

[³H]Azidodantrolene Photoaffinity Labeling, Synthetic Domain Peptides and Monoclonal Antibody Reactivity Identify the Dantrolene Binding Sequence on RyR1

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Running Title: Identification of the dantrolene binding sequence on RyR1

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Summary

Dantrolene is a drug that suppresses intracellular Ca^{2+} release from sarcoplasmic reticulum in normal skeletal muscle and is used as a therapeutic agent in individuals susceptible to malignant hyperthermia. Though its precise mechanism of action has not been elucidated, we have identified the N-terminal region (amino acids 1-1400) of the skeletal muscle isoform of the ryanodine receptor (RyR1), the primary Ca^{2+} release channel in sarcoplasmic reticulum, as a molecular target for dantrolene using the photoaffinity analog [^3H]azidodantrolene(1). Here, we demonstrate that heterologously expressed RyR1 retains its capacity to be specifically labeled with [^3H]azidodantrolene, indicating that muscle specific factors are not required for this ligand-receptor interaction. Synthetic domain peptides of RyR1, previously shown to affect RyR1 function *in vitro* and *in vivo*, were exploited as potential drug binding site mimics and used in photoaffinity labeling experiments. Only DP1 and DP1-2, peptides containing the amino acid sequence corresponding to RyR1 residues 590-609, were specifically labeled by [^3H]azidodantrolene. A monoclonal anti-RyR1 antibody which recognizes RyR1 and its 1400 amino acid N-terminal fragment, recognizes DP1 and DP1-2 in both Western blots and immunoprecipitation assays, and specifically inhibits [^3H]azidodantrolene photolabeling of RyR1 and its N-terminal fragment in sarcoplasmic reticulum. Our results indicate that synthetic domain peptides can mimic a native, ligand binding conformation *in vitro*, and that the dantrolene binding site and the epitope for the monoclonal antibody on RyR1 are equivalent and composed of amino-acids 590-609.

Introduction

Dantrolene is a hydantoin derivative used to treat malignant hyperthermia (MH), a rare, pharmacogenetic disorder of skeletal muscle characterized by uncontrolled Ca^{2+} release from sarcoplasmic reticulum (SR) stores in response to volatile anesthetics. Triggering of MH results in hypercontracture, hyperthermia and eventually death. Dantrolene's therapeutics results from its effective suppression of skeletal muscle SR Ca^{2+} release, presumably by modulating the activity of the ryanodine receptor (RyR1), the primary Ca^{2+} release channel in skeletal muscle *via* which Ca^{2+} stored in the SR is released into the myoplasm to initiate muscle contraction in response to membrane depolarization (2). The genetic defect that causes MH in humans has been linked to point mutations in RyR1 in about 50% of patients. To date, some 26 MH-linked mutations in RyR1 have been identified: 9 in the extreme N-terminal region, 16 in the central region, and 1 in the extreme C-terminal region (3-8). Interestingly, a single amino acid deletion, rather than mutation, in the central region of RyR1 (Glu2347) has also been found to be associated with MH susceptibility (9).

Whether dantrolene suppression of Ca^{2+} release occurs *via* direct interaction with RyR1 is not entirely clear. Some have found evidence suggesting that RyR1 is not the target (10;11) and others have found evidence that it is (2;12;13). Given the controversy as to the mechanism and, hence, the targets of dantrolene action, we have embarked on a project to directly identify the molecular target(s) of dantrolene by photoaffinity labeling. Knowing the target(s) of dantrolene binding would allow genetic and physiological

manipulation of these molecular entities to not only elucidate the mechanism of action of this drug, but provide insights into the *in vivo* mechanisms controlling RYR1 channel Ca^{2+} release in skeletal muscle excitation-contraction coupling (ECC) and the pathophysiology of MH.

Recently, we have demonstrated that [^3H]azidodantrolene, a pharmacologically active, photoaffinity analog of dantrolene, specifically labels the N-terminal, 1400 amino acid residue fragment of RyR1 cleaved by n-calpain, a tissue specific isoform of this Ca^{2+} - and thiol-activated protease (1). Several studies have demonstrated that this portion of the RyR plays a significant role in the regulation of channel function (14-18). Additionally, the nine, N-terminal mutations in RyR1 linked to MH alluded to above are localized within this region of the channel.

In the present study, we identify the amino acid sequence of the dantrolene binding site on RyR1 using specific [^3H]azidodantrolene photolabeling of synthetic domain peptides from RyR1 and the II-III loop of the α -1 subunit of the skeletal muscle dihydropyridine receptor (DHPR). Specific photolabeling is demonstrated only with those peptides containing the sequence corresponding to amino-acid residues 590-609 on RyR1. Moreover, a monoclonal anti-RyR1 antibody raised against rabbit terminal cisternae (19) that recognizes both the intact rabbit RyR1 and the 172 kDa, n-calpain-cleaved, N-terminal fragment of this channel (1), also recognizes synthetic RyR1 peptides. This antibody specifically inhibited [^3H]azidodantrolene photolabeling of RyR1 in SR in a

concentration dependent manner. These results indicate, therefore, that the dantrolene binding site on RyR1 is comprised of amino acids 590-509.

