Antibodies to junctional sarcoplasmic reticulum proteins: probes for the Ca^{2+} -release channel

Francesco ZORZATO,*§ Alice CHU† and Pompeo VOLPE*‡

*Centro di Studio per la Biologia e la Fisiopatologia Muscolare del Consiglio Nazionale delle Ricerche, Istituto di Patologia Generale dell'Università di Padova, via Loredan 16, 35100 Padova, Italy,

†Department of Medicine, Cardiovascular Sciences Section, Baylor College of Medicine, Houston, TX 77030, U.S.A., and ‡Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, TX 77550, U.S.A.

The junctional face membrane plays a key role in excitation–contraction coupling in skeletal muscle. A protein of 350 kDa, tentatively identified as a component of the junctional feet, connects transverse tubules to terminal cisternae of sarcoplasmic reticulum [Kawamoto, Brunschwig, Kim & Caswell (1986) *J. Cell Biol.* **103**, 1405–1414]. The membrane topology and protein composition of sarcoplasmic reticulum Ca^{2+} -release channels of rabbit skeletal muscle were investigated using an immunological approach, with anti-(junctional face membrane) and anti-(350 kDa protein) polyclonal antibodies. Upon preincubation of the terminal cisternae with anti-(junctional face membrane) antibodies, Ca^{2+} -ATPase and Ca^{2+} -loading activities were not affected, whereas anti-(350 kDa protein) antibodies stimulated Ca^{2+} -ATPase activity by 25% and inhibited Ca^{2+} -loading activity by 50% (at an antibody/terminal cisternae protein ratio of 1:1). Specific photolabelling of terminal cisternae proteins with [^{14}C]doxorubicin was prevented by both anti-(junctional face membrane) and anti-(350 kDa protein) antibodies. Stimulation of Ca^{2+} release by doxorubicin was prevented by both anti-(junctional face membrane) and anti-(350 kDa protein) antibodies. Half-maximal inhibition was obtained at an antibody/terminal cisternae protein ratio of 1:1. Kinetic measurements of Ca^{2+} release indicated that anti-(350 kDa protein) antibodies prevented Ca^{2+} -induced Ca^{2+} release, whereas the ATP-stimulation and the inhibition by Mg^{2+} were not affected. These results suggest that: (i) Ca^{2+} - and doxorubicin-induced Ca^{2+} release is mediated by Ca^{2+} channels which are selectively localized in the junctional face membrane; (ii) the 350 kDa protein is a component of the Ca^{2+} -release channel in native terminal cisternae vesicles; and (iii) the Ca^{2+} -activating site of the channel is separate from other allosteric sites.

INTRODUCTION

The sarcoplasmic reticulum is an intracellular network of membranes which controls the contraction–relaxation cycle of skeletal muscle by raising and lowering the myoplasmic free Ca^{2+} concentration. The sarcoplasmic reticulum consists of two morphologically and functionally distinct portions. The ‘free’ sarcoplasmic reticulum is formed by longitudinally oriented tubules and non-junctional membranes of terminal cisternae, and is endowed with a Ca^{2+} pump (Jorgensen *et al.*, 1982; Inesi, 1985). The junctional face membrane is the portion of terminal cisternae directly facing the transverse tubules, the invaginations of the sarcolemma. The transverse tubules and terminal cisternae are connected by feet structures, which cross a gap of 10 nm separating the two membrane compartments (Franzini-Armstrong, 1980). The subunit of the feet structures is a junctional component having a molecular mass of 350 kDa, recently identified as the ryanodine receptor (Campbell *et al.*, 1987; Inui *et al.*, 1987; Lai *et al.*, 1987). The molecular mass of the feet protein has not yet been determined, and estimates range from 325 (Seiler *et al.*, 1984) to 450 kDa (Imagawa *et al.*, 1987). Throughout the text, the feet protein is also referred to as 350 kDa spanning protein

(Kawamoto *et al.*, 1986), doxorubicin-binding protein (Zorzato *et al.*, 1986) and ryanodine receptor (Imagawa *et al.*, 1987; Lai *et al.*, 1988; Hymel *et al.*, 1988).

Skeletal muscle contraction is initiated following the release of calcium from terminal cisternae (Somlyo *et al.*, 1985). Single channel recording studies on sarcoplasmic reticulum membranes incorporated in planar lipid bilayers have shown the existence of two types of Ca^{2+} channels: (i) a low-conductance channel distributed in both free and junctional sarcoplasmic reticulum, and (ii) a high-conductance channel which is selectively localized in terminal cisternae (Smith *et al.*, 1986). Ca^{2+} flux rates through Ca^{2+} channels of terminal cisternae are compatible with those expected to occur *in vivo* (Meissner *et al.*, 1986). The high-conductance Ca^{2+} channel is activated by *cis* Ca^{2+} and adenine nucleotide and is inhibited by *cis* (myoplasmic) Mg^{2+} and Ruthenium Red. Single channel recording studies have shown that the only component of such a channel is the ryanodine receptor (Lai *et al.*, 1988; Hymel *et al.*, 1988; Smith *et al.*, 1988). The kinetics and pharmacological properties of the reconstituted ryanodine receptor Ca^{2+} channel are very similar to those of the Ca^{2+} channel of the native sarcoplasmic reticulum vesicles.

