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Chemical crosslinking and enzyme kinetics provide no evidence for a regulatory role for the 53 kDa glycoprotein of sarcoplasmic reticulum in calcium transport

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m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) was used to cross-link the protein components of rabbit skeletal muscle sarcoplasmic reticulum. Analysis of cross-linked material by SDS-polyacrylamide gel electrophoresis showed that both the (Ca^{2+}-Mg^{2+})-ATPase and the 53 kDa glycoprotein could be cross-linked, since the amount of protein at the locations on the gel corresponding to uncross-linked material was reduced in the presence of 1.0 mM MBS. Cross-linked products of 130 kDa, 200–260 kDa and approx. 300 kDa were identified. Probing the cross-linked products with monoclonal antibodies against ATPase, 53 kDa glycoprotein and calsequestrin revealed no cross-linked products containing the ATPase and either calsequestrin or the 53 kDa glycoprotein over the range of molecular weights examined here. Possible interactions between the ATPase and calsequestrin or the 53 kDa glycoprotein were also investigated by studying the ATPase activity for the purified ATPase and for the ATPase in sarcoplasmic reticulum vesicles made permeable to Ca^{2+} with A23187. Effects of Ca^{2+} and ATP on the two systems were indistinguishable, providing no evidence for a major modulatory role of calsequestrin or the 53 kDa glycoprotein on the ATPase.

Introduction

The control of Ca^{2+} ion concentration in skeletal muscle fibres is mediated by the intracellular membrane system known as the sarcoplasmic reticulum (SR). The major protein component of the SR membrane, the (Ca^{2+}-Mg^{2+})-ATPase (110 kDa), is responsible for ATP-dependent Ca^{2+} uptake into the lumen of the SR on muscle relaxation [1]. The Ca^{2+} transport function of the ATPase has been characterised in detail [2–4] and, more recently, structural models for the transport protein have been proposed based on the primary amino acid sequence [5].

Other protein components of the SR membrane include calsequestrin (63 kDa [6,7]), the high affinity Ca^{2+} binding protein (55 kDa [8]), the ryanodine-sensitive Ca^{2+} release channel (400–370 kDa [9–11]) and the 53 and 160 kDa glycoproteins [12,13]. The primary sequences of many of these proteins have now been determined [14–18] and the functional roles of both calsequestrin and the Ca^{2+} release channel are well established. However, the functions of the 53 and 160 kDa glycoproteins remain to be determined.

Leonards and Kutchai [19] have suggested that the role of the 53 kDa glycoprotein could be in regulating

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Abbreviations: CALSQ, calsequestrin; DSP, dithiobis(succinimidylpropionate); DST, disuccinimidyl tartrate, ethylene glycolbis(succinimidylsuccinate); FCA, Freund's complete adjuvant; GP, glycoprotein; mAb, monoclonal antibody; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; SR, sarcoplasmic reticulum.

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the coupling of Ca^{2+} transport to ATP hydrolysis by the (Ca^{2+}-Mg^{2+})-ATPase in SR. They reported that if SR vesicles were reconstituted under conditions where the 53 kDa glycoprotein was lost, then the degree of coupling of ATP hydrolysis to Ca^{2+} transport was less than for SR reconstituted under conditions where the 53 kDa glycoprotein was retained [19]. Chiesi and Carafoli [20] found that trifluoperazine bound tightly to the 53 kDa glycoprotein, and attributed the inhibitory effect of trifluoperazine on Ca^{2+} uptake by SR to an effect on the 53 kDa glycoprotein, modifying its presumed interaction with the ATPase. More recently, binding of antiserum against the 53 kDa glycoprotein has been reported to uncouple Ca^{2+} transport from hydrolysis of ATP in SR vesicles without having any effect on ATP hydrolysis [21]. Kutchai and Campbell [21] have also reported that removal of the 53 kDa glycoprotein from SR vesicles produces significant changes in the dependence of ATPase activity on the concentrations of Ca^{2+} and ATP. These results were interpreted as suggesting that the function of the 53 kDa glycoprotein in SR was analogous to that of the β subunit of the (Na^+-K^+)-ATPase [19–21,35].