Experimental Procedures

Materials – Dantrolene sodium $\cdot$ 3.5H₂O, and azumolene sodium $\cdot$ 2H₂O, were generous gifts of Proctor & Gamble, Norwich, NY. Polyclonal sheep anti-rabbit RyR1, monoclonal mouse (IgM) anti-rabbit RyR1 XA7 (mAb) and polyclonal rabbit anti-rabbit RyR1 C-terminal (raised against a synthetic C-terminal RyR1 peptide corresponding to amino-acids 5023-5037) antibodies were generous gifts of Dr. K. P. Campbell (University of Iowa, Iowa City, IA). Rabbit fast-twitch skeletal muscle was supplied by Dr. H. Weiss (UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ).

[³H]Azidodantrolene Synthesis – [³H]Azidodantrolene was synthesized, purified and characterized exactly as described (20), and specific activity determined to be 28 Ci/mmol.

Peptide Synthesis – Rabbit RyR1 domain peptides (DP1, DP1-2, DP4, DP3, DP7), DP1 scrambled peptide SCR1, and rabbit skeletal muscle DHPR α 1 subunit peptides (PepA and PepC) were synthesized on an Applied Biosystems model 431 A synthesizer and purified by reversed-phase HPLC, as described (21;22). DP1 scrambled peptide SCR4, was synthesized by Biosynthesis Inc., Lewisville, TX. Scrambled DP1 sequences were generated by computer program, and SCR1 and SCR4 sequences picked for subsequent synthesis.

Cloning and Expression of RyR1 in CHO Cells– The entire cDNA of RyR1 was cloned into the eukaryotic expression vector pRRS11 under the control of the SV40 promoter. Plasmid pRRS11 was introduced into CHO cells using lipofectamine-mediated gene transfection method, as described previously (28;29). Stable clones of CHO cells permanently transfected with RyR1 were selected using G418(23;24).

Membrane Preparation – Crude SR vesicles were prepared from rabbit fast twitch skeletal muscle in the presence of protease inhibitors (200 μ M PMSF, 0.3 μ M aprotinin, 10 μ g/ml soybean trypsin inhibitor, and 2.8 μ M pepstatin A) as described by (25). Microsomal membranes from CHO cells were obtained by ultracentrifugation of cell lysates prepared after sonication of cultured cells, as described (23). Leupeptin was specifically omitted from buffers used in membrane preparation to specifically allow for n-calpain cleavage of RyR1 and subsequent identification of its 172 kDa, N-terminal fragment, if present (1;26).

Photolabeling with [3 H]Azidodantrolene – SR vesicles (100 :g) and CHO microsomal membranes (200 :g) were photolabeled with [3 H]azidodantrolene (100-200 nM) in binding buffer (20 mM PIPES, pH 7, containing 0.5 mM AMP-PCP) in the absence (T, total binding) or presence (N, nonspecific binding) of azumolene (150-300 :M), as described previously (1). For inhibition of photolabeling by mAb anti-RyR1, mouse ascites were added to the binding buffer

at a dilution of 1:50, 1:25 and 1:10 in the presence of protease inhibitors (see *Membrane Preparation* above, with the addition of 1 mM leupeptin). Synthetic peptides (12.5 :M) were photolabeled with 50-100 nM [³H]azidodantrolene in binding buffer containing 10 µg of BSA in the absence of AMP-PCP. Following photolabeling, samples were resolved by SDS-PAGE (5% acrylamide for SR samples, and 20% for synthetic peptides) (27).

Autoradiography and Western Blot Analysis – For autoradiography and Western blot analyses, the SDS-PAGE resolved proteins were electroblotted onto PVDF membranes (Sequi-Blot, Bio-Rad) (28). Autoradiography was performed as described (1). For Western blots, the membranes were probed with primary antibody at appropriate dilution/concentration (polyclonal anti-RyR1, 1:5000; mAb, 1:1000), followed by alkaline phosphatase conjugated secondary antibody. Immunoreactive bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate/*p*-nitro blue tetrazolium using standard procedures.

Quantification of Radiolabeled Bands – The autoradiograph was scanned using Photoshop 6.0 (Adobe) software and the image was saved as a .tif file. The saved image was analyzed using ImageQuant for Windows NT™ software (Molecular Dynamics, Amersham Biosciences), and the radiolabeled bands quantified. The arbitrary values were normalized after subtracting for background and non-specific binding, and expressed as a percentage of control values.

Results

[³H]Azidodantrolene photolabels expressed RyR1 in CHO cells – Of the 5037 amino acids that constitute the RyR1 sequence, we have shown that a dantrolene binding site resides between amino-acids 1-1400 of the N-terminal region of the channel (1). In an attempt to devise a molecular biological approach to define the amino acids that comprise the dantrolene binding site, we first asked whether RyR1 is capable of specifically interacting with dantrolene in the absence of a muscle cell background. We reasoned that if it did, a) this would corroborate our finding that the RyR1 is a pharmacological target of dantrolene, and b) we might reasonably attempt to express a region of the N-terminal portion of the channel which would retain its ability to bind dantrolene and assist us in localization of the drug binding site.

