The Skeletal Muscle Ryanodine Receptor Identified as a Molecular Target of [^{3}H]Azidodantrolene by Photoaffinity Labeling†

Kalanethee Paul-Pletzer,‡ Sanjay S. Palnitkar,‡ Leslie S. Jimenez,§ Hiromi Morimoto,|| and Jerome Parness*,‡,⊥,#

Departments of Anesthesia, Pharmacology, and Pediatrics, UMDNJ−Robert Wood Johnson Medical School, Piscataway, New Jersey 08854, Department of Chemistry, Rutgers University, Piscataway, New Jersey 08854, and National Tritium Labelling Facility and Physical Biosciences Division, E. O. Lawrence Berkeley National Laboratory, One Cyclotron Road, Berkeley, California 94720

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ABSTRACT: Dantrolene is a skeletal muscle relaxant which acts by inhibiting intracellular Ca^{2+} release from sarcoplasmic reticulum (SR). It is used primarily in the treatment of malignant hyperthermia (MH), a pharmacogenetic sensitivity to volatile anesthetics resulting in massive intracellular Ca^{2+} release. Determination of the site and mechanism of action of dantrolene should contribute to the understanding of the regulation of intracellular Ca^{2+} release in skeletal muscle. Photoaffinity labeling of porcine SR with [^{3}H]azidodantrolene, a photoactivatable analogue of dantrolene, has identified a 160 kDa SR protein with immunologic cross-reactivity to skeletal muscle ryanodine receptor (RyR) as a possible target [Palnitkar et al. (1999) *J. Med. Chem. 42*, 1872−1880]. Here we demonstrate specific, AMP-PCP-enhanced, [^{3}H]-azidodantrolene photolabeling of both the RyR monomer and a 160 or 172 kDa protein in porcine and rabbit SR, respectively. The 160/172 kDa protein is shown to be the NH_2-terminus of the RyR cleaved from the monomer by an endogenous protease activity consistent with that of n-calpain. MALDI-mass spectrometric analysis of the porcine 160 kDa protein identifies it as the 1400 amino acid NH_2-terminal fragment of the skeletal muscle RyR reportedly generated by n-calpain [Shevchenko et al. (1998) *J. Membr. Biol. 161*, 33−34]. Immunoprecipitation of solubilized, [^{3}H]azidodantrolene-photolabeled SR protein reveals that the cleaved 160/172 kDa protein remains associated with the C-terminal, 410 kDa portion of the RyR. [^{3}H]Dantrolene binding to both the intact and the n-calpain-cleaved channel RyR is similarly enhanced by AMP-PCP. n-Calpain cleavage of the RyR does not affect [^{3}H]dantrolene binding in the presence of AMP-PCP, but depresses drug binding in the absence of nucleotide. These results demonstrate that the NH_2-terminus of the RyR is a molecular target for dantrolene, and suggest a regulatory role for both n-calpain activity and ATP in the interaction of dantrolene with the RyR in vivo.

Dantrolene sodium is a hydantoin derivative whose major known action is to prevent Ca^{2+} release from skeletal muscle sarcoplasmic reticulum (SR)[1] (*1*−*3*). Significantly, it is the only approved drug for the treatment of MH, a rare, inherited, pharmacogenetic disorder of skeletal muscle, in which uncontrolled, intracellular Ca^{2+} release is triggered by volatile anesthetics, resulting in a hypermetabolic, hyperthermic syndrome with high potential for lethality [for reviews, see (*4*−*6*)]. The genetic defect in both porcine and the majority of human cases of MH has been linked to the major calcium release channel of skeletal muscle SR, the RyR, which is likely the Ca^{2+} channel whose function dantrolene modifies.

There are three isoforms of this Ca^{2+} channel: RyR1, expressed predominantly in skeletal muscle; RyR2, expressed predominantly in cardiac muscle; and RyR3, expressed predominantly in brain, smooth muscle, and epithelial cells, and somewhat in skeletal muscle. The skeletal muscle RyR is a homotetrameric, transmembrane protein of monomeric molecular mass ∼565 kDa, whose structure and function have been extensively reviewed (*7*, *8*). The carboxy-terminal one-fifth of this protein contains the transmembrane, calcium release channel/pore, while the amino-terminal four-fifths of

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* To whom correspondence should be addressed at the Department of Anesthesia, UMDNJ−Robert Wood Johnson Medical School, 125 Paterson St., Clinical Academic Building, Suite 3100, New Brunswick, NJ 08901. Voice: (732)235-4824. FAX: (732)235-4073. E-mail: parness@umdnj.edu.

‡ Department of Anesthesia, UMDNJ−Robert Wood Johnson Medical School.

§ Department of Chemistry, Rutgers University.

|| E. O. Lawrence Berkeley National Laboratory.

⊥ Department of Pharmacology, UMDNJ−Robert Wood Johnson Medical School.

Department of Pediatrics, UMDNJ−Robert Wood Johnson Medical School.

[1] Abbreviations: AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; CAPN3, monoclonal antibody to n-calpain (p94); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DHPR, dihydropyridine receptor; DMF, dimethylformamide; DTT, dithiothreitol; ECC, excitation−contraction coupling; HSR, heavy sarcoplasmic reticulum; FKBP12, the immunophilin FK-binding protein of 12 kDa molecular mass; MALDI, matrix-assisted laser desorption; MH, malignant hyperthermia; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinyl difluoride; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; TBS, Tris-buffered saline.

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the molecule, whose function is presumed to be structural and regulatory, is cytoplasmic (*9*−*13*). As a major component of the ECC pathway, this channel is known to interact, directly or indirectly, with a number of other ECC pathway proteins such as the DHPR and calsequestrin, regulatory proteins such as FKBP12 and calmodulin, and a number of other proteins, such as triadin and junctin, whose functions are less well-defined (*7*, *8*). The large cytoplasmic region of the RyR has been identified as the likely target of many endogenous and exogenous ligands that regulate this calcium channel (*8*, *14*). Point mutations in this portion of RyR1 have been linked to MH and to the even rarer myopathy, central core disease, though 50% of genotyped, MH-susceptible humans have no demonstrable mutation in the gene for the RyR (*4*, *15*). Dantrolene is effective in truncating episodes of MH irrespective of the underlying genotype, suggesting that dantrolene acts at a final common pathway of Ca^{2+} release likely involving the skeletal muscle RyR. Whether the drug interacts directly with the RyR, however, has been a matter of some controversy.

Using [^{3}H]dantrolene as a probe in equilibrium binding studies on isolated, porcine skeletal muscle HSR enriched for the RyR, we identified a single class of drug binding sites (*16*). We demonstrated that pharmacological modulators of both RyR channel activity and [^{3}H]ryanodine binding had no effect on [^{3}H]dantrolene binding. Furthermore, following centrifugation of SR vesicles on linear sucrose gradients, we were able to demonstrate separate peaks of [^{3}H]dantrolene and [^{3}H]ryanodine binding, suggesting that the receptors for these compounds were not identical (*17*). Others, however, have reported dantrolene−RyR1 interactions. For example, a biphasic effect of dantrolene on the Ca^{2+} currents of porcine skeletal muscle RyR in lipid bilayers, first activating, then inhibiting, has been demonstrated (*18*). Furthermore, it has been reported that dantrolene both inhibited Ca^{2+} release from normal and MH-susceptible porcine skeletal muscle SR vesicles in vitro, and inhibited [^{3}H]ryanodine binding to these vesicles (*3*). Both reports suggested that the effects of dantrolene on SR Ca^{2+} release resulted from a direct drug interaction with the RyR.

To directly determine the molecular target of dantrolene, we synthesized a pharmacologically active, photoactivatable analogue of dantrolene, [^{3}H]azidodantrolene, and have used this compound to identify a 160 kDa porcine HSR protein target that is immunologically cross-reactive with a polyclonal anti-skeletal muscle RyR antibody (*19*). Recently, Shoshan-Barmatz et al. (*20*) reported that the thiol- and Ca^{2+}-activated protease, n-calpain, is located in rabbit SR membrane and specifically cleaves the RyR to generate a ∼150 kDa amino-terminal fragment. Here we show that the immunoreactive 160 kDa porcine protein and its 172 kDa rabbit counterpart identified by [^{3}H]azidodantrolene photoaffinity labeling are n-calpain-derived fragments of the RyR. Specific photolabeling of the RyR and the n-calpain-derived fragments is enhanced by AMP-PCP, though the nucleotide has no effect on n-calpain cleavage of the RyR.

EXPERIMENTAL PROCEDURES

Materials. Dantrolene sodium·3.5H_2O and azumolene sodium·2H_2O were generous gifts of Proctor & Gamble, Norwich, NY. [^{3}H]Dantrolene (10.2 Ci/mmol) was custom-synthesized by Vitrax, Placentia, CA. The protease inhibitors PMSF, leupeptin, aprotinin, and pepstatin A, alkaline phosphatase-conjugated donkey anti-sheep IgG, Protein A−agarose beads, and all other reagents and chemicals (high purity grade) were from Sigma. Polyclonal sheep anti-rabbit skeletal muscle RyR antibody and purified bovine brain calmodulin were gifts of Dr. Kevin P. Campbell (University of Iowa, Iowa City, IA) and Dr. Donald J. Wolff (UMDNJ−Robert Wood Johnson Medical School, Piscataway, NJ), respectively. Monoclonal anti-CAPN3 antibody and CAPN3 antigen were generously donated by Dr. Luc Camoin (Institut Cochin de Genetique Moleculaire, Laboratoire d'Immunopharmacologie Moleculaire, Paris, France). Polyclonal (IgG) rabbit anti-rabbit RyR synthetic C-terminal peptide (sequence corresponding to amino acids 5023−5037 of RyR1) was obtained from the Developmental Studies Hybridoma Bank, University of Iowa, and mouse monoclonal anti-rabbit skeletal muscle RyR (IgM) antibody was obtained from Upstate Biotechnology, Lake Placid, NY.