An analogy between the 53 kDa glycoprotein and the β-subunit of the (Na^+-K^+)-ATPase was made more likely by the report that the 53 kDa glycoprotein was transmembranous [36], like the β-subunit of the (Na^+-K^+)-ATPase [37]. However, the sequence of the 53 kDa glycoprotein [17] includes no likely transmembranous regions, and it has been suggested that the 53 kDa glycoprotein is associated with the inner surface of the SR. Further, it was found that transfecting COS-1 cells with cDNA encoding for the (Ca^{2+}-Mg^{2+})-ATPase or co-transfecting with cDNA encoding for both the 53 kDa glycoprotein and the (Ca^{2+}-Mg^{2+})-ATPase led to microsomes with identical ability to pump Ca^{2+}, arguing against any regulatory role for the 53 kDa glycoprotein [17].

Chemical cross-linking reagents have been used extensively to investigate interactions between membrane proteins [22] and, in particular, interactions between the α and β subunits of the (Na^+-K^+)-ATPase have been clearly established by chemical cross-linking [38]. In complex membrane systems, reliable detection methods are required to visualise the cross-linked products. In this study, chemical cross-linkers have been used in conjunction with monoclonal antibodies (mAbs) to investigate any interactions between the ATPase and other SR protein components including the 53 kDa glycoprotein and calsequestrin. In addition, we have examined the effect of varying the ATP and Ca^{2+} concentration on the ATPase activity of the purified ATPase, where the modulatory influences of other SR components has been removed, and SR treated with calcium ionophore A23187 where such influences should be apparent.

Materials and Methods

Preparation of SR vesicles

SR vesicles were prepared from rabbit white skeletal muscle as described by McWhirter et al. [23].

Monoclonal antibodies

The anti-ATPase monoclonal antibody (mAb 1/2H7) was raised as outlined in Colyer et al. [24]. Calsequestrin was purified from whole rabbit skeletal muscle homogenate as described by Slupsky et al. [25]. DEAE cellulose fractions containing calsequestrin were pooled, dialysed against phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) and frozen at −70°C. Female BALB/C mice were immunised by intraperitoneal injections of 100 μg purified calsequestrin in Freund's complete adjuvant (FCA). After 4 weeks mice were boosted with 100 μg calsequestrin in PBS. Three days later, one animal was culled and the spleen was removed. Spleen cells were fused with P3/NS1/1-Ag4-1 myeloma cells at a ratio of 3:1 in 50% poly(ethylene glycol) (Merck), as described by Galfrè and Milstein [26]. Positive hybridoma cell lines were detected by enzyme-linked immunosorbant assay (ELISA, [27]), using purified calsequestrin as the antigen and were cloned twice by limiting dilution. The mAb used here was designated C/1F9.

A mAb (designated L/5F8) was raised against the 53 kDa glycoprotein by a method based on that of Hockfield [28] involving the immunisation of newborn mice. To reduce the numbers of mAbs produced against (Ca^{2+}-Mg^{2+})-ATPase, newborn mice received intraperitoneal injections of 1 mg ATPase in PBS on the day of birth (day 1) and every other day until day 8. Following a 3 week recovery period, mice were immunised with 100 μg SR protein in FCA. Intraperitoneal booster injections were carried out every 7 days until a positive response was detected in ELISA tests of blood serum. After a final boost injection, the fusion of spleen cells with myeloma cells was carried out 3 days later as described above.

Chemical cross-linking

SR vesicles (2 mg/ml) were incubated with varying concentrations of *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS, Pierce; dissolved in DMSO [29]), in 10 mM sodium phosphate (pH 7.5) for 30 min on ice. Reactions were stopped by denaturation in sample buffer for 30 min at 37°C in the presence of 2% sodium dodecyl sulphate (SDS), 9% glycerol, 10% 2-mercaptoethanol, 75 mM Tris-HCl (pH 6.8) with either 0.01% Bromophenol blue or 0.01% Pyronin Y as tracker dye. Cross-linked SR components were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on linear 4–12% polyacrylamide gradients [30]. Resolved SR

