Lumenal Sites and C Terminus Accessibility of the Skeletal Muscle Calcium Release Channel (Ryanodine Receptor)*

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The membrane topology of the skeletal muscle ryanodine receptor (RyR1) was investigated using site-directed antibodies directed against amino acid sequences 2804–2930, 4581–4640, 4860–4886, and 4941–5037. Ab(2804–2930) bound with identical affinity to either closed or permeabilized sarcoplasmic reticulum vesicles, confirming the cytoplasmic location of this segment. Ab(4581–4640) did not bind to closed vesicles but bound well to permeabilized vesicles, supporting a lumenal location for this segment. Ab(4860–4886) did not bind to closed vesicles but exhibited weak binding to the permeabilized vesicles, suggesting that a portion of the epitope may be exposed on the lumenal surface. The C-terminal antibody (Ab(4941–5037)) bound weakly to closed vesicles, and binding was not significantly enhanced by permeabilizing vesicles with low concentrations of non-denaturing detergent. However, the C-terminal antibodies bound efficiently to vesicles which were transiently incubated at alkaline pH or subjected to trypsinolysis, conditions where few of the vesicles were permeabilized. These results support a model for the membrane topology of the ryanodine receptor as proposed by Takeshima *et al.* (Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T., and Numa, S. (1989) *Nature* 339, 439–445). The results also suggest that the native conformation of the C terminus is inaccessible to antibodies.

The process of excitation-contraction coupling in skeletal muscle involves the rapid release of Ca^{2+} from the sarcoplasmic reticulum (SR).[1] This release is mediated by the ryanodine receptor (RyR), a high conductance Ca^{2+} release channel in the SR membrane (1, 2). The RyR functions as a cation-specific channel which can be modulated by a variety of ligands including Ca^{2+}, Mg^{2+}, calmodulin, and ATP (2, 3). Structurally and functionally related forms of the RyR are also found in cardiac muscle (4–6), smooth muscle (7, 8), and brain (9–11). Together with the related inositol 1,4,5-trisphosphate receptor (IP₃R), the RyR isoforms represent a distinct family of ligand gated ion channels.

The skeletal muscle RyR has been shown to be a high molecular weight homotetramer (12–14). The primary sequence of the rabbit skeletal muscle isoform (RyR1) has been deduced from cDNA cloning (15, 16). Each subunit of the RyR is composed of 5037 (or 5032) amino acids with a predicted molecular mass of ~565-kDa, making it one of the largest catalytic polypeptides yet identified. Recent studies suggest that each high molecular mass subunit may be tightly associated with a 12-kDa subunit identified as FK506 binding protein, an immunophilin (17).

The identification and structural characterization of the membrane spanning segments of the RyR are central to understanding the mechanisms of channel action and regulation. The ion conduction pathway is expected to be formed by the membrane-associated portion of the protein. Studies of the IP₃R have also implicated the membrane-associated domain as an important region for tetramer formation (18, 19). Structural studies and sequence analysis of the RyR have suggested that only a small region of the protein is integral to the SR membrane. It can also be estimated that approximately 15% or less of the total protein mass contributes to the transmembrane assembly, based on the low resolution three-dimensional structure determined from electron microscopy (20, 21). The most hydrophobic regions of the sequence, which may correspond to membrane spanning segments, are clustered in a domain of approximately 1000 residues near the C terminus. The pattern of hydrophobic segments in the C-terminal domain is well conserved among the three mammalian RyR isoforms (22) and across species from mammals to arthropods (23).

The absence of any identifiable signal sequence at the N terminus implies that the large, hydrophilic N-terminal domain is localized in the cytoplasm. This is consistent with the distribution of mass on the cytoplasmic face of the SR observed in electron micrographs (24–26). Several sites in the N-terminal domain have been identified as cytoplasmic. Serine 2843 has been shown to be the site of phosphorylation by several kinases (27). Arginines 426, 1508, 2401, 2840, and 3119 have been identified as sites of trypsin cleavage in closed SR vesicles (28). An antibody recognizing residues 2–15 at the N terminus was also shown to bind to the cytoplasmic face of SR vesicles (29).

The membrane topology for the RyR is not yet well defined. On the basis of hydrophobicity plots, predictions for the number of membrane spanning segments range from four (15) to 12 (16) segments. All of the published models predict an even number of membrane spanning segments. Consequently, the extreme C terminus is predicted to be cytoplasmic. The cytoplasmic localization of the extreme C terminus is supported by antibody binding studies of the RyR (29) and the IP₃R (30). Other sites in the C-terminal domain identified as cytoplasmic include a putative regulatory site in the sequence 4489–4499 identified by antibody binding (31) and tryptic cleavage sites at arginine 4475 and arginine 4756 (28).

[1] The abbreviations used are: SR, sarcoplasmic reticulum; RyR, ryanodine receptor; JFM, junctional face membrane; IP₃R, inositol 1,4,5-trisphosphate receptor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate; ELISA, enzyme-linked immunosorbent assay; [^{125}I]TID, 3-(trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine.

In this study we have used site-directed antibodies to derive evidence for the topological location of several segments of the ryanodine receptor, in particular in the region predicted to be membrane-associated. The differential binding of the antibodies to intact and permeabilized SR vesicles was used to assign cytoplasmic or lumenal localization. We show evidence for two lumenal segments in the C-terminal domain of the RyR and confirm the cytoplasmic localization of the C terminus. In addition, we provide evidence that the C terminus is folded in an inaccessible conformation in the native receptor.

EXPERIMENTAL PROCEDURES

Materials—Vent Polymerase was obtained from New England Bio-labs (Beverly, MA). The protease inhibitor Pefabloc, restriction endonucleases and other DNA modifying enzymes were obtained from Boehringer Mannheim. pATH vectors and *Escherichia coli* strain RR1 were obtained from the American Type Culture Collection (Rockville, MD). Trypsin (Type III) and the catalytic subunit of calpain II (rabbit skeletal muscle) were obtained from Sigma. Anti-TrpE antibody was obtained from Oncogene Science Inc. (Uniondale, NY) and anti-β-galactosidase antibody was obtained from 5 Prime → 3 Prime Inc (Boulder, CO). Anti-calsequestrin (chicken) antibody was a kind gift from Dr. Dennis O. Clegg, University of California at Santa Barbara (32). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG antibodies were obtained from Calbiochem (La Jolla, CA). Immobilon-P was obtained from Millipore Inc. (Bedford, MA).

Preparation of Heavy SR Vesicles, Junctional Face Membranes, and Purification of Ryanodine Receptor—Heavy SR vesicles were prepared from the back and leg muscle of rabbits in the presence of protease inhibitors as described previously (33). Vesicles were rapidly frozen and stored at -80 °C and then thawed immediately prior to use. Junctional face membranes (JFM) were prepared by incubating heavy SR vesicles in 1% Triton X-100, 2 mM $CaCl_2$, pH 7.5, pelleting at 90,000 revolutions/min in a Beckman TL100.3 rotor followed by extraction of calsequestrin in 0.5 M NaCl, 2 mM EDTA, pH 6.9 (25). Purified RyR was prepared from heavy SR vesicles as described previously (12). All preparations were carried out in the presence of 20 μM leupeptin and either 0.1 mM DIFP or 1 mM Pefabloc. Leupeptin was omitted from the final steps of purification in preparations subsequently subjected to calpain digestion.

JFM and SR vesicle protein content was determined by BCA assay (34) and purified RyR protein content by the Amido Black assay (35).