[^{3}H]Azidodantrolene Synthesis. [^{3}H]Azidodantrolene was synthesized, purified, and characterized exactly as described (*19*), and the specific activity was determined to be 24.4 Ci/mmol.

Preparation of HSR Vesicles. Porcine and rabbit fast twitch skeletal muscles were supplied by Drs. Sheila Muldoon and Paul Mongan, Uniformed Services University of the Health Sciences, Bethesda, MD, and Dr. Harvey Weiss, UMDNJ−Robert Wood Johnson Medical School, Piscataway, NJ, respectively. Freshly dissected paraspinal (porcine) or back and hindlimb (rabbit) muscles were immediately frozen in dry ice−isopentane and then in liquid nitrogen, and kept at −80 °C until use. HSR vesicles were prepared by cycles of differential centrifugation: from pig skeletal muscles, as reported previously (*16*); and from rabbit, according to the method of Hawkes et al. (*21*). The only differences between these two methods of HSR preparation are minor: in the latter, solid KCl is added to a final concentration of 0.5 M in the salt wash step, as opposed to resuspension of microsomes in buffer containing 0.6 M KCl in the former, and the final storage buffer pH is 7.0 for pig HSR, and 7.4 for rabbit HSR. The following protease inhibitors were used in buffers at all stages of membrane preparation: PMSF (200 μM), aprotinin (0.3 μM), and pepstatin A (2.8 μM). To maintain activity of endogenous n-calpain and allow for cleavage of the RyR, leupeptin was deliberately omitted from the cocktail of protease inhibitors during these preparations. Porcine cardiac SR preparations, kindly supplied by Drs. Hector H. Valdivia (University of Wisconsin−Madison) and Bradley Fruen (University of Minnesota), were prepared in the presence of leupeptin and stored as above.

[^{3}H]Dantrolene Binding Assay. [^{3}H]Dantrolene binding to skeletal muscle HSR was assayed as previously described (*16*) with the following modifications: 20−50 μg of HSR vesicles (pig or rabbit) was incubated with 200 nM [^{3}H]-dantrolene, for inhibition binding assays, or with increasing concentrations of radioligand, for equilibrium accretion binding curves, in binding buffer (20 mM Na-PIPES, pH 7, 150 mM KCl, 0.5 mM AMP-PCP, 0.5 mM $MgCl_2$) at 37 °C for 90 min, in triplicate. Free ligand was separated from bound ligand by rapid filtration on Whatman GF/C glass fiber filters, and bound radioactivity was determined by liquid scintillation counting, as described (*16*, *19*). Nonspecific

binding was determined in the presence of 150 μM azumolene. Specific binding was obtained by subtracting nonspecific binding from total binding in the absence of azumolene. Equilibrium radioligand saturation binding data were analyzed by nonlinear regression analysis, and nonparametric data were analyzed using the Student's *t*-test (Prism 3.0, GraphPad Software, Inc.).

Photoincorporation of [^{3}H]Azidodantrolene into HSR Proteins. [^{3}H]Azidodantrolene is extremely photosensitive, and all reactions involving this compound were performed under red light or in the dark (*19*). A 100−150 μg sample of HSR vesicle protein (pig or rabbit) was incubated with 400 nM [^{3}H]azidodantrolene in [^{3}H]dantrolene binding buffer (see above) in the absence or presence of 25 μM dantrolene or 150 μM azumolene as specific inhibitors, or 150 μM atropine, as negative control, at 37 °C for 90 min. A 1000-fold excess of the unlabeled drugs, dantrolene and azumolene, could not be used here because the aqueous solubility limits of these drugs are ∼30 and 300 μM, respectively (*16*). The samples were then irradiated at room temperature with a hand-held UV lamp (366 nm) at a distance of 2 cm for 2 × 2 min with a 1 min pause. Unless specifically noted otherwise, the vesicles were then concentrated by centrifugation in a microfuge at 13 400 rpm (16400*g*, 15 min). Membrane pellets were then resuspended in Laemmli sample buffer containing 2% SDS, heated at 60 °C for 10 min, and subjected to SDS−polyacrylamide gel electrophoresis, as described below. The efficiency of photoincorporation was previously determined to be ∼1.5% (*19*).

SDS−Polyacrylamide Gel Electrophoresis, Electroblotting, Autoradiography, and Western Blot Analysis. Samples of HSR photoreacted with [^{3}H]azidodantrolene were analyzed by SDS−PAGE (4% stacking, 5 or 6% resolving gels) according to Laemmli (*22*). Protein bands were visualized by staining with Coomassie Blue. For autoradiography and Western blot analyses, the resolved proteins were transferred onto PVDF membranes (Sequi-Blot, Bio-Rad) using Towbin buffer (*23*) containing 5% methanol in an Idea Scientific (Minneapolis, MN) transfer apparatus. Air-dried blots were exposed to BioMax-MS film (Kodak) using a TranScreen LE intensifying screen (Kodak) at −80 °C, and radiolabeled protein bands were detected after 2 days. After autoradiography, the blots were then probed with primary antibody at appropriate dilution:concentration (polyclonal sheep anti-skeletal muscle RyR, 1:5000; polyclonal rabbit anti-C-terminus RyR1, 1:1000; mAb-CAPN3, 2 μg/mL) in TBS containing 5% nonfat dry milk, overnight at 4 °C. The blots were then washed 3 times with TBS and incubated at room temperature for 2 h with alkaline phosphatase-conjugated secondary antibody, and immunoreactive bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate in 100% DMF and *p*-nitro blue tetrazolium in 70% DMF using standard procedures. Monoclonal anti-CAPN3 antibody used to identify the presence of muscle-specific n-calpain in our HSR preparations was produced to a peptide corresponding to the unique sequence represented by amino acids 607−628 in the IS2 domain of human n-calpain/calpain3, and shown to be specific (*24*). The specificity of the polyclonal sheep anti-rabbit skeletal muscle RyR antibody used in our Western blots has been extensively characterized (*25*−*27*).

Immunoprecipitation of Photolabeled HSR Proteins. The procedure for immunoprecipitation of detergent-solubilized rabbit skeletal muscle HSR proteins with anti-RyR antibodies was adapted from Zhang et al. (*66*). Rabbit skeletal muscle HSR vesicles (1 mg of protein per condition) were photolabeled with [^{3}H]azidodantrolene in the presence or absence of excess azumolene, as described above, and pelleted in a microfuge at 13 400 rpm (16400*g*) for 15 min. The pellet was resuspended in 20 mM Tris-HCl, pH 7.5, containing 1 M NaCl, 3% CHAPS, and protease inhibitors (10 μM leupeptin, 2 μM pepstatin A, 2 μM aprotinin, 200 μM PMSF) and incubated on ice for 2 h. This mixture was then centrifuged in a Beckman TL-100 rotor at 100 000 rpm for 5 min, and the supernatant containing the solubilized proteins was diluted 10-fold in 20 mM Tris-HCl, pH 7.5, containing the above protease inhibitors, to reduce the salt and detergent concentrations. The diluted proteins were preincubated with 100 μL of packed protein A−agarose beads equilibrated in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.3% CHAPS, (Equilibration Buffer) in a total volume of 200 μL for 2 h at 4 °C with rotary shaking and then sedimented to remove nonspecifically interacting proteins. Anti-RyR antibody [polyclonal sheep anti-rabbit RyR1 (IgG, 1:100), polyclonal rabbit anti-RyR1 C-terminal (IgG, 1:100), or mouse monoclonal anti-RyR1 (IgM,1:200)] was then added to the supernatants and incubated overnight with rotary shaking at 4 °C. Nonimmune immunoglobulin (IgG or IgM, as appropriate) was used as negative control. Protein A−agarose beads (200 μL total, 100 μL packed) were then added to the solubilized protein−antibody mixture together with protease inhibitors and incubated for another 2 h. Beads were then washed 5 times by centrifugation through Equilibration Buffer, and bound proteins were eluted by adding 80 μL of 5× Laemmli sample buffer and incubated for 30 min at room temperature. The eluted proteins from the three different immunoprecipitations were resolved simultaneously on a 5% SDS−PAGE gel and transferred onto a PVDF membrane, and autoradiography and Western blotting were performed, as described above.

