1 mL of water was added dropwise, and the resulting solution was stirred at 2−5 °C for 40 min. Some precipitation formed. A solution of 520 mg (5.41 mmol) of 2-furaldehyde in 3 mL of acetone was then added dropwise, followed by the addition of 20 mg of $CuCl_2$ in 0.2 mL of water, and the mixture was allowed to stir at room temperature in the dark for 3 days. Some brownish solid formed. The solid was then filtered and washed with water twice. The solid residue was dissolved in a minimum amount of CH_2Cl_2 and chromatographed on silica using PE:EtOAc (3:1) as eluent. A total of 220 mg (59%) of a light-gray solid was obtained.

Procedure B: To a solution of 4.0 mg (0.019 mmol) of **4a** in 1 mL of THF was added MnO_2 (16 mg, 0.19 mmol). The reaction mixture was stirred at room temperature under a nitrogen atmosphere for 5 h. The reaction mixture was then filtered through a Celite pad, and the Celite cake was rinsed with THF three times. The filtrate was concentrated and chromatographed on silica using 20% EtOAc in hexane as eluent. A total of 2.9 mg (72%) of a light-gray solid was obtained. Mp: 104−105 °C. ^{1}H NMR ($CDCl_3$): δ 9.64 (s, 1H), 7.82 (d, 2H, J = 8.5 Hz), 7.32 (d, 1H, J = 3.8 Hz), 7.10 (d, 2H, J = 8.5 Hz), 6.81 (d, 1H, J = 3.8 Hz). MS (CI): *m/e* 214 ($M + H^+$). Anal. ($C_{11}H_7N_3O_2$) C, H, N.

1-{[5-(4-Azidophenyl)furfurylidene]amino}-2,4-imidazolidinedione (6a). A total of 20 mg (0.09 mmol) of **5a** was dissolved in 4 mL of CH_3CN and then added to a 25-mL round-bottom flask. A solution of 25 mg (0.16 mmol) of 1-amino-2,4-imidazolidinedione hydrochloride in 3 mL of water was then added to the flask, 3 mL of water was added, and the solution was stirred at room temperature for 1 h. After some precipitate formed, 10 mL of water was added to the suspension and the flask was chilled in an ice bath for 5 min. The solid was collected by filtration, washed with water several times, rinsed with methanol once, and then air-dried. A total of 22 mg (75%) of a yellow solid was obtained. Mp: >320 °C dec. ^{1}H NMR (DMSO-d_6): δ 11.26 (s, 1H), 7.80 (d, 2H, J = 8.4 Hz), 7.71 (s, 1H), 7.21 (d, 2H, J = 8.4 Hz), 7.11 (d, 1H, J = 3.5 Hz), 6.94 (d, 1H, J = 3.5 Hz), 4.34 (s, 2H). MS (FAB$^+$): *m/e* 311 ($M + H^+$). Anal. ($C_{14}H_{10}N_6O_3$) C, H, N.

1-{[5-(4-Azidophenyl)furfurylidine]amino}-2,4-imidazolidinedione Sodium Salt. To a slurry of 7.2 mg (0.023 mmol) of **6a** in 1.0 mL of anhydrous methanol under a nitrogen atmosphere was added 5.3 mg (0.024 mmol) of $NaOCH_3$ (25 wt % solution in methanol). The mixture was stirred at room temperature in the dark overnight. The solution was still clear, and the solvent was removed in vacuo resulting in a yellow solid. The solid was washed with acetone twice and was then dissolved in absolute ethanol. The ethanol solution was filtered, the solid residue was washed with ethanol three times, and the filtrate was concentrated in vacuo giving 7.0 mg (91%) of a yellow solid. Mp: >320 °C dec. ^{1}H NMR (DMSO-d_6) δ 7.77 (d, 2H, J = 8.4 Hz), 7.40 (s, 1H), 7.18 (d, 2H, J = 8.4 Hz), 7.02 (d, 1H, J = 3.3 Hz), 6.71 (d, 1H, J = 3.3 Hz), 3.66 (s, 2H). MS (FAB$^-$): *m/e* 309 (M^-), 281 ($M^- - N_2$). HRMS (FAB$^-$): calcd for $C_{14}H_9N_6O_3$, 309.0736; obsd, 309.0733. Anal. ($C_{14}H_{10}N_6O_3\cdot 1.5H_2O$) C, H; N: calcd, 23.39; found, 22.44.