In the last few years, certain functional and structural

Abbreviation used: PBS, phosphate-buffered saline (0.1 M-sodium phosphate, pH 7.2/0.15 M-NaCl).

§ Present address: C.H. Best Institute, University of Toronto, 112 College Street, Toronto, Ontario, Canada M5G 1L6.

properties of cation channels have been elucidated by means of specific antibodies (Meiri *et al.*, 1986; Malouf *et al.*, 1987; Morton *et al.*, 1988; Fitzpatrick *et al.*, 1988). In the present series of experiments, we used two polyclonal antibodies raised against junctional face membrane proteins and the 350 kDa protein (Volpe *et al.*, 1988) to investigate membrane topology and protein composition of the Ca^{2+} -gated Ca^{2+} channel of isolated terminal cisternae. After active loading, Ca^{2+} release was elicited by doxorubicin, Ca^{2+} or ATP. Both anti-(junctional face membrane) and anti-(350 kDa protein) antibodies inhibited doxorubicin-induced Ca^{2+} release, and anti-(350 kDa protein) antibodies prevented the Ca^{2+} activation of the Ca^{2+} -release channel. The present results indicate that the Ca^{2+} -gated Ca^{2+} channel is localized in the junctional face membrane and that the Ca^{2+} -activating site(s) on the channel is separate from other allosteric sites.

EXPERIMENTAL

Materials

Antipyrilazo III, sodium phosphocreatine, Ruthenium Red, creatine kinase, phosphoenolpyruvate and the ionophore A23187 were obtained from Sigma. Pyruvate kinase, lactate dehydrogenase and NADH were obtained from Boehringer-Mannheim. Doxorubicin and [^{14}C]-doxorubicin were a gift from Farmitalia Carlo Erba (Nerviano, Italy). Sepharose-Protein A was from Pharmacia. [^3H]Ryanodine and ^{45}Ca were from New England Nuclear. Ryanodine was a gift from Dr. L. R. Jones, University of Indiana, Indianapolis, IN, U.S.A. All other chemicals were reagent grade.

Isolation of sarcoplasmic reticulum fractions

Sarcoplasmic reticulum of fast-twitch rabbit skeletal muscle was isolated and fractionated into light and heavy fractions according to Saito *et al.* (1984). Sarcoplasmic reticulum fractionation was carried out in the presence of 100 μM -phenylmethanesulphonyl fluoride. Calsequestrin-containing junctional face membrane was isolated by either Triton X-100 or octaethyleneglycolmono-*n*-dodecylether (C_{12}E_8) treatment of the terminal cisternae fraction as previously described (Costello *et al.*, 1986). Calsequestrin was extracted from calsequestrin-containing junctional face membrane by incubation in 10 mM-tris(hydroxymethyl)aminomethane (Tris)/2 mM-EDTA, pH 8.0 (Duggan & Martonosi, 1970). All fractions were resuspended in 0.3 M-sucrose/5 mM, imidazole, pH 7.4 (buffer A) and stored at -70°C until used. The protein concentration was determined according to Lowry *et al.* (1951), using bovine serum albumin as standard.

Preparation of anti-(junctional face membrane) and anti-(350 kDa protein) polyclonal antibodies

The anti-(junctional face membrane) serum was raised in hen, by weekly injections into breast muscle of approx. 300 μg of calsequestrin-free junctional face membrane in buffer A diluted (1:1) with 1 ml of incomplete Freund's adjuvant. The animal was bled 35 days after beginning the immunization. The immunoglobulins were extracted from preimmune and immune sera as previously described by Orlans *et al.* (1961).

The anti-(350 kDa protein) serum was raised in guinea pig as previously described by Volpe *et al.* (1988). The

anti-(350 kDa protein) polyclonal antibodies were affinity purified according to Bisson & Schiavo (1986). Preimmune immunoglobulins were purified from guinea pig serum by Sepharose-Protein A chromatography (Goudswaard *et al.*, 1978).

Immunoblot

Proteins of sarcoplasmic reticulum fractions were electrophoretically resolved according to Laemmli (1970) in SDS/polyacrylamide linear gradient (5–15%) gels, and then transferred on to nitrocellulose sheets (blots) according to Gershoni *et al.* (1985). The transfer was carried out at constant current (50 V) for 16–18 h in a buffer containing 25 mM-Tris/192 mM-glycine, pH 8.3. The blot was preincubated for 60 min at room temperature with 0.1 M-sodium phosphate, pH 7.2/0.15 M-NaCl (PBS) and 10% low-fat milk to block binding of non-specific antibodies, and then incubated for 150 min with either anti-(junctional face membrane) antibodies or anti-(350 kDa protein) affinity-purified antibodies diluted with PBS (5 $\mu\text{g}/\text{ml}$). After a 30 min wash in PBS/10% low-fat milk, the blot was incubated either with anti-(chicken immunoglobulin) or anti-(guinea pig immunoglobulin) antibodies conjugated with alkaline phosphatase. Other steps were carried out as described by Damiani *et al.* (1986).