We used recombinant RyR1, stably expressed in CHO cells, as a target for our photoaffinity labeling experiments. Microsomal membranes prepared from these cells, along with rabbit skeletal muscle SR vesicles as positive control, were photolabeled with [³H]azidodantrolene in the presence or absence of AMP-PCP and excess azumolene, as described in “Experimental Procedures”. As shown in Fig. 1A, specific photolabeling of a 565 kDa protein corresponding to the RyR1 monomer in SR was observed in CHO microsomal membranes expressing RyR1. This photolabeling was dependent on the presence of AMP-PCP. This is identical to the requirements for photolabeling of RyR1 and its 172 kDa fragment in SR reported previously (1). The Western blot (Fig 1B) of the same membrane used for autoradiography, probed with a polyclonal anti-RyR1

antibody, demonstrates equivalent protein loading in all the experimental lanes and confirms that the radiolabeled band in CHO cell extracts is indeed RyR1. No specific photolabeling or anti-RyR1 immunoreactive bands were observed in membranes prepared from untransfected CHO cells. These results demonstrate (a) that dantrolene can bind to heterologously expressed RyR1 in the absence of other muscle-specific proteins, and (b) that this binding is pharmacologically specific and dependent on adenine nucleotide triphosphate, a known regulator of the channel protein.

Specific [3 H]azidodantrolene photolabeling of synthetic domain peptides of RyR1

– In order to determine the amino acids on RyR1 that constitute the binding site for dantrolene, we attempted to specifically photolabel a heterologously expressed, GFP-tagged, RyR1-N-terminal fragment containing amino acids 182-1608. This fragment did not label, presumably due to incorrect folding or processing (data not shown). We then turned to synthetic domain peptides of RyR1 as possible surrogate targets for the dantrolene binding site. We synthesized four peptides with amino-acid residues from within the N-terminal, 1400 amino-acid region of RyR1 (DP1, DP1-2, DP3, DP7), as well as peptides from the central region of RyR1 (DP4) and from the II-III loop of the DHPR- α 1 subunit (PepA and PepC)(Fig. 2). These peptides were photoreacted with [3 H]azidodantrolene in the presence or absence of excess azumolene, and the peptides analyzed for specific photolabeling by autoradiography. The

autoradiograph in Fig. 3A demonstrates specific photolabeling of DP1 (amino-acid residues 590-609) and an elongated version of DP1, DP1-2 (amino-acid residues 590-628). DP7 (amino-acid residues 543-576) was non-specifically labeled, while the other peptides were not labeled at all. The pharmacological specificity of [³H]azidodantrolene labeling of DP1 was demonstrated by the ability of dantrolene and azumolene, but not the unrelated drug, atropine, to inhibit this interaction (Fig. 3B). In addition, scrambling of the DP1 sequence (Fig. 3D, SCR1 and SCR4) led only to non-specific labeling of varying intensity (Fig. 3C). These results suggest that DP1 might represent an *in vitro* model of the dantrolene-binding site on RyR1.

A monoclonal anti-RyR1 antibody recognizes DP1 – Our previous work has shown that a monoclonal anti-RyR1 antibody (mAb) immunoprecipitates [³H]azidodantrolene-labeled RyR1 and its 172 kDa fragment from solubilized SR (1). Given the epitope specificity of monoclonal antibodies and that the epitope of this monoclonal seemed to be on the 172 kDa protein, we reasoned that it was possible that one of our domain peptides corresponding to sequences from the N-terminal region of RyR1 might interact with this antibody. If it did, it would demonstrate the feasibility of using synthetic peptides to mimic a protein epitope in this channel.

First, to confirm our earlier results described above, we demonstrated that only intact RyR1 and its 172 kDa N-terminal fragment were reactive with the monoclonal antibody by photolabeling duplicate SR samples with

[³H]azidodantrolene, electroblotting these onto PVDF membranes after SDS-PAGE, and probing one sample with mAb anti-RyR1 and the other with polyclonal anti-RyR1 C-terminal antibody. Fig. 4A clearly demonstrates that the mAb recognizes [³H]azidodantrolene labeled RyR1 monomer and its 172 kDa fragment but not the C-terminal, 410 kDa portion of the channel. The anti-RyR1 C-terminal antibody, on the other hand, recognizes the [³H]azidodantrolene labeled RyR1 monomer and its 410 kDa, C-terminal fragment but not its 172 kDa N-terminal fragment (Fig. 4B). This indicated to us that the epitope of mAb, hitherto unknown, must lie within the first 1400 residues of the N-terminal region of RyR1. To determine if any of our RyR1 domain peptides could be recognized by this antibody, we resolved the peptides on a 20 % SDS-PAGE gel, transferred them onto PVDF membrane and probed the latter with mAb anti-RyR1. Only DP1 and DP1-2, both of which share the same corresponding core of amino-acid residues (590-609) of RyR1, were recognized by the mAb (Fig. 5A). Scrambled DP1 peptides, SCR1 and SCR4, were not recognized by the antibody (Fig. 5B). These results indicate that the epitope for the mAb is contained within the DP1 sequence.