[^{3}H]-5-(4-Azidophenyl)-2-furanmethanol (4b). Lithium triethylborotritide (0.075 mmol) was prepared and dissolved in dry THF (500 μL) and cooled to 0 °C. Methyl 5-(4-azidophenyl)-2-furoate (**3**) (8.6 mg, 0.035 mmol) was added, and the reaction was allowed to proceed for 30 min. After quenching with 2 drops of ammonium chloride in methanol, the solvent was removed and the residue was extracted with ether. The ether layer was washed with water, dried by passage through magnesium sulfate, and removed by a stream of nitrogen gas. Yield: 825 mCi. ^{1}H NMR (THF-d_8): δ 7.65 (d, J = 8.2 Hz, 2H), 7.04 (d, J = 8.2 Hz, 2H), 6.62 (d, J = 2.4 Hz, 1H), 6.27 (d, J = 2.4 Hz, 1H). ^{3}H NMR (THF-d_8): δ 4.41 (s, C^3H_2OH).

[^{3}H]-5-(4-Azidophenyl)-2-furaldehyde (5b). The THF-d_8 was removed by a stream of nitrogen gas and replaced with dry THF. Manganese(IV) oxide (100 mg) was added and the reaction mixture stirred for 4.5 h. The MnO_2 was removed by filtration through GF/C borosilicate microfiber filter (MFS, Dublin, CA) and rinsed with ethyl acetate. Yield: 530 mCi, 20% conversion to the aldehyde. ^{1}H NMR (THF-d_8): δ 7.85 (d, J = 8.4 Hz, 2H), 7.35 (d, J = 3.8 Hz, 1H), 7.14 (d, J = 8.4 Hz, 2H), 6.98 (d, J = 3.8 Hz, 1H). ^{3}H NMR (THF-d_8): δ 9.61 (s, C^3HO), 4.41 residual alcohol (s, C^3H_2OH).

[^{3}H]-1-{[5-(4-Azidophenyl)furfurylidene]amino}-2,4-imidazolidinedione (6b). Ethyl acetate was removed by a stream of nitrogen gas, the residue was dissolved in acetonitrile (1 mL), and 1-amino-2,4-imidazolidenedione hydrochloride (6.9 mg, 1 mL H_2O) was added. After stirring overnight, the solvent was removed and the residue suspended in methanol (1 mL). The cloudy suspension was centrifuged in a clinical centrifuge and the pellet dissolved in THF-d_8 (750 μL). Yield: 184 mCi. The product was purified by HPLC using a Vydac LC 18 column at 3 mL/min using a linear gradient of 0−100% acetonitrile over 40 min. Yield: 20 mCi. ^{1}H NMR (THF-d_8): δ 7.76 (d, J = 8.5 Hz, 2H), 7.09 (d, J = 8.5 Hz, 2H), 6.84 (s, 2H), 4.20 (s, 2H). ^{3}H NMR (THF-d_8): δ 7.89 (s, $C^3H{=}N$).

[^{3}H]-1-{[5-(4-Azidophenyl)furfurylidine]amino}-2,4-imidazolidinedione Sodium Salt. To a methanol solution of the [^{3}H]-1-{[5-(4-azidophenyl)furfurylidine]amino}-2,4-imidazolidinedione was added 25% sodium methoxide in methanol (20 μL, 87 mmol), and the reaction was stirred overnight. HPLC purification was performed on Vydac LC-18, as previously. Yield: 12 mCi. The ^{1}H and ^{3}H NMR spectra in THF-d_8

showed **6b**, so the sodium salt apparently becomes protonated under these conditions.