Constructs for Expression of Fusion Proteins—All recombinant DNA manipulations were carried out by standard procedures (36). Fragments of the RyR1 cDNA in pBluescript vectors were prepared by antibody screening of a rabbit skeletal muscle cDNA library or by reverse transcribed-polymerase chain reaction of rabbit skeletal muscle mRNA. Clone CRC6 was provided as a generous gift by Dr. Andrew Marks, Mt. Sinai School of Medicine. The *trpE* fusion construct pATH(2804–2930) was produced by ligation of a *Bgl*II-*Pst*I fragment of clone CRC6, corresponding to RyR1 sequence 8539–8919, to *Pst*I digested pATH1 (37). Constructs pATH(4581–4640), pATH(4860–4886), and pATH(4941–5037) were prepared by polymerase chain reaction amplification of RyR1 cDNA fragments from positions 13872–14052, 14709–14790, and 14951–15245, respectively. The polymerase chain reaction primers for each sequence included additional sequences at the 5' end to generate an *Eco*RI site (forward primers) or *Hind*III site (reverse primers) to facilitate in frame ligation to pATH10 ((4581–4640)) or pATH3 ((4860–4886), (4941–5037)). The construct pATH-(5006–5037) was prepared by digestion of the *Eco*RI-*Hind*III insert from pATH(4941–5037) with *Hae*III. The gel purified 103-base pair product, corresponding to ryr1 sequence 15147–15245, was ligated to *Sma*I-*Hind*III digested vector pWR-590-2 (38). The resulting *lacZ* fusion construct, pWR(5006–5037), was digested with *Eco*RI-*Hind*III and insert was ligated to *Eco*RI-*Hind*III digested pATH1. The sequences and reading frames of all the recombinant plasmids were confirmed by DNA sequencing (39). The *lacZ* fusion construct pWR(2804–2930) was constructed by *Eco*RI-*Hind*III digestion of pATH(2804–2930) followed by ligation of the gel purified fragment into *Eco*RI-*Hind*III digested pWR-590-2. Constructs pWR(4581–4640), pWR(4860–4886), pWR-(4860–4917), and pWR(4941–5037) were similarly constructed by ligation of *Eco*RI-*Hind*III inserts from the corresponding pATH plasmids to pWR-590 (38).

Expression and Purification of TrpE Fusions—The TrpE fusion proteins were prepared by expression in *E. coli* strain RR1 as described (37). Overnight cultures grown in M9 + glucose media containing tryptophan were diluted 10-fold into the same media without trypto-

phan and then further induced after 1 h growth by the addition of 5 μg/ml indoleacrylic acid. Cells were washed in 10 mM Tris-Cl, pH 7.5. Inclusion bodies were prepared from cultures expressing TrpE(2804–2930), TrpE(4581–4640), and TrpE(4941–5037). Cleared lysates were prepared from cultures expressing TrpE' (no insert DNA), TrpE(4860–4886), and TrpE(5006–5037) by sonication on ice in 50 mM Tris-Cl, 5 mM EDTA, 0.1% Nonidet P-40, pH 7.5, followed by centrifugation to remove insoluble material at $300,000 \times g$ for 15 min. All preparations were carried out at 4 °C in the presence of 0.5 mM phenylmethylsulfonyl fluoride. The expression of the fusion proteins was confirmed by 10% SDS-PAGE. The identity of each expressed protein was confirmed by immunoblotting with an anti-TrpE monoclonal antibody.

Gel purification of fusion proteins was carried out by preparative SDS-agarose electrophoresis (ProSieve, FMC BioProducts, Rockland, ME). The band corresponding to the fusion protein was identified by Coomassie Blue staining of a parallel lane of the gel and excised. The excised band was melted at 65 °C in 50 mM Tris-Cl, 1 mM EDTA, 0.1% SDS, pH 8.0, frozen at -70 °C, thawed at 0 °C, and spun at 14,000 revolutions/min to remove the agarose. The supernatant containing fusion protein was concentrated with a Centriprep-10 concentrator (Amicon, Beverly, MA).

β-Galactosidase fusion proteins were prepared by expression from pWR constructs in *E. coli* strain MV1189. Overnight cultures of the appropriate recombinants were diluted 10-fold in LB media and grown for 2 h followed by induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were washed in 10 mM Tris-Cl, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.5 and extracted by boiling in SDS denaturing buffer. The expression of the *lacZ* fusion protein was confirmed by 8% SDS-PAGE analysis and immunoblotting with a polyclonal anti-β-galactosidase antibody.

Synthetic Peptides and Conjugates—Peptides corresponding to the ryr1 sequences (148–162) and (4965–4979) were prepared by solid-phase synthesis by Dr. D. Klapper, University of North Carolina. The peptides were prepared with an additional N-terminal cysteine to facilitate coupling. Peptides were coupled to keyhole limpet hemocyanin by Immuno-Dynamics Inc. (La Jolla, CA).

Preparation of Site-directed Antibodies—A polyclonal antibody against the entire rat skeletal muscle RyR has been previously described (40). Rabbits were immunized by intramuscular injection of antigenic peptides in complete Freund's adjuvant. When the antigens were fusion proteins, 300–800 μg of either gel-purified fusion protein or bacterial inclusion bodies containing approximately 50% of the desired fusion protein were injected. For synthetic peptide antigens, 1 mg of each keyhole limpet hemocyanin-conjugated peptide was injected. Booster injections were made intradermally and subcutaneously with equal amounts of protein in incomplete Freund's adjuvant after 4–6 weeks and then at 10–14 day intervals. Antisera were collected 10–14 days post-injection.

Antisera to fusion proteins were affinity purified by absorption to immobilized fusion protein by the method of Lillie and Brown (41). Bacterial inclusion bodies (TrpE(2804–2930), TrpE(4581–4640), TrpE(4941–5037)) or cleared cell lysates (TrpE(4860–4886), TrpE-(5006–5037)) were separated on 10% SDS-PAGE and transferred to Immobilon-P membranes (42). The position of the fusion protein band was determined by staining a portion of the blot and a strip of the blot at that location was cut out from the blot. The strip was blocked in 5% non-fat dry milk in PBS and a 0.5–1-ml aliquot of antisera applied to the strip for 1–2 h at 25 °C. Nonspecific antibodies were removed by washing in PBS-T (phosphate-buffered saline, 0.05% Tween-20). Bound antibodies were eluted in 0.5–1 ml of 0.1 M glycine, pH 2.7, which was immediately neutralized with 1/10 volume of 1 M Tris-Cl, pH 8.0. Antisera against synthetic peptides were affinity purified using a peptide-agarose column prepared using Sulfo-Link gel from Pierce (Rockford, IL). Approximately 10 mg of each peptide was coupled to a 2-ml column.

Electrophoresis and Western Blotting—SR protein samples (20–50 μg) were denatured in Laemmli sample buffer at 100 °C for 3 min and separated in 3–12% SDS-polyacrylamide gels by the method of Laemmli (43). The following molecular mass standards (kDa) were used: myosin (205,000), β-galactosidase (116), phosphorylase b (97.4), bovine serum albumin (66), ovalbumin (45), and carbonic anhydrase (29). The proteins were either stained by the colloidal Coomassie Blue method (44) or transferred to Immobilon-P membranes at 400 mA at 12 °C for 1–3 h followed by 1 A for 16 h (45). The membranes were blocked overnight in 5% nonfat dry milk, PBS, and then incubated with specific antibodies in 1% milk dissolved in PBS-T. After washing in PBS-T, the bound antibody was detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG and visualized by incubation with 3,3-diaminobenzidine and H_2O_2.
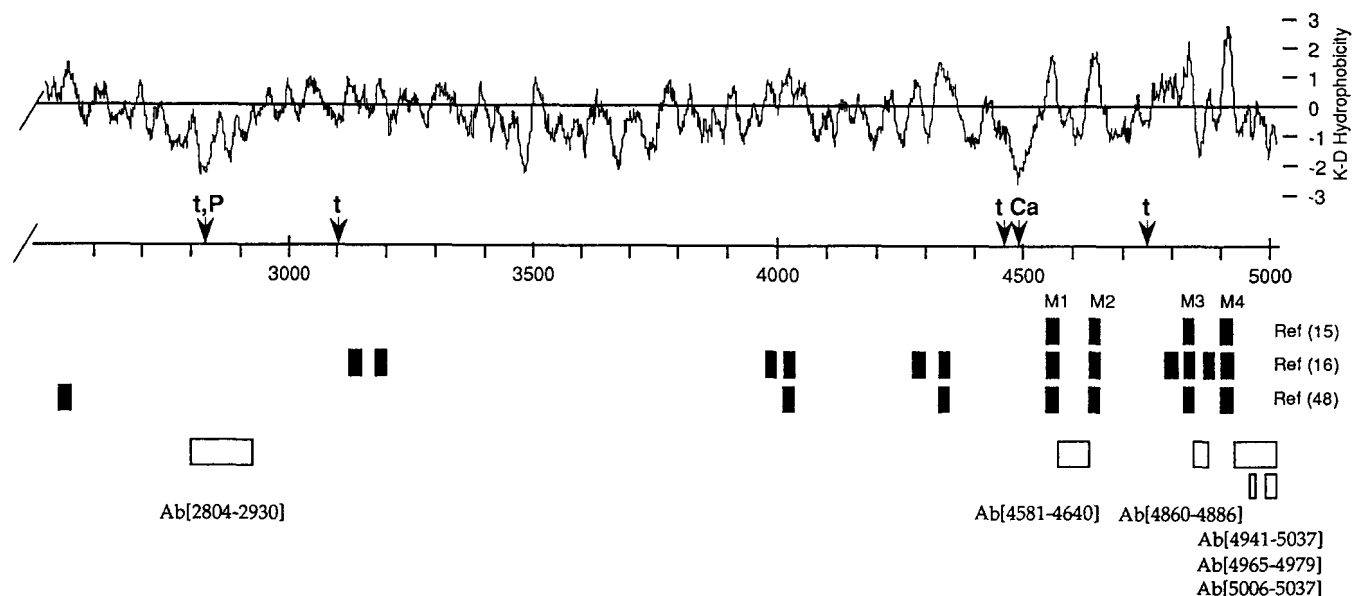
FIG. 1. **Map of putative transmembrane segments and antigenic peptides in the C-terminal region of the skeletal RyR.** *First line* (*top*), hydrophobicity plot of the C-terminal sequence of the RyR from amino acid 2537–5037 (15) calculated using the scale of Kyte and Doolittle (63). *Second line*, scale bar with amino acid sequence. The location of a cytoplasmic phosphorylation site at serine 2843 (*P*) (27), a putative cytoplasmic calcium regulatory site (*Ca*) (31), and four cytoplasmic tryptic cleavage sites (*t*) (28) are indicated. Schematic of the locations of predicted membrane-spanning segments are shown as *closed bars* in the third through fifth lines. *Third line*, segments M1-M4 predicted by Takeshima *et al.* (15). *Fourth line*, 12 segments predicted by Zorzato *et al.* (16). *Fifth line*, seven of the 10 segments predicted by Brandt *et al.* (48). *Sixth line*, the locations of sequences used as antigenic peptides (*open boxes*). The nomenclature of the site-directed antibodies prepared from the peptides is indicated below the corresponding boxes.