Monoclonal antibody inhibition of [³H]azidodantrolene photolabeling of RyR1 –

Significantly, the results presented above demonstrate a parallelism between mAb recognition and specific [³H]azidodantrolene photolabeling of a sequence in the N-terminal of RyR1. The intersection of these two sets of data predicts that mAb anti-RyR1 should inhibit [³H]azidodantrolene labeling of RyR1 in SR. To test

this hypothesis, we photolabeled SR in the absence or presence of increasing concentrations of mAb-containing mouse ascites. The autoradiograph (Fig. 6A) reveals a concentration dependent inhibition of specific photolabeling of both the RyR1 monomer, as well as its 172 kDa fragment, this despite equivalent anti-RyR1 immunoreactive protein loading in each lane (Fig. 6B). Quantitation of the inhibition of photolabeling revealed near linear inhibition over the mAb concentration range tested, particularly for the 172 kDa fragment (Fig. 6C). The linearity of inhibition of photolabeling of intact RyR1 by mAb anti-RyR1 is less evident in this autoradiograph due to its high concentration and degree of photolabeling relative to the N-terminal fragment. Optimizing exposure for the inhibition of photolabeling of the 172 kDa results in near saturation of the sensitivity of the X-ray film for the labeling of the 565 kDa RyR1 monomer (data not shown). As a result, the two inhibition curves are not entirely parallel. This does not negate the phenomenon of specific inhibition of [³H]azidodantrolene photolabeling of both forms of the channel protein - n-calpain-cleaved and uncleaved. Moreover, inhibition of labeling is not due to non-specific effects of IgM itself, as purified mouse IgM at the highest concentration used in mouse ascites did not affect photolabeling of either the intact channel, or its 172 kDa fragment (Fig. 6A).

Discussion

It is our hypothesis that understanding the molecular mechanism of action of dantrolene will lead to a better understanding of the physiology of MH, the mechanism of excitation-contraction coupling, and the regulation of intracellular Ca^{2+} release from SR. A prerequisite for elucidating molecular mechanism requires the identification of molecular target(s). The biochemical identification of amino acids involved in drug binding allows for the design of mutational experiments to test their functional significance in drug action. Here, we have amassed evidence that the amino acid sequence of the dantrolene binding site on RyR1 corresponds to amino acids 590-609, corresponding to the synthetic domain peptide DP1.

The most compelling evidence for the DP1 sequence being the dantrolene binding sequence in RyR1 comes from our experiments with the monoclonal antibody, mAb anti-RyR1. In 1987, Campbell and colleagues produced a monoclonal (IgM) anti-RyR1 antibody (mAb), clone XA7, of uncharacterized epitope, that specifically immunoprecipitates [^3H]ryanodine-bound RyR1 (19). Our present studies demonstrate that this antibody recognizes its epitope not only on the RyR1 monomer and its 172 kDa, N-terminal, calpain cleavage fragment, but also on the synthetic peptides, DP1 and DP1-2s, containing the core RyR1 sequence 590-609. Most significantly, this antibody specifically inhibits [^3H]azidodantrolene photolabeling of RyR1 and its 172 kDa fragment in SR in a concentration dependent manner. This result is consistent with our earlier experiments demonstrating the absence of a dantrolene binding site on RyR1 C-

terminal to the n-calpain cleavage site between amino acids 1400-1401 (1). The fact that we were able to demonstrate virtually complete inhibition of [³H]azidodantrolene photolabeling of the 172 kDa fragment with a monoclonal antibody is a strong argument that the epitope defined by this antibody, amino acids 590-609, represents the only dantrolene binding site on RyR1. The relatively high concentrations (<1:50 dilution) of mouse ascites required to bring about inhibition of specific photolabeling is likely due to the low antigen affinities of IgM antibodies, which are part of the body's early immune response, relative to mature IgG antibodies (29). These data also strengthen the experimental evidence that, *in vitro*, synthetic domain peptides are capable of assuming the native conformation(s) of domains present in the parent protein - RyR1 in this case (30-32).

Previous studies have already shown that heterologously expressed RyR1 in CHO cells forms functional channels (33), and allowed us to address the question of whether dantrolene binding to RyR1 requires the presence of muscle specific factors in heterologously expressed channels. Here, we have demonstrated that RyR1 can interact directly with dantrolene in the absence of a muscle-specific milieu. That this interaction is modulated by AMP-PCP, as it is in SR (1), substantiates our assertion that this drug-channel interaction is pharmacologically relevant. It further indicates that this nucleotide analog affects RyR1 directly in enhancing the ability of this channel to bind dantrolene. Specific photolabeling of the synthetic peptide, DP1, on the other hand, should be independent of all specific regulators of RyR1 activity, since none of the putative

nucleotide or protein regulatory sites on RyR1 would be expected to be associated with this 20 amino acid peptide. Indeed, our data demonstrate that [³H]azidodantrolene photolabeling of the two DP1 sequence-containing peptides is independent of AMP-PCP (data not shown). Though we have presented strong evidence that dantrolene binding site on RyR1 is modulated by this nucleotide analog, it is not yet clear how ATP might modulate the activity of this drug *in vivo*.

