Transmembrane orientation of the N-terminal and C-terminal ends of the ryanodine receptor in the sarcoplasmic reticulum of rabbit skeletal muscle

Isabelle MARTY,*‡ Michel VILLAZ,* Gérard ARLAUD,† Isabelle BALLY† and Michel RONJAT*

*Laboratoire de Biophysique Moléculaire et Cellulaire, Département de Biologie Moléculaire et Structurale, CENG 85X, 38041 Grenoble Cédex, and †Laboratoire d'Enzymologie Moléculaire, Institut de Biologie Structurale, 41 Av. des Martyrs, 38027 Grenoble Cédex 1, France

Antibodies were raised against synthetic peptides corresponding to the N-terminal (residues 2–15) and the C-terminal (residues 5027–5037) parts of the rabbit skeletal muscle ryanodine receptor. The specificity of the antibodies generated was tested by e.l.i.s.a., Western blotting and immunofluorescence. All these tests demonstrated the specificity of the antibodies and their ability to react with both the native and the denaturated ryanodine receptor. Both the anti-N-terminus and the anti-C-terminus antibodies bound to sarcoplasmic reticulum vesicles, indicating that each end of the membrane-embedded ryanodine receptor is exposed to the cytoplasmic side of the vesicles. These immunological data were complemented with proteolysis experiments using carboxypeptidase A. Carboxypeptidase A induced degradation of the C-terminal end of the ryanodine receptor in sarcoplasmic reticulum vesicles and a concomitant loss of reactivity of the anti-C-terminus antibodies in Western blots, providing extra evidence for the cytoplasmic localization of the C-terminal end of the ryanodine receptor.

INTRODUCTION

Depolarization of the skeletal muscle plasma membrane induces release of calcium contained in the sarcoplasmic reticulum (SR). This rapid intracellular calcium release occurs via a calcium release channel localized to the terminal cisternae of the SR, which has been purified and identified as the ryanodine receptor (RyR) (Inui et al., 1987; Imagawa et al., 1987; Lai et al., 1987). The primary sequence of the protein has been deduced by cloning and sequencing of the cDNA (Takeshima et al., 1989; Zorzato et al., 1990). The rabbit skeletal muscle RyR is composed of 5037 amino acids, with a molecular mass of about 565 kDa. Electron microscopy pictures of the RyR show four-leaf clover structures (Lai et al., 1988, 1989; Block et al., 1988), corresponding to the association of four identical subunits of 565 kDa, and a tridimensional structure of the isolated RyR with a 3 nm resolution has been proposed (Wagenknecht et al., 1989; Radermacher et al., 1992).

At this time, very little is known about the membrane topography of the RyR, or about the localization of the different parts of the protein on the cytoplasmic side or on the luminal side of the SR membrane. The models proposed are all derived from structure prediction algorithms (Takeshima et al., 1989; Zorzato et al., 1990), but the experimental evidence to support these predictions remains to be obtained. Recently, Chen et al. (1992), using fusion proteins, have identified a region of the protein as a possible calcium binding site, and suggested that this portion of the protein, between amino acids 4478 and 4512, could be surface-exposed.

In the present study, two peptides corresponding respectively to the N-terminal and the C-terminal ends of the rabbit skeletal muscle RyR have been synthesized and used to produce polyclonal anti-peptide antibodies. Immunological studies with these antibodies and proteolytic degradation with carboxypeptidase A were performed on the membrane-embedded RyR, and allowed us to determine the accessibility and the orientation of the N-terminal and C-terminal ends of the RyR within the SR membrane.

EXPERIMENTAL

Membrane preparation

Heavy SR (HSR) vesicles were prepared according to the procedure of Kim et al. (1983), slightly modified as follows. All membrane preparations were carried out in the presence of the following protease inhibitors: leupeptin (10 μM), pepstatin (7 μM), phenylmethanesulphonyl fluoride (PMSF) (1 mM), EDTA (200 μM). Lower concentrations were used for the vesicles prepared for proteolytic degradation with carboxypeptidase A: leupeptin (1 μM), pepstatin (1 μM), PMSF (200 μM), EDTA (200 μM). The supernatant of the first centrifugation, after filtration through Whatman no. 4 paper, was incubated for 1 h at 4 °C in the presence of 1 M NaCl. The filtrate was centrifuged for 40 min at 10000 *g*. Pellets were suspended in a buffer containing 150 mM KCl, 300 mM sucrose, 20 mM Pipes (pH 7.1) and 2.5 mM EGTA (buffer A). HSR vesicles were collected by a 40 min centrifugation at 17000 *g*, and washed twice in the same buffer but without EGTA. Final pellets were resuspended in buffer A without EGTA at a protein concentration of approx. 20–30 mg/ml, and stored in liquid nitrogen. Protein concentration was determined by the Biuret technique (Gornall et al., 1949).