Competition ELISA Assay—For detergent permeabilization, SR vesicles were incubated in 125 mM Tris-Cl, 750 mM NaCl, pH 7.5, 10 mM Pefabloc, 100 μM leupeptin, 1 mM CaCl₂, 1 mM EGTA, and either 0.25 or 0.1% (w/v) CHAPS at 23 °C for 0–60 min, as indicated in the figure legends. The vesicles were then diluted 5-fold with deionized water. For transient alkalinization, SR vesicles were incubated for 0–60 min in 25 mM Tris-Cl, 150 mM NaCl, 2 mM Pefabloc, 20 μM leupeptin, 200 μM EGTA, pH 8.5–9.5 at 23 °C. Control SR vesicles were incubated in parallel at pH 7.5. At the end of the alkaline incubation, 200 μM CaCl₂ was added, and the alkaline solution was adjusted to pH 7.5 with HCl. Control vesicles were incubated in parallel in 25 mM Tris-Cl, 150 mM NaCl, 2 mM Pefabloc, 20 μM leupeptin, 200 μM CaCl₂, 200 μM EGTA, pH 7.5, at 23 °C. The treated vesicles were serially diluted with 25 mM Tris-Cl, 150 mM NaCl, pH 7.5, with or without CHAPS at the same concentration in the diluted incubation media. The final concentration of detergent in CHAPS-treated samples was ≤0.06% and the same in each titration at all vesicle concentrations. CHAPS did not interfere with the binding of antibody to the immobilized RyR at the final concentrations used (data not shown).

To the serial dilutions of either control or treated SR vesicles was added an equal volume of antibody solution in 2% dry milk, 25 mM Tris-Cl, 150 mM NaCl, pH 7.5. In some experiments 5% normal goat serum or 50 μg/ml of cleared bacterial lysate from *E. coli* expressing the TrpE' protein was included in the incubation. These additional blocking reagents resulted in a small reduction in the background of the ELISA assay but did not significantly change the results of the binding assays. The antibodies were incubated with vesicles for 12–16 h at 4 °C. Antigen-coated wells were prepared by drying 0.3 μg/well of purified RyR from deionized water in polystyrene microtiter plates (Falcon) followed by fixation with methanol. Alternatively, 5 μg/well of JFM were incubated for 3 h at 23 °C in 50 mM NaCO₃, pH 9. Coated plates were blocked by incubation with 5% dry milk in PBS overnight at 4 °C. The antibodies not bound to vesicles were detected by adding 100 μl of antibody-vesicle mixture to antigen-coated microtiter wells for 2 h at 23 °C. The wells were washed thrice with PBS-T and then the antibodies bound to the wells were quantitated using horseradish peroxidase-conjugated secondary antibody followed by color development with *o*-phenylenediamine. The dilution of each anti-RyR antibody was adjusted such that the free antibody concentration in the absence of added vesicles produced a signal of approximately 0.05–0.10 OD(490 nm), within the linear range for detection with the antigen-coated plate. The fraction of free antibody was calculated as the ratio of OD(490 nm) for antibody incubated with and without SR vesicles.

Calpain II Digestion of Purified RyR—Calpain II digestions were

carried out in 100 mM NaCl, 20 mM sodium PIPES, pH 7.5, 2 mM dithiothreitol, 4 mM CaCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 0.1 μM aprotinin at 23 °C. Calpain II (1 unit/ml) was preactivated by incubation for 10 min at 0 °C in 1 mM Ca²⁺ before addition of purified RyR (100 μg/ml). Reactions were terminated after 0–60 min by the addition of 100 μM leupeptin and excess EGTA. The preparation of rabbit muscle calpain used in this study (Sigma) had a specific activity of 20 units/mg protein. In addition to the 80-kDa catalytic subunit of calpain II, the enzyme preparation exhibited significant contamination by a protein at ~95 kDa.

Trypsin Digestion of Heavy SR Vesicles—SR vesicles were incubated with trypsin at varying SR protein/trypsin ratios in 0.3 M sucrose, 1 mM CaCl₂, 20 mM potassium PIPES, pH 7.0, for 30 min at 23 °C. The digestion was stopped by addition of 1.01 volume of 100 mM Pefabloc, 200 μM aprotinin, 200 μM leupeptin, 15 mM EGTA. The digested vesicles were then pretreated with 0.1% CHAPS or at pH 8.5 for 10 min at 23 °C followed by incubation with antibodies as described above at a final concentration of 300 μg/ml SR protein. The unbound antibody was detected as above. Parallel aliquots of trypsin-digested vesicles were analyzed by SDS-PAGE and immunoblotting.

RESULTS

Preparation and Characterization of Site-directed Antibodies—The analysis of membrane protein topology based on antibody binding relies on the specificity of the antibodies used. For this study, polyclonal antibodies were raised against peptides whose sequences were identical to segments of the deduced amino acid sequence of the rabbit skeletal muscle RyR (15). The antigenic peptides used in this study are shown schematically in Fig. 1. The peptide sequences were selected as potential antigens based on the predicted location of transmembrane segments (16). Each sequence has a high calculated surface probability and antigenic index according to the algorithms of Emini *et al.* (46) and Jameson and Wolf (47), respectively. Each antibody was affinity purified by absorption with the appropriate peptide antigen. Ab(4941–5037) was further purified to higher specificity by absorption to fusion protein TrpE(5006–5037) to give Ab(5006–5037).

The peptide-directed antibodies specifically recognize the RyR as indicated by immunoblot analysis of SR proteins (Fig. 2). The regional specificity of the antibodies was evaluated by
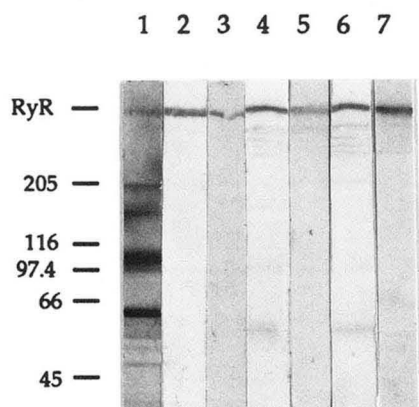
1 2 3 4 5 6 7

FIG. 2. **Immunoblot analysis of site-directed antibodies.** SR vesicles (10 μg/lane) were solubilized in SDS denaturing buffer, separated on 3–12% SDS-PAGE, and stained with Coomassie Blue (*lane 1*) or transferred to Immobilon-P membranes (*lanes 2–7*) as described under "Experimental Procedures". The blots were incubated with the following antibodies: Ab(RyR) (*lane 2*), Ab(2804–2930) (*lane 3*), Ab(4581–4640) (*lane 4*), Ab(4860–4886) (*lane 5*), Ab(4941–5037) (*lane 6*), or Ab(5006–5037) (*lane 7*). The mobility of the RyR and molecular mass standards (kDa) is indicated.

examining their reactivity with other RyR fusion proteins and proteolytic fragments of the RyR. Four antibodies raised against TrpE fusion proteins (Ab(2804–2930), Ab(4860–4886), Ab(4941–5037), and Ab(5006–5037)) and anti-peptide antibody Ab(4965–4979) reacted specifically on immunoblots with β-galactosidase fusion proteins expressing the cognate RyR sequence (data not shown).