Endogenous Calpain Cleavage of RyR. Skeletal muscle SR is reported to contain a membrane-bound, endogenous thiol protease, n-calpain (also known as p94, calpain 3, or CAPN3), in addition to the ubiquitous, m- and μ-calpains present in the myoplasm. This enzyme is reported to specifically cleave the skeletal muscle RyR to produce a carboxy-terminal ∼410 kDa fragment and an amino-terminal ∼150 kDa fragment (1400 amino acids) in the presence of a highly reducing environment (≥10 mM DTT) and nanomolar Ca^{2+} (*20*, *28*). The ubiquitous calpains are activated by reducing environments and millimolar (m-calpain) or micromolar (μ-calpain) concentrations of Ca^{2+} (*28*, *29*). To determine the degree of susceptibility of pig and rabbit skeletal muscle RyR to calpain cleavage, HSR membrane proteins (100 μg) were incubated with endogenous Ca^{2+} in ligand binding buffer described above at 37 °C for 30 min, in the absence or presence of 10 mM DTT, varying concentrations of EGTA, the thiol-dependent protease inhibitor leupeptin, or calmodulin, a known inhibitor of calpain-induced cleavage of the RyR. The proteins were then resolved on a 7.5% SDS−polyacrylamide gel, and either stained with Coomassie Blue or transferred to PVDF membranes for autoradiography and/or probing with various anti-RyR1 antibodies. Additionally, the presence of n-calpain in our SR preparations was detected by Western blots using

anti-CAPN3 monoclonal antibody as probe, as described above.

MALDI-Mass Spectrometry. To positively identify the 160 kDa RyR fragment by MALDI-mass spectrometry, we labeled duplicate samples of pig HSR with [^{3}H]azidodantrolene, and resolved the proteins on a 7.5% SDS−polyacrylamide gel in adjacent lanes. The gel was then stained with Coomassie Blue, and the 160 kDa bands from the two lanes were excised. One band was dissolved in 30% hydrogen peroxide, and the presence of radioactivity (i.e., photo-cross-linked, radiolabeled protein) was ascertained by liquid scintillation counting, while the other band and an adjacent, non-protein-containing portion of the gel, as control, were sent to the W. M. Keck Foundation, Biotechnology Resource Laboratory, Yale University, New Haven, CT, for protein identification via MALDI-mass spectrometry. Criteria used to identify proteins by this approach include: (1) a match of >25% of the predicted protein sequence within a peptide mass tolerance of ≤0.015%, (2) a ProFound search score of 1.0e+00, with a clear break between this score and the following nonrelated protein, and (3) a Peptide search that matches the same protein.

Protein Estimation. Protein concentration in the HSR preparations was determined by the Bradford assay using bovine serum albumin as reference protein (*30*).

RESULTS

Specific Photolabeling of the RyR, and a 160/172 kDa HSR Protein with [^{3}H]Azidodantrolene. We previously reported the identification of a 160 kDa protein in porcine HSR that was both specifically photo-cross-linked by [^{3}H]azidodantrolene in the presence of AMP-PCP and immunoreactive with a polyclonal rabbit anti-skeletal muscle RyR antibody (*19*). These results are most consistent with the supposition that this 160 kDa protein is a proteolytic fragment of the RyR, possibly the result of a previously reported, endogenous, n-calpain cleavage of the RyR (*20*). Proof of this hypothesis requires (a) the demonstration of specific [^{3}H]-azidodantrolene photolabeling of the RyR 565 kDa monomer, (b) the demonstration of endogenous calpain activity in our SR membrane preparations, and (c) the n-calpain-dependent loss of photolabeled 565 kDa protein and enhanced generation of a photolabeled 160 kDa protein. Our previous report did not demonstrate [^{3}H]azidodantrolene-dependent photolabeling of the 565 kDa channel monomer, a finding inconsistent with the above hypothesis (*19*). Further experimentation revealed, however, that little RyR monomer transferred out of 7.5% SDS−PAGE gels onto PVDF membranes using standard buffer containing 10% methanol. Near-quantitative transfer, however, was obtained when the methanol concentration was reduced to 5%, the transfer time was increased from 1.5 to 3 h, and 5 or 6% resolving SDS−polyacrylamide gels were used (data not shown). This increase in transfer time did not result in a loss of labeled proteins through the PVDF membrane, since a second membrane placed behind the first did not contain any radiolabeled proteins (data not shown). Accordingly, our earlier results not assigning photolabeling of [^{3}H]azidodantrolene to the RyR monomer were likely secondary to lack of protein transfer from gel to membrane, not lack of specific interaction. Additionally, an irreversible thiol protease inhibitor, leupeptin, is usually present in all buffers during standard skeletal muscle membrane fractionation procedures which would preclude the demonstration of an endogenous calpain activity in SR. Therefore, all experiments reported below were optimized for quantitative protein transfer to PVDF membranes, and all preparations of HSR were made in the absence of leupeptin.

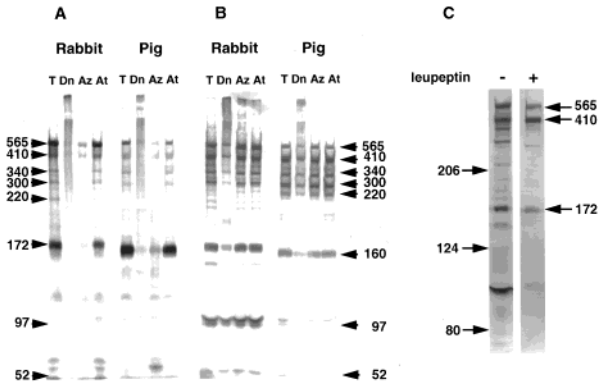

FIGURE 1: Specific [^{3}H]azidodantrolene photolabeling of HSR. [^{3}H]-Azidodantrolene (400 nM) was incubated with 150 μg of HSR from rabbit or pig in the absence (T, total) or presence of 25 μM dantrolene (Dn), 150 μM azumolene (Az), or 150 μM atropine (At). Following UV irradiation, samples were centrifuged and pellets resolved on 6% SDS−polyacrylamide gels and transferred onto PVDF membranes. (A) Autoradiography and (B) Western blotting of the same PVDF membrane were performed with polyclonal anti-rabbit skeletal muscle RyR antibody. The specifically photolabeled 565 and 410 kDa RyR doublet and the 160/172 kDa protein bands are indicated by the arrows. (C) Western blot of SR membranes prepared in the presence or absence of leupeptin probed with polyclonal anti-rabbit skeletal muscle RyR, as in (B).

To directly determine whether [^{3}H]azidodantrolene specifically interacted with the 565 kDa RyR monomer, the 160 kDa protein, and/or other HSR proteins, rabbit or pig membranes were incubated in the presence of AMP-PCP in binding buffer and photoreacted with [^{3}H]azidodantrolene, in the absence or presence of dantrolene, azumolene, or atropine, and processed as described under Experimental Procedures. The results of a representative experiment (n = 5), shown in Figure 1A, demonstrate specific photoincorporation of [^{3}H]azidodantrolene primarily into prominent protein bands of 565 and 160 kDa in pig, and 565, 410, and 172 kDa in rabbit, respectively. Additionally, there is low-level-specific photolabeling of bands at 340, 300, and 220 kDa and possibly other even lower molecular mass bands. Unlabeled dantrolene and azumolene inhibited [^{3}H]azidodantrolene photoincorporation into these protein bands while atropine, as expected, did not. The specific labeling of the 410 kDa band in rabbit HSR is inconsistent from experiment to experiment and from preparation to preparation, while in pig, the labeling of this band is invariably weak.

The same PVDF membranes used to generate the autoradiograms above were probed with a polyclonal anti-rabbit skeletal muscle RyR antibody, revealing prominent immunoreactivity of bands of 565, 410, and 160/172 kDa, as well as a host of other bands (Figure 1B). The 565 and 410 kDa bands are consistent with the molecular masses of the well-characterized skeletal muscle RyR doublet, the former being the monomer and the latter, a proteolytic product (*31*, *32*). Given the fact that leupeptin was not present in these

preparations, the immunoreactive bands are likely to have been derived from the RyR monomer by proteolytic degradation (*28*, *29*). Indeed, Western blots comparing rabbit HSR prepared in the presence or absence of leupeptin using the same polyclonal anti-RyR antibody reveal a greater diversity of immunoreactive protein bands present in SR membranes prepared in the absence of leupeptin than in its presence (Figure 1C). The data suggest that the prominently labeled 160 (pig)/172 (rabbit) kDa bands are proteolytically derived from the 565 kDa protein, the RyR monomer.

Note that in the autoradiograms, in the lanes of samples incubated in the presence of unlabeled dantrolene, a smear of label is seen above the RyR monomer (i.e., in the stacking gel) with some material not even entering the gel (Figure 1A). This smear demonstrates cross-reactivity to anti-RyR antibody (Figure 1B). Further inspection of the Western blots reveals attenuation of the intensity of the anti-RyR immunoreactive bands only in the sample incubated with unlabeled dantrolene. These results suggest that UV irradiation of samples with dantrolene induces multimerization of the RyR and other HSR proteins, resulting in high molecular mass entities which either do not enter into or are poorly resolved on the gel. Nitrophenyl groups, such as those present in dantrolene or the dihydropyridines, are known to be photoactive (*33*, *34*). It is likely, therefore, that the combination of photoactivated dantrolene and azidodantrolene catalyzed cross-linking of HSR proteins, including the RyR, in our reactions. Reduction in the photolabeling of proteins by [3H]-azidodantrolene incubated in the presence of dantrolene is likely an artifact of drug-induced, protein−protein photocross-linking. Therefore, all further specificity determinations in our [3H]azidodantrolene photolabeling experiments were done in the presence of azumolene, which does not contain a nitrophenyl group and does not cause such cross-linking.