A recent, detailed examination of the possible physiological site(s) of dantrolene action in skeletal muscle has shown that dantrolene suppresses, but never completely eliminates intracellular Ca²⁺ release in whole and skinned muscle fibers, or from SR vesicles, but has no effect on purified RyR1 incorporated into lipid bilayers (11). These results led those authors to conclude that RyR1 is not likely the site of action of dantrolene. This result is in stark contrast to our results above and those of Nelson and colleagues (12). Possible unifying explanations of these disparate results are likely to be methodologic and could include the following: 1) the dantrolene binding site on RyR1 is uniquely sensitive to modification during the purification process rendering it dantrolene insensitive under certain, as yet undefined, conditions, or 2) the physiological effects of dantrolene binding to RyR1 requires the interaction of other RyR1 interacting factors which might be removed during purification or whose interactions are not modeled well in the artificial lipid bilayer.

Comparing the DP1 sequence in RyR1 with the other rabbit isoforms reveals that there is an identical sequence in RyR2 (601-619) and a nearly identical one in RyR3 (577-597), with a conservative amino acid substitution (Val

to Leu) at position 596 (34). If this sequence is the dantrolene binding site, all three should be sensitive to this drug. Yet, existing evidence indicates that only RyR1 and RyR3 isoforms are sensitive to dantrolene. We have shown by [³H]azidodantrolene photolabeling of SR vesicles prepared from skeletal or cardiac muscle that only RyR1 is a good target for dantrolene, not RyR2 (1). Similarly, other laboratories have shown that dantrolene can inhibit [³H]ryanodine binding to RyR1 and RyR3, but not RyR2 (2;35). If the DP1 sequence is the dantrolene binding-site, why is the RyR2 isoform insensitive to this drug when it contains the identical sequence? The most likely explanation at our present state of knowledge is that the domain-domain interactions within RyR2 constrain the conformation around this sequence so that dantrolene is no longer able to bind with high affinity to this site. Other possible explanations include RyR2 associated proteins, or post-translational modifications of the channel that block access to the site. The underlying reasons for the differences in dantrolene sensitivity of RyR2 relative to RyR1 are under active investigation in our laboratory.

As noted above, the DP1 sequence of RyR1 is nearly identical in RyR3. Whether this isoform is responsive to dantrolene in the organs in which this isoform is expressed remains to be clarified. Early studies indicated that dantrolene has no effect on smooth muscle contractility (36), though more recent studies contradict that (37). Indeed, the latter studies are strongly supported by the demonstration that heterologously expressed RyR3 has been shown to be responsive to dantrolene (35). If RyR3 is indeed a target of dantrolene, then this

last result might explain the various effects of dantrolene seen in non-muscle tissues which express RyR3, i.e., neuronal and immune cells (38;39).

Recently, Ikemoto and co-workers have demonstrated that DP1 added to SR vesicles activates RyR1, as measured by enhanced [^3H]ryanodine binding (21;22). These authors have postulated that DP1 may be assuming the native conformation of the identical sequence in the channel (domain x), thereby competing with this domain for its interacting or “mating” domain (domain y) on RyR1, resulting in “unzipping” of the native RyR1 domain x-domain y interactions (40-42). If, under normal resting conditions, domain x-domain y interactions would keep the channel in a closed state, unzipping these interactions should result in activation of the channel. It follows, then that the insinuation of DP1 into the normal domain x position, “mating” or “zipping” to domain y of RyR1, would result in loss of the conformational constraints imparted by these “zipped” domains, and lead to channel opening. If the above is true, then the most parsimonious explanation of the results to date is that dantrolene inhibits RyR1-mediated Ca^{2+} release by *stabilizing* the interdomain x-y interactions. Moreover, dantrolene stabilization of these interdomain interactions may even result in stabilization of other protein domain-domain interactions, either within RyR1 or with another interacting protein(s) or both, thus reducing the likelihood of channel openings and inhibiting Ca^{2+} release. Elucidation of the identity of the putative interacting domain(s) and modeling of the dantrolene binding site in the three channel isoforms would greatly contribute to our understanding of the regulation of RyR channel function.

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Abbreviations

The abbreviations used are: MH, malignant hyperthermia; SR, sarcoplasmic reticulum; RyR, ryanodine receptor; ECC, excitation-contraction coupling; DHPR, dihydropyridine receptor; mAb, monoclonal antibody; DP, domain peptide; SCR, scrambled peptide; HPLC, high performance liquid chromatography; CHO, Chinese Hamster Ovary; PMSF, phenyl-methanesulfonyl fluoride; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); AMP-PCP, β,γ -methylenadenosine 5'-triphosphate; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinyl difluoride

Figure Legends

FIG. 1. Specific [³H]azidodantrolene photolabeling of recombinant RyR1.