Calpain II Digestion Pattern—As the β-galactosidase fusion proteins prepared represent only a limited portion of the RyR1 sequence (307 amino acids), the specificity of the antibodies was also probed by examining the reactivity of Ab(2804–2930), Ab(4581–4640), Ab(48860–4886), Ab(4941–5037), Ab(5006–5037), and anti-peptide antibody Ab(4965–4979) with either calpain II (Fig. 3) or tryptic (see Fig. 8) fragments of the RyR. The calcium-dependent protease calpain II selectively degrades the RyR giving rise to a well-defined pattern of fragments (48–50). Calpain II digestion of the purified RyR produced major fragments of ~400, ~300, ~165, and ~100 kDa which were detected on Coomassie Blue-stained gels (Fig. 3A) or by immunoblotting with a polyclonal antibody against the intact RyR (Fig. 3B). Time course analysis of fragment formation suggests that the ~300- and ~100-kDa fragments are produced by the breakdown of the ~400 kDa (data not shown). The ~165-kDa fragment was recognized specifically by Ab(148–162) (Fig. 3C), consistent with its previous assignment as the N-terminal fragment (48, 50). A secondary fragment of ~150 kDa is derived from the ~165-kDa fragment (Fig. 3C). The ~400-kDa fragment was recognized by Ab(2804–2930) (Fig. 3D), Ab(4581–4640) (Fig. 3E), and Ab(4941–5037) (Fig. 3F), identifying it as the C-terminal fragment. Secondary cleavage of the ~400-kDa fragment produced a ~300-kDa fragment from the N-terminal portion recognized by Ab(2804–2930) (Fig. 3D) and a C-terminal 100-kDa fragment recognized by Ab(4581–4640) and Ab(4941–5037) (Fig. 3, E and F). Anti-peptide antibody Ab(4965–4979) reacted with calpain-digested fragments of membrane-bound RyR identical to Ab(4941–5037) (data not shown). In addition, Ab(4581–4640), Ab(4860–4886), and Ab(5006–5037) reacted with previously identified tryptic fragments (see Fig. 8, E-G). The pattern of recognition of complementary calpain and tryptic fragments by the antibodies is consistent with single-site specificity.

Binding of Antibodies to Intact and Permeabilized SR Vesicles—Heavy SR vesicles containing the RyR have been shown

previously to be predominantly oriented with the extravesicular face corresponding to the cytoplasmic face of the SR (51). Permeation studies have also shown that heavy SR vesicles are well-sealed (29, 52). The binding of an antibody to heavy SR vesicles therefore provides an indication that the epitope recognized by the antibody is localized on the cytoplasmic face of the SR membrane. We tested the ability of antibodies to bind to intact and permeabilized SR vesicles. Intact or permeabilized SR vesicles at varying concentrations were incubated with antibodies and allowed to reach equilibrium. Under the conditions where the antibody concentration is limiting, the binding of the antibody to the RyR should deplete the pool of free antibodies. The depletion of free antibodies was detected by an antibody-capture ELISA assay using microtiter plates coated with denatured RyR.

Antibodies Recognizing Cytoplasmic Epitopes—The antigenic peptide recognized by Ab(2804–2930) spans a region in the N-terminal domain which contains the cytoplasmic phosphorylation site at serine 2843 and a cytoplasmic trypsin cleavage site at arginine 2840 (Fig. 1). When incubated with SR vesicles, Ab(2804–2930) binds equally well to intact vesicles and vesicles permeabilized with the zwitterionic detergent CHAPS (0.25%) (Fig. 4A). Similar results are also found for a monoclonal antibody, RyRD286, which has been epitope mapped to the region 2785–2802.[2] The absence of any additional binding upon permeabilization confirms that the epitopes for these antibodies are localized on the cytoplasmic surface of the protein. The results also confirm that the vesicles are substantially oriented with the cytoplasmic face out. An increase in binding with permeabilization would be expected if there is a significant population of vesicles with inverted orientation.

Antibodies Recognizing Lumenal Epitopes—Two of the antibodies raised against sequences in the C-terminal domain of the receptor, Ab(4581–4640) and Ab(4860–4886), exhibit little or no binding to intact vesicles (Fig. 4, B and C). However, there was a significant increase in the extent of binding when vesicles were permeabilized with 0.25% CHAPS. The extent of binding by Ab(4860–4886) to permeabilized vesicles was variable and exhibited low avidity for the soluble RyR as compared to denatured RyR. Differential binding with permeabilization suggests that the sequences 4581–4640 and 4860–4886 are located on the lumenal face of the SR membrane. To our knowledge, these results are the first reported evidence for the lumenal location of RyR sequences.

Antibodies Recognizing the C Terminus—The C terminus of the RyR, from position 4941–5037, is predicted to be cytoplasmic in the three published topology models. Ab(4941–5037) bound to intact vesicles (Fig. 4D). However, the binding was significantly less than that observed for Ab(2804–2930) and was variable and dependent on the SR vesicle preparation (data not shown). Moreover, Ab(4941–5037) exhibited enhanced binding to the CHAPS permeabilized SR vesicles (Fig. 4D). An increase in binding after CHAPS treatment was observed for all preparations tested. The differential binding of this C-terminal directed antibody was at variance with the results of Marty *et al.* (29) and was further investigated.

Since Ab(4941–5037) was raised against a relatively large peptide (97 amino acids), the differential binding may reflect the polyspecific character of this antibody. Accordingly, a smaller peptide corresponding to the sequence of the last 32 amino acids at the C terminus was used to affinity purify a subset of antibodies (Ab(5006–5037)) from Ab(4941–5037). In addition, we independently prepared Ab(4965–4979) against a 15-amino-acid peptide corresponding to a hydrophilic sequence

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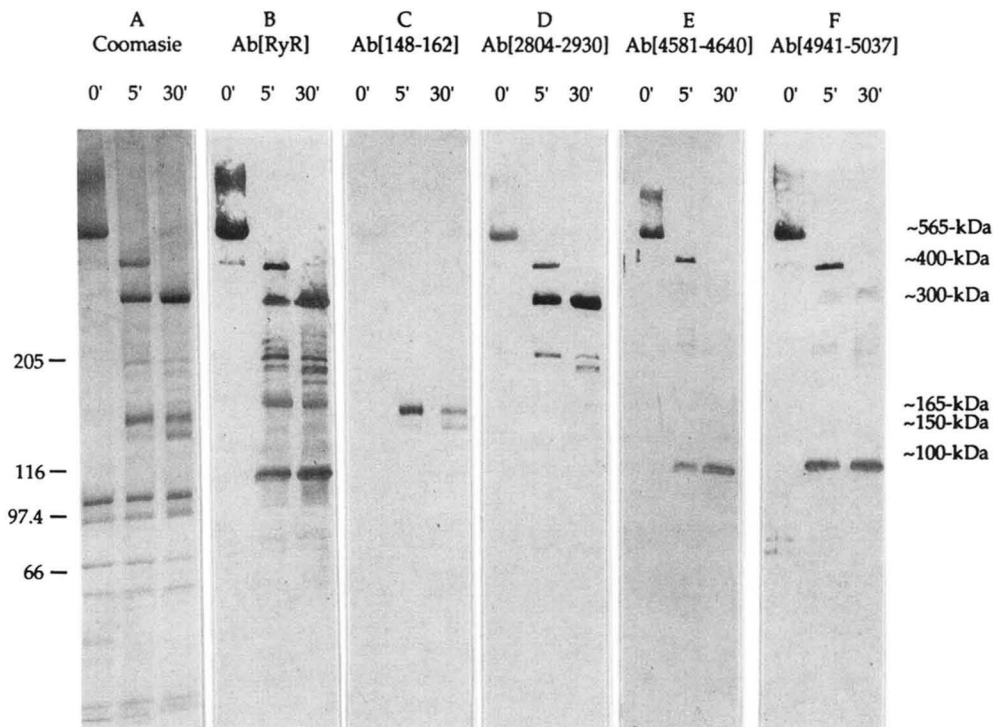[2] L. Gao and G. Meissner, unpublished results.