Endogenous Calpain Cleavage of Skeletal Muscle RYR. Exogenously added m-calpain has also been shown to first cleave the RyR monomer into fragments of 410 and 150−172 kDa before degrading the channel protein further (*35*, *36*). Given the fact that leupeptin was not present in these preparations, we suspected that the 160/172 kDa band was likely to have been derived from the RyR monomer by proteolytic degradation via muscle-specific n-calpain, or contaminating μ- or m-calpains, described above. Therefore, we endeavored to determine whether the [3H]azidodantrolene-photolabeled 160 kDa pig and 172 kDa rabbit protein bands seen in our autoradiograms are the amino-terminal fragments of the intact RyR derived by endogenous n-calpain cleavage.

We first determined whether an endogenous, RyR-degrading, protease activity consistent with that of n-calpain was present in our preparations. We postulated that this enzyme activity would degrade the 565 kDa RyR monomer in response to exogenous thiol and endogenous Ca^{2+}, producing an increase in the amount of the 410 and 160/172 kDa bands, and be sensitive to inhibition by the calpain inhibitor, leupeptin, and by EGTA. Pig and rabbit HSR membranes were incubated under calpain activating conditions, and proteins were resolved by SDS−PAGE and stained with Coomassie Blue. In both pig and rabbit HSR (Figure 2A), the addition of DTT in the presence of endogenous Ca^{2+} is sufficient to generate both a loss of staining in the 565 kDa band and a concomitant increase in the staining intensity of the 160/172 and the 410 kDa bands. Leupeptin inhibited

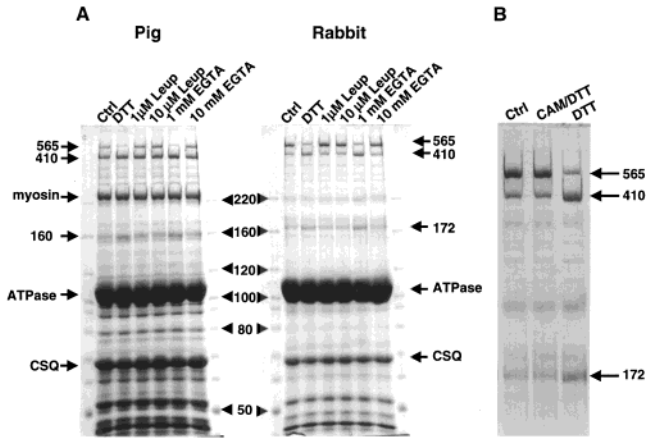

FIGURE 2: Generation of 160 and 172 kDa protein RyR fragments by endogenous n-calpain activity. (A) Pig and rabbit HSR proteins (100 μg) were incubated in the absence (Ctrl) or presence of 10 mM DTT (DTT) and leupeptin (Leup, 1 or 10 μM) or EGTA (1 or 10 mM). The reactions were terminated by diluting aliquots of reaction mixtures into Laemmli sample buffer and heating at 60 °C for 10 min. Proteins were resolved on a 7.5% SDS−polyacrylamide gel and stained with Coomassie Blue. The 565 and 410 kDa RyR doublet and the 160 or 172 kDa protein bands are indicated on the left and right of the figure. Molecular weight markers are in the middle. The positions of myosin, Ca^{2+}-ATPase, and calsequestrin (CSQ) are also indicated. (B) The addition of 100 μM calmodulin to rabbit HSR under n-calpain activating conditions inhibits the generation of the 410 and the 172 kDa proteins.

generation of the 160 and 172 kDa bands to levels equivalent to background. Significantly, EGTA at 1 mM was insufficient to inhibit the endogenous calpain activity: a minimum of 10 mM EGTA was required to inhibit this reaction, as increases in the Coomassie Blue staining intensity of the 160/172 kDa band were observed even in the presence of 8 mM EGTA (data not shown). Therefore, extremely low Ca^{2+} concentrations are sufficient to activate this calpain-like activity in the presence of thiol. Furthermore, exogenously added bovine brain calmodulin (100 μM), another inhibitor of calpain activity (*35*), also inhibited the generation of the 160 and 172 kDa fragments in both rabbit (Figure 2B) and pig (data not shown). Time course experiments revealed that maximal cleavage of the 565 kDa protein by this endogenous n-calpain activity was reached by 30 min, both at room temperature and at 37 °C (data not shown). Despite this, there is never complete conversion of the presumed RyR monomer to the 410 and 160/172 kDa proteins. Furthermore, in the absence of exogenously added reducing agent, incubation of HSR at 37 °C for up to 90 min resulted in no cleavage of the 565 kDa protein beyond that already present in our preparations (data not shown). These results indicate that an endogenous calpain activity consistent with that of n-calpain (i.e., activated by reducing agents and submicromolar Ca^{2+} concentrations) is present in our SR membrane preparations that results in cleavage of the 565 kDa RyR monomer, generating primarily ~410 and 160 or 172 kDa protein bands in pig and rabbit, respectively. We hypothesized that the 160/172 kDa fragments likely represent the [3H]azidodantrolene binding protein we previously reported (*19*).

To corroborate the presence of n-calpain in our HSR preparations, pig and rabbit skeletal muscle HSR proteins resolved by SDS−PAGE were transferred to PVDF membranes, and probed with a monoclonal antibody to a unique

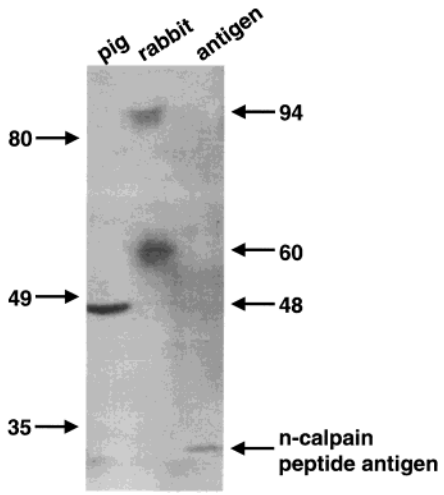

FIGURE 3: Pig and rabbit HSR probed with anti-CAPN3. Western blot of rabbit and pig HSR proteins (100 μg per lane) resolved on a 7.5% SDS−polyacrylamide gel, transferred onto a PVDF membrane, and probed with monoclonal anti-CAPN3 antibody. The immunoreactive bands in pig and rabbit HSR are denoted by the arrows. The antigen, a peptide containing the unique sequence of amino acids 607−628 located in the IS2 region of human CAPN3, was run as positive control.

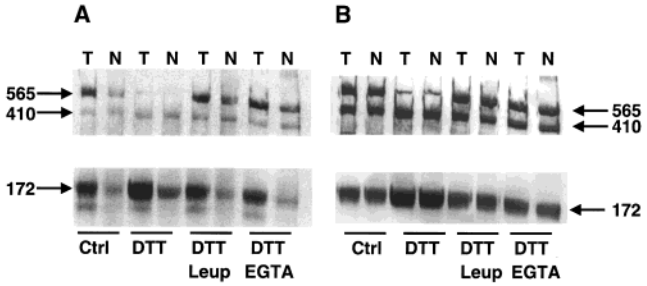

FIGURE 4: [^{3}H]Azidodantrolene photolabeling of the skeletal muscle RyR followed by n-calpain cleavage. Rabbit HSR membranes were incubated with [^{3}H]azidodantrolene in the absence (T, total) or presence (N, nonspecific) of excess azumolene. Following UV irradiation, the vesicles were pelleted and resuspended in 20 mM Na-PIPES, pH 7. DTT (10 mM), leupeptin (1 μM), and EGTA (10 mM) were added, where indicated, and the samples were incubated for a further 30 min at 37 °C. The reactions were terminated by diluting the samples in Laemmli sample buffer and heating at 60 °C for 10 min. Proteins were resolved on a 6% SDS−polyacrylamide gel and transferred to a PVDF membrane. The same PVDF membrane was subjected to autoradiography (A) and Western blotting with polyclonal rabbit skeletal muscle anti-RyR antibody (B). The 565 kDa RyR monomer and the 410 and 172 kDa RyR fragments are indicated. Note that the specific photolabel in the 565 kDa RyR monomer is "chased" into the 172 kDa band in an n-calpain-dependent manner.

sequence in human n-calpain, anti-CAPN3 (Figure 3). In pig HSR, only a single band of ~48 kDa was detected, while in rabbit HSR, two immunoreactive bands of ~94 and ~60 kDa were detected. The 94 kDa band corresponds to the known molecular mass of the intact n-calpain catalytic subunit (*29*, *37*). The 60 (rabbit) and 48 (pig) kDa bands are likely proteolytic products of this notoriously autolytic enzyme, known to generate fragments ranging from 60 to 45 kDa (*24*, *29*, *38*). Despite the failure to detect the 94 kDa n-calpain catalytic subunit in the pig HSR preparation, our results demonstrate that the enzymatic activity of this enzyme is maintained. This is consistent with reports that some autolytic fragments of n-calpain retain their proteolytic activity (*39*).