Preparation of SR Membranes from Porcine Skeletal Muscle. Freshly dissected porcine fast twitch skeletal muscle (*Longissimus dorsi*), a gift of Drs. Sheila Muldoon and Paul Mongan of the Uniformed Services University of the Health Sciences, Bethesda, MD, was immediately frozen in liquid nitrogen and maintained at −72 °C until use. SR membranes were prepared essentially as described previously.[8] Briefly, defrosted skeletal muscle was homogenized using a Waring blender and a crude membrane preparation derived by removing the cell debris and nuclear material using differential centrifugation. The crude microsomes were further fractionated by ultracentrifugation on discontinuous sucrose density gradients to separate fractions enriched in sarcolemma, transverse tubules, and both light and heavy SR. Light SR are enriched in Ca^{2+}-ATPase (SERCA1), and the heavy SR are enriched in the ryanodine receptor. Heavy SR were used exclusively in the experiments reported herein, as this fraction contains the greatest concentration of both dantrolene and ryanodine binding sites.[8] The SR membranes were stored at −72 °C until use.

Inhibition Assay for [^{3}H]Azidodantrolene Binding to SR Membranes. All experiments with azidodantrolene, labeled or unlabeled, were performed under red light or in the dark. Porcine skeletal muscle SR membranes (50 μg of protein) were incubated with 200 nM [^{3}H]dantrolene (10.2 Ci/mmol) or [^{3}H]azidodantrolene (24.4 Ci/mmol) and increasing concentrations of unlabeled congener for 60 min at 37 °C, in a buffer[20] containing AMP-PCP (500 μM), $MgCl_2$ (500 μM), $CaCl_2$ (100 nM), calmodulin (1 μM), potassium propionate (150 mM), and Na-PIPES (20 mM, pH 7.0). After incubation, the assay mixture was rapidly filtered through Whatman GF/C filters using a Hoefer filtration unit (model FH225V) and washed with 5 mL of ice-cold buffer to remove free [^{3}H]dantrolene. The filters were then transferred into scintillation vials and incubated overnight in the presence of hyamine hydroxide to hydrolyze the proteins. CytoScint-ES was added to the vials, and radioactive counts were determined using an LKB-RacBeta scintillation counter. Statistical analysis of the binding data was carried out using Inplot (version 4.0, GraphPad Inc.).

Photoincorporation of [^{3}H]Azidodantrolene in SR Membrane Proteins. One hundred micrograms of SR membrane protein was incubated in the dark (1 h at 37 °C) with 250 nM [^{3}H]azidodantrolene in the absence or presence of 25 μM dantrolene or 100 μM azumolene, as positive controls, or 100 μM atropine, as negative control, in the binding buffer described above. The aqueous solubility limits of dantrolene and azumolene at room temperature are ~30 and 300 μM, respectively.[8] The samples were then irradiated with a handheld UV lamp (366 nm) at a distance of 2 cm for 2 × 1 min at room temperature (at 4 °C, the [^{3}H]azidodantrolene/dantrolene mixture precipitates out of solution).

SDS−PAGE, Electroblotting, and Fluorography of Photo-Cross-Linked Proteins. Photo-cross-linked proteins were resolved using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).[23] Briefly, Laemmli sample buffer containing 2% SDS was added to the reaction mixture containing the [^{3}H]azidodantrolene photo-cross-linked proteins and allowed to stand at room temperature for 1 h. Samples (~50 μg of protein) were loaded onto 7.5% SDS−polyacrylamide gels and run at 175 V until the dye front was within 1 cm of the bottom. Resolved proteins in the gel were then electroblotted onto poly(vinyl difluoride) (PVDF) membranes using a wet protein-transfer apparatus (Idea Scientific, Minneapolis, MN) following the manufacturer's protocol. Following protein transfer, the PVDF membrane was air-dried and exposed to BioMax-MS film (Kodak) using a TranScreen LE intensifying screen (Kodak) at −80 °C. The film was developed after 5 days using standard film development technique. The resultant fluorogram was digitally scanned (UMAX V-12, Umax Corp.) and visualized using Photoshop (version 4.0, Adobe Systems, Inc.).

Western Blotting of Photo-Cross-Linked Proteins. After SDS−PAGE and electroblotting of resolved photo-cross-linked proteins described above, the PVDF membrane was blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature and washed three times with TBS and the membrane probed with a polyclonal sheep anti-rabbit skeletal muscle RyR antibody (a gift of Dr. Kevin Campbell) at a 1:5000 dilution in TBS containing 5% nonfat dry milk for 1 h at room temperature. After being washed three more times with TBS, the membrane was incubated with the secondary antibody, donkey anti-sheep IgG conjugated to alkaline phosphatase (Sigma Immunochemicals), at 1:5000 dilution overnight at 4 °C. Color was developed using 5-bromo-4-chloro-3-indolyl phosphate in 100% dimethylformamide and *p*-nitro blue tetrazolium chloride in 70% dimethylformamide using standard procedures.