SR vesicles (100 µg protein) and CHO microsomes (200 µg protein) expressing full-length rabbit RyR1 were photolabeled with [³H]azidodantrolene in the absence (T) or presence (N) of azumolene and in the absence or presence of AMP-PCP. Following photolabeling, proteins were fractionated by SDS-PAGE, electroblotted onto PVDF membrane and subjected to autoradiography. (A) Specific photolabeling of the 565 kDa RyR1 monomer was obtained in SR and in CHO cells expressing full-length RyR1, only in the presence of AMP-PCP. No specific photolabeling was obtained in mock-transfected control cells (*ctrl*). The N-terminal, 172 kDa proteolytic fragment of RyR1 was photolabeled only in SR. The Western blot (B) of the same membrane used for autoradiography, probed with polyclonal anti-RyR1 antibody, reveals the presence of RyR1 proteins in all samples except in control samples. Note the lack of immunoreactive 172 kDa protein in the RyR1 transfected CHO cells.

FIG. 2. Sequences of synthetic RyR1 and α 1-DHPR subunit domain peptides.

Various domain peptides of RyR1 and the skeletal muscle, α 1 subunit of the DHPR were synthesized to test in our in vitro photoaffinity labeling and antibody immunoreactivity assays: DP3, DP1, DP1-2 and DP7 from the N-terminal region of RyR1, DP4, from its central portion, and PepA and PepC, from the II-III loop region of the α 1 subunit of skeletal muscle DHPR.

FIG. 3. [³H]Azidodantrolene photolabeling of synthetic peptides.

(A) Synthetic peptides were photolabeled with [³H]azidodantrolene in the absence (T) or presence of azumolene (N), resolved on 20 % SDS-PAGE, electroblotted onto PVDF membrane and subjected to autoradiography. (B) Pharmacological specificity of photolabeling of DP1 was determined in the absence (T) or presence of 150 μM azumolene (Az), 25 μM dantrolene (Dn), 150 μM atropine (At), an unrelated drug as negative control. (C) Photolabeling of DP1 and scrambled peptides of DP1, SCR1 and SCR4. (D) Sequence comparison of DP1 and scrambled peptides.

FIG. 4. Autoradiograph and Western blot of SR proteins probed with anti-RyR1 antibodies.

100 :g of [³H]azidodantrolene labeled, skeletal SR proteins, were resolved by SDS-PAGE and electroblotted onto PVDF membrane. (A) Autoradiograph (left panel) and Western blot (right panel) probed with mAb anti-RyR1 (clone XA7). (B) Autoradiograph (left panel) and Western blot (right panel) probed with polyclonal anti-RyR1 C-terminal antibody. Immunoreactive bands and corresponding relative molecular masses (kDa) are indicated by arrows. Note that immunoreactivity of the 172 kDa RyR1 N-terminal fragment occurs only with mAb anti-RyR1, while immunoreactivity of the 410 kDa RyR C-terminal fragment is reactive only to the polyclonal anti-RyR1 C-terminal antibody.

FIG. 5. Immunorecognition of DP1-sequence containing peptides by mAb anti-RyR1. (A) RyR1 domain peptides, DP1, DP1-2, DP7, and DP4, and DHPR II-III loop peptide, pepA, and (B) scrambled DP1 peptides, SCR1 and SCR4, were resolved on a 20 % SDS-PAGE gel and transferred onto PVDF membrane and probed with mAb anti-RyR1. Only the DP1-sequence containing peptides are recognized.

FIG. 6. Inhibition of [³H]azidodantrolene photolabeling of RyR1 by mAb anti-RyR1. (A) SR vesicles were photolabeled in the absence (T) or presence (N) of azumolene and in the absence or presence of increasing dilutions of mAb ascites in binding buffer (1:10; 1:25; 1:50). Photolabeling in the presence of an equivalent concentration of IgM as total protein in the 1:10 diluted mouse ascites, was used as negative control. (B) The blot used for autoradiography was probed with polyclonal anti-RyR1 antibody. (C) Quantification of radiolabeled bands as a function of mAb dilution in (A) using ImageQuant for Windows NT™ software.