Effect of anti-(junctional face membrane) and anti-(350 kDa protein) polyclonal antibodies on Ca^{2+} loading, Ca^{2+} -dependent ATPase, and Ca^{2+} release

Aliquots of each sarcoplasmic reticulum fraction were incubated with different amounts of the anti-(junctional face membrane) antibodies and anti-(350 kDa protein) affinity-purified antibodies for 10 min at room temperature.

Ca^{2+} loading was measured as previously described by Mitchell *et al.* (1983), following the differential absorbance change (710–790 nm) of the Ca^{2+} indicator Antipyrilazo III in a Hewlett Packard 8451A diode array spectrophotometer. The assay was carried out at room temperature (approx. 20–22 $^\circ\text{C}$) in a medium containing, in a final volume of 1 ml, 92 mM-potassium phosphate, pH 7.0/200 μM -Antipyrilazo III/1 mM-MgATP/30 μg of sarcoplasmic reticulum protein preincubated in the presence or in the absence (control) of antibodies. The reaction was started by adding 25 nmol of CaCl_2 .

Ca^{2+} -dependent ATPase was measured by an enzyme-coupled assay following the rate of NADH oxidation in a Perkin Elmer 551 S spectrophotometer (Warren *et al.*, 1974). The assay was carried out at 37 $^\circ\text{C}$ in a medium containing, in a final volume of 3 ml, 20 mM-histidine, pH 7.2/100 mM-KCl/5 mM-MgSO $_4$ /2 mM-ATP/150 mM-NADH/0.5 mM-phosphoenolpyruvate/5 units of pyruvate kinase/5 units of lactate dehydrogenase, in the presence and absence of 1.5 μg of A23187/ml (Zorzato *et al.*, 1985). The reaction was started by adding approx. 10 μg of sarcoplasmic reticulum protein preincubated in the presence or in the absence (control) of antibodies.

Doxorubicin-induced Ca^{2+} release from terminal cisternae was measured as previously described by Palade (1987) by following the differential absorbance (710–790 nm) of the Ca^{2+} indicator Antipyrilazo III in a Hewlett Packard 8451A diode array spectrophotometer. The assay was carried out at 37 $^\circ\text{C}$ in a medium containing, in a final volume of 1 ml, 7.5 mM-potassium

pyrophosphate, pH 7.0/1.5 mM-MgATP/250 μ M-Antipyrilazo III/100 mM-KCl/5 mM-creatine phosphate, 20 μ g of creatine phosphokinase/ml/40 μ g of terminal cisternae protein preincubated in the presence or in the absence (control) of antibodies. Pulses of 20 nmol of CaCl₂ were administered to load terminal cisternae vesicles with approx. 2.5 μ mol of Ca²⁺ per mg of protein. When steady state was obtained, Ca²⁺ release was triggered by 100 μ M-doxorubicin.

Kinetic measurements of ⁴⁵Ca²⁺ release were carried out as described by Chu *et al.* (1988). Terminal cisternae vesicles previously incubated with anti-(350 kDa protein) antibodies (see above) were actively loaded at 25 °C in a medium containing, in a final volume of 1 ml, 30 μ g of sarcoplasmic reticulum protein/100 μ M-⁴⁵CaCl₂ (specific activity about 7000 c.p.m./nmol)/10 mM-MgCl₂/80 mM-KCl/20 mM-Tris/Mops, pH 7.0/2 mM-acetyl phosphate. After 2 min, the entire assay medium (1 ml) was filtered through a HAWP 0.45 μ m Millipore filter, which was then washed with 2 ml of a Mg²⁺ medium [10 mM-MgCl₂/1 mM-Tris/EGTA, 80 mM-KCl/20 mM-Tris/Mops, pH 7.0] at room temperature (22 °C) to remove residual uptake medium and acetyl phosphate. The filters retaining the loaded terminal cisternae vesicles were then flushed with various releasing and non-releasing media at different times. The velocity and time of flushing through the filters were electronically controlled by a rapid filtration apparatus (Cosmologic, Olympia, WA, U.S.A.). The radioactivity in the filters was then counted by liquid scintillation spectrometry. The release media contained 80 mM-KCl/20 mM-Tris/Mops, pH 7.0, and either 5 μ M free Ca²⁺ (51.51 μ M-CaCl₂/50 μ M-Tris/EGTA), ATP (1 mM-Na₂ATP/1 mM-Tris/EGTA), or 5 μ M-free Ca²⁺ and ATP (69.7 μ M-CaCl₂/50 μ M-Tris/EGTA/1 mM-Na₂ATP). Free ligand concentrations were calculated based on a computer program (Fabiato & Fabiato, 1979).