Endogenous n-Calpain Cleavage of [^{3}H]Azidodantrolene-Labeled RyR. If the intact RyR is the target of [^{3}H]-azidodantrolene, and the radiolabeled 160/172 kDa proteins of pig and rabbit skeletal muscle are generated by the action of endogenous n-calpain, one should be able to "chase" radioactively labeled protein from the 565 kDa monomer into the 160 or 172 kDa proteins only under conditions that favor n-calpain activation. There should be a concomitant loss of anti-RyR immunoreactivity with the 565 kDa band, and an increase in reactivity with the 410 and 160/172 kDa bands. Furthermore, it is conceivable that the photolabeling of the RyR with [^{3}H]azidodantrolene prior to activation of n-calpain might alter the conformation of the RyR such that this enzyme might not be able to cleave the RyR.

To determine whether we could indeed generate an increase in photolabeled 172 kDa protein by activating n-calpain, rabbit HSR membrane proteins were first photolabeled with [^{3}H]azidodantrolene in the absence or presence of azumolene, collected by centrifugation, and subjected to n-calpain activating conditions. Autoradiography and Western Blot analysis of these photolabeled proteins (Figure 4) demonstrate specific basal incorporation of label into the 565, 410, and 172 kDa bands, similar to the results presented above (Figure 1). Incubation with DTT and endogenous Ca^{2+} results in an increase in the [^{3}H]azidodantrolene-labeled 172 kDa band. This is accompanied by a concomitant decrease in the radiolabeled 565 kDa RyR monomer. Generation of a specifically radiolabeled 172 kDa band is inhibited by leupeptin and 10 mM EGTA (Figure 4A). In contrast with the results in Figure 1, there is no specific basal incorporation of photolabel into the 410 kDa RyR fragment, and there is no increase in the labeling of this band in DTT-treated samples in this experiment (Figure 4A).

To demonstrate that the "chasing" of photolabel from the 565 kDa protein into the 172 kDa protein was due to degradation of the RyR monomer, the same PVDF membrane used to generate the autoradiogram was probed with a polyclonal sheep anti-rabbit skeletal muscle RyR antibody. The Western blot shows that there is a decrease in the levels of the 565 kDa band, and an increase in the levels of the 410 and 172 kDa bands only in the presence of DTT and endogenous Ca^{2+} (Figure 4B). Leupeptin and 10 mM EGTA inhibited the generation of these RyR fragments. EGTA, therefore, has no inhibitory effect on [^{3}H]azidodantrolene photolabeling of the RyR, only on the degree of cleavage of the RyR. Virtually identical photolabeling, n-calpain cleavage, and anti-RyR immunoreactivity results were obtained with pig HSR, except that, as noted above, the pig RyR fragment runs as a 160 kDa protein in SDS−PAGE (data not shown). These findings confirm that, in the presence of AMP-PCP, [^{3}H]azidodantrolene is capable of specifically photolabeling the intact RyR 565 kDa monomer. n-Calpain, under the appropriate conditions of a reducing environment and sufficient Ca^{2+}, cleaves the RyR to generate 410 and 160/172 kDa proteins, the latter of which is specifically photolabeled with [^{3}H]azidodantrolene. The likeliest target for [^{3}H]azidodantrolene photolabeling on the RyR monomer, therefore, is on that portion of channel protein released by n-calpain cleavage.

Endogenous n-Calpain Cleavage of the Skeletal Muscle RyR followed by [^{3}H]Azidodantrolene Labeling. The results

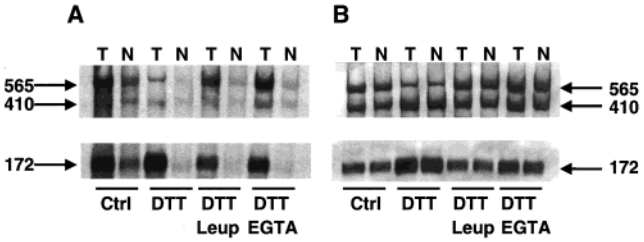

FIGURE 5: n-Calpain cleavage of the skeletal muscle RyR followed by [3H]azidodantrolene photolabeling. Rabbit HSR membranes were treated with DTT and either leupeptin or EGTA, as indicated, collected by centrifugation, resuspended, and incubated with [3H]-azidodantrolene in the absence (T, total) or presence (N, nonspecific) of excess azumolene. Membranes were then centrifuged and resuspended in Laemmli sample buffer; proteins were resolved by SDS−PAGE and electroblotted onto PVDF membranes. These were subjected to autoradiography (A) and then probed with polyclonal rabbit anti-skeletal muscle RyR antibody by Western blotting (B).

above (Figures 1 and 4) indicate that these HSR membranes contain a mixture of both intact and cleaved RyRs, and that [3H]azidodantrolene can interact specifically with both. It is not clear from the above experiments whether [3H]azidodantrolene photolabeling of the 160/172 kDa fragments of the cleaved RyR occurs once all the RyR has undergone n-calpain clipping, or whether the fragment photolabeling occurs only in populations where intact-RyR/cleaved-RyR interactions might stabilize a dantrolene-receptive conformation in the cleaved RyR. To answer this question, we first converted virtually all the RyR to the calpain-nicked form by first incubating HSR under n-calpain activating conditions, and then proceeded with [3H]azidodantrolene photolabeling, autoradiography, and Western blotting, as described above. The autoradiogram demonstrates (Figure 5A) that in the absence of n-calpain activation specific incorporation of [3H]-azidodantrolene into both the 565 and 172 kDa bands is consistent with the levels of photolabeling seen above. Activation of n-calpain activity prior to [3H]azidodantrolene photolabeling results in near total loss of photolabeling of the 565 kDa RyR monomer, and is accompanied by an increase in specific label incorporation into the 172 kDa band. This shift in labeling pattern is inhibited by the presence of either leupeptin or EGTA. Once again, there is little, if any, specific incorporation of label into the 410 kDa band. Examination of the Western blot (Figure 5B) reveals that there is virtually identical loading of anti-RyR reactive protein from lane to lane, and only in the reaction containing exogenous DTT and endogenous Ca^{2+} is there a loss of 565 kDa protein and a concomitant increase in 172 kDa protein. Virtually identical results were obtained with pig HSR (data not shown). These data demonstrate not only that both the intact and n-calpain-cleaved RyR are specifically photolabeled by [3H]azidodantrolene, but also that the cleaved form likely does not require association with the intact form to be photolabeled. Since there is a centrifugation and membrane resuspension step between the n-calpain degradation of the RyR and [3H]azidodantrolene photolabeling, these results also indicate that after being cleaved, the 160/172 kDa fragment of the RyR remains associated with the SR and still retains the functional ability to bind [3H]azidodantrolene.

Immunoprecipitation of [3H]Azidodantrolene-Photolabeled HSR. To determine whether the 410 and 160 kDa proteins retain a physical association after n-calpain cleavage, we

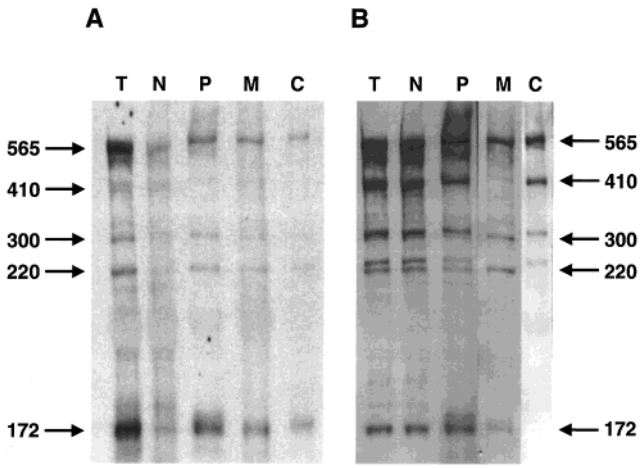

FIGURE 6: Immunoprecipitation of HSR photolabeled with [3H]-azidodantrolene. Rabbit HSR membrane proteins were photolabeled with [3H]azidodantrolene, in the absence (T, total) or presence (N, nonspecific) of excess azumolene. Membrane proteins were then solubilized, immunoprecipitated with a sheep polyclonal anti-skeletal muscle RyR antibody (P), a mouse monoclonal anti-RyR antibody (M), or a polyclonal anti-RyR C-terminal antibody (C), resolved by SDS−PAGE, transferred to PVDF membranes, and subjected to autoradiography (A) and Western blot analysis (B).

examined photolabeled rabbit HSR proteins immunoprecipitated using a battery of anti-rabbit skeletal muscle RyR antibodies. [3H]Azidodantrolene-photolabeled proteins were immunoprecipitated from CHAPS-solubilized rabbit HSR with three separate antibodies against RyR1: the polyclonal sheep IgG antibody used above; a second, rabbit polyclonal anti-RyR1 C-terminal antibody (IgG); and a third, mouse monoclonal, anti-RyR1 antibody (IgM), and resolved. All three antibodies immunoprecipitated the specifically photolabeled 565 kDa RyR1 monomer and the 172 kDa protein, as well as faintly labeled proteins at ~220 and 300 kDa (Figure 6A). The faintly labeled 410 kDa protein present in the starting material (Figure 6A, lanes T, N) is immunoprecipitated (Figure 6B, lanes P, M, C), but the level of labeling is too low to be detected in the autoradiograph (Figure 6A, lanes P, M, C). Nonimmune immunoglobulin did not precipitate any photolabeled or immunoreactive protein (data not shown). Furthermore, Figure 6B demonstrates that the three antibodies used herein have distinct yet overlapping epitopes. The polyclonal anti-RyR recognizes all the bands recognized by both the monoclonal anti-RyR and the anti-C-terminal antibodies. With this antibody, the bands at ~220 and ~300 kDa are shown to be doublets whose epitopes are discriminated between by the monoclonal and anti-C-terminal antibodies. The anti-C-terminus recognizes only the 565 and 410 kDa bands and the upper bands of both doublets, and does not recognize the 172 kDa band. The mouse monoclonal antibody recognizes the 565 kDa band, the lower bands of both doublets, the 172 kDa band, and the 410 kDa band only faintly. Superimposition of the autoradiogram and the Western blots reveals that the specifically photolabeled bands in the 220 and 300 kDa region are the lower bands in both cases, i.e., the ones recognized by the mouse monoclonal antibody. These data indicate that even an antibody which only recognizes an RyR1 epitope(s) distinct from the 172 kDa band (the C-terminus) is still capable of immunoprecipitating this photolabeled fragment.