components were either stained with Coomassie brilliant blue or transferred electrophoretically to nitrocellulose (0.45 μ, Amersham International) by the method of Towbin et al. [34]. The nitrocellulose was blocked overnight at 4°C in 5% low fat dried milk, PBS (pH 7.2) before sequential incubations with mAb (1 : 10 dilution culture medium; 1 : 2000, 2 h, room temperature), followed by horseradish peroxidase conjugated rabbit anti-mouse Ig (Serotec; 1 2000 dilution; 1 h, room temperature) and, finally, substrate, 3,3′-diaminobenzidine (Sigma), 0.02% H_2O_2, PBS (pH 7.2). All antibodies were diluted in 0.05% Tween 20, PBS (pH 7.2) and between each incubation the nitrocellulose was washed with the Tween 20, PBS buffer.

Cross-linking with dithiobis(succinimidylpropionate) (DSP, Pierce) and disuccinimidyl tartrate, ethylene glycolbis(succinimidylsuccinate) (DST, Pierce) were carried out essentially as above except that the incubation conditions were 10 mM sodium phosphate (pH 8.3) at 25°C for 60 min and 0.25 M sucrose, 50 mM tetraethlyammonium (pH 8.0) at 25°C for 60 min, respectively. The reactions were quenched with 0.125 M Tris (pH 8.0) for 15 min 20°C and 0.1 M ammonium acetate for 10 min at 20°C, respectively. The proteins were then solubilised in sample buffer as above before performing PAGE. Iodoacetamide (24 mM) was incorporated into the sample buffer when DSP was used.

For two-dimensional gels, the SR vesicles were cross-linked with DSP and separated by PAGE as above. A track of the gel was then excised and soaked in 3% 2-mercaptoethanol, 1% SDS, 50 mM Tris-HCl, (pH 6.8) for 20 min at 65°C. The gel was then washed in four changes of 0.1% SDS, 0.001% Bromophenol blue, Tris-HCl (pH 6.8) and DSP before being cast into the stacking gel of a polyacrylamide gel identical to the one outlined above. Following electrophoresis the proteins were transferred to a sheet of nitrocellulose which was probed sequentially with mAbs L/5F8 C/1F9 and 1/2H7 as above.

ATPase activity

(Ca^{2+}-Mg^{2+})-ATPase activity was determined using a coupled enzyme assay [24]. Each assay was performed with 12.5 μg of SR or ATPase in a final volume of 2.5 ml in a medium containing 40 mM Hepes-KOH, 100 mM KCl, 0.42 mM phospho*enol*pyruvate, 0.15 mM NADH, 5.0 mM $MgSO_4$, 1.02 mM EGTA, rabbit muscle pyruvate kinase (18 IU), pig heart lactate dehydrogenase (7.5 IU) and, unless otherwise stated, 2.0 mM ATP (pH 7.2). The reaction was started by the addition of an aliquot of $CaCl_2$ to give the required free concentration of Ca^{2+}. Unless stated otherwise, the free Ca^{2+} concentration was that giving maximal activity; free Ca^{2+} concentrations were calculated using the binding constants given in Gould et al. [4]. Assays were carried out at 25°C by measuring the decrease in absorbance at 340 nm due to the oxidation of the NADH. A23187 was added as a 1 mg/ml solution to give a final concentration of 0.78 μM where appropriate.