Binding of [³H]ryanodine

The binding of ryanodine was carried out as previously described by Fleischer *et al.* (1985) with slight modifications. The assay was carried out at 37 °C for 30 min in a medium containing, in a final volume of 0.2 ml, 0.15 M-KCl/10 mM-Hepes, pH 7.4/3 mM-ATP/0.2 mg of sarcoplasmic reticulum protein/100 nM-[³H]ryanodine (specific activity 14 500 c.p.m./pmol) in the presence and absence of 10 μ M unlabelled ryanodine. The samples were filtered through GSWP 0.22 μ m Millipore filters, washed with 4 ml of assay buffer without ryanodine and then twice with 4 ml of ice-cold 10% ethanol. The radioactivity on filters was counted in a Packard β -counter. Longitudinal sarcoplasmic reticulum and terminal cisternae bound 0.6 and 7 pmol of ryanodine respectively/mg of protein. Terminal cisternae fractions containing the ryanodine receptor were used for Ca²⁺ release studies and for obtaining junctional face membrane as well as the 350 kDa protein antigen.

Photolabelling of terminal cisternae with [¹⁴C]doxorubicin

Photolabelling was carried out as previously described (Zorzato *et al.*, 1986) with 50 μ M-[¹⁴C]doxorubicin in the absence and presence of either anti-(junctional face membrane) or anti-(350 kDa protein) antibodies. Terminal cisternae (150 μ g of protein) were preincubated with antibodies for 10 min at room temperature at an

antibody/terminal cisternae protein ratio of 1:1, before photoactivation. After SDS/polyacrylamide-gel electrophoresis (5–15% linear gradient gel), each gel lane was sliced, digested with Soluene and radioactivity counted.

Anti-(junctional face membrane) antibodies comprise the total immunoglobulin fraction, which contains both specific antibodies and other immunoglobulins. We do not exactly know the relative content of specific antibodies against each junctional face membrane protein. Thus, the actual anti-(junctional face membrane) antibodies/terminal cisternae ratio is lower than that reported. Throughout the text, when we indicate the anti-(junctional face membrane) antibodies/terminal cisternae ratio, we refer to the protein/protein ratio between the total immunoglobulin fraction and the terminal cisternae.

RESULTS

Characterization of anti-(junctional face membrane) and anti-(350 kDa protein) polyclonal antibodies

Polyclonal antibodies against calsequestrin-free junctional face membrane and the 350 kDa protein were produced in hens and guinea pigs respectively. The specificity of both antibodies was ascertained by Western blot. Fig. 1 shows the indirect immunoenzymatic staining with anti-(junctional face membrane) (lanes a, b and c) and anti-(350 kDa protein) antibodies (lanes d and e) and the Ponceau Red staining (f and g) of longitudinal sarcoplasmic reticulum (a, d and f), terminal cisternae (b) and calsequestrin-containing junctional face membrane (c, e and g). Comparison between lanes (Fig. 1) clearly indicates that anti-(junctional face membrane) antibodies stained most of the proteins of junctional

Fig. 1. Specificity of the anti-(junctional face membrane) and anti-(350 kDa protein) polyclonal antibodies

Each sarcoplasmic reticulum fraction (15 μ g) was separated in a SDS/polyacrylamide linear gradient (5–15%) gel and then transferred on to nitrocellulose sheets as described in the Experimental section. Indirect immunoenzymatic staining with anti-(junctional face membrane) (lanes a, b, and c) and anti-(350 kDa protein) antibodies (d and e) and Ponceau Red staining (f and g) of longitudinal sarcoplasmic reticulum (a, d and f), terminal cisternae (b) and junctional face membrane (c, e and g). Apparent molecular masses were determined as described in Zorzato *et al.* (1986). Abbreviations: ATPase, Ca²⁺-ATPase; CS, calsequestrin.

Fig. 2. Effect of anti-(junctional face membrane) and anti-(350 kDa protein) antibodies on photoaffinity labelling of terminal cisternae with [^{14}C]doxorubicin

Photoactivation and SDS/polyacrylamide-gel electrophoresis were carried out as described in the Experimental section. About 150 μg of protein were applied per lane. Each gel lane was sliced and each slice was digested with Soluene and radioactivity counted. Control terminal cisternae ($\circ$); plus anti-(junctional face membrane) antibodies (antibody/terminal cisternae protein ratio of 1:1; $\bullet$); plus anti-(350 kDa protein) antibodies (antibody/terminal cisternae protein ratio of 1:1; $\blacksquare$). Numbers represent molecular mass (kDa) of labelled peptides. The 60 kDa polypeptide was labelled to a greater extent than in previous experiments (Zorzato *et al.*, 1986).

sarcoplasmic reticulum; in particular, the immuno-reactivity was noticeable with components of molecular mass 350, 200, 118, 63 and 60 kDa. The terminal cisternae fraction exhibited an immunoreactive profile with anti-(junctional face membrane) antibodies similar to that of calsequestrin-containing junctional face membrane, but, as expected, it was less marked. In longitudinal sarcoplasmic reticulum (non-junctional sarcoplasmic reticulum) two bands were weakly stained by the anti-(junctional face membrane) antibodies (lane a): a negligible amount of contaminating calsequestrin and a 38 kDa polypeptide, i.e. aldolase (E. Damiani & P. Volpe, unpublished work), uniformly distributed throughout the sarcoplasmic reticulum membrane.