FIG. 3. **Site-directed antibody recognition of calpain II-digested RyR.** Purified RyR (100 µg/ml) was digested with 1 unit/ml calpain II. The reactions were terminated at the indicated times with the addition of excess EGTA (25 mM) and leupeptin (100 µM). The reactions were denatured in SDS denaturing buffer and separated by 3–12% SDS-PAGE. *A*, Coomassie Blue-stained gel. The mobility of molecular mass standards (kDa) is indicated to the *left*. Some of the RyR migrated as a diffuse band above the position of the RyR monomer. The majority of minor bands below 100-kDa in the unproteolyzed (*0'*) lane arose from protein in the calpain preparation. The RyR preparation also contained residual CaATPase, apparent as a band at ~105-kDa. *B-G*, immunoblot of calpain-digested RyR with Ab(RyR) (*B*), Ab(148–162) (*C*), Ab(2804–2930) (*D*), Ab(4581–4640) (*E*), and Ab(4941–5037) (*F*). The predicted molecular weight of the native RyR (565,000) and the estimated molecular weights of the major digestion products are indicated on the right of *panel F*. Note that the ~100-kDa fragment is obscured in the Coomassie Blue-stained gel shown in *A* by proteins in the calpain II preparation.
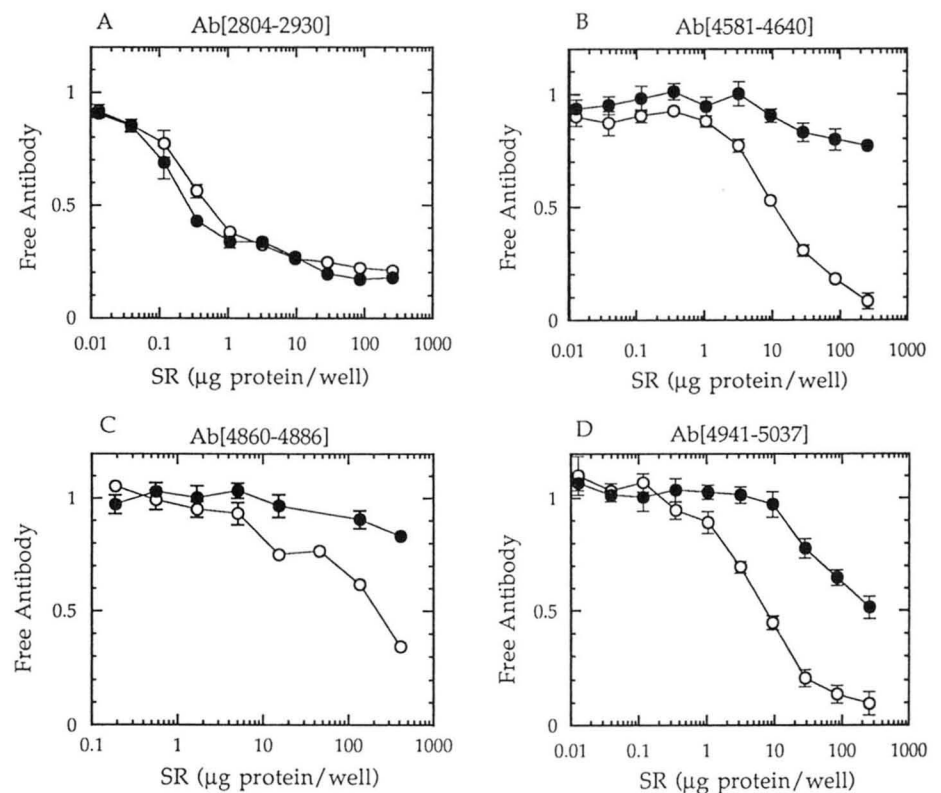

FIG. 4. **Antibody binding to intact and permeabilized SR vesicles.** SR vesicles were incubated with (*open circles*) or without (*closed circles*) 0.25% CHAPS at pH 7.5 for 60 min and then diluted, as described under "Experimental Procedures." The final concentration of CHAPS after dilution was 0.025% at all concentrations of CHAPS-treated vesicles. Diluted vesicles were incubated at 4 °C for 16 h with Ab(2804–2930) (*A*), Ab(4581–4640) (*B*), Ab(4860–4886) (*C*), and Ab(4941–5037) (*D*). The free antibodies were detected by binding to denatured RyR immobilized on microtiter plates, as described under "Experimental Procedures." The free antibodies are equivalent to the unbound antibody detected and plotted as a fraction of the total antibody detected in the absence of SR vesicles. Values are means ± S.E. of two to three experiments performed in triplicate.

in the C-terminal region. The specificity of Ab(4965–4979) was confirmed by a characteristic pattern of reactivity on immunoblots of SR proteins, RyR calpain proteolytic fragments, and bacterial fusion proteins (data not shown). Additionally, we investigated alternative methods for permeabilization of the SR vesicles. Extended exposure to alkaline pH in the absence of
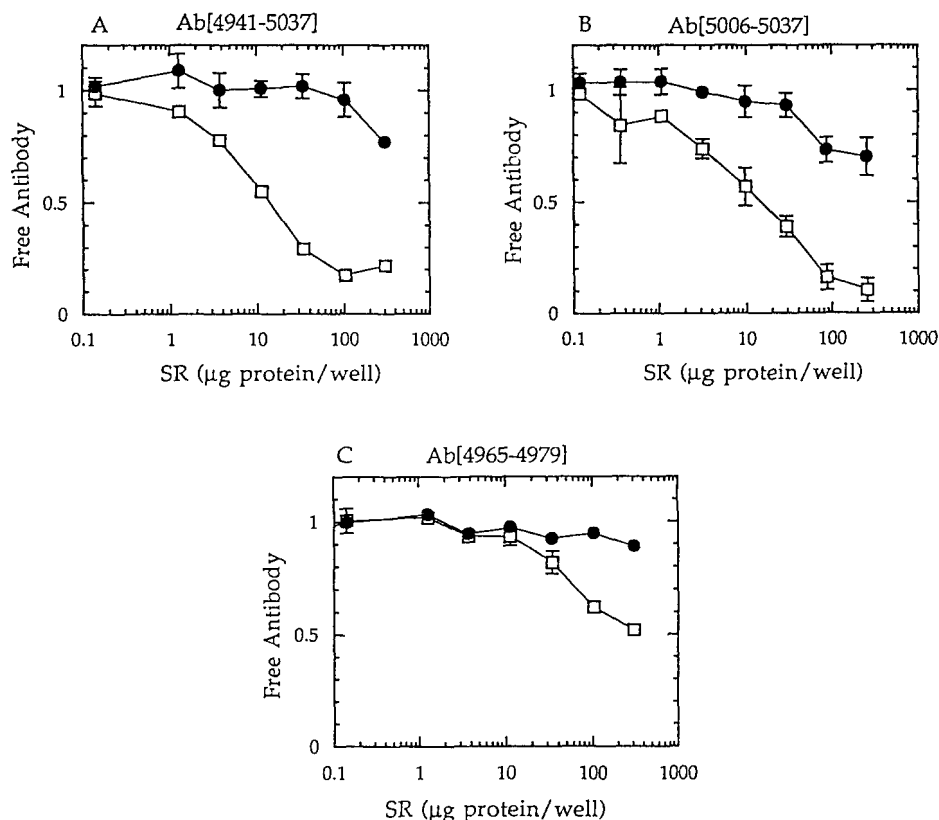
FIG. 5. **Differential binding of C-terminal-directed antibodies to intact and alkaline-treated SR vesicles.** SR vesicles were incubated in Tris-buffered saline at pH 7.5 (*closed circles*) or pH 8.5 (*open squares*) for 60 min. The pH was then adjusted to 7.5 with HCl, as described under "Experimental Procedures." The vesicles were diluted and incubated at pH 7.5 with Ab(4941–5037) (*A*), Ab(5006–5037) (*B*), or Ab(4965–4979) (*C*). The free antibodies were detected as in Fig. 4. Values are means ± S.E. of triplicate determinations of a representative experiment.

calcium renders SR vesicles irreversibly permeable to macromolecules (52). Therefore, we examined binding of the C-terminal antibodies to SR vesicles permeabilized by transient exposure to pH 8.5 at low calcium.