RyR purification

RyR was purified on a sucrose gradient, as described by Lai et al. (1988). The fractions corresponding to the peak of bound [^{3}H]ryanodine were pooled and concentrated by ultrafiltration on a YM30 membrane (Amicon Corp.).

Abbreviations used: ACter, anti-(C-terminal peptide) antibody; ANter, anti-(N-terminal peptide) antibody; FITC, fluorescein 5′-isothiocyanate; HSR, heavy sarcoplasmic reticulum; PMSF, phenylmethanesulphonyl fluoride; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

‡ To whom correspondence should be addressed.

Peptide synthesis

The two peptides corresponding to the N-terminal and C-terminal ends of the RyR were synthesized chemically by the stepwise solid-phase method (Barany and Merrifield, 1980), using an Applied Biosystems 430A automated synthesizer. The N-terminal peptide (Gly-Asp-Gly-Gly-Glu-Gly-Glu-Asp-Glu-Val-Gln-Phe-Leu-Arg-Tyr) corresponds to residues 2–15 of the rabbit skeletal muscle RyR (Takeshima et al., 1989), with an extra Tyr added at the C-terminus. The C-terminal peptide (Cys-Phe-Arg-Lys-Gln-Tyr-Glu-Asp-Gln-Leu-Ser) corresponds to residues 5027–5037. The t-Boc group was used for protection of the N_{α}-amino group of all amino acids. All couplings were performed by the dicyclohexyl carbodi-imide/1-hydroxybenzotriazole method, using *N*-methylpyrrolidone and dimethyl sulphoxide as coupling solvents, according to the protocol defined by Applied Biosystems. All amino acids were systematically double-coupled, and amino groups left unreacted at the end of each coupling cycle were capped with acetic anhydride. Deprotection and cleavage of the peptides from the resin was performed with either HF (N-terminal peptide) or trifluoromethane sulphonic acid (C-terminal peptide). Both peptides were purified by reverse-phase h.p.l.c., using a 300 Å (30 nm) Deltapak C_{18} column (1.9 cm × 30 cm) (Waters Associates). Each purified peptide was characterized by amino acid analysis and by fast-atom bombardment mass spectrometry, as described previously (Thielens et al., 1990).

Coupling of peptides to ovalbumin and generation of anti-peptide antibodies

The N-terminal peptide (residues 2–15) was coupled to ovalbumin using bis-diazotided benzidine through the hydroxy group of the Tyr residue added at the C-terminal end of the peptide. The coupling reaction was performed as described by Tamura and Bauer (1982). The C-terminal peptide (residues 5027–5037) was coupled to maleimidobenzoic acid *N*-hydroxysuccinimide ester-activated ovalbumin (Pierce) through the SH group of Cys-5027.

Rabbits were immunized with three intradermal injections followed by three intramuscular injections of 100 μg of the conjugated peptide, at 3 week intervals. The rabbits were bled 3 weeks after the last injection.

The antibodies were purified by affinity chromatography on a column containing the corresponding peptide coupled to Sepharose-4B.

Antibody assays by e.l.i.s.a.

The ability of the antisera to react with the synthetic peptides, the purified RyR and the membrane-bound RyR in HSR vesicles was tested by e.l.i.s.a., using microtitration plates (Nunc; ref. 4-42404), as previously described (Marty et al., 1992). The antigen was diluted at the appropriate concentration in PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4), and coated by an overnight incubation at 4 °C. After each incubation, the wells were washed with PBS-T [PBS supplemented with 0.05% (w/w) Tween 20]. The remaining binding sites were saturated by a 1 h incubation with PBS-T/BSA (1%, w/w). The antibodies were then allowed to react, for 2 h at room temperature, at the chosen dilution in PBS-T/BSA. The second antibody, goat anti-(rabbit IgG) conjugated to horseradish peroxidase (Biosys), was diluted 2000-fold in PBS-T/BSA and added to each well; incubation was continued for a further 2 h at room temperature. The immune complex was revealed with 3,3′,5,5′-tetramethylbenzidine (TMB), and the absorbance was measured at 450 nm after addition of H_2SO_4.