As illustrated in lanes e and g, the anti-(350 kDa protein) affinity-purified antibodies recognized a polypeptide of 350 kDa. Longitudinal sarcoplasmic reticulum did not show any reactivity (lane d). Preimmune sera from either hen or guinea pig did not stain any sarcoplasmic reticulum protein (results not shown).

Effect of anti-(junctional face membrane) and anti-(350 kDa protein) antibodies on photoaffinity labelling of terminal cisternae with [^{14}C]doxorubicin

Before photoactivation, terminal cisternae were preincubated in the absence or presence of either anti-

Fig. 3. Effect of anti-(junctional face membrane) antibodies on the functional properties of terminal cisternae

Terminal cisternae (TC) were preincubated with increasing amounts of antibodies for 10 min at room temperature. Data are expressed as % of control values. Ca^{2+} release rate: at the end of the preincubation, terminal cisternae were actively loaded with approx. 2.5 μmol of Ca^{2+} /mg of protein. After completion of loading, Ca^{2+} release was triggered by 100 μM -doxorubicin. Data from three different terminal cisternae preparations are plotted ($\circ$, $\bullet$, $\odot$). Control Ca^{2+} release rate was 2.85 μmol of Ca^{2+} /min per mg of protein. Ca^{2+} -dependent ATPase activity: ATPase activity was measured spectrophotometrically in the presence of the Ca^{2+} ionophore A23187 (1.5 $\mu\text{g}/\text{ml}$) at 37 $^{\circ}\text{C}$, in an enzyme-coupled assay as described in the Experimental section ($\blacksquare$). Control Ca^{2+} -ATPase rate was 5.70 μmol of P_i /min per mg of protein. Ca^{2+} loading rate: Ca^{2+} loading was measured in the presence ($\triangle$) and in the absence ($\blacktriangle$) of 10 μM -Ruthenium Red, as described in the Experimental section. Data from two different terminal cisternae preparations were averaged. Control Ca^{2+} loading rates were 0.25 and 1.04 μmol of Ca^{2+} /min per mg of protein in the absence and in the presence of Ruthenium Red respectively. JFM, junctional face membrane.

(junctional face membrane) or anti-(350 kDa protein) antibodies (at an antibody/terminal cisternae protein ratio of 1:1). After SDS/polyacrylamide-gel electrophoresis, each gel lane was sliced and radioactivity counted. The photoaffinity labelling profile (Fig. 2) of control terminal cisternae ($\circ$) shows four radioactive peaks (350, 170, 80 and 60 kDa). Anti-(junctional face membrane) antibodies ($\bullet$) markedly decreased the incorporation of [^{14}C]doxorubicin into the 350, 170 and 80 kDa polypeptides. Anti-(350 kDa protein) antibodies ($\blacksquare$) abolished photolabelling of the 350 kDa polypeptide only. The results indicate that both antibodies prevent interaction of doxorubicin with (some of) its binding site(s).

Doxorubicin activation of Ca^{2+} release from terminal cisternae (Zorzato *et al.*, 1985) might occur via specific doxorubicin-binding proteins (Zorzato *et al.*, 1986). This possibility was tested by studying the effect of anti-

(junctional face membrane) and anti-(350 kDa protein) antibodies on the functional properties of terminal cisternae.

Effect of anti-(junctional face membrane) antibodies on doxorubicin-induced Ca²⁺ release, Ca²⁺-dependent ATPase and Ca²⁺ loading rates

Fig. 3 shows that the anti-(junctional face membrane) antibodies inhibited the Ca²⁺-releasing action of doxorubicin. A half-maximal effect was obtained by preincubating terminal cisternae at an antibody/terminal cisternae protein ratio of 1:1. In addition, Fig. 3 shows that the anti-(junctional face membrane) antibodies did not affect the Ca²⁺ dependent ATPase rate in the presence of the Ca²⁺ ionophore A23187, and the Ca²⁺ loading rate in the presence or in the absence of Ruthenium Red. Doxorubicin-induced Ca²⁺ release was either slightly reduced (5% at an immunoglobulin/terminal cisternae protein ratio of 5:1) or unaffected when terminal cisternae were preincubated with preimmune immunoglobulin and junctional face membrane-preadsorbed anti-(junctional face membrane) antibodies (antibody/junctional face membrane protein ratio of 1:1) respectively.

The inhibition of doxorubicin-induced Ca²⁺ release by anti-(junctional face membrane) antibodies might be due to a negligible amount (undetectable by immunoblot) of antibodies against non-junctional sarcoplasmic reticulum protein(s). This possibility was ruled out by carrying out an experiment in which anti-(junctional face membrane) antibodies were adsorbed with longitudinal sarcoplasmic reticulum (antibodies/longitudinal sarcoplasmic reticulum protein ratio of 1:1). After such treatment, the anti-(junctional face membrane) antibodies were still able to block doxorubicin-induced Ca²⁺ release, with a slightly reduced effectiveness (results not shown).