Each of the C-terminal-directed antibodies bound poorly to the intact vesicles in the preparation of SR shown in Fig. 5. Ab(4941–5037) bound well to vesicles treated for 60 min at pH 8.5 (Fig. 5A). Ab(5006–5037) exhibited a similar differential binding to alkaline treated vesicles (Fig. 5B). The binding to treated vesicles by Ab(4965–4979) is also increased over binding to intact vesicles but does not approach saturation (Fig. 5C), presumably due to the lower affinity of this antibody for the membrane-bound receptor. The C terminus is therefore exposed to antibodies by alkaline treatment as well as by 0.25% CHAPS, and this exposure is not peculiar to a single antibody preparation.

Exposure of the C Terminus: Effect of CHAPS and pH—The increase in binding of an antibody under conditions of permeabilization may reflect an increase in exposure of an epitope which is topologically cytoplasmic but sterically inaccessible under native conditions. The detergent CHAPS has been used previously to solubilize the RyR with retention of functional properties, suggesting that there are no major changes in the tertiary or quaternary structure of the solubilized complex (12). However, CHAPS may disrupt low affinity interactions with other proteins or produce subtle changes in protein structure (local denaturation) not detected by functional assays. In order to sort out conditions for permeabilization from denaturation, we examined the effects of transient exposure of SR vesicles to either a low concentration of detergent (0.1% CHAPS) or alkaline pH. We examined the effects on vesicle integrity, as gauged by the retention and protection of the soluble lumenal SR protein calsequestrin.

SR vesicles were treated with 0.1% CHAPS or at alkaline pH (pH 8.5 or 9.5) for 10 min before diluting the detergent 10-fold or adjusting the pH to 7.5. The vesicles were pelleted, and the release of protein from the vesicles was analyzed by SDS-

PAGE. Treatment with 0.1% CHAPS for 10 min resulted in only partial solubilization of the RyR. The major portion of calsequestrin, however, was released from the vesicles (Fig. 6B). Permeabilization with 0.3% CHAPS resulted in almost complete loss of calsequestrin from the vesicles (data not shown). In contrast, transient treatment at pH 8.5 resulted in no significant release of calsequestrin in the supernatant, relative to the untreated vesicles (Fig. 6C). Transient exposure to higher pH (pH 9.5) resulted in the partial extraction of calsequestrin (Fig. 6D). Calsequestrin was also protected from tryptic digestion in untreated vesicles and vesicles subjected to transient exposure at pH 8.5, but was accessible to trypsin after treatment with 0.1% CHAPS (data not shown). These results suggested that the SR permeability barrier toward proteins as small as trypsin (24-kDa) remained substantially intact after transient exposure at pH 8.5. In contrast, brief exposure to CHAPS as low as 0.1% was sufficient to permeabilize the vesicles. We therefore re-examined the binding of C-terminal-directed antibodies to CHAPS and alkaline-treated SR vesicles.

The C terminus becomes accessible to Ab(5006–5037) after 15 min of exposure to pH 8.5 or 9.5 (Fig. 7A, *squares* and *triangles*). In contrast, the binding of Ab(5006–5037) to vesicles treated with 0.1% CHAPS is only marginally increased over the low level of binding to control vesicles (Fig. 7A, *open circles*). However, the binding to the detergent-treated vesicles tends to increase, to a maximal extent of binding equivalent to that of pH 8.5-treated vesicles, as the vesicles are held at 0.1% CHAPS for increasing periods of time before dilution (Fig. 7B, *open circles*). The half-time for exposure is ~30 min at room temperature. This process is much slower than the rate of partial solubilization of SR vesicles under the same conditions (≤10 s), as detected by 90 °C light scattering (data not shown). The rate of detergent-mediated exposure of the C terminus is increased by approximately 3-fold when the salt concentration during the incubation at 0.1% CHAPS is reduced from 0.75 M NaCl to 0 (data not shown).

FIG. 6. **SDS-PAGE analysis of CHAPS and alkaline-treated SR vesicles.** SR vesicles were treated for 10 min at room temperature at pH 7.5 (*A*), pH 7.5, 0.1% CHAPS (*B*), pH 8.5 (*C*), or pH 9.5 (*D*). The incubations were terminated by dilution or adjustment of pH to 7.5. An aliquot from each incubation was pelleted (15 min at 150,000 × *g*, 4 °C), and the pellets were resuspended to an equivalent volume. Equal volumes of the total incubation mixture (*T*), supernatant (*S*), and resuspended pellet (*P*) were solubilized in SDS denaturing buffer, separated on 3–12% SDS-PAGE, and stained with Coomassie Blue. *Lanes* labeled *T* contained 10 µg of SR protein. The position of bands corresponding to RyR (*RyR*), CaATPase (*CA*), and calsequestrin (*Caseq*) are indicated.
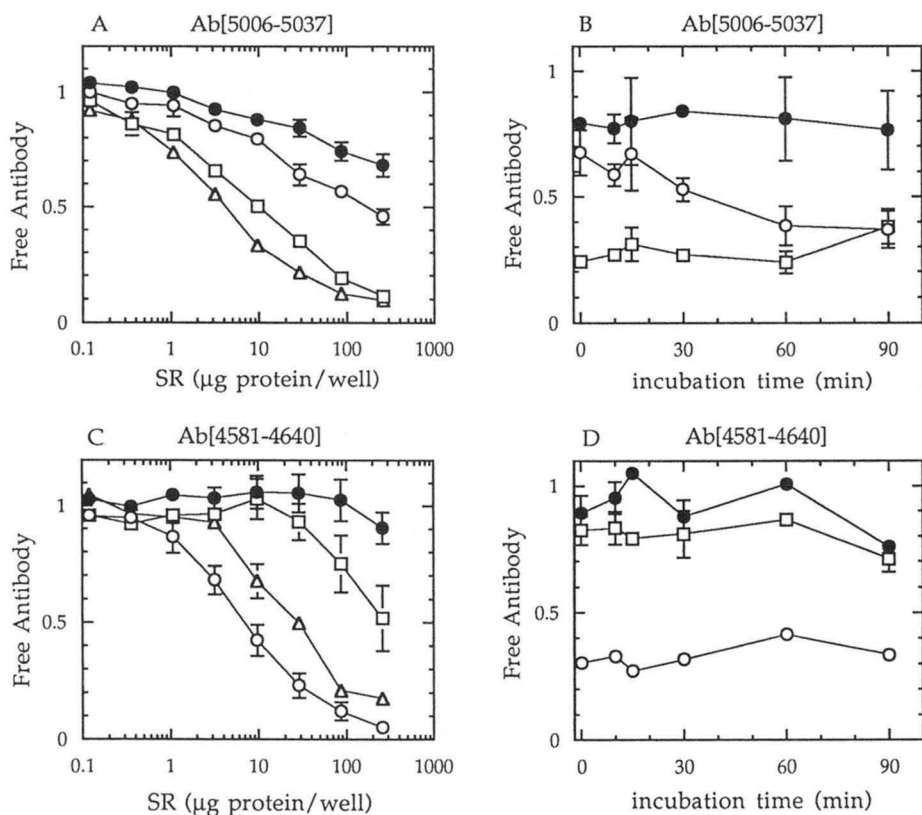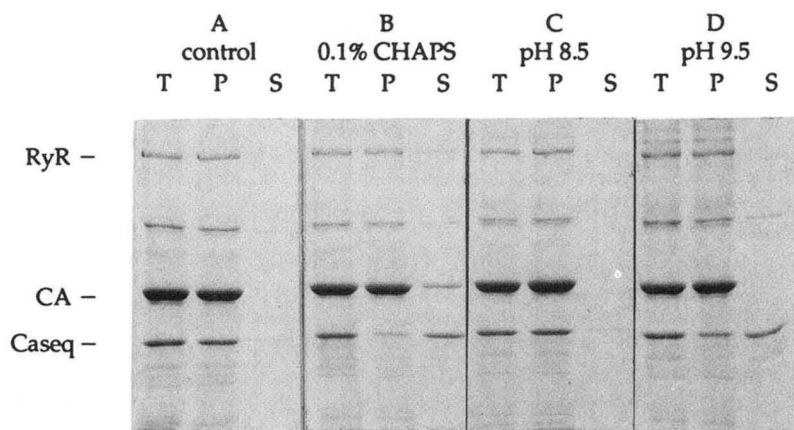

FIG. 7. **Differential binding of site-directed antibodies to CHAPS and alkaline-treated SR vesicles.** *A* and *C*, increasing concentrations of SR vesicles were incubated for 15 min at pH 7.5 (*closed circles*), pH 8.5 (*squares*), pH 9.5 (*triangles*), or with 0.1% CHAPS (*open circles*) before adjusting the pH to 7.5 or diluting the detergent, respectively. The treated vesicles were then incubated with Ab(5006–5037) (*A*) or Ab(4581–4640) (*C*). Values are the mean ± S.E. of three to four experiments performed in duplicate. *B* and *D*, time course of exposure of binding sites for Ab(5006–5037) (*B*) and Ab(4581–4640) (*D*). SR vesicles were incubated as in *A* and *C* for the indicated times at pH 7.5 (*closed circles*), pH 8.5 (*squares*), or with 0.1% CHAPS (*open circles*). The first time point in *B* and *D* was taken after 5–10 s which reflects the mixing time for the addition of diluent or HCl. The treated vesicles were incubated overnight with antibodies at a final SR protein concentration of 0.3 mg/ml, equivalent to 30 µg/well SR protein in the ELISA assay. Values are the mean ± S.E. of two experiments performed in duplicate.