Acknowledgment. L.S.J. thanks the donors of the Petroleum Research Fund, administered by the American Chemical Society, the National Science Foundation (CHE-9502149), and the Charles and Johanna Busch Memorial Fund for support of this work. J.P. is indebted to the Foundation for Anesthesia Education and Research for an Anesthesiology Young Investigator Award, to the National Institutes of Health (NIAMS) for Grant AR45593-01, and to the Department of Anesthesia, UMDNJ−Robert Wood Johnson Medical School, for financial support. P.G.W. and H.M. thank the Biomedical Technology Area, National Center for Research Resources, U.S. National Institutes of Health, for their support under Grant P41 RR01237, through Department of Energy Contract DE-AC03-76SF00098 with the University of California.

References

(1) MacLennan, D. H.; Phillips, M. S. Malignant Hyperthermia. *Science* **1992**, *256*, 789−794.
(2) Mickelson, J. R.; Louis, C. F. Malignant Hyperthermia: Excitation-Contraction Coupling, Ca^{2+} Release Channel, and Cell Ca^{2+} Regulation Defects. *Physiol. Rev.* **1996**, *76*, 537−592.
(3) Van Winkle, W. B. Calcium Release from Skeletal Muscle Sarcoplasmic Reticulum: Site of Action of Dantrolene Sodium? *Science* **1976**, *193*, 1130−1131.
(4) Harrison, G. G. Dantrolene − Dynamics and Kinetics. *Br. J. Anaesth.* **1988**, *60*, 279−286.
(5) Meissner, G. Ryanodine Receptor/Ca^{2+} Release Channels and their Regulation by Endogenous Effectors *Annu. Rev. Physiol.* **1994**, *56*, 485−508.
(6) Coronado, R.; Morrissette, J.; Sukhareva, M.; Vaughan, D. M. Structure and Function of Ryanodine Receptors. *Am. J. Physiol.* **1994**, *266*, C1485−C1504.
(7) Sutko, J. L.; Airey, J. A. Ryanodine Receptor Ca^{2+} Release Channels: Does Diversity in Form Equal Diversity in Function? *Physiol. Rev.* **1996**, *76*, 1027−1071.
(8) Parness, J.; Palnitkar, S. S. Identification of Dantrolene Binding Sites in Porcine Skeletal Muscle Sarcoplasmic Reticulum. *J. Biol. Chem.* **1995**, *270*, 18465−18472.
(9) Bayley, H. *Photogenerated Reagents in Biochemistry and Molecular Biology. Laboratory Techniques in Biochemistry and Molecular Biology;* Elsevier: Amsterdam, 1983; Vol. 12, pp 1−187.
(10) Ruoho, A. E.; Rashidbaigi, A.; Roeder, P. E. In *Membranes, Detergents, and Receptor Solubilization*; Venter, J. C., Harrison, L. C., Eds.; Alan R. Liss: New York, 1984; pp 119−160.
(11) Brunner, J. New photolabeling and cross-linking methods. *Annu. Rev. Biochem.* **1993**, *62*, 483−514.
(12) Snyder, H. R., Jr.; Davis, C. S.; Bickerton, R. K.; Halliday, R. P. 1-[(5-Arylfurfurylidene)amino]hydantoins. A new class of Muscle Relaxants. *J. Med. Chem.* **1967**, *10*, 807−810.
(13) Evans, E. A. In *Tritium and Its Compounds*; Wiley: New York, 1974; pp 1−822.
(14) Starkey, E. B. *p*-Dinitrobenzene. *Org. Synth.* **1943**, *II*, 225−227.
(15) Doyle, M. P.; Siegfried, B.; Dellaria, J. F., Jr. Alkyl Nitrite-Metal Halide Deamination Reactins. 2. Substitutive Deamination of Arylamines by Alkyl Nitrites and Copper(II) Halides. *J. Org. Chem.* **1977**, *42*, 2426−2431.
(16) Andres, H.; Morimoto, H.; Williams, P. G. Preparation and Use of $LiEt_3BT$ and $LiAlT_4$ at Maximum Specific Activity. *J. Chem. Soc., Chem. Commun.* **1990**, 627−628.