Chicken anti-(rabbit calsequestrin) antibodies were a gift from Dr. F. Zorzato (Ferrara, Italy). When the e.l.i.s.a. test was performed with these antibodies, an incubation with rabbit anti-(chicken IgG) (Sigma ImmunoChemicals; 1:50000 dilution) was performed before the incubation with goat anti-(rabbit IgG) horseradish peroxidase-conjugated antibodies.

For back-titration experiments, the HSR vesicles were incubated overnight at 4 °C with the antibodies, and the supernatants were collected after centrifugation at 6500 *g* for 30 min. The unreacted antibodies present in the supernatant were assayed by e.l.i.s.a. against the corresponding peptides coated on to microtitre plates as described above.

Western blot analysis

The reactivity of the anti-peptide antibodies was tested against the RyR or its proteolytic fragments using the Western blot technique (Towbin et al., 1979). HSR protein (50 μg) or purified RyR (30 μg) was loaded on a 5–15% polyacrylamide gel. After electrophoretic separation, the proteins were transferred to a nitrocellulose sheet over 4 h at 1.5 A.

After saturation of the remaining binding sites with BSA, the nitrocellulose sheets were incubated overnight at 4 °C with the antibodies (1:20 to 1:100 dilution, depending on the antibody), then with ^{125}I-labelled Protein G, and the reactive proteins were detected by autoradiography.

Immunofluorescence microscopy

Bundles of frog or mouse skeletal muscle fibres were fixed with 4% paraformaldehyde in PBS at 4 °C for 1 h, washed in 10% and then 20% sucrose in PBS, embedded in Tissue-Tek O.C.T. compound (Miles Inc.), and frozen in liquid nitrogen. Cryosections (10 μm thickness) on microscope slides were permeabilized with 0.25% Triton X-100 in PBS, incubated for 1 h at 37 °C with anti-peptide antibodies diluted 1:100 in PBS supplemented with 0.1% Triton X-100 and 2% lamb serum, and washed three times at 4 °C with PBS/0.1% Triton X-100/0.5% BSA. The sections were then incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-(rabbit IgG) antibody (ref. 111-096-008; Jackson Immunoresearch Lab. Inc.) (1:150 dilution) for 30 min at 37 °C. After three washes in PBS (10 min each), samples were mounted and examined with a microscope equipped with fluorescence optics. To assess antibody specificity, samples were incubated according to the same protocol but without primary antibody.

Measurement of the permeability of HSR vesicles

HSR vesicles were diluted to a final concentration of 2 mg/ml in 100 mM KCl, 20 mM Tris, pH 7.2, and incubated for 2 h at room temperature with [^{3}H]glucose (4 μCi/ml; 115 nM final concentrations) or [^{3}H]inulin (1 μCi/ml; 87 nM final concentrations). Glucose is a permeant sugar (Kometani and Kasai, 1978), whereas inulin, a much larger molecule (molecular mass 5200 Da), is supposed to be non-permeant. An aliquot fraction of the HSR vesicles incubated with [^{3}H]glucose was filtered on a Millipore filter (HA 0.45 μm), and then washed with 3 × 5 ml of 100 mM KCl, 20 mM Tris, pH 7.2. As glucose is slowly permeant, this allows the washes of the filter to eliminate the glucose retained outside the vesicles. The internal volume (V_i) of the HSR vesicles was calculated by measuring the radioactivity retained on the filter. On the other hand, 3 ml of the HSR vesicle solutions ([^{3}H]glucose or [^{3}H]inulin) was centrifuged for 30 min at 100000 *g*. The supernatants were discarded, and the tubes were

carefully wiped. The pellets were then dissolved in 1 ml of 4% cholate, and the radioactivity contained in the pellets was measured by liquid scintillation counting. The amount of [^{3}H]glucose incorporated in the pellets indicated the total volume of the pellet (V_T) (internal volume of the vesicles plus external volume of the vesicles in the pellet). The external volume was then obtained as the difference between the internal volume (V_I, determined by filtration), and the total volume (V_T, determined by centrifugation) of the pellet. The volume accessible to [^{3}H]inulin, directly measured as the amount of [^{3}H]inulin in the pellet, was compared with the external volume.