In contrast to the C terminus, the putative lumenal epitope for Ab(4581–4640) is made accessible after very brief exposure (<10 s) to 0.1% CHAPS (Fig. 7D, *open circles*), consistent with exposure of the epitope being dependent on permeabilization. At reduced salt concentration there is an approximately 40% decrease in the rate of detergent-mediated exposure (data not shown). Transient alkalinization to pH 8.5 results in relatively little exposure of the same epitope (Fig. 7, *C* and *D*, *squares*), consistent with the observation that this condition does not substantially permeabilize the SR vesicles (see above). Transient alkalinization to pH 9.5 (Fig. 7C, *triangles*) was nearly as effective as 0.1% CHAPS in exposing the epitope of Ab(4581–4640).

Exposure of the C Terminus: Effect of Proteolysis—The requirement for either detergent or pH-mediated denaturation for exposure of the C terminus suggests that this region of the protein may be buried within the native RyR complex. We tested whether this region may become exposed as a consequence of proteolytic degradation of the RyR. Limited tryptic digestion of intact SR vesicles resulted in the apparently complete exposure of the epitope for Ab(5006–5037) (Fig. 8A).

Complete exposure was achieved after digestion for 30 min at 23 °C at trypsin/SR protein of 1:100 (w/w). The extent of trypsin-dependent exposure was not affected by subsequent treatment with detergent or alkaline pH. In contrast to the C terminus, the putative lumenal epitope for Ab(4581–4640) was not exposed by proteolysis alone under the same conditions, but could be exposed only after treatment of the proteolyzed vesicles with 0.1% CHAPS (Fig. 8B). Calsequestrin was not degraded by trypsin (Figs. 8, *C* and *D*), consistent with a previous report (28). Therefore, it appears that the permeability barrier of the vesicles was not broken down during trypsin digestion. After proteolysis at trypsin/SR protein of 1:100 the epitopes for Ab(4581–4640) are recovered from the proteolyzed vesicles primarily in fragments of ~76, ~66, and ~37 kDa (Fig. 8E), and the epitopes for Ab(4860–4886) and Ab(5006–5037) are recovered as ~76-, ~66-, and ~27-kDa fragments (Fig. 8, *F* and *G*, respectively). These fragments have been previously identified by Callaway *et al.* (28). The ~76- and ~66-kDa fragments appear to be derived from the C terminus by cleavage at arginine 4475 and another undetermined site, respectively. The
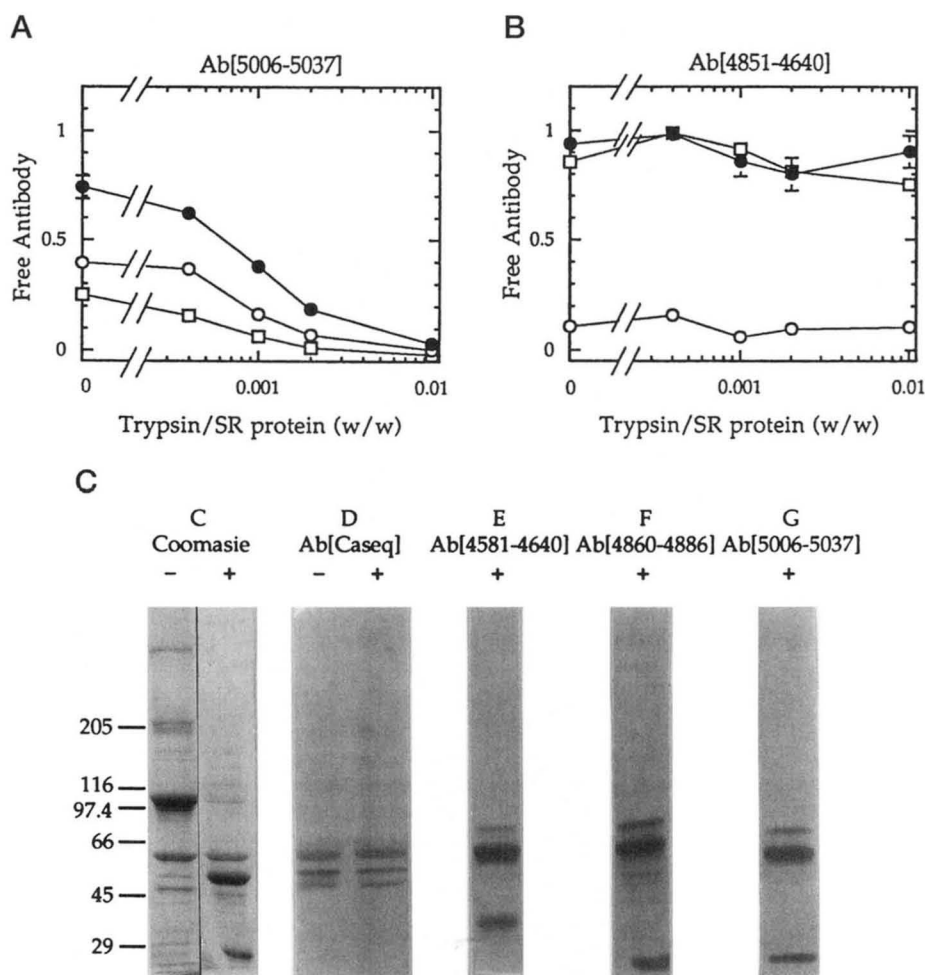
FIG. 8. **Exposure of C-terminal epitopes by trypsin digestion.** *A* and *B*, SR vesicles were incubated with trypsin at the indicated trypsin/SR protein (w/w) ratios for 30 min at 23 °C. The digestion was stopped with the addition of trypsin inhibitors. The digested vesicles were then incubated at pH 7.5 (*closed circles*), pH 8.5 (*squares*), or with 0.1% CHAPS (*open circles*) for 10 min. The vesicles were diluted or adjusted to pH 7.5, respectively, and then incubated with Ab(5006–5037) (*A*) or Ab(4581–4640) (*B*) at a final concentration of 0.3 mg/ml SR protein. Values are the mean ± S.E. of two experiments performed in triplicate. *C*, Coomassie Blue-stained 3–12% SDS-PAGE of 15 µg of SR proteins from undigested vesicles (– *lane*) or vesicles digested for 30 min at 23 °C at a trypsin/SR protein (w/w) of 0.01 (+ *lane*). Parallel samples were blotted with anti-calsequestrin antibody (*D*), Ab(4581–4640) (*E*), Ab(4860–4886) (*F*), or Ab(5006–5037) (*G*).

~27-kDa fragment appears to be derived from the C terminus by cleavage at arginine 4756, consistent with recognition by Ab(4860–4886) and Ab(5006–5037). The ~37-kDa fragment appears to be derived from the N-terminal portion of the ~66-kDa fragment, presumably by cleavage at arginine 4756, consistent with its recognition by Ab(4581–4640).

DISCUSSION

Topological Assignment of Epitopes—The present study examined the topological location of RyR sequences in the C-terminal portion of the molecule, a region which is believed to constitute the channel forming domain of the RyR. The topology of several sites was probed by competition ELISA assay using site-directed antibodies. Heavy SR vesicles are well oriented, with the extravesicular face corresponding to the cytoplasmic side of the SR. The intravesicular space, corresponding to the lumen of the SR, was exposed by solubilization of the membrane with detergent or by disruption at alkaline pH (52). The differential exposure of antibody-binding sites in SR vesicle has been used previously to define a lumenal sequence of the SR CaATPase (53).