Effect of anti-(350 kDa protein) affinity-purified antibodies on Ca²⁺-dependent ATPase and Ca²⁺ loading rates

In order to test whether the 350 kDa protein, one of the doxorubicin-binding proteins, is involved in the mechanism of Ca²⁺ release, we studied the effect of anti-(350 kDa protein) antibodies on the functional properties of terminal cisternae. The Ca²⁺ loading rate of terminal cisternae was partially inhibited by anti-(350 kDa protein) antibodies (Fig. 4b, ○). The Ca²⁺ loading rate is the net difference between Ca²⁺ influx mediated by the Ca²⁺ pump and Ca²⁺ efflux via the Ca²⁺ channel and/or other efflux pathways. Thus, the effect of anti-(350 kDa protein) antibodies on Ca²⁺ loading might be due either to inhibition of the Ca²⁺-ATPase, activation of Ca²⁺ efflux, or both. The experiment of Fig. 4(b) (▲) shows the effect of anti-(350 kDa protein) antibodies on the Ca²⁺-dependent ATPase rate of terminal cisternae. At an antibody/terminal cisternae ratio of 0.5, the Ca²⁺-dependent ATPase rate was stimulated by 25%. Anti-(350 kDa protein) antibodies did not affect Ca²⁺-dependent ATPase rate of terminal cisternae in the presence of A23187 (results not shown). These results suggest that the anti-(350 kDa protein) antibodies did not interact directly with the Ca²⁺ pump and might, instead, interact with protein domain(s) relevant to activation of Ca²⁺ efflux. We tested this possibility by studying the effect of anti-(350 kDa protein) on stimulated Ca²⁺ release.

Fig. 4. Effect of anti-(350 kDa protein) affinity-purified polyclonal antibodies on the functional properties of terminal cisternae

Terminal cisternae (TC) were preincubated with antibodies as described in the legend to Fig. 3. Two different sarcoplasmic reticulum preparations were used and the data were averaged. (a) Control terminal cisternae and terminal cisternae preincubated with antibodies were preloaded with approx. 2.5 μmol of Ca²⁺/mg of protein. Ca²⁺ release from terminal cisternae was triggered by 100 μM-doxorubicin. (b) Ca²⁺ loading (○) was carried out as described in the Experimental section. Ca²⁺-dependent ATPase activity (▲) was measured spectrophotometrically as described in the Experimental section.

Effect of anti-(350 kDa protein) antibodies on doxorubicin-induced Ca²⁺ release

Both control terminal cisternae and terminal cisternae preincubated with antibodies were loaded with about 2.5 μmol of Ca²⁺/mg of protein. Doxorubicin-induced Ca²⁺ release was measured spectrophotometrically according to Palade (1987). Fig. 4(a) shows that anti-(350 kDa protein) antibodies prevent doxorubicin stimulation of Ca²⁺ release. Half-maximal inhibition was obtained at an antibody/terminal cisternae ratio of 0.5. Doxorubicin acts on caffeine-sensitive Ca²⁺ channels (Zorzato *et al.*, 1985; Palade, 1987) and induces Ca²⁺ release via channels which are allosterically regulated by Ca²⁺, Mg²⁺ and ATP. Thus, the effect of anti-(350 kDa protein) antibodies on doxorubicin-induced Ca²⁺ release might be due, at least in part, to changes in Ca²⁺-channel regulation.

Kinetic resolution of Ca²⁺ release: effect of anti-(350 kDa protein) antibodies on Ca²⁺- and ATP-induced Ca²⁺ release

Kinetic resolution of Ca²⁺ release was obtained using a fast filtration apparatus. Both control terminal cisternae and terminal cisternae preincubated with antibodies were actively loaded with the same amount of Ca²⁺ (approx. 80 nmol of Ca²⁺/mg of protein), as determined by back extrapolation of the Ca²⁺ release curves (Fig. 5). Complete removal of the Ca²⁺ loading medium (see the Experimental section for details) allowed us to study the effect of anti-(350 kDa protein) antibodies on stimulated

Fig. 5. Effect of anti-(350 kDa protein) antibodies on rapid Ca^{2+} release elicited by Ca^{2+} and ATP