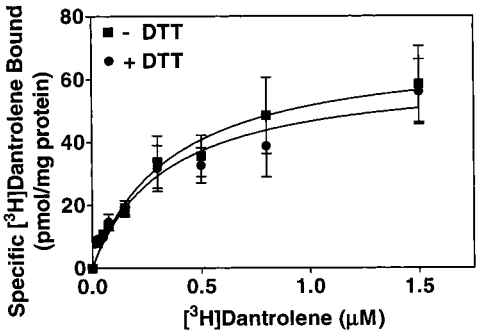

FIGURE 7: Specific binding of [^{3}H]dantrolene to HSR in the absence or presence of DTT. Rabbit HSR membranes were incubated in the absence (■) or presence (●) of 10 mM DTT in 20 mM Na-PIPES, pH 7, at 37 °C for 30 min. The rest of the components of the binding buffer (see Experimental Procedures), including incremental concentrations of [^{3}H]dantrolene ± excess azumolene, were then added. Incubation continued for a further 90 min before rapid filtration, and specific binding was calculated. Each data point represents the mean of triplicate measurements, and the error bars represent the standard error of measurement.

MALDI-Mass Spectrometric Analysis of the Porcine 160 kDa Fragment. To positively identify the n-calpain-cleaved, RyR fragment, we subjected the porcine, SR-derived, 160 kDa protein fragment to MALDI-mass spectrometry, as described in Experimental Procedures. The 160 kDa [^{3}H]-azidodantrolene-photolabeled protein was positively identified as the amino-terminal fragment of the pig skeletal muscle RyR, containing the first 1400 amino acids of this protein (data not shown).

Specific Binding of [^{3}H]Dantrolene to Rabbit HSR in the Absence or Presence of DTT. The data above demonstrate that [^{3}H]azidodantrolene is able to interact with either the intact or the n-calpain-nicked RyR, implying that this aspect of channel pharmacological activity is maintained independent of endogenous calpain activity. However, photolabeling experiments cannot detect quantitative differences in the equilibrium binding parameters of the radioligand for the two forms of the RyR, cleaved or intact. Furthermore, DTT, the reducing agent used to activate n-calpain in these experiments, is also capable of reducing sulfhydryls critical to the activity of the channel (*40*, *41*). To determine whether DTT had any effects on the binding affinity (K_d) or the binding capacity (B_{max}) of [^{3}H]dantrolene for the RyR, rabbit HSR was preincubated under n-calpain-activating conditions, and radioligand binding was determined. Specific [^{3}H]-dantrolene binding curves (Figure 7) analyzed by nonlinear regression analysis are most consistent with a single radioligand binding site in HSR in both cases. Calculated K_d and B_{max} values for [^{3}H]dantrolene binding to rabbit HSR were 360 ± 65 nM and 70 ± 5 pmol/mg of protein in the absence of DTT, and 336 ± 97 nM and 62 ± 7 pmol/mg of protein in the presence of DTT, respectively. These values are not significantly different from each other. Moreover, they are virtually identical to those obtained with rabbit HSR prepared in the presence of leupeptin (data not shown). The K_d values reported above are slightly higher than those we obtained with pig HSR prepared in the presence of leupeptin, in the absence (~275 nM) or presence (210 nM) of AMP-PCP (*16*, *19*). However, the B_{max} values are substantially higher than those we reported in the absence (~13 pmol/mg of protein) (*16*) and presence (~40 pmol/mg of protein) (*19*) of AMP-PCP, and may be due to species differences. We conclude

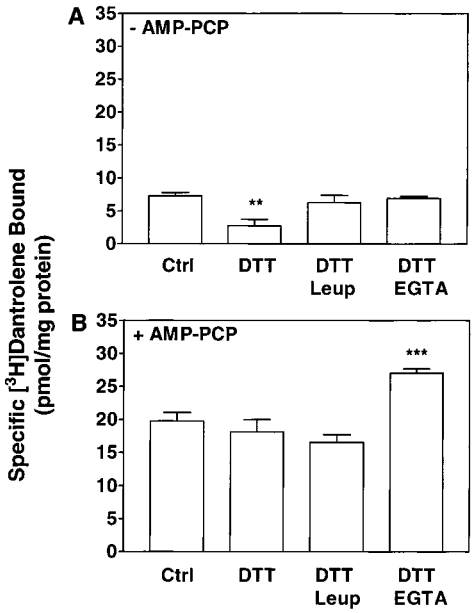

FIGURE 8: Effect of AMP-PCP on the specific binding of [^{3}H]-dantrolene to HSR in the absence or presence of DTT. Rabbit HSR membranes were incubated in the absence or presence of DTT, or DTT and leupeptin, or DTT and EGTA, in binding buffer without nucleotide at 37 °C for 30 min. [^{3}H]Dantrolene (200 nM) was then added to the reaction mixture and incubated for a further 90 min in the absence (A) or presence (B) of 0.5 mM AMP-PCP before rapid filtration. Nonspecific binding was determined in the presence of excess azumolene and specific binding calculated. Bars indicate the mean ± SE ($n = 6$, $^{**}p < 0.02$ compared with all other values in panel A, $^{***}p < 0.005$ compared with all other values in panel B).

that the presence of DTT and/or calpain cleavage of the RyR has no effect on the equilibrium binding parameters of [^{3}H]-dantrolene for rabbit HSR in vitro. When compared to values achieved in the absence of nucleotide, AMP-PCP had a stimulatory effect (~3-fold) on specific [^{3}H]dantrolene binding to HSR.

Effect of n-Calpain Cleavage on Specific Binding of [^{3}H]-Dantrolene to Rabbit HSR in the Absence or Presence of AMP-PCP. Both AMP-PCP and calpains are reported to stimulate RyR activity and/or SR Ca^{2+} release (*20*, *42*, *43*). Though AMP-PCP is reported to enhance [^{3}H]dantrolene binding to SR (*19*), nothing is known about the effects of n-calpain activation on radioligand binding in the absence of this nucleotide. To examine this in more detail, we first incubated rabbit HSR under n-calpain activating conditions, ±leupeptin or EGTA as controls, and then determined specific [^{3}H]dantrolene binding in the absence or presence of AMP-PCP by a rapid filtration assay. Figure 8A shows that in the absence of AMP-PCP, there is a significant decrease (~60%) in specific [^{3}H]dantrolene binding to HSR treated with DTT relative to control membranes incubated in the absence of DTT. This decrease is not due to a direct inhibitory effect of DTT on [^{3}H]dantrolene binding, nor to the reduction of intramolecular disulfide bridges, for no decrease is seen in samples where n-calpain activity is inhibited, i.e., in the presence of DTT and either leupeptin or EGTA. On the other hand, and in agreement with the results in Figure 7, the presence or absence of DTT had no effect on [^{3}H]dantrolene binding in the presence of AMP-PCP (Figure 8B). Moreover, the presence of leupeptin had no effect on [^{3}H]dantrolene binding, while EGTA actually

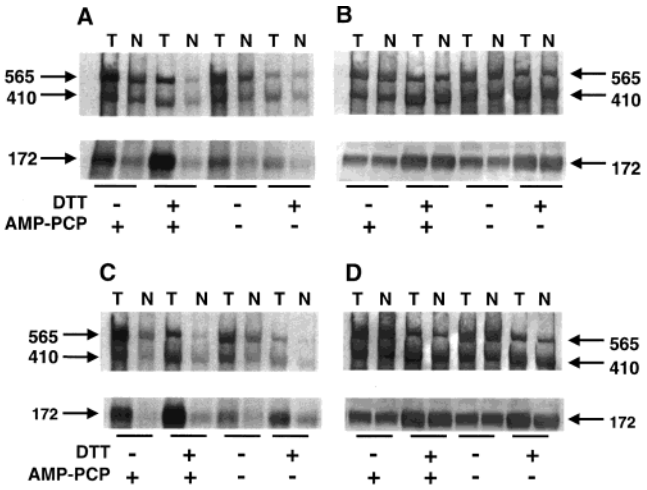

FIGURE 9: Effect of AMP-PCP and order of n-calpain cleavage and [3H]azidodantrolene photoactivation on specific photolabeling of RyR. Autoradiograph (A) and Western blot (B) of rabbit HSR treated first with DTT to activate n-calpain, followed by [3H]-azidodantrolene photolabeling in the presence (+) or absence (−) of AMP-PCP. Autoradiograph (C) and Western blot (D) of rabbit HSR first labeled with [3H]azidodantrolene in the presence (+) or absence (−) of 0.5 mM AMP-PCP and then treated with 10 mM DTT. Total (T) photolabeling was obtained in the presence of [3H]-azidodantrolene alone, and nonspecific (N) photolabeling was that obtained in the presence of [3H]azidodantrolene and excess azumolene. Western blots were probed with polyclonal rabbit skeletal muscle anti-RyR antibody. The 565, 410, and 172 kDa RyR bands are indicated.

enhanced specific binding. Further experimentation revealed that the presence or absence of DTT had no effect on the degree of EGTA-induced enhancement in [3H]dantrolene binding to rabbit HSR, as long as AMP-PCP was present during incubation (data not shown).