However, this approach is confounded by possible effects of detergent or alkaline treatment on protein structure, and consequently antibody affinity, independent of permeabilization. We have therefore attempted to examine antibody binding under conditions in which vesicles are permeabilized with minimal disruption of structure or, conversely, conditions in which epitopes are exposed by treatments which do not permeabilize the vesicles. Our results suggest that two regions including amino acids 4581–4640 and 4860–4886 are located in the lumen of the SR and that the C-terminal sequence which includes

amino acids 4941–5037 is located on the cytoplasmic face of the SR but inaccessible to antibody under native conditions.

Lumenal Loop 4581–4640—The sequence recognized by antibody Ab(4581–4640) is predicted by the three published topology models (15, 16, 48) to be a loop located in the lumen of the SR. The homologous site is also predicted to be lumenal in one model of the IP₃R (18) but cytoplasmic in another (30). The results of this study are most consistent with a lumenal location of the sequence 4581–4640. The antibody binds if and only if the SR vesicles are permeabilized. The sequence characteristics of this region are consistent with a surface accessible structure. The sequence is poorly conserved between RyR isoforms and between species, in particular in the central region from amino acid 4583 to 4627. The sequence identities between isoforms in this region are below 20% and there are deletions (RyR2, RyR3, and frog alpha) or insertions (*Drosophila*) in other species and isoforms. The region is hydrophilic and contains a glycine-rich cluster in the middle, consistent with a flexible or disordered structure. The remainder of the sequence is highly acidic, a property shared by the RyR3 and Drosophila isoforms but not by the RyR2 isoform. These features suggest that this region may have a functional role related to a high density of negative surface charge on the lumenal face of the channel, a function not conserved in RyR2.

Lumenal Loop 4860–4886—The region from 4860–4878 is predicted to be lumenal in the topology models of Takeshima *et al.* (15) and Brandt *et al.* (48). In contrast, the model of Zorzato *et al.* (16) predicts a cytoplasmic location for this sequence. The homologous region is also predicted to be lumenal in one model of the IP₃R (30) but cytoplasmic in another (18). Our results

indicate that Ab(4860–4886) bound, albeit with low apparent affinity, to a lumenal site when SR vesicles are permeabilized.

The region recognized by Ab(4860–4886) is well conserved among the RyR isoforms, and the adjacent hydrophobic segments (4789–4820, 4837–4856, 4879–4898, and 4923–4937) are among the most highly conserved sequences between the RyR and the IP_3R. The IP_3R, however, contains a 47–48-amino-acid insertion at a location homologous to tyrosine 4863 of the RyR. This inserted sequence, which is highly variable between IP_3R isoforms, has recently been shown to be located in the lumen of the ER by antibody labeling and to be glycosylated at two sites, consistent with a lumenal localization (54).

Like the IP_3R, the RyR also contains a potential recognition site (NKS) for N-linked glycosylation in this region at asparagine 4864. However, glycosylation of the RyR has not been detected in lectin overlay studies of SR[3] or JFM proteins (55). The potential glycosylation site in the RyR falls within a comparatively short and well conserved region which may be sterically inaccessible to the ER glycosylation machinery. A relatively inaccessible conformation may explain, in part, the poor affinity of Ab(4860–4886) for the native receptor in permeabilized vesicles. That this polar and acidic region is apparently located within a confined space may reflect a role as part of the conduction pathway, perhaps contributing to the negative surface charge which has been found near the lumenal mouth of the channel (56).

C Terminus 4941–5037: Cytoplasmic and Buried—A recent report by Marty *et al.* (29) found that an antibody directed at the last 11 amino acids of the RyR could bind to intact SR vesicles, consistent with the predicted cytoplasmic location of the C terminus. The results of the current study suggest that the binding sites for antibodies directed toward the last 100 residues of the RyR are relatively inaccessible in SR vesicles but are exposed when the vesicles are subjected to several treatments including prolonged incubation with detergent, alkaline pH, or proteolysis. Exposure of the C terminus under conditions where the vesicles are not permeabilized, *e.g.* by trypsin digestion, is consistent with a cytoplasmic localization of the C terminus.

The C terminus is likely to be exposed by conformational changes which are related to the structural and functional destabilization of the RyR. Limited trypsin digestion, which exposes the C terminus, has a dramatic effect on RyR function (28, 40), despite the apparent retention of the tryptic fragments in a membrane-bound complex (28, 57, 58). High NaCl, a condition which favors solubilization of the functional RyR complex in CHAPS (59), renders the solubilized RyR partially resistant to trypsin (60) and favors ryanodine binding (2, 3), appears to stabilize the C terminus in an antibody-inaccessible conformation.

The apparent inaccessibility of the C terminus in the native RyR suggests that this region may be well structured and localized either in the folded core of the protein or at a subunit interface. The amino acid sequence of this region is well conserved among the RyR isoforms and contains two hydrophobic sequences, suggesting a structurally conserved region. The absence of protease-sensitive sites in this region (28, 57, 61) is also consistent with a buried domain.

Significance for Topology Models—Four (15), 10 (48), or 10–12 (16) membrane-spanning segments have been predicted for the RyR based on sequence analysis. In addition to segments in the C-terminal domain, Brandt *et al.* (48) predicted at least four membrane-spanning segments in the N-terminal domain. They predict two segments, at amino acids 1514–1530

and 1839–1855, based in part on their assignment of the calpain cleavage sites (48). Their cleavage site assignments were based in part on hydrophobic affinity labeling using [^{125}I]TID (48). However, protein labeling with [^{125}I]TID has been shown to occur in hydrophobic regions of proteins which are not membrane associated (62), potentially resulting in the erroneous assignments of membrane-associated protein domains. In this study we find a different arrangement of secondary calpain cleavage sites, based on antibody labeling. We suggest that the evidence presented by Brandt *et al.* (48) does not support the prediction the N-terminal membrane-spanning segments. Indeed, Chen *et al.* (57) found that a tryptic fragment which includes these predicted N-terminal segments was extracted by Na_2CO_3, indicating that these segments are not integral to the membrane.

The results of this study support a model with an even number of membrane-spanning segments resulting in a cytoplasmic C terminus and a cytoplasmic N-terminal domain. The four membrane-spanning segments identified as M1-M4 by Takeshima *et al.* (Fig. 1) at positions 4564–4580, 4640–4664, 4836–4859, and 4918–4937 are consensus predictions of all three topology models (Fig. 1). The model of Zorzato *et al.* (16) differs significantly from those of Takeshima *et al.* (15) and Brandt *et al.* (48) in the prediction of membrane-spanning segments at position 4789–4820 and 4879–4898. These segments have relatively low mean hydrophobicity scores (<0.3) and as such are weak candidates for membrane-spanning segments. The evidence for a lumenal location of the region 4860–4886 presented in this study and the lumenal localization of the homologous region in the IP_3R (54) suggest that the region including 4879–4898 cannot be a membrane-spanning segment.

The segment predicted by Zorzato *et al.* (16) at position 4789–4820 is also unlikely to be a membrane-spanning segment. If the M3 segment (4836–4859) traverses from cytoplasm to lumen, as discussed above, then an adjacent segment from position 4789–4820 would have to traverse the membrane from lumen to cytoplasm. Consequently, the region from 4665–4788 would be predicted to have a lumenal location. However, Callaway *et al.* (28) identified a cytoplasmic tryptic cleavage site at arginine 4756. Moreover, the data from this study demonstrate the lumenal location of the region 4581–4640, consistent with the existence of membrane-spanning segment M2 (4641–4664) which traverses the membrane from lumen to cytoplasm.

There is as yet no direct evidence for additional membrane-spanning segments beyond the four consensus segments closest to the C terminus. The models of Zorzato *et al.* (16) and Brandt *et al.* (48) predict as many as six additional segments. Indirect evidence for the existence of upstream membrane-spanning segments comes from the observations of Chen *et al.* (57) that a tryptic fragment which extends from approximately residue 3370–4475 is retained as an apparently integral membrane protein.

Taken together, the data from the current study and studies from other laboratories support a minimal membrane topology model for the RyR. The model includes at least four membrane-spanning segments near the C terminus, corresponding to the consensus segments first identified by Takeshima *et al.* (15) from sequence analysis of the skeletal muscle RyR.

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