Terminal cisternae (TC) (0.6 mg of protein/ml) were preincubated with (●, ▲) and without (○, △) antibodies (0.6 mg of protein/ml) in buffer A for 10 min at 25 °C, prior to dilution into the $^{45}\text{Ca}^{2+}$ loading medium, as described in the Experimental section. The filters retaining the loaded terminal cisternae vesicles were exposed to various release media for different times as indicated on the abscissa. Results of a representative experiment are shown. Similar effects were observed when terminal cisternae were preincubated with antibodies in PBS or in $^{45}\text{Ca}^{2+}$ loading medium (at an antibody/terminal cisternae protein ratio of 1:1). (a) The release media contained 80 mM-KCl/20 mM-Tris/Mops, pH 7.0, and either 10 mM- MgCl_2 /1 mM-EGTA (Mg^{2+} medium; ●, ○), or 51.51 μM - CaCl_2 /50 μM -Tris/EGTA (5 μM -free Ca^{2+} medium; ▲, △). (b) The release media contained 80 mM-KCl/20 mM-Tris/Mops, pH 7.0, and either 1 mM-ATP/1 mM-Tris/EGTA (ATP medium; ●, ○), or 69.7 μM - CaCl_2 /50 μM -Tris/EGTA/1 mM-ATP (5 μM -free Ca^{2+} /ATP medium; ▲, △).

Ca^{2+} release under conditions in which the Ca^{2+} pump is uninfluent. Fig. 5 shows that Ca^{2+} -induced Ca^{2+} release from control terminal cisternae was inhibited by Mg^{2+} and potentiated by ATP. In the absence of Ca^{2+} , ATP itself was a potent Ca^{2+} releasing agent (Fig. 5b). Preincubation of terminal cisternae vesicles with anti-(350 kDa protein) antibodies (at an antibody/terminal cisternae protein ratio of 1:1) decreased both the rate and extent of Ca^{2+} -induced Ca^{2+} release (Fig. 5a) by $60 \pm 6\%$ (control first-order rate constant approx. 3 s^{-1}) and $40 \pm 20\%$ respectively ($n = 3$). Anti-(350 kDa protein) antibodies affected neither the ATP stimulation of Ca^{2+} release (Fig. 5b) nor Mg^{2+} inhibition of Ca^{2+} release (Fig. 5a).

DISCUSSION

The ryanodine receptor is comprised of four 350 kDa feet protein subunits, which form a cation channel in planar lipid bilayer (Lai *et al.*, 1988). The pharmacological sensitivity, conductance and selectivity of the ryanodine receptor channel closely match those of the Ca^{2+} channels of intact sarcoplasmic reticulum vesicles (Lai *et al.*, 1988; Smith *et al.*, 1988). However, other experimental approaches have suggested that additional sarcoplasmic reticulum proteins might be part of, or regulative components of, the native sarcoplasmic reticulum Ca^{2+} channels (Campbell & MacLennan, 1982; Kim & Ikemoto, 1986; Morii *et al.*, 1986; Meszaros *et al.*, 1987; Shoshan-Barmatz, 1987; Rubstov & Murphy, 1988). In a previous report, Zorzato *et al.* (1986) provided indirect evidence that two junctional sarcoplasmic reticulum proteins, having molecular masses of 350 and 170 kDa, might be constituents of the Ca^{2+} release channel as indicated by [^{14}C]doxorubicin photolabelling.

In the present experiments, we implemented an immunological approach to identify component(s) of the native sarcoplasmic reticulum Ca^{2+} channel. In the first set of experiments, we studied whether antibodies against the junctional face membrane proteins had any effect on

the functional properties of terminal cisternae and on the photoaffinity labelling of terminal cisternae with [^{14}C]doxorubicin. Anti-(junctional face membrane) antibodies inhibited both the Ca^{2+} releasing action of doxorubicin and the photoaffinity labelling of terminal cisternae by [^{14}C]doxorubicin. Such an effect is specifically due to a population of antibodies against the junctional face membrane proteins, since after preabsorption with longitudinal sarcoplasmic reticulum, anti-(junctional face membrane) antibodies were still able to block the effect of doxorubicin. In the absence of doxorubicin, anti-(junctional face membrane) antibodies did not affect Ca^{2+} loading and Ca^{2+} -dependent ATPase rates of terminal cisternae in the presence of A23187. We do not know exactly how the heterogeneous population of antibodies against a variety of junctional face membrane proteins affect the mechanism of Ca^{2+} release. A possibility is that the antibodies interact with protein domain(s) which are directly and/or indirectly relevant to doxorubicin binding only, and do not modify the open/closed state of the Ca^{2+} channel. The major conclusion which can be inferred from the present results is that the one and/or more junctional doxorubicin-binding protein(s) is(are) component(s) of the molecular complex of the native Ca^{2+} channels.