[3H]Azidodantrolene Labeling of the Intact and n-Calpain-Cleaved RyR in the Absence or Presence of AMP-PCP. To demonstrate equivalence between [3H]dantrolene binding and [3H]azidodantrolene photolabeling, one should verify that conditions which enhance the binding of one enhance the extent of photolabeling by the other and vice versa. In light of the results in Figures 7 and 8 above, we compared the effects of both AMP-PCP and DTT (i.e., n-calpain cleavage of the RyR) on the specific photolabeling of the RyR and its n-calpain-derived fragment by [3H]azidodantrolene. To do this, we first incubated rabbit HSR under n-calpain activating conditions, collected the membranes by centrifugation, and then photolabeled with [3H]azidodantrolene in the presence or absence of AMP-PCP. The resultant autoradiogram and Western blot, shown in Figure 9A,B, allow a number of conclusions to be drawn. Despite equivalent amounts of protein loaded in each lane, the addition of AMP-PCP imparts a striking enhancement of specific photoincorporation of [3H]azidodantrolene into both the 565 and the 172 kDa bands. This is in agreement with the results in Figure 1. Significantly, preincubation with DTT, irrespective of the presence of AMP-PCP, resulted in near total disappearance of the anti-RyR immunoreactive 565 kDa band, producing an increase in the immunoreactive 410 and 172 kDa bands (Figure 9B). Thus, though AMP-PCP has no effect on the activity of endogenous n-calpain on the RyR, it allows for enhanced photolabeling of the 172 kDa band. Surprisingly, in the absence of AMP-PCP, there is less specific photolabeling of all the major RyR bands in the sample pretreated with DTT than in the one not treated with the reducing agent. Though the reasons for this last result are not clear, the totality of these effects demonstrates a correlation between the photoincorporation of [3H]azidodantrolene and the degree of [3H]dantrolene binding to HSR described above.

In the previous experiment, it is possible that conformational changes in the RyR induced by AMP-PCP were sufficient to override potential inhibitory effects of the photocross-linked [3H]azidodantrolene on the access of n-calpain to the RyR. To test this possibility, we first photolabeled HSR with [3H]azidodantrolene ± excess azumolene, in the presence or absence of AMP-PCP, then treated the samples with DTT, and resolved the pelleted proteins as above. The results are shown in Figure 9C,D. Qualitatively, the addition of DTT after specific photolabeling results in reduced labeling of the 565 kDa band and a concomitant increase in labeling of the 172 kDa protein. The results are the same whether initial photolabeling occurred in the absence or presence of AMP-PCP. Quantitatively, however, the degree of labeling of these bands is significantly enhanced in the presence of AMP-PCP. Once again, the Western blots show no effect of AMP-PCP on the ability of DTT to activate n-calpain cleavage of the labeled RyR. Interestingly, in this experiment, the 410 kDa band is specifically photolabeled, though at much lower levels than the 565 and 172 kDa bands.

[3H]Azidodantrolene Labeling of Porcine Cardiac SR in the Absence or Presence of AMP-PCP. Finally, we examined the ability of [3H]azidodantrolene to interact with porcine cardiac SR prepared in the presence of leupeptin. Cardiac SR membranes were incubated with [3H]azidodantrolene in binding buffer, in the presence or absence of AMP-PCP and excess azumolene. Photolabeling, SDS−PAGE, electroblotting onto PVDF membranes, and autoradiography were then performed, as described above, except that the entire reaction mixture was loaded onto the gels without centrifugation. The results, shown in Figure 10, demonstrate that there is very low level specific photolabeling of cardiac RyR by this ligand, and no effect of AMP-PCP is evident. Virtually identical results were obtained with two separate preparations from two different animals. No other specifically [3H]-azidodantrolene-photolabeled proteins are present in these experiments with cardiac SR.

DISCUSSION

Despite both its importance in the treatment of MH and the demonstrations both in vivo and in vitro that dantrolene acts by inhibiting the release of SR Ca^{2+}, the molecular mechanism of dantrolene action remained murky. While it has been suggested that dantrolene acts directly on the skeletal muscle RyR Ca^{2+} channel to inhibit calcium release (*44*, *45*), direct evidence for this interaction has not heretofore been presented. Our earlier data failed to demonstrate an interaction between activators or inhibitors of [3H]ryanodine binding and [3H]dantrolene binding, suggesting a lack of interaction between the binding sites for these two ligands (*17*). Furthermore, we were able to demonstrate separable, yet overlapping peaks of [3H]dantrolene and [3H]ryanodine binding to HSR (*17*), suggesting that dantrolene and ryanodine binding sites might lie on separate, interacting mol-

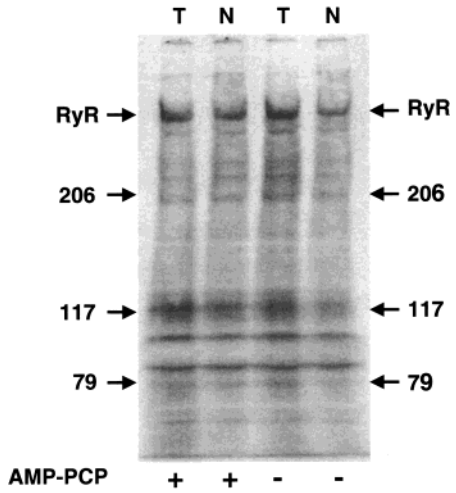

FIGURE 10: Photolabeling of porcine cardiac SR with [^{3}H]-azidodantrolene in the presence or absence of AMP-PCP. Porcine cardiac SR membranes (100 μg) were incubated with [^{3}H]-azidodantrolene in binding buffer ± excess azumolene, ± AMP-PCP, and processed for autoradiography. Total (T) photolabeling was that obtained in the presence of [^{3}H]azidodantrolene alone, and nonspecific (N) photolabeling was that obtained in the presence of [^{3}H]azidodantrolene and excess azumolene. Note the barely detectable specific photolabeling of the 565 kDa RyR monomer.

ecules. On the other hand, it has been demonstrated that dantrolene inhibited [^{3}H]ryanodine binding to HSR and inhibited Ca^{2+} release from skeletal muscle SR vesicles (*3*). Moreover, Nelson et al. (*18*) reported a biphasic action of dantrolene on highly purified porcine skeletal muscle RyR in lipid bilayers: first activating, then inhibiting. These data suggested that the RyR itself is a likely target of dantrolene. The data presented herein demonstrate unequivocally that the RyR is a molecular target of dantrolene. Furthermore, we have localized the [^{3}H]azidodantrolene binding site to the 1400 amino acid region of the N-terminus of the RyR that is cleaved by n-calpain.

The fact that dantrolene interacts with the N-terminus of the RyR is consistent with a significant role for this region of the channel in the regulation of SR Ca^{2+} release. First, the porcine genetic defect associated with MH sensitivity (Arg615Cys) is in the N-terminus, as are most of the described human RyR mutations associated both with this syndrome and with the rare, familial myopathy, central core disease [for reviews, see (*4, 15, 46*)]. Second, a number of lines of evidence point to this region as one that may regulate the ability of the RyR to interact with appropriate portions of the DHPR, the T-tubule voltage sensor, in skeletal muscle ECC (*4*). Deletion of N-terminal residues 1303−1406 of the skeletal muscle RyR in functional expression experiments led to the loss of ECC rather than Ca^{2+} channel activity, while its reinsertion led to restoration of ECC (*47*). Skeletal muscle RyR N-terminal peptides corresponding to Arg1076−Asp1112 and Lys954−Asp1112 interact in vitro with the II−III and III−IV loops of the DHPR α1 subunit likely involved in skeletal muscle ECC (*48, 49*). Indeed, the RyR-activating peptide of the scorpion toxin, Imperatoxin A, contains a sequence nearly identical to Glu666−Leu690 of the RyR-activating II−III loop segment of the DHPR α1 subunit (*50*), and binds to a cytoplasmic (N-terminal) domain of its target channel (*51*). More recently, synthetic peptides corresponding to subdomains of the near-amino-terminal region of both cardiac and skeletal muscle RyR have also been shown to activate the Ca^{2+} release channel (*52, 53*). Third, critical sulfhydryl groups whose redox states play a significant role in the regulation of the RyR are located in the amino terminus and central portions of the RYR (*32, 40, 41, 54−56*). Finally, the cytoplasmic (N-terminal-containing) region of RyR is the likely site of action of many endogenous and exogenous effectors [for reviews, see (*8, 14*)]. We now add dantrolene and azumolene to the long list of effectors likely to interact directly with the N-terminus of the RyR.