Proteolytic digestion

HSR vesicles, prepared in the presence of low amounts of protease inhibitors, were diluted to 4 mg/ml in a medium composed of 0.27 M sucrose, 10 mM Tris, pH 7.5, at a temperature of 37 °C. Carboxypeptidase A (Boehringer) was added at time zero, at a protease/total protein ratio of 1:100 (w/w). After 1 min, 10 min, 30 min, 1 h and 4 h, a portion of the reaction medium was withdrawn and the reaction stopped by addition of EDTA (pH 6; 5 mM final concentration) followed 1 min later by Laemmli's dissociation medium (62.5 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, Bromophenol Blue). The samples were stored at −20 °C and further analysed by immunoblotting. In control experiments, HSR vesicles were incubated for 4 h under the same conditions, but in the absence of proteases.

RESULTS

Characterization of the anti-peptide antibodies by e.l.i.s.a., Western blot and immunofluorescence

The reactivity of the anti-(N-terminal peptide) antibody (ANter) and of the anti-(C-terminal peptide) antibody (ACter) was first determined against the N-terminal peptide or the C-terminal peptide respectively by e.l.i.s.a. on microtitration plates coated with the corresponding peptide. The results (Figure 1) show that each antibody reacted strongly with the corresponding peptide. In both cases, no reaction was found using pre-immune serum. The ANter and ACter antisera were then assayed by e.l.i.s.a. against purified RyR coated on the microtitration plates. The data presented in Figure 2 demonstrate that both ANter and ACter antibodies reacted with RyR purified from rabbit skeletal muscle.

Figure 3 shows the reactivity in a Western blot of ANter and ACter against the proteins contained in HSR vesicles or against purified RyR. The ANter antibodies reacted with a high-molecular-mass protein (apparent mass of approx. 430 kDa) in HSR vesicles (Figure 3, lane 1), and with two bands of about 430 kDa and 160 kDa in the purified RyR (lane 2). The ACter antibodies reacted with a single high-molecular-mass band (approx. 430 kDa) in HSR vesicles (lane 3) and with two bands (approx. 430 kDa and 350 kDa) in purified RyR (lane 4). The reactivity of both antibodies was completely abolished when the corresponding free peptide was added as a competitor (results not shown). The high-molecular-mass band (430 kDa) corresponds to intact RyR. The 350 kDa band, recognized only by ACter, corresponds to a C-terminal fragment of the RyR. Conversely, the 160 kDa band, recognized only by ANter, corresponds to a N-terminal fragment of the RyR. These fragments result from proteolytic degradation of the RyR during the purification steps, as confirmed by the fact that the same degradation pattern was observed in HSR vesicles when the vesicles were prepared with lower amounts of protease inhibitors (see e.g. Figure 7, lane 1). Similar degradation of the RyR has already been described by different groups, of the RyR either in purified form (Lai et al., 1988) or in HSR membranes (Gilchrist et al., 1992; Brandt et al., 1992).

The two antibodies were also tested by immunofluorescence with different preparations of skeletal muscle. The patterns of immunostaining obtained with ANter are shown in Figure 4 for amphibian (frog) and mammalian (mouse) skeletal muscle longitudinal sections. Identical patterns were obtained with ACter (results not shown). No staining appeared in sections incubated without primary antibody, as illustrated for a frog fibre section in Figure 4(c), confirming the specificity of the antibody–antigen reactions (identical results were obtained using a mouse fibre

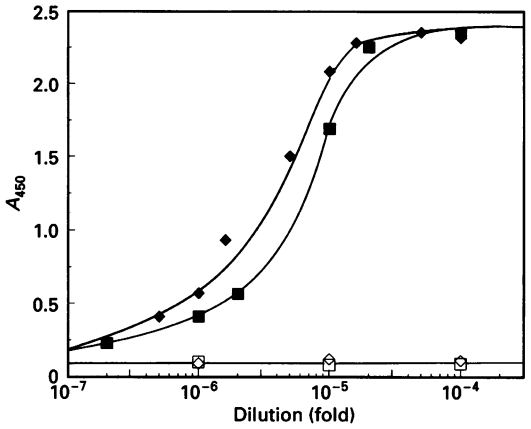

Figure 1 Reactivity of ANter, ACter and preimmune sera (PINter or PICter) with N-terminal and C-terminal peptides of the RyR, as assessed by e.l.i.s.a.

Microtitration plates were coated with the N-terminal peptide (100 ng/ml) or the C-terminal peptide (50 ng/ml) diluted in PBS. The corresponding serum (■, ANter; □, PINter; ◆, ACter; ◇, PICter) was added at different dilutions. After incubation with peroxidase-conjugated second antibody, the reactivity of each antiserum was measured by a chromogenic reaction, as described in the Experimental section.

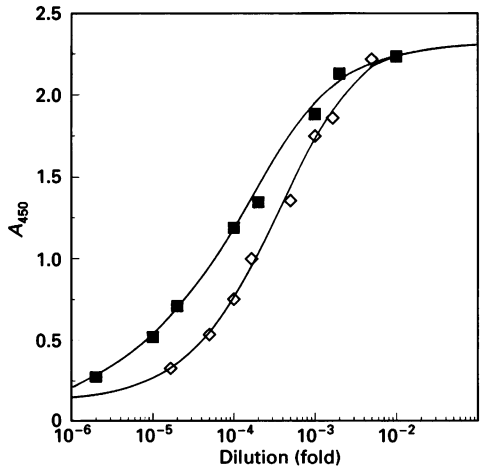

Figure 2 Reactivity of ANter and ACter antibodies with purified RyR, as assessed by e.l.i.s.a.

Microtitration plates were coated with purified RyR at 500 ng/ml in PBS. The antisera (■, ANter; ◇, ACter) were then added at the different dilutions, and their reactivity was determined as described in the legend to Figure 1.

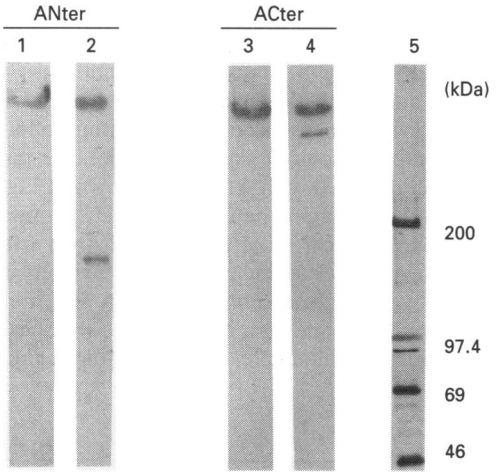

Figure 3 Reactivity of the anti-peptide antibodies against the RyR, as assessed by Western blot analysis

Samples of 50 μg of solubilized HSR proteins (lanes 1 and 3) or 30 μg of purified RyR (lanes 2 and 4) were separated by SDS/PAGE on a 5–15% polyacrylamide gel. After electrotransfer to nitrocellulose, the sheets were treated with ANter antibody (diluted 1:50; lanes 1 and 2) or with ACter antibody (diluted 1:20; lanes 3 and 4), as described in the Experimental section. After incubation with ^{125}I-labelled Protein G (Amersham), the immunoreactive proteins were detected by autoradiography. Lane 5 contains ^{14}C-labelled Rainbow molecular mass standards (Amersham).

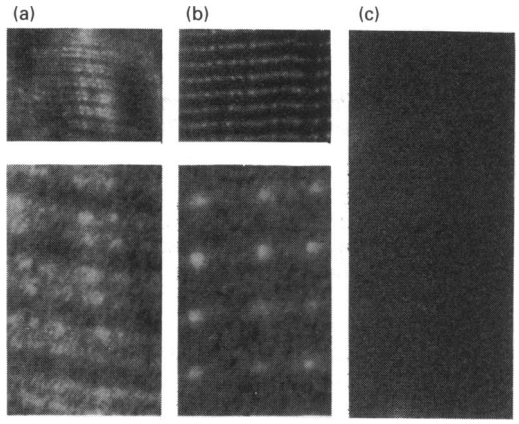

Figure 4 Patterns of immunostaining of skeletal muscle fibres with ANter antibodies

Fixation and sectioning were carried out as described in the Experimental section. Sections (**b**) and (**c**) were obtained from frog leg muscle, and section (**a**) was from mouse muscle. The two lower panels in (**a**) and (**b**) are a $\times 4$ magnification of the upper sections. The scale is given by the striation periodic space of about 2 μm. Section (**c**) is a frog fibre section not incubated with the primary antibodies. Primary antibodies were used at a dilution of 1:100, and secondary antibodies at a dilution of 1:150.

section). Immunostaining patterns obtained with ANter and ACter showed, longitudinally to the fibre, a ratio of 1 dot per sarcomere in the frog section (Figure 4b) and of 2 dots per sarcomere in the mouse section (Figure 4a). These results confirm that both antibodies recognize a protein that is specifically located in the triad regions of skeletal muscle, since the patterns observed with frog and mouse muscles perfectly match the distribution of triad structures observed in amphibians and mammals (Uehara et al., 1976).

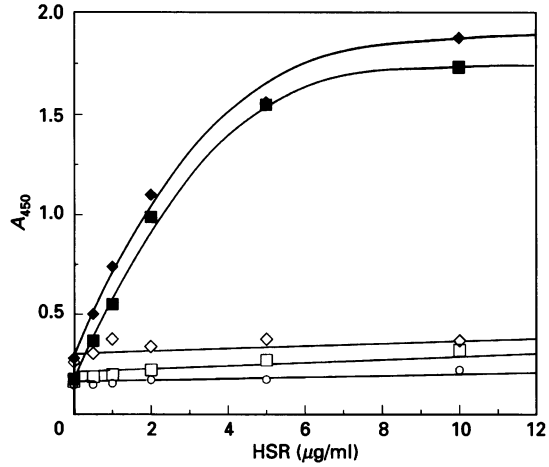

Figure 5 Reactivity of the ANter, ACter and anti-calsequestrin antibodies toward HSR vesicles, as assessed by e.l.i.s.a.

The HSR vesicles were diluted in a medium composed of 0.27 M sucrose, 10 mM Tris, pH 7.2, and coated overnight onto the microtitration plates. The anti-peptide antibodies were diluted 1:10000 in PBS-T/BSA (see the Experimental section), and incubated with increasing concentrations of HSR vesicles coated on the microtitration plate, in the presence (□, ANter + Nter; ◇, ACter + Cter) or absence (■, ANter; ◆, ACter) of the corresponding peptide at 5 μg/ml, as a competitor. Anti-calsequestrin antibodies (○) were used at a dilution of 1:50000 in PBS-T/BSA.

Reactivity of the anti-peptide antibodies with membrane-bound RyR

Having demonstrated the specificity of the ANter and ACter antibodies, and their ability to react with the RyR, using e.l.i.s.a., immunoblot or immunofluorescence techniques, we used these antibodies to investigate the transmembrane arrangement of the RyR in the SR membrane. These tests were performed using HSR vesicles, which have an orientation similar to that of the SR in the muscle cell: the outside of the vesicles corresponds to the cytoplasmic side of the SR, and the inside of the vesicles corresponds to the luminal side of the SR (Saito et al., 1978, 1984). The reactivity of the ANter and ACter antibodies against the membrane-bound RyR was determined by e.l.i.s.a. using HSR vesicles coated on microtitration plates. The results (Figure 5) showed that the amount of antibodies retained on the plate increased with the amount of HSR vesicles coated, with the signal reaching saturation when the wells were saturated with the HSR vesicles. Moreover, the binding of the antibodies was completely abolished by the presence of the corresponding peptide added as a competitor. These results indicate that the two extremities of the RyR are accessible to the antibodies on the cytoplasmic side of the HSR vesicles.

When the same test was carried out with antibodies directed against an internal muscle protein, calsequestrin (Jorgensen et al., 1983; Franzini-Armstrong et al., 1987), no reactivity was observed (Figure 5), confirming the integrity and the orientation of the HSR vesicles. In order to further investigate the porosity of the HSR vesicles used in this study, we measured the permeability of the vesicles to inulin. Inulin, as a large molecule (5200 Da), is supposed to be non-permeant. We measured the internal volume (V_I) of the HSR vesicles by a filtration technique (see the Experimental section), and the total volume (V_T) or the inulin-accessible volume (V_{inulin}) by centrifugation. The results presented in Table 1 show that the inulin-accessible volume was identical to the external volume ($V_{ext.} = V_T - V_I$) of the HSR

Table 1 Examination of the integrity of the HSR vesicles

The HSR vesicles (6 mg of protein) were incubated with [^{3}H]glucose or [^{3}H]inulin and then filtered or centrifuged, and the different volumes were calculated as described in the Experimental section. V_i, internal volume of the vesicles; V_T, total volume of the pellet; $V_{ext.}$, external volume of the vesicles in the pellet; V_{inulin}, inulin-accessible volume. The results presented here are from a single experiment.

Parameter	Volume (μl)
V_i	12.2
V_T	46
$V_{ext.}$	33.8
V_{inulin}	33.4

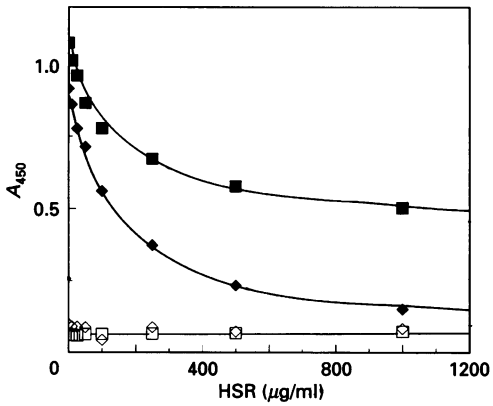

Figure 6 Back-titration by e.l.i.s.a. of ANter or ACter antibodies after reaction with membrane-bound RyR (HSR vesicles)

The antibodies, diluted 1:200000, were incubated with increasing concentrations of HSR vesicles, in the presence (□, ANter+Nter; ◇, ACter+Cter) or absence (■, ANter; ◆, ACter) of the corresponding peptide (3 μg/ml). The vesicles were sedimented by centrifugation, and the antibodies present in the supernatant were assessed by e.l.i.s.a. against the corresponding peptide, coated at 50 ng/ml.

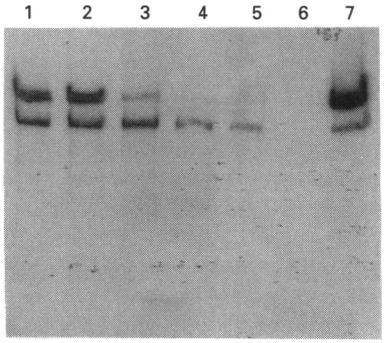

Figure 7 Reactivity of ACter antibodies with the RyR in HSR vesicles treated with carboxypeptidase A

HSR vesicles (4 mg/ml) were incubated with carboxypeptidase A (protease/protein ratio; 1:100), as described in the Experimental section. Lanes 1–6, proteolytic degradation for 0, 1 min, 10 min, 30 min, 1 h and 4 h respectively; lane 7, control (4 h incubation of the vesicles under the same conditions, but without protease). The proteins, after proteolysis, were separated by electrophoresis and analysed by Western blotting with ACter antibodies (diluted 1:20).

vesicles in the pellet. As a control, we checked that, on addition of digitonin (1 mg/mg of protein), the vesicles became permeant to inulin, since the inulin-accessible volume corresponded to the total volume of the pellet. These results demonstrate that the vesicles were completely impermeable to a molecule of 5200 Da and therefore also to antibodies.

In order to eliminate the possibility that disruption of the HSR membrane is induced by coating on to the microtitration plate, resulting in exposure of the N- and/or C-terminal ends of the RyR, the reactivity of the antibodies was tested by using back-titration e.l.i.s.a. In these experiments, increasing concentrations of HSR vesicles were incubated in solution with a fixed concentration of antibodies. After centrifugation, the unreacted antibodies were titrated against the corresponding coated peptide by e.l.i.s.a. (see the Experimental section). In these experiments, adsorption of the HSR vesicles on to the microtitration plate and the potential resulting modification of the membrane were avoided. The more the antibodies have reacted with the HSR vesicles, the less abundant they are in the supernatant after centrifugation, and the lower is their reactivity against the coated peptide observed in the subsequent e.l.i.s.a. This is exactly what is observed in Figure 6: the amount of ANter or ACter antibodies in the supernatant, and their resulting reactivity against the coated N-terminal or C-terminal peptide respectively, markedly decreased as the concentration of HSR vesicles was increased. For the ANter antibodies we observed a residual signal even at high HSR concentrations; this signal was totally abolished by the free peptide. A likely explanation for this residual signal is the presence of a population of antibodies recognizing the peptide but not the protein (because of a conformational difference). When free peptide was added at the same time as HSR vesicles to the antibodies, all of the antibodies were trapped and no further reactivity was detected in the back-titration experiments. This clearly demonstrates that the adsorption of the HSR vesicles on to the microtitration plate does not modify the accessibility of the two ends of the RyR.

Accessibility of the C-terminal end of the RyR to carboxypeptidase A

The accessibility of the C-terminal end of the RyR was assayed with another kind of tool: the ability of carboxypeptidase A to degrade it in the membranes of HSR vesicles. The extent of proteolytic digestion was determined by means of immunoblotting with ACter antibodies (Figure 7). As the incubation time with carboxypeptidase A increased, the signal observed with ACter antibodies decreased, indicating that the C-terminal end of the RyR was degraded and thereby became unable to react with the antibodies. The intact RyR (apparent molecular mass 430 kDa) appeared to be more sensitive to degradation than did the 350 kDa fragment, probably because of a slightly different organization of the fragment in the membrane due to the loss of a quite large portion of the N-terminal end (about 160 kDa).

This demonstrates that the C-terminal end of the RyR is accessible to carboxypeptidase A added externally to HSR vesicles, providing further evidence that the C-terminal end of the RyR is cytosolic.

DISCUSSION

In this study we have used immunological techniques combined with proteolysis to localize the two ends of the RyR within the membrane of HSR vesicles. These techniques have already been used to study the topography of numerous proteins, including the mitochondrial phosphate carrier (Capobianco et al., 1991),

the (Ca^{2+}–Mg^{2+})-ATPase of the SR (Matthews et al., 1989), the H^+-ATPase of *Neurospora* plasma membranes (Mandala and Slayman, 1989), the multidrug transporter (Yoshimura et al., 1989) the mitochondrial ADP/ATP carrier (Brandolin et al., 1989), the glucose transporter of the human erythrocyte membrane (Davies et al., 1987) and the acetylcholine receptor (Ratnam et al., 1986) (for a review on the different techniques and proteins studied, see Jennings, 1989). In the study presented here, two peptides, corresponding to the N-terminal and C-terminal ends of the RyR, were synthesized and used to produce polyclonal anti-peptide antibodies, specific to the RyR. These antibodies were then used to determine the localization of the two extremities of the RyR within the reticulum membrane.

In the first part of this work we have demonstrated the specificity of the antibodies for the RyR either by e.l.i.s.a. using the peptides and the purified RyR or by immunoblot analysis against total HSR protein and the purified RyR. Moreover, we show that these antibodies can be used for immunolocalization of the RyR in muscle sections. We have also demonstrated that the HSR vesicles are completely sealed and impermeable to rather small molecules such as inulin (5200 kDa), and therefore also to molecules such as antibodies and carboxypeptidase A. We also show that antibodies directed against calsequestrin, a protein known to be specifically localized in the intraluminal part of the SR, are unable to react with the membrane vesicles. Finally, we show (i) that the antibodies directed against the N-terminal or C-terminal regions of the RyR are able to react with the RyR in the membrane of the SR vesicles, and (ii) that the C-terminal region of the RyR in HSR vesicles can be degraded by carboxypeptidase A. Taken together, these results allow us to conclude that both the N-terminal and the C-terminal regions of the RyR are located on the cytoplasmic side of the SR membrane.

Two theoretical models have been proposed for the topography of the RyR in the SR membrane. One features four transmembrane segments (Takeshima et al., 1989), and the other proposes between 10 and 12 transmembrane α-helices (Zorzato et al., 1990). Although our results do not allow us to discriminate between the two models, we show that the two extremities of the RyR are localized on the same side of the membrane, thus disallowing a model with an odd number of transmembrane segments.

Different proteolysis experiments have been reported either with the purified RyR (Marks et al., 1990) or with the membrane-embedded RyR (Chu et al., 1988; Shoshan-Barmatz and Zarka, 1988; Meissner et al., 1989). However, the experiments performed on the membrane-embedded RyR were not followed by the sequencing of the proteolytic fragments and thus could not lead to the identification and the localization of the sites of proteolysis. The experiments performed on the purified RyR were followed by the identification of the proteolytic fragments, but it is not possible to conclude that the sites that are accessible on the purified protein are also accessible on the membrane-embedded protein, even with the use of an algorithm to predict the probability of the proteolysis sites being located on the surface.

More recently, a calcium binding and regulatory site has been identified on the RyR (Chen et al., 1992). In that study, antibodies were raised against different fusion proteins, including the segment between residues 4478 and 4512. Different results with these antibodies (immunoprecipitation of the purified RyR, and effect of the antibodies on the RyR re-incorporated in an artificial lipid bilayer) have led the authors to conclude that this part of the protein is surface-exposed. Nevertheless, none of these results was obtained with RyR in native SR membranes.

Only few experimental data concerning the identification and localization of the different regions of the RyR are available at present, and the topographic study of this large intrinsic protein is still at an early stage.

In conclusion, the results presented in this paper provide the first experimental evidence that both ends of the RyR are located on the cytoplasmic side of the SR membrane. Anti-peptide antibodies such as those used in our study are tools not only for the localization of the different parts of the protein, but also for a more precise definition of the proteolytic pattern of the RyR and for the identification of the functionally important regions involved in regulation of the calcium channel.

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