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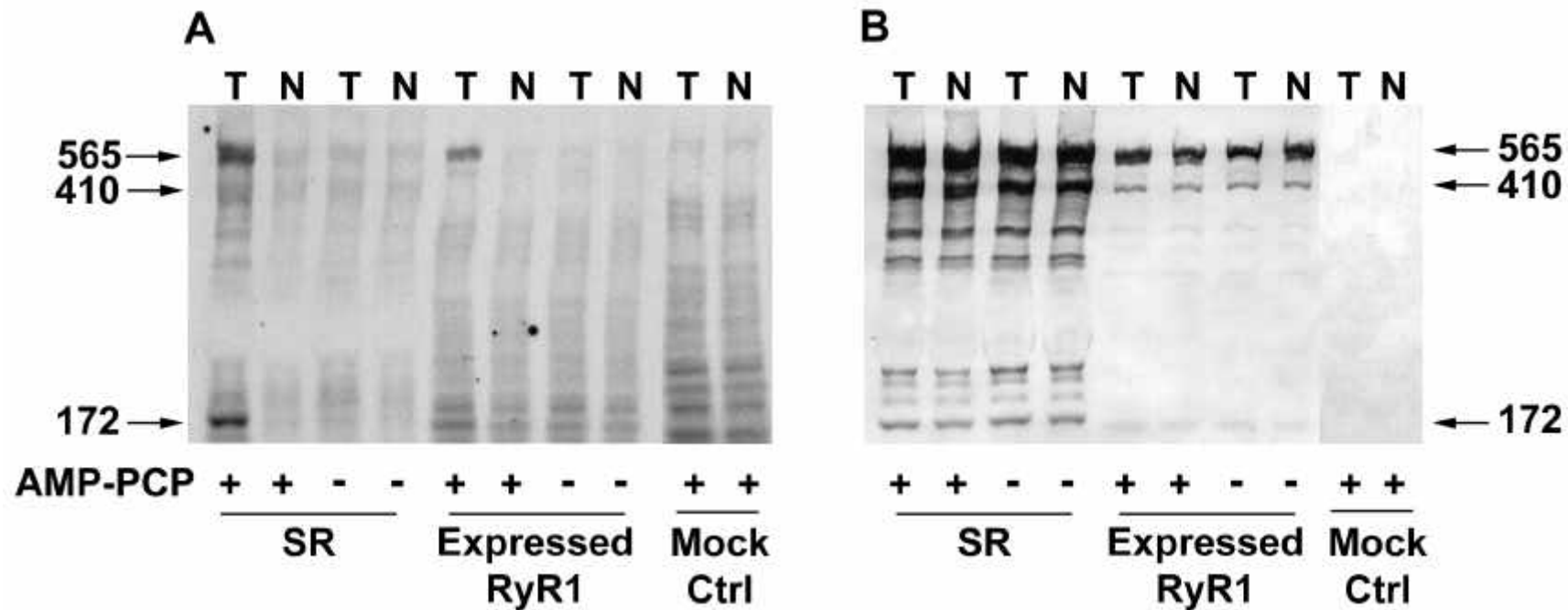


Figure 1

PEPTIDE	AMINO ACID #	SEQUENCE
RyR1		
DP3	324-51	DTAPKRDVEGMDPPEIKYGESLCFVQHV
DP7	543-76	NLDWVVSKLDRLEASSGILEVLYCVLIESPEVLN
DP4	2442-77	LIQAGKGGEALRIRAILRSLVPLDDLVDIISDPQIP
DP1	590-609	LDKHGRNHKVLDVLCSLCVC
DP1-2s	590-628	LDKHGRNHKVLDVLCSLCVCNGVAVRSNQDLITENLLPG
DHPR II-III LOOP		
PeptA	671-690	TSAQKAKAEERKRRKMSRGL
PeptC	724-760	EFESNVNEVKDPYPSADFPGDDEEDEPEIPVSPRPRP