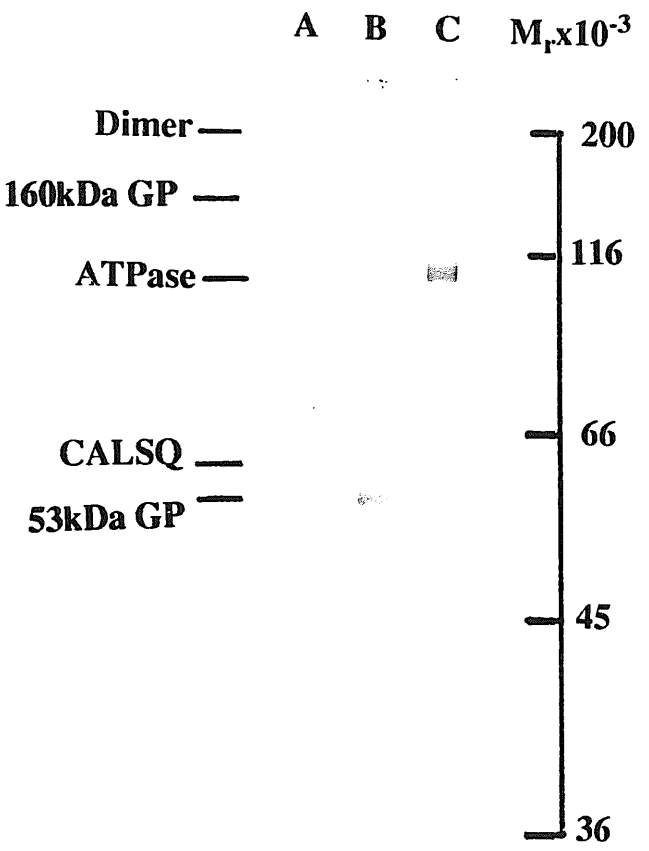


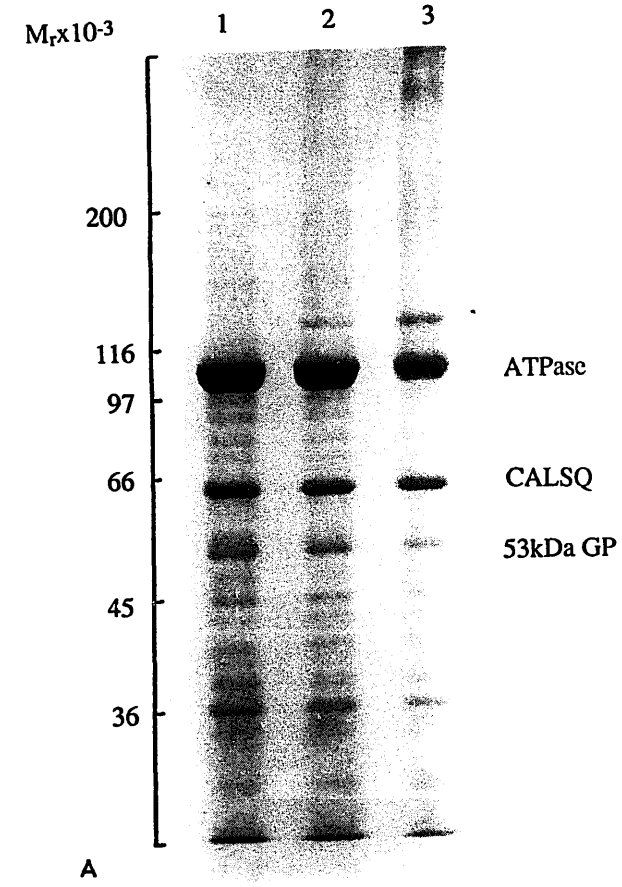
Fig. 1. Western blot of SR with mAbs 1/2H7, C/1F9 and L/5F8. SR (30 μg protein per track) was separated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was blocked overnight with low fat dried milk before incubation with mAb C/1F9 (lane A), L/5F8 (lane B), or 1/2H7 (lane C) followed by rabbit anti-mouse Ig conjugated with horseradish peroxidase and finally substrate (H_2O_2 and 4-chloro-1-naphthol). CALSQ = calsequestrin, GP = glycoprotein.