The requirement for AMP-PCP for enhanced dantrolene binding to both the n-calpain-cleaved and intact RyR implicates ATP as a regulator of dantrolene binding to the RyR. ATP is a known activator of the Ca^{2+} release channel, and dantrolene is a known inhibitor of SR Ca^{2+} release (*8, 14, 57*). ATP would therefore be involved in two competing RyR-dependent processes—intrinsic activation of Ca^{2+} release and dantrolene inhibition of Ca^{2+} release. Interestingly, Nelson et al. (*18*) demonstrated both an activating and an inhibiting activity for dantrolene. Our results, along with theirs, suggest that certain conditions which activate the channel might enhance interaction of dantrolene and azumolene with the RyR.

Our previously reported binding data in the absence of AMP-PCP are consistent with a single site in SR (*16*). Given the fact that there is only low-level-specific [^{3}H]azidodantrolene photolabeling of the RyR in the absence of AMP-PCP, it seems likely that there is a single site per tetramer. Since AMP-PCP enhances [^{3}H]dantrolene binding ∼3−4-fold, we postulate that the nucleotide conformationally generates four equivalent sites per tetramer. Indeed, we have reported that AMP-PCP enhances the B_{max} for [^{3}H]dantrolene binding to porcine skeletal muscle SR 3−4-fold without a significant effect on the K_d (*58*).

On the other hand, our previously reported sucrose density gradient data seem to support a protein(s) with a lower *S* value than the RyR as a target of dantrolene (*17*). Inspection of those data reveals that the peak of [^{3}H]dantrolene binding in the gradient is associated with the rise in [^{3}H]ryanodine binding, not its absence. The data presented herein demonstrate that dantrolene interacts with the RyR both in the presence and in the absence of AMP-PCP, but to a greater extent in its presence. We interpret this to mean that though dantrolene may interact with and modify the activity of the RyR, such interaction may be modified by adenine nucleotide triphosphate and, possibly, the presence or absence of other SR proteins. Indeed, we have preliminary results indicating that [^{3}H]azidodantrolene specifically photolabels two other SR proteins which do not cross-react with any of the anti-RyR antibodies used above (K. Paul-Pletzer and J. Parness, unpublished results). The identity of these proteins and whether they have a role in regulating the interaction of dantrolene with the RyR are under active investigation.

We present evidence that an endogenous protease activity degrades both native RyR and [^{3}H]azidodantrolene-photolabeled RyR equivalently to primarily 410 and 160/172 kDa proteins, and that [^{3}H]dantrolene binding is equivalent in the presence and absence of DTT. This protease is almost certainly n-calpain because (1) it is activated by DTT and endogenous Ca^{2+}; (2) inhibition of enzymatic activity requires high concentrations of EGTA (≥10 mM); (3) it is

inhibited by leupeptin; and (4) immunoblotting with an n-calpain-specific monoclonal antibody, anti-CAPN3, demonstrates its presence. Indeed, n-calpain has been demonstrated only in skeletal muscle (*20*, *37*) and in a restricted subset of tissues (*29*, *59*). From the data presented above, neither n-calpain cleavage of the RyR nor the redox state of the peri-SR environment appears to play a significant role in the ability of dantrolene to interact with its binding site on the RyR. Rather, we would predict that the energy state of the myocyte and resultant ATP levels control the access of dantrolene to its conformationally sensitive binding site on the N-terminus of the RyR.

Our autoradiograms and Western blots demonstrate that not all of the RyR monomer is degraded under in vitro n-calpain activating conditions. This may be due either to the presence of an n-calpain-resistant subpopulation of RyRs, to the presence of a substoichiometric amount of a calpain inhibitor such as calpastatin (*60*), or, most likely, to autolytic destruction of enzymatic activity in the course of the experiment (*61*). Additionally, since the 160/172 kDa fragments are observed in SDS−PAGE gels of HSR membranes prepared in the presence of leupeptin, this leads us to believe that n-calpain cleavage of the RyR occurs in vivo, and that this cleavage may play a significant regulatory role in the biology of skeletal muscle RyR. Indeed, mutations in the gene for this enzyme are causative for limb−girdle muscular dystrophy, type 2A (*62*).

The n-calpain binding site is reported to be beyond amino acid position 1356 on the rabbit skeletal muscle RyR, and cleavage occurs between amino acids 1400 and 1401 (*20*). The fragment so generated corresponds exactly to the 160 kDa fragment we have obtained in pig. We expect that the interaction of a ligand with a specific binding site would locally perturb the structure of that site. Hence, were the dantrolene binding site to be somewhere between positions 1356 and 1401, we would expect this ligand to inhibit n-calpain activity, by perturbing either its binding or its access to its cleavage site. Since our evidence does not support an effect of dantrolene or azumolene on n-calpain activity, we consider it unlikely that dantrolene binds to the RyR within this region. Characterization of the dantrolene binding site on the N-terminus of the RyR awaits definitive clarification of the amino acids involved in the binding. Given the fact that incorporation of photolabel into the RyR is less than 1.5% (*19*), mapping of the site will have to proceed by other than direct chemical means.

Despite the inconsistent specific [^{3}H]azidodantrolene labeling of the 410 kDa fragment in our experiments, the absence of high-level, specific [^{3}H]azidodantrolene labeling of any anti-RyR-reactive protein fragments in our gel system other than the amino-terminal 160 (pig)/172 (rabbit) kDa segments of the RyR is strong evidence that this portion of the channel contains the dantrolene binding site. The inconsistent, low-level labeling of the 410 kDa protein and what are likely proteolytic fragments at ~300 and 220 kDa has a number of possible explanations. It is possible that the 410 kDa band may contain a small amount of a secondary, n-calpain-derived cleavage fragment of the intact RyR that is generated from a site near the C-terminus that runs along with the 410 kDa band, thereby leaving a small amount of N-terminal photolabeled product to be identified in our autoradiograms. Second, even if there is no endogenous n-calpain cleavage site on the C-terminus of the RyR, low-level photolabeling of the 410 kDa band might still occur, resulting from the proposed dynamic interactions of the N-terminus with the central domains of the channel (*52*, *53*). If these interactions with the proposed dantrolene binding site on the N-terminus were sufficiently dynamic over the lifetime of the photoactivated radioligand, one might expect low-level, specific labeling of the C-terminal portion of the channel protein. Alternatively, skeletal muscle has been reported to contain low levels of RyR3 (*63*) which may also have a dantrolene binding site. If this labeled channel runs along with the 410 kDa band, it might be detected both by autoradiography and by the anti-RyR antibodies used herein.

AMP-PCP enhances binding of [^{3}H]dantrolene to skeletal muscle SR and, in parallel, the photolabeling of the RyR by [^{3}H]azidodantrolene irrespective of the degree of n-calpain cleavage of the RyR. Since equivalent degrees of n-calpain cleavage occur in the presence or absence of AMP-PCP, this nucleotide analogue does not control the activity of n-calpain or its access to the RyR. On the other hand, in the absence of AMP-PCP, n-calpain cleavage of the RyR suppresses specific [^{3}H]dantrolene binding to HSR. These data suggest that n-calpain cleavage of the RyR results in a conformational change of the channel that inhibits dantrolene binding in an AMP-PCP reversible manner. Surprisingly, high concentrations of EGTA in the presence of AMP-PCP stimulate [^{3}H]-dantrolene binding to HSR. The reasons for this are not clear, but may relate to the effects of high extraluminal EGTA concentrations on intraluminal SR Ca^{2+} concentrations which reportedly regulate RyR activity (*64*, *65*). Indeed, when SR vesicles are preincubated in 10 mM EGTA and resuspended in binding buffer lacking EGTA, specific [^{3}H]dantrolene binding remains enhanced relative to control (K. Paul-Pletzer and J. Parness, unpublished results), suggesting that loss of intraluminal Ca^{2+} enhances dantrolene binding.

The data above demonstrate that cardiac RyR is a poor target for [^{3}H]azidodantrolene photoaffinity labeling. This suggests that this form of the RyR is unlikely to be pharmacologically affected by dantrolene, or that there are subsets of dantrolene-sensitive, cardiac cell types not picked up by the global method of cardiac SR membrane preparation used in these studies.

The photoaffinity labeling results presented herein definitively establish that the skeletal muscle RyR is a molecular target of dantrolene. The following question remains: How does dantrolene inhibit SR Ca^{2+} release? The most parsimonious explanation of the results to date is that at physiological ATP concentrations dantrolene binds to a specific site on the N-terminus of the RyR, modifying its activity, thereby suppressing Ca^{2+} release. The molecular basis of this action of dantrolene awaits mapping of the dantrolene binding site and functional studies of the RyR.

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