(17) Cherif, A.; Farquhar, D. J. N-(5,5-diacetoxypent-1-yl)doxorubicin: a New Intensely Potent Doxorubicin Analogue. *J. Med. Chem.* **1992**, *35*, 3208−3214.
(18) Elmore, C. Investigation of the Stereochemistry of the Methyl-to-Methylene Elimination in the Biosynthesis of Several Terpenes Through the Use of Chiral Methyl Labeled Compounds. Ph.D. Thesis, Department of Chemistry, University of Illinois at Urbana−Champaign, 1997.
(19) Nelson, T. E.; Lin, M.; Zapata-Sudo, G.; Sudo, R. T. Dantrolene Sodium can Increase or Attenuate Activity of Skeletal Muscle Ryanodine Receptor Calcium Release Channel. *Anesthesiology* **1996**, *84*, 1368−1379.
(20) Fruen, B. R.; Mickelson, J. R.; Louis, C. F. Dantrolene Inhibition of Sarcoplasmic Reticulum Ca^{2+} Release by Direct and Specific Action at Skeletal Muscle Ryanodine Receptors. *J. Biol. Chem.* **1997**, *272*, 26965−26971.
(21) Palnitkar, S. S.; Mickelson, J. R.; Louis, C. F.; Parness, J. Pharmacological Distinction between Dantrolene and Ryanodine Binding Sites: Evidence from Normal and Malignant Hyperthermia-Susceptible Porcine Skeletal Muscle. *Biochem. J.* **1997**, *326*, 847−852.
(22) Morimoto, H.; Williams, P. G. Design and Operations at the National Labeling Facility. *Fusion Technol.* **1992**, *21*, 256−261.
(23) Laemmli, U. K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(24) Brandt, N. R.; Caswell, A. H.; Brandt, T.; Brew, K.; Mellgren, R. L. Mapping of the calpain proteolysis products of the junctional foot protein of the skeletal muscle triad junction. *J. Membr. Biol.* **1992,** *127*, 35−47.
(25) Shevchenko, S.; Feng, W.; Varsanyi, M.; Shoshan-Barmatz, V. Identification, characterization and partial purification of a thiol-protease which cleaves specifically the skeletal muscle ryanodine receptor/Ca^{2+} release channel. *J. Membr. Biol.* **1998**, *161*, 33−43.
(26) Wu, Y.; Aghdasi, B.; Dou, S. J.; Zhang, J. Z.; Liu, S. Q.; Hamilton, S. L. Functional interactions between cytoplasmic domains of the skeletal muscle Ca^{2+} release channel. *J. Biol. Chem.* **1997**, *272*, 25051−25061.
(27) Futatsugi, A.; Kuwajima, G.; Mikoshiba, K. Tissue-specific and developmentally regulated alternative splicing in mouse skeletal muscle ryanodine receptor mRNA. *Biochem. J.* **1995**, *305*, 373−378.
(28) Leeb, T.; Brenig, B. cDNA cloning and sequencing of the human ryanodine receptor type 3 (RYR3) reveals a novel alternative splice site in the RYR3 gene. *FEBS Lett.* **1998**, *423*, 367−370.
(29) Zaidi, M.; Shankar, V. S.; Tunwell, R.; Adebanjo, O. A.; Mackrill, J.; Pazianas, M.; O'Connell, D.; Simon, B. J.; Rifkin, B. R.; Venkitaraman, A. R.; Huang, C. L. H.; Lai, F. A. A ryanodine receptor-like molecule expressed in the osteoclast plasma membrane functions in extracellular Ca^{2+}. *J. Clin. Invest.* **1995**, *73*, 1582−1590.
(30) Mygland, A.; Kuwajima, G.; Mikoshiba, K.; Tysnes, O. B.; Aarli, J. A.; Gilhus, N. E. Thymomas express epitopes shared by the ryanodine receptor. *J. Neuroimmunol.* **1995**, *62*, 79−83.

JM9805079