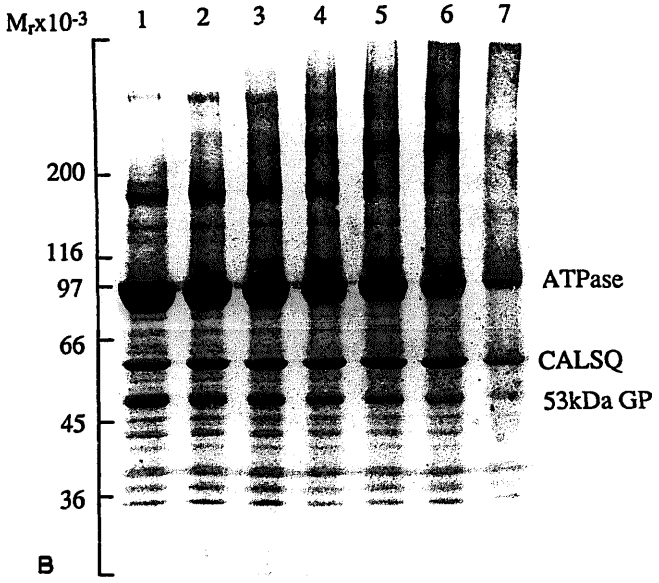
Results

Fig. 1 shows a Western blot of SR using mAbs 1/2H7, C/1F9 and L/5F8. It can be seen that 1/2H7 specifically recognises the (Ca^{2+}-Mg^{2+})-ATPase and its dimer, whereas C/1F9 recognises calsequestrin (CALSQ). MAb L/5F8 is specifically directed against the 53 kDa glycoprotein (53 kDa GP) and the closely related 160 kDa glycoprotein.

Fig. 2A (track 1) shows an SDS PAGE gel of rabbit skeletal muscle sarcoplasmic reticulum (SR). The major protein components, (Ca^{2+}-Mg^{2+})-ATPase, calsequestrin (CALSQ) and the 53 kDa glycoprotein (53 kDa GP) were readily identified. Cross-linking for 30 min with increasing amounts of MBS (tracks 2 and 3) re-

sulted in a reduction in the amount of ATPase observed around 100 kDa and a reduction in the amount of the 53 kDa glycoprotein. In addition, two new bands appeared on the gel, most noticeably at the higher concentration of MBS used (track 3); one sharp band with an apparent molecular mass of around 130 kDa and a much more diffuse band close to the top of the gel. The molecular weight of this broad band is difficult to estimate but is certainly of 300 kDa and above. There was no evidence of a cross-linked product of 150–160 kDa, which might correspond to ATPase cross-linked to the 53 kDa glycoprotein or calsequestrin. Similar cross-





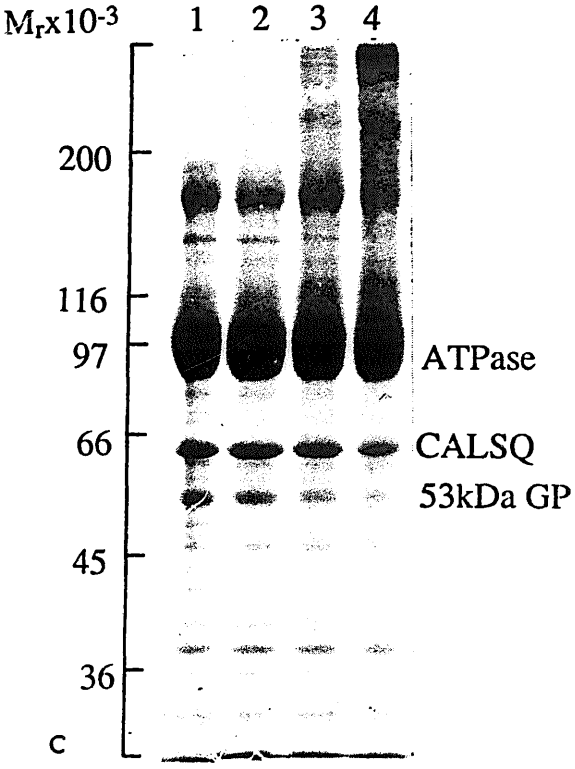


Fig. 2. Coomassie blue stained gels of cross-linked SR proteins. SR vesicles (2 mg ml^{-1}) were incubated with varying concentrations of (A) MBS, (B) DST, (C) DSP for 30–60 min and the cross-linked products were separated by SDS PAGE on 4–12% gradient gels (80 μg protein per track). Proteins were stained on the gel with Coomassie blue. (A) Lane 1, 0 mM MBS; lane 2, 0.1 mM MBS; lane 3, 1.0 mM MBS. (B) Lane 1, 0 mM DST; lane 2, 0.1 mM DST; lane 3, 0.25 mM DST; lane 4, 0.5 mM DST; lane 5, 0.75 mM DST; lane 6, 1.0 mM DST; lane 7, 2.5 mM DST. (C) Lane 1, 0 mM DSP; lane 2, 0.1 mM DSP; lane 3, 0.25 mM DSP; lane 4, 0.5 mM DSP. CALSQ = calsequestrin, GP = glycoprotein.