On a molar basis, the 350 and 170 kDa junctional proteins exhibit the highest incorporation of [^{14}C]doxorubicin (Zorzato *et al.*, 1986), and, on this account, were deemed putative components of sarcoplasmic reticulum Ca^{2+} channel. Kawamoto *et al.* (1986) have reported that the 350 kDa spanning protein, later referred to as ryanodine receptor, is very sensitive to proteolytic degradation by Ca^{2+} -activated proteases. They also found that the major proteolytic fragment of the spanning protein is a polypeptide of 170 kDa. These observations raise the possibility that the 170 kDa doxorubicin-binding protein may be a proteolytic byproduct of the 350 kDa protein. Our present results do not favour this hypothesis since anti-(350 kDa protein) antibodies did not inhibit the photoaffinity labelling of the 170 kDa protein with

[¹⁴C]doxorubicin (Fig. 2), and affinity-purified anti-(350 kDa protein) antibodies did not cross-react with proteins in the 170 kDa range on Western blot of terminal cisternae (Volpe *et al.*, 1988; Fig. 1). Thus, it would seem that the 170 kDa doxorubicin-binding protein is distinct from the major proteolytic fragment of the spanning protein, and is possibly a unique component of the native sarcoplasmic reticulum Ca²⁺ channel. In this context, it is worth mentioning that a protein with a similar molecular mass, selectively localized in the heavy sarcoplasmic reticulum, has been proposed to be the caffeine receptor of the sarcoplasmic reticulum Ca²⁺ channel (Rubstov & Murphy, 1988).

In the second set of experiments, we studied the effect of affinity-purified anti-(350 kDa protein) antibodies on Ca²⁺ release. We have focused our attention on the 350 kDa doxorubicin-binding protein, since it is most probably the same polypeptide which binds other modulators of Ca²⁺ release, including ryanodine (Lai *et al.*, 1987; Inui *et al.*, 1987; Campbell *et al.*, 1987), ATP (Lai *et al.*, 1988; Imagawa *et al.*, 1987) and calmodulin (Seiler *et al.*, 1984). Upon preincubation of terminal cisternae with anti-(350 kDa protein) antibodies, Ca²⁺ loading became partially uncoupled from ATP hydrolysis (Fig. 4b). The uncoupling effect was selective for terminal cisternae because the anti-(350 kDa protein) antibodies did not affect the Ca²⁺-dependent ATPase of longitudinal sarcoplasmic reticulum, both in presence and absence of A23187 (results not shown), and specific because preimmune IgG did not influence the Ca²⁺ loading rate of terminal cisternae. Under the prevailing experimental conditions, anti-(350 kDa protein) antibodies seem to interact with epitopes likely to be localized on the activating domain of the channel, to increase Ca²⁺ efflux and reduce net Ca²⁺ loading. The reduced transmembrane Ca²⁺ gradient, in turn, would remove back-inhibition on the Ca²⁺ pump and enhance Ca²⁺-dependent ATPase activity. Our interpretation is that some of the anti-(350 kDa protein) antibodies open a few Ca²⁺ channels which are in the closed or activatable state.

Anti-(350 kDa protein) antibodies prevented photolabelling of the antigen by [¹⁴C]doxorubicin (Fig. 2), thereby compromising the stimulation of Ca²⁺ release by doxorubicin (Fig. 4a). The inhibition of doxorubicin-induced Ca²⁺ release appears to be a consequence of antibodies binding to their epitopes, since preimmune IgG did not have any effect. The ability of the anti-(350 kDa protein) antibodies to activate Ca²⁺ efflux (Fig. 4b) and to prevent stimulation of Ca²⁺ release by doxorubicin seems, at first, contradictory. The 'blocking' and 'enhancing' effects of anti-(350 kDa protein) antibodies on the Ca²⁺ efflux might represent the existence of at least two populations of antibodies, and are reminiscent of the 'double' effect exerted by polyclonal antibodies on sarcolemmal Ca²⁺ channels of isolated myocytes (Morad *et al.*, 1988). Another plausible speculation is that anti-(350 kDa protein) antibodies interact with an activating domain of the channel and cannot be displaced by doxorubicin.

Doxorubicin-induced Ca²⁺ release occurs via Ca²⁺/ATP-gated Ca²⁺ channels (Palade, 1987). Thus, it might be supposed that the partial inhibition of doxorubicin-induced Ca²⁺ release by anti-(350 kDa protein) antibodies is due to changes of the allosteric regulation of the channels by Ca²⁺ and/or ATP. This interpretation is supported by the observation that anti-(350 kDa

protein) antibodies prevent the Ca²⁺-dependent activation of the channels (Fig. 5a). When terminal cisternae were preincubated with anti-(350 kDa protein) antibodies, the first-order rate constant of Ca²⁺-induced Ca²⁺ release was reduced, on average, by 60%. Interestingly, anti-(350 kDa protein) antibodies affected neither the stimulation by ATP nor the inhibition by millimolar concentrations of Mg²⁺ (Fig. 5). If the inhibition of Ca²⁺-induced Ca²⁺ release is due to the interaction of the anti-(350 kDa protein) antibodies with the Ca²⁺-activating site(s) of the channel, it is tempting to speculate that the distance between the Ca²⁺-activating site(s) and the other allosteric sites roughly corresponds to the size of an Ig molecule. In addition, these results indicate that the doxorubicin and Ca²⁺ activating site(s) is (are) in close proximity to each other.

In conclusion, this study shows that the native Ca²⁺-gated Ca²⁺ channels are restricted to the junctional face membrane of sarcoplasmic reticulum. It remains to ascertain, however, if the ryanodine receptor is the only subunit of such a Ca²⁺ release channel.

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