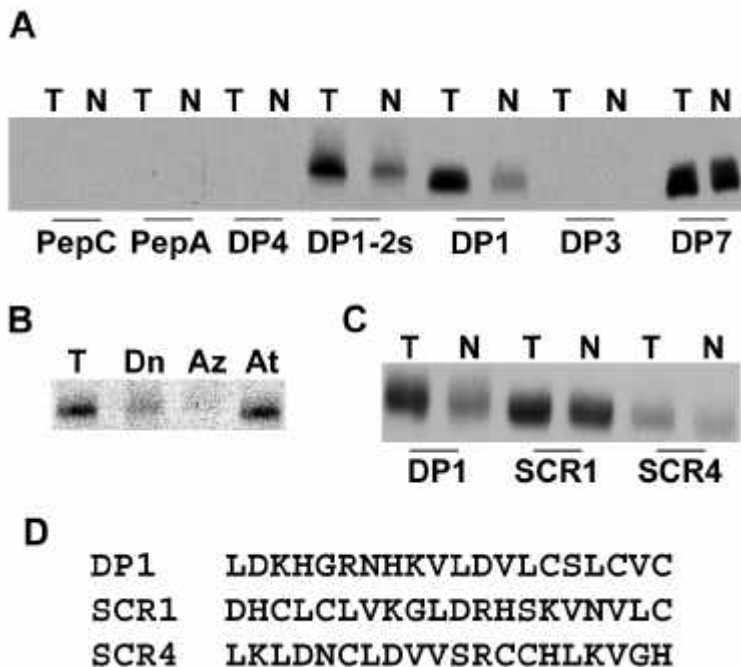


Figure 3

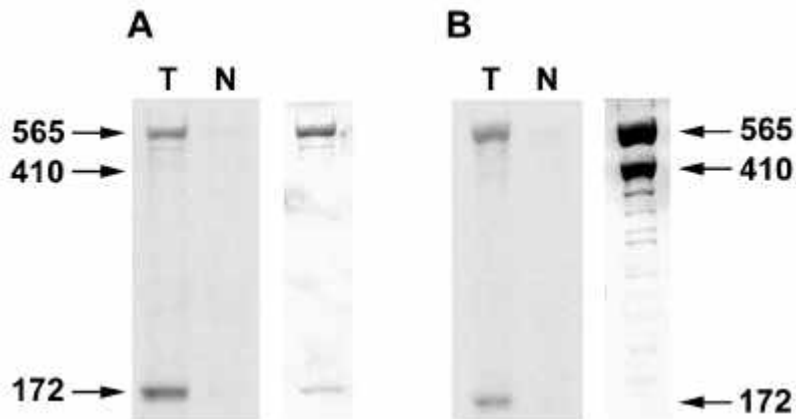


Figure 4

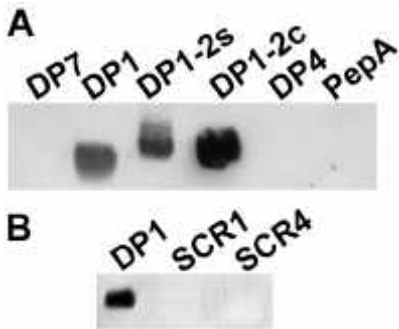


Figure 5

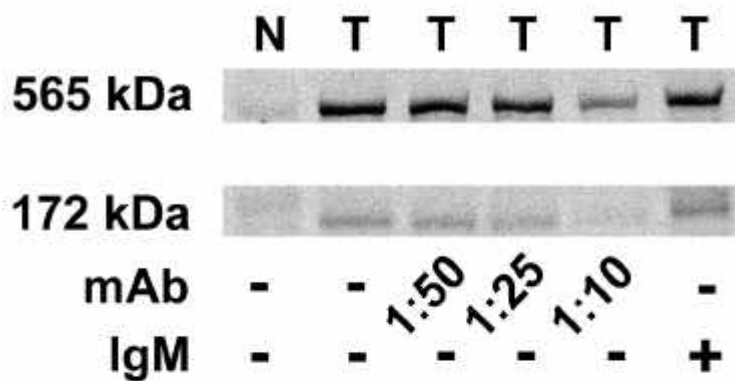
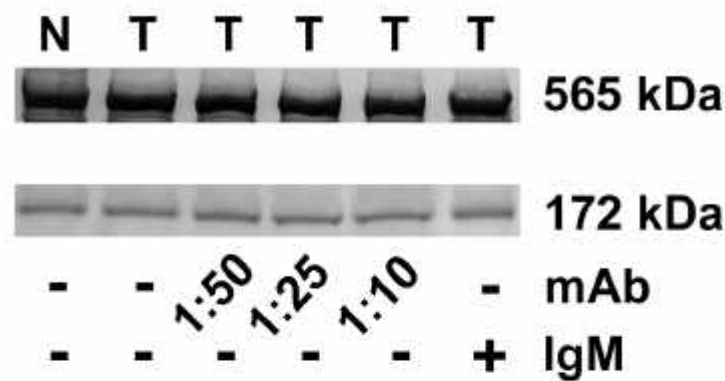
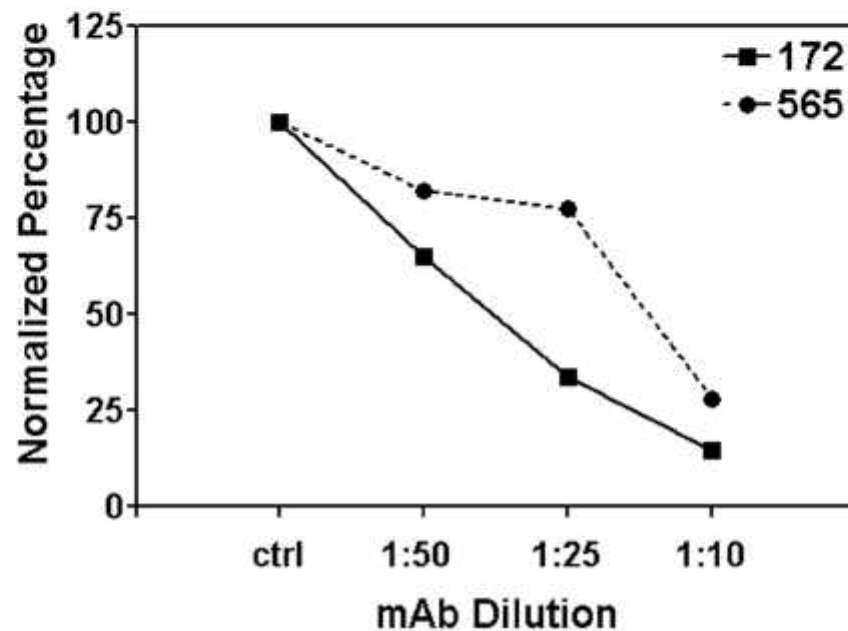
A**B****C**

Figure 6