Identification of the domain recognized by anti-(ryanodine receptor) antibodies which affect Ca^{2+} -induced Ca^{2+} release

Susan TREVES, Paola CHIOZZI and Francesco ZORZATO

Istituto di Patologia Generale, Università degli Studi di Ferrara, Via Borsari 46, 44100 Ferrara, Italy

In the present paper we have defined putative functional domains of the ryanodine receptor Ca^{2+} channel. cDNA fragments of the skeletal muscle ryanodine receptor were fused in-frame with the *Escherichia coli* trpE protein and the resulting fusion proteins were evaluated for their ability to react with anti-(ryanodine receptor) antibodies, which are known to block Ca^{2+} -dependent activation of the Ca^{2+} -release channel. Anti-(ryanodine receptor) antibodies react with epitopes lying within a 245-amino-acid-long polypeptide which is located in a region (residues 4380–4625) encompassing most of myoplasmic loop 2, the predicted trans-membrane segment M_5 and part of the next luminal loop (45

residues). Purification of the anti-(ryanodine receptor) antibodies by affinity chromatography led to the isolation of a population of antibodies which was capable of decreasing (by > 30 %) the doxorubicin-induced Ca^{2+} release from isolated terminal cisternae. Polyclonal antibodies raised against a ryanodine receptor fusion encompassing part (198 out of 245 residues) of the immunopositive polypeptide decreased by 2-fold the first-order rate constant of Ca^{2+} -induced $^{45}\text{Ca}^{2+}$ efflux from isolated terminal cisternae. These results suggest strongly that the Ca^{2+} -activating domain of the skeletal muscle Ca^{2+} -release channel is close to, or associated with, myoplasmic loop 2.

INTRODUCTION

The sarcoplasmic reticulum is an intracellular membrane compartment which controls the myoplasmic Ca^{2+} concentration, thereby playing an important role in the excitation–contraction coupling mechanism (Endo, 1985). Skeletal muscle contraction is initiated by Ca^{2+} release from terminal cisternae (Somlyo et al., 1985), the portion of the sarcoplasmic reticulum that is functionally associated, via feet structures, to invaginations of the plasmalemma called the transverse tubules (Franzini-Armstrong, 1980; Kawamoto et al., 1986). Electrophysiological studies have shown the existence of two types of Ca^{2+} -release channels in sarcoplasmic reticulum membranes: (i) a low-conductance channel evenly distributed throughout the sarcoplasmic reticulum membrane; and (ii) a high-conductance channel which appears to be selectively localized in the junctional sarcoplasmic reticulum (Smith et al., 1986). The high-conductance channel is modulated by a variety of agents (Smith et al., 1986; Palade, 1987) including Ca^{2+} , ATP, Mg^{2+} , doxorubicin and ryanodine, a plant alkaloid that has been used to identify and purify the molecular component of the Ca^{2+} -release channel (Pessah et al., 1986; Inui et al., 1987; Imagawa et al., 1987; Lai et al., 1988; Hymel et al., 1988). The ryanodine receptor (RyR) is comprised of four subunits, each having a molecular mass of 565 kDa (Takeshima et al., 1989; Zorzato et al., 1990; Otsu et al., 1990; Nakai et al., 1990; Fujii et al., 1991). After reconstitution in the planar lipid bilayer, the RyR forms a cation channel whose pharmacological characteristics, conductance and ion selectivity match closely those of the native channel (Smith et al., 1988; Lai et al., 1988). Morphologically, the purified RyR is identical to the feet structures (Inui et al., 1987) whose protein constituents had previously been identified as the high-molecular-mass junctional foot protein by Kawamoto et al. (1986). Because of the functional and structural properties of the RyR, it has been postulated that it plays a crucial role in both transverse-tubule–sarcoplasmic-reticulum functional coupling and Ca^{2+} release (Rios and Pizarro, 1991).

The study of the structural and functional relationship of the RyR is hampered by its size; in fact, it is the largest protein whose primary and predicted secondary structures have been determined (Takeshima et al., 1989; Zorzato et al., 1990; Otsu et al., 1990; Nakai et al., 1990; Fujii et al., 1991). Nevertheless, useful information regarding this channel may be obtained by comparing the molecular and functional properties of naturally occurring mutants (Knudson et al., 1990; Fill et al., 1990; Fujii et al., 1991) or by defining functional domains by means of sequence-specific probes. We have previously used anti-RyR antibodies (Abs) to study the functional properties of the Ca^{2+} -release channel (Zorzato et al., 1989; Fill et al., 1991). We showed that polyclonal Abs raised against the SDS-denatured 565 kDa subunit of the RyR were capable of affecting the Ca^{2+} -dependent gating of the channel. In the present paper we have mapped the antigenic domain recognized by these anti-RyR Abs. Furthermore, we studied the effect on Ca^{2+} release of a polyclonal Ab raised against a RyR fusion protein covering part of the immunopositive polypeptide. Our results provide evidence concerning a domain that may be involved in the Ca^{2+} -dependent regulation of the skeletal muscle RyR Ca^{2+} channel.

MATERIALS AND METHODS

Materials

Nitrocellulose was from Schleicher and Schuell; $^{45}\text{Ca}^{2+}$ was from Du Pont; restriction enzymes and DNA-modifying enzymes were from Boehringer Mannheim and Pharmacia LKB Biotechnology; doxorubicin was a gift from Dr. P. Volpe (Institute of General Pathology, University of Padova, Padova, Italy); Ruthenium Red was from Fluka; alkaline phosphatase-conjugated anti-(guinea pig IgG), anti-(chicken IgG) and protein molecular mass markers were from Sigma; the Bluescript cloning vector was from Stratagene; pATH vectors were gifts from Dr. C. Wayne and Dr. D. H. MacLennan (Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada). All chemicals were reagent grade.

DNA manipulations and production of the *trpe* fusion protein

DNA manipulations were carried out according to standard protocols as described in Maniatis et al. (1989). cDNA fragments from the rabbit skeletal muscle RYR cloned in Bluescript (Zorzato et al., 1990) were fused in-frame with the *trpe* gene by ligation into the appropriate pATH vector restriction enzyme sites (Koerner et al., 1991). *Escherichia coli* (JM 101 strain) were transformed with the pATH constructs. Cells containing the correct construct were induced to produce fusion proteins as described by Koerner et al. (1991). The cultures were sedimented at 2500 g_{max} for 10 min in an ALC 4246A centrifuge and the cell pellet was resuspended in one-tenth the initial volume in a solution containing 10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM dithioerythritol (DTE), 1 μ g/ml leupeptin, 100 μ M phenylmethanesulphonyl fluoride (PMSF) and 3 mg/ml lysozyme. The suspension was incubated at room temperature (18–20 °C) for 30 min, and then homogenized for 2 min at 4 °C with a Sorvall omnimixer at setting 8. The homogenate was then sedimented at 2500 g_{max} for 15 min and the resulting pellet was resuspended in 10 mM Tris/HCl, pH 7.5, 0.3 M NaCl, 1 mM DTE, 1 μ g/ml leupeptin, 100 μ M PMSF, 10 μ g/ml DNAase and 10 μ g/ml RNAase. After a 10 min incubation at room temperature, the extract was sedimented at 2500 g_{max} . The final pellet was resuspended in 1.0–1.5 ml of 10 mM Tris/HCl, pH 7.5, 100 μ M PMSF and 1 μ g/ml leupeptin. The fractions were stored at –20 °C until used. Protein concentration was determined by the Lowry et al. (1951) or the Coomassie G250 (Bradford, 1976) methods using BSA as a standard.

SDS/PAGE and immunological techniques

Slab gel electrophoresis was carried out as described by Laemmli (1970). Slab gels were stained with Coomassie Brilliant Blue and destained in 50 % methanol/10 % acetic acid. Western blots of proteins in the bacterial extracts were carried out as described by Gershoni et al. (1985). Indirect immunoenzymic staining of Western blots was carried out as described by Young et al. (1985), with a slight modification: non-specific binding sites on the nitrocellulose were blocked by incubating the membranes overnight at 4 °C in a solution containing 10 mM Tris/HCl pH 8.0, 150 mM NaCl and 10 % fresh low-fat milk. To eliminate background staining, both the anti-RYR Abs (Volpe et al., 1988; Zorzato et al., 1989) and alkaline phosphatase-conjugated anti-guinea pig Abs were preadsorbed on *E. coli* JM 101 extracts and then incubated with the blotted proteins (Maniatis et al., 1989). Anti-RYR Abs affinity-purified on fusion protein gels were obtained as described by Bisson and Schiavo (1986). Briefly, 3 ml of a solution containing 100 μ g/ml anti-RYR Abs was incubated with nitrocellulose membranes on to which fusion protein PC8 (see Figure 1) had previously been blotted. The Abs which bound to the fusion protein were eluted with 0.2 M glycine, pH 2.8. The same anti-RYR Ab solution was subjected to five cycles of purification. The eluted Abs were pooled, dialysed against PBS and concentrated by using Amicon Centricon-30 micro-concentrators. Anti-(fusion protein PC15) Abs were raised in chickens as previously described (Zorzato et al., 1989) using electrophoretically purified fusion protein PC15 as antigen. Immunoglobulins were extracted from serum by Na_2SO_4 precipitation as described by Orlans et al. (1961), and further purified by using DEAE-cellulose anion-exchange column chromatography as described by Harlow and Lane (1988). The immunoglobulin fraction was dialysed against PBS and batch-extracted for 3 h at room temperature with CNBr-Sepharose coupled to the bacterial protein *trpe*. The suspension was centrifuged for 10 min at 3000 g_{max} , and the resulting supernatant

was incubated with an equal volume of 36 % Na_2SO_4 . The precipitates were resuspended in 1 ml of PBS and dialysed overnight at 4 °C against PBS.

Ca^{2+} -release assays

Sarcoplasmic reticulum was isolated and fractionated into light and terminal cisternae fractions according to Saito et al. (1984). Isolated terminal cisternae were incubated at 4 °C for 10 min with the fusion-protein–affinity-purified anti-RYR Ab. Doxorubicin-induced Ca^{2+} release was measured as described by Palade (1987) in a Beckman DU 7400 diode array spectrophotometer by monitoring the change in absorbance (710–790 nm) of antipyrylazo III. $^{45}\text{Ca}^{2+}$ efflux from isolated terminal cisternae was measured as described by Nakamura et al. (1986) with slight modifications. Briefly, terminal cisternae vesicles (3–4 mg/ml) were incubated with either preimmune IgG or anti-(PC15 fusion protein) Abs at 0–4 °C for 15–16 h in a solution containing 50 mM K-Mops, pH 7.0, 90 mM KCl, 5 mM CaCl_2 and $^{45}\text{Ca}^{2+}$ (approx. 50000 c.p.m./nmol). Passively loaded vesicles were diluted 200-fold in an ice-cold (0–1 °C) solution containing 50 mM Mops, pH 7.0, 90 mM KCl, 5 mM EGTA and different quantities of CaCl_2 and MgCl_2 , so that Ca^{2+} efflux was measured either at pCa 4.5 or at pCa 8/pMg 2. Aliquots of diluted terminal cisternae vesicles were filtered (approx. 3 ml/s) through Whatmann GF/B filters, which were rapidly washed with 2 $\times$ 4 ml of an ice-cold solution containing 90 mM KCl, 5 mM MgSO_4 and 10 μ M Ruthenium Red. $^{45}\text{Ca}^{2+}$ trapped in terminal cisternae vesicles was measured by scintillation counting. Free Ca^{2+} and Mg^{2+} concentrations were determined using the computer program of Fabiato (1988).

RESULTS

Figure 1 shows the strategy used to carry out in-frame fusion of rabbit skeletal muscle RYR cDNA fragments to the *E. coli trpe* gene contained in the pATH vectors. To cover the entire coding region of the RYR we made 14 constructs which contain cDNA fragments defined by the following residues: PC1, *Bam*H1 (844)–*Eco*R1 (vector/3516); PC2, *Eco*R1 (2395)–*Eco*R1 (vector/3516); PC3, *Eco*R1 (vector/3231)–*Eco*R1 (vector/4909); PC4, *Bam*H1 (4894)–*Eco*R1 (vector/9140); PC5, *Xho*I (6464)–*Eco*R1 (11973); PC6, *Bam*H1 (10983)–*Sph*I (13143); PC7, *Eco*R1 (11973)–*Eco*R1 (vector/15241); PC8, *Bam*H1 (10983)–*Clal* (14313); PC9, *Bgl*II (14328)–*Eco*R1 (vector/15241); PC10, *Eco*R1 (vector/121)–*Bam*H1 (844); PC11, *Sma*I (13290)–*Clal* (14313); PC12, *Sac*I (14825)–*Eco*R1 (vector/15241); PC13, *Bam*H1 (844)–*Eco*R1 (2395); PC14, *Acc*I (13885)–*Clal* (14313) (Takeshima et al., 1989). The fusion proteins produced by the cells contained at their N-termini 323 residues encoded by the *E. coli trpe* gene, followed by the translation product of the fused cDNA. The production of fusion proteins was monitored by SDS/PAGE analysis of cell extracts from induced cultures. As indicated in Figure 2, the fusion proteins appear to be the prominent protein components of the cell extracts and can be identified on the basis of their abundance and absence in extracts from induced and uninduced cultures respectively (Figure 2a) (Koerner et al., 1991). A further criterion used to define the identity of the fusion proteins was the evaluation of their apparent molecular mass. Lanes 3 and 4 of Figure 2(a) show the protein compositions of bacteria which have been transformed with different pATH vectors containing the same PC9 cDNA fragment. When the cDNA is fused out-of-frame (Figure 2a, lane 3), the resulting protein has an apparent molecular mass that does not correspond to the expected one, while the proteins encoded

Figure 1 Fusion protein production strategy

The top line indicates the size of the full-length rabbit RYR cDNA (Zorzato et al., 1990); the numbering is positive beginning at the first nucleotide of the initiator methionine. The underlying segments indicate the sizes of the rabbit RYR cDNA fragments cloned into pATH vectors. Synthesis of fusion proteins PC7, PC9 and PC12 was terminated at the stop codon of the RYR cDNA. The signs (+ and -) on the right indicate positive or negative reactivity of the fusion proteins with anti-RYR Abs.

Figure 2 SDS/PAGE of terminal cisternae and bacterial cell extracts

Proteins of the terminal cisternae fraction and bacterial cell extracts were electrophoretically separated in a 7.5% (a and b) or 10% (c), 1.5 mm-thick, SDS/polyacrylamide gel and stained with Coomassie Brilliant Blue. Asterisks indicate fusion proteins. G, 160 kDa glycoprotein; ATPase, Ca^{2+} -ATPase; CS, calsequestrin. (a) Lane 1, 30 μg of terminal cisternae; lane 2, 50 μl of *E. coli* JM 101 extracts from uninduced culture; lane 3, 71 μg of bacterial cell extracts in which the PC9 fragment is inserted out of frame. Lanes 4-11 contained the following amounts of the indicated fusion proteins: 4, 77 μg ; 5, 36 μg ; 6, 60 μg ; 7, 74 μg ; 8, 80 μg ; 9, 70 μg ; 10, 77 μg ; 11, 81 μg . (b) Lanes: 1, 30 μg of terminal cisternae; 2, 20 μg of PC10; 3, 20 μg of PC13; 4, 55 μg of PC2; 5, 18 μg of PC11. (c) Lanes: 1, 30 μg of terminal cisternae; 2, 48 μg of PC14; 3, 50 μg of PC12.

by the constructs having the cDNA fused in-frame to the *trpe* gene (Figure 2a, lanes 4-11; Figure 2b, lanes 2-5; Figure 2c, lanes 2 and 3) exhibit, within experimental error, the expected sizes. We succeeded in efficiently expressing very large fusion proteins; nevertheless, proteolysis occurred even in the presence of anti-proteolytic agents. PC7, a fusion protein containing 10 of the 12 putative transmembrane segments, displayed a high degree of degradation.

Immunological characterization of the *trpe*-RYR fusion protein

The purpose of these experiments was to determine precisely the antigenic domain(s) recognized by polyclonal anti-RYR Abs which have previously been used to study the functional and structural properties of Ca^{2+} release from the sarcoplasmic reticulum (Zorzato et al., 1989; Fill et al., 1991). Figure 3 shows the indirect immunoenzymic staining of Western blots of terminal cisternae and cell extracts containing fusion proteins. As expected, the anti-RYR Abs recognized their antigen after blotting on to nitrocellulose (Figure 3a, lane 1). Comparison

between Figure 1 and Figures 3(a) and 3(b) indicates that the major domains recognized by the anti-RYR Abs are located within fusion proteins PC11 and PC8, in particular in a region defined by residues 4380-4625 (Zorzato et al., 1990). There was some immunoreactivity with the fusion protein PC7, in a region that is also common to PC11 and PC8. The lanes containing bacterial cell extracts were overloaded so that the amount of protein present was greater than the amount of RYR [compare the content of the RYR band in the terminal cisternae fraction (Figures 2a-2c, lane 1) with the band of fusion proteins]. This procedure and the lability of the fusion proteins probably account for the smear of immunological reactivity observed with PC8 and PC7. In fact, when smaller quantities of PC8 were loaded on the gel, a discrete band was stained by the anti-RYR Ab (Figure 3a, lane 11).

Effect of PC8-affinity-purified anti-RYR Abs on Ca^{2+} release

To investigate the role of the region encompassed by residues 4380-4621 in Ca^{2+} release, we studied the effect of PC8-affinity-

Figure 3 Indirect immunoenzymic staining of Western blots

Proteins present in the terminal cisternae fraction and bacterial cell extracts were separated on an SDS/7.5%-polyacrylamide gel and transferred on to a nitrocellulose filter as described in the Materials and methods section. Indirect immunoenzymic staining of Western blots was carried out using guinea pig anti-RYR antibodies (final concentration 2 $\mu\text{g}/\text{ml}$). Abbreviations and protein concentrations are as in Figure 2. A 10 μg portion of protein was loaded on lane 11 of (a) and the blot was probed with anti-RYR Ab (5 $\mu\text{g}/\text{ml}$).

purified anti-RYR Abs on doxorubicin-induced Ca^{2+} release (Zorzato et al., 1986). The PC8-affinity-purified Abs were first probed on a Western blot of terminal cisternae; as indicated in

Table 1 Effect of PC8-affinity-purified anti-RYR antibodies on the initial rate of doxorubicin-induced Ca^{2+} release

Values are means $\pm$ S.E.M. of n measurements in parentheses. Doxorubicin-induced Ca^{2+} release was measured as described in Figure 4B at an Ab/terminal cisternae protein ratio of 0.4.

	Preimmune IgG		PC8-affinity-purified anti-RYR Ab
	–	+	
Doxorubicin-induced Ca^{2+} release ($\mu\text{mol}/\text{min}$ per mg of protein)	1.15 ± 0.05 (3)	1.13 ± 0.09 (3)	0.79 (2)

Figure 4(a), these Abs recognized a high-molecular-mass band which corresponds to the RYR monomer. The procedure used to affinity purify the Abs was apparently highly efficient, since no immunoreactive RYR band was visible when terminal cisternae were probed with the unbound antibody solution (results not shown). Figure 4(b) shows the recordings of a Ca^{2+} -release experiment from both control terminal cisternae (trace A) and terminal cisternae preincubated with PC8-affinity-purified anti-RYR Ab (trace B). Terminal cisternae were loaded with Ca^{2+} (approx. 1.3 μmol of Ca^{2+} /mg of protein) and, after steady state was reached, Ca^{2+} release was elicited by adding 100 μM doxorubicin to the spectrophotometer cuvette. Doxorubicin-induced Ca^{2+} release is Ca^{2+} -dependent and is fully activated in the presence of a free Ca^{2+} concentration ranging from submicromolar to 8–10 μM (Abramson et al., 1988). The estimated external free $[\text{Ca}^{2+}]$ following active Ca^{2+} loading into terminal cisternae vesicles (Palade, 1987) should fall within the submicromolar range, a concentration at which doxorubicin elicits maximal stimulation of Ca^{2+} release. Preincubation of terminal cisternae with preimmune IgG did not affect the ability

Figure 4 Effect of PC8-fusion-protein-affinity-purified anti-RYR Ab on doxorubicin-induced Ca^{2+} release

(a) Terminal cisternae were electrophoretically separated on an SDS/polyacrylamide gel and blotted on to nitrocellulose as in Figure 3. The blot was probed with PC8-affinity-purified anti-RYR Ab (0.5 $\mu\text{g}/\text{ml}$). (b) Where indicated, 45 μg of terminal cisternae (TC) was added to the cuvette. Three consecutive additions of 15 nmol of CaCl_2 were administered (arrows). After completion of Ca^{2+} loading, 100 μM doxorubicin was added. A downward deflection indicates Ca^{2+} accumulation; an upward deflection indicates Ca^{2+} release. The ordinate axis is $A_{710} - A_{790}$. At the end of the experiment, 15 nmol of CaCl_2 was added to calibrate the dye response. Trace A, control terminal cisternae; trace B, terminal cisternae pretreated with PC8-affinity-purified anti-RYR Ab at an Ab/protein ratio of 0.4.

Figure 5 SDS/PAGE and indirect immunoenzymic staining of the PC15 fusion protein

(a) A 40 μ g sample of PC15 bacterial extracts were separated in an SDS/10% polyacrylamide gel and stained with Coomassie Brilliant Blue. Also shown is the full-length rabbit RYR cDNA. Numbering of nucleotides is positive, beginning at the first nucleotide of the initiator codon. The underlying segment indicates the size of the cDNA cloned into the pATH vector. (b) Indirect immunoenzymic staining of the Western blot with anti-(PC15 fusion protein) Ab (final concentration 2 μ g/ml). Lanes: 1, 20 μ g of terminal cisternae; 2, 20 μ g of PC15 fusion protein.

Table 2 Effect of anti-(PC15 fusion protein) Ab on $^{45}\text{Ca}^{2+}$ efflux from isolated terminal cisternae

Values are means $\pm$ S.E.M. of n values shown in parentheses; * $P < 0.01$ compared with control. Terminal cisternae were incubated with Ab overnight at 0–4 $^{\circ}\text{C}$ at an Ab/terminal cisternae ratio of 1. Ca^{2+} loading and measurement of $^{45}\text{Ca}^{2+}$ efflux were carried out as described in the legend to Figure 6. Free $[\text{Ca}^{2+}]$ and $[\text{Mg}^{2+}]$ were calculated by the program of Fabiato (1988). The first-order rate constants were determined by a computer fit of the data points using ENZFITTER program.

	k for Ca^{2+} efflux (s^{-1})	
	Preimmune IgG	Anti-(PC15 fusion protein) Ab
pCa8/pMg 2	0.0095 ± 0.0030 (5)	0.010 ± 0.003 (3)
pCa 4.5	0.25 ± 0.02 (5)	0.11 ± 0.01 (5)*

Figure 6 Effect of anti-(PC15 fusion protein) Ab on $^{45}\text{Ca}^{2+}$ efflux from passively loaded terminal cisternae vesicles

Terminal cisternae vesicles were passively loaded with $^{45}\text{Ca}^{2+}$ in the presence of preimmune IgG and anti-(PC15 fusion protein) Ab as described in the Materials and methods section. Ca^{2+} efflux was carried out at pCa 4.5. Curves were obtained by a computer fit of the data points using the ENZFITTER program. $^{45}\text{Ca}^{2+}$ trapped by terminal cisternae vesicles at the time of dilution was determined by back-extrapolation of a Ca^{2+} efflux curve in the presence of 10 nM free Ca^{2+} and 10 mM free Mg^{2+} . The free Ca^{2+} and Mg^{2+} concentrations were determined by using the computer program of Fabiato (1988). ■, Terminal cisternae incubated with anti-(PC15 fusion protein) Ab; ▲, terminal cisternae incubated with preimmune IgG.

of doxorubicin to elicit Ca^{2+} release from terminal cisternae (Table 1). PC8-affinity-purified anti-RYR Abs at an Ab/terminal cisternae ratio of 0.4 caused a decrease of approx. 30% in the initial rate of net doxorubicin-induced Ca^{2+} release. Doxorubicin induces Ca^{2+} release via Ca^{2+} -regulated Ca^{2+} channels (Palade, 1987; Abramson et al., 1988). The decrease in doxorubicin-induced Ca^{2+} release could be due to an alteration of the allosteric regulation of the channel. Thus we raised a polyclonal Ab against fusion protein PC15, which contains part (residues 4425–4621) of the immunopositive polypeptide (residues 4380–4621), and tested the effect of these Abs on Ca^{2+} -induced Ca^{2+} release from passively loaded terminal cisternae vesicles.

Effect of anti-(PC15 fusion protein) Abs on Ca^{2+} -induced Ca^{2+} release

Figure 5(a) shows a Coomassie Blue-stained SDS gel of PC15 fusion protein. Anti-(PC15 fusion protein) Ab stained the RYR band of terminal cisternae (Figure 5b, lane 1) and PC15 fusion protein (lane 2). We also observed immunological reactivity with a protein band having an electrophoretic mobility slightly higher than that of PC15 fusion protein; this may represent a proteolytic product of the PC15 fusion protein. The labelling was specific, as the preimmune IgG did not stain any band in the fraction (results not shown). Terminal cisternae vesicles were passively loaded (approx. 60 nmol of Ca^{2+} /mg of protein) in the presence of either preimmune IgG or anti-(PC15 fusion protein) Abs. In the latter case the first-order rate constant of Ca^{2+} -induced Ca^{2+} release decreased by 2.3-fold (Table 2 and Figure 6). Anti-(PC15 fusion protein) Abs did not affect the inhibition of Ca^{2+} release by Mg^{2+} (Table 2).

DISCUSSION

Recent studies have shown that the RYR is a large homotetrameric oligomer which acts as a Ca^{2+} -release channel. This protein is also the major constituent involved in coupling

Figure 7 Antigenic domain identified by anti-RYR Ab

The antigenic domain defined by residues 4380–4625 is indicated by the thick line in (b). Transmembrane segments are indicated by open rectangles (M' , M'' and M_1 – M_{10} from left to right). (a) Hopp and Woods (1981) plot relative to the antigenic domain was carried out with a window of six amino acids.

the transverse tubules to the junctional sarcoplasmic reticulum (Kawamoto et al., 1986; Inui et al., 1987; Smith et al., 1988; Lai et al., 1988).

Analysis of the primary and predicted secondary structure of the RYR monomer indicates that the last 1000 C-terminal residues of the molecule may contain up to 10 transmembrane segments, while the N-terminal hydrophilic portion of the molecule is predicted to contain: (a) four repeats arranged in two tandem pairs, separated by a 1600-residue-long region containing an acidic domain; and (b) an unstructured region enriched in glycine, alanine and proline residues (GAP-rich); a second GAP-rich region is also present between the transmembrane segments M_4 and M_5 . The predicted primary sequence of the skeletal muscle RYR, however, has shed little light on the localization of putative functionally relevant domains (e.g. regions which may interact with known channel modulators such as Ca^{2+} , ATP, Mg^{2+} and calmodulin) (Takeshima et al., 1989; Otsu et al., 1990; Nakai et al., 1990). In this study we have defined a RYR domain which seems to be involved in the Ca^{2+} -dependent gating of the Ca^{2+} -release channel. We show that the major antigenic domain of the anti-RYR Ab capable of affecting Ca^{2+} -dependent channel activation (Zorzato et al., 1989; Fill et al., 1991) is defined by a 245-residue-long sequence which includes 179 residues of myoplasmic loop number 2, the transmembrane segment M_5 and 45 N-terminal residues of the next luminal loop (Zorzato et al., 1990). This result cannot rule out the co-existence of other Abs directed against complex conformational epitopes located in a region crucial for channel gating, which may not be revealed by Western blotting. We believe that this possibility is unlikely, however, since we raised the anti-RYR Ab using the SDS-denatured RYR 565 kDa monomer as antigen (Volpe et al., 1988). Various pieces of direct and indirect evidence suggest that the domain defined by residues 4380–4625 contains regions which are relevant to the Ca^{2+} gating of the RYR Ca^{2+} channel.

First, Abs against PC15, a fusion protein containing 199 out of the 245 residues of the immunopositive polypeptide, decreased Ca^{2+} -induced Ca^{2+} release from passively loaded terminal

cisternae vesicles. The effect of the anti-(PC15 fusion protein) Ab appears to be specific for the Ca^{2+} regulatory sites, since the Ab did not affect the inhibition of Ca^{2+} release by Mg^{2+} .

Secondly, within the region defined by residues 4380–4625 there are amino acid stretches which are predicted to be antigenic (Figure 7) and unstructured, in view of the high content of glycine and proline residues (Otsu et al., 1990).

Thirdly, according to the analysis of the primary structure of the RYR by Takeshima et al. (1989), the antigenic domain defined in the present study encompasses putative high-affinity Ca^{2+} -binding sites.

Fourthly, the antigenic domain recognized by anti-RYR and anti-(PC15 fusion protein) Abs overlaps with a sequence that has been suggested to be exposed towards the myoplasmic space (Marks et al., 1990). This result implies that, in the native RYR, the antigenic domain is accessible to both the myoplasmic free Ca^{2+} and the anti-RYR Ab.

Finally, PC8-affinity-purified anti-RYR Ab decreased by 30 % the initial rate of doxorubicin-induced Ca^{2+} release, indicating that the functional effect of this Ab mimics that originally observed with the 'whole' anti-RYR Ab fraction (Zorzato et al., 1989). Doxorubicin activates the Ca^{2+} -regulated channel, and thus the effect of PC8-affinity-purified Ab on doxorubicin-induced Ca^{2+} release might be interpreted as an alteration of the allosteric regulation of the channel. This hypothesis is consistent with the data of Figure 6 and Table 2, which show that Abs raised against the immunopositive polypeptide alter the Ca^{2+} -dependent activation of the Ca^{2+} channel. We cannot exclude the possibility that the effect of the PC8-affinity-purified anti-RYR Ab on doxorubicin-induced Ca^{2+} release might be due to an inhibition of the binding of the agonist to the channel. If this is so, it is plausible that such an effect is secondary to the interaction of the Ab with a region involved in the allosteric regulation of the channel by Ca^{2+} rather than with doxorubicin binding site(s), since it has been shown that both the effect of the Ab and the binding of doxorubicin to the channel are Ca^{2+} -dependent (Zorzato et al., 1986; Abramson et al., 1988).

The exact three-dimensional conformation of the RYR is not known. Therefore one cannot rule out the possibility that the interaction of the anti-RYR Abs with the polypeptide defined by residues 4380–4625 might influence the gating domain, which is adjacent in the three-dimensional structure but may be distant in the linear sequence. In either case, the present experiments indicate that the Ca^{2+} -dependent gating domain of the RYR channel lies near the junctional sarcoplasmic reticulum membrane at the level of, or closely associated with, myoplasmic loop 2. This implies that the Ca^{2+} -gating domain is located far from the surface of the RYR, in contact with the transverse tubules, the membrane compartment that generates the trigger signal for channel opening during E–C coupling.

We thank Professor F. Di Virgilio for helpful discussions. S.T. is the recipient of a Banca Popolare fellowship. This work was supported by grants from Telethon-Italy, C.N.R. 91.02429.CT14 to F.Z. Part of these experiments were also supported by MURST 40% and 60% funds to Professor T. Pozzan and Professor P. Melandri, respectively.

REFERENCES

- Abramson, J. J., Buck, E., Salama, G., Casida, J. E. and Pessah, I. N. (1988) *J. Biol. Chem.* **263**, 18750–18758
- Bisson, R. and Schiavo, G. (1986) *J. Biol. Chem.* **261**, 4373–4376
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Endo, M. (1985) *Curr. Topics Membr. Transp.* **25**, 181–229
- Fabiato, A. (1988) *Methods Enzymol.* **157**, 378–417
- Fill, M., Coronado, R., Mickelson, J. R., Vilven, J., Ma, J., Jacobson, B. A. and Louis, C. F. (1990) *Biophys. J.* **273**, 449–457

- Fill, M., Mejia-Alvarez, R., Zorzato, F., Volpe, P. and Stefani, E. (1991) *Biochem. J.* **273**, 449–457
- Franzini-Armstrong, C. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 2403–2409
- Fujii, J., Otsu, K., Zorzato, F., DeLeon, S., Khanna, V. J., Weiler, J. E., O'Brien, P. J. and MacLennan, D. H. (1991) *Science* **253**, 448–451
- Gershoni, J. M., Davis, F. E. and Palade, G. (1985) *Anal. Biochem.* **144**, 32–40
- Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Hopp, T. P. and Woods, K. R. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3824–3828
- Hymel, L., Inui, M., Fleisher, S. and Schindler, H. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 441–445
- Imagawa, T., Smith, J. S., Coronado, R. and Campbell, K. (1987) *J. Biol. Chem.* **262**, 16636–16643
- Inui, M., Saito, A. and Fleisher, S. (1987) *J. Biol. Chem.* **262**, 1740–1747
- Kawamoto, R. M., Brunschwig, J. P., Kim, K. C. and Caswell, A. H. (1986) *J. Cell Biol.* **103**, 1405–1414
- Knudson, C. M., Mickelson, J. R., Louis, C. F. and Campbell, K. P. J. (1990) *J. Biol. Chem.* **265**, 2421–2424
- Koerner, T. J., Hill, J. E., Myers, A. M. and Tzagoloff, A. (1991) *Methods Enzymol.* **194**, 477–490
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lai, F. A., Erickson, H. P., Rosseau, E., Liu, Q. Y. and Meissner, G. (1988) *Nature (London)* **331**, 315–319
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Maniatis, T., Fritsch, E. F. and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Marks, A. R., Fleisher, S. and Tempst, P. (1990) *J. Biol. Chem.* **265**, 13143–13149
- Nakai, J., Imagawa, T., Hakamata, Y., Shigekawa, M., Takeshima, H. and Numa, S. (1990) *FEBS Lett.* **271**, 169–177
- Nakamura, Y., Kobayashi, J., Gilmore, J., Mascal, M., Rinehart, K., Nakamura, H. and Oshizumi, Y. (1986) *J. Biol. Chem.* **261**, 4139–4142
- Orlans, E., Rose, E. and Marrak, J. (1961) *Immunology* **5**, 262–267
- Otsu, K., Willard, H. F., Khanna, V. J., Zorzato, F., Green, N. M. and MacLennan, D. H. (1990) *J. Biol. Chem.* **265**, 13472–13483
- Palade, P. (1987) *J. Biol. Chem.* **262**, 6136–6141
- Pessah, I. N., Francini, A. O., Scales, D. J., Waterhouse, A. L. and Casida, J. E. (1986) *J. Biol. Chem.* **261**, 8643–8648
- Rios, E. and Pizarro, G. (1991) *Physiol. Rev.* **71**, 849–908
- Saito, A., Seiler, S., Chu, A. and Fleisher, S. (1984) *J. Cell. Biol.* **99**, 975–985
- Smith, J. S., Coronado, R. and Meissner, G. (1986) *Biophys. J.* **50**, 921–928
- Smith, J. S., Imagawa, T., Ma, J., Fill, M., Campbell, K. P. and Coronado, R. (1988) *J. Gen. Physiol.* **92**, 1–26
- Somlyo, A. V., McLellan, G., Gonzales-Serratos, H. and Somlyo, A. P. (1985) *J. Biol. Chem.* **260**, 6801–6807
- Takeshima, H., Nishimura, S., Matsumoto, H., Ishida, K., Kangawa, N., Minamino, H., Matsuo, M., Ueda, M., Hanakoa, M., Horise, T. and Numa, S. (1989) *Nature (London)* **339**, 439–445
- Volpe, P., Bravin, M., Zorzato, F. and Margreth, A. (1988) *J. Biol. Chem.* **263**, 9901–9907
- Young, R. A., Bloom, B. R., Grosskinsky, G. M., Ivanyi, J., Thomas, D. and Davis, R. W. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2583–2587
- Zorzato, F., Margreth, A. and Volpe, P. (1986) *J. Biol. Chem.* **261**, 13252–13257
- Zorzato, F., Chu, A. and Volpe, P. (1989) *Biochem. J.* **261**, 863–870
- Zorzato, F., Fujii, J., Otsu, K., Phillips, M., Green, N. M., Lai, F. A., Meissner, G. and MacLennan, D. H. (1990) *J. Biol. Chem.* **265**, 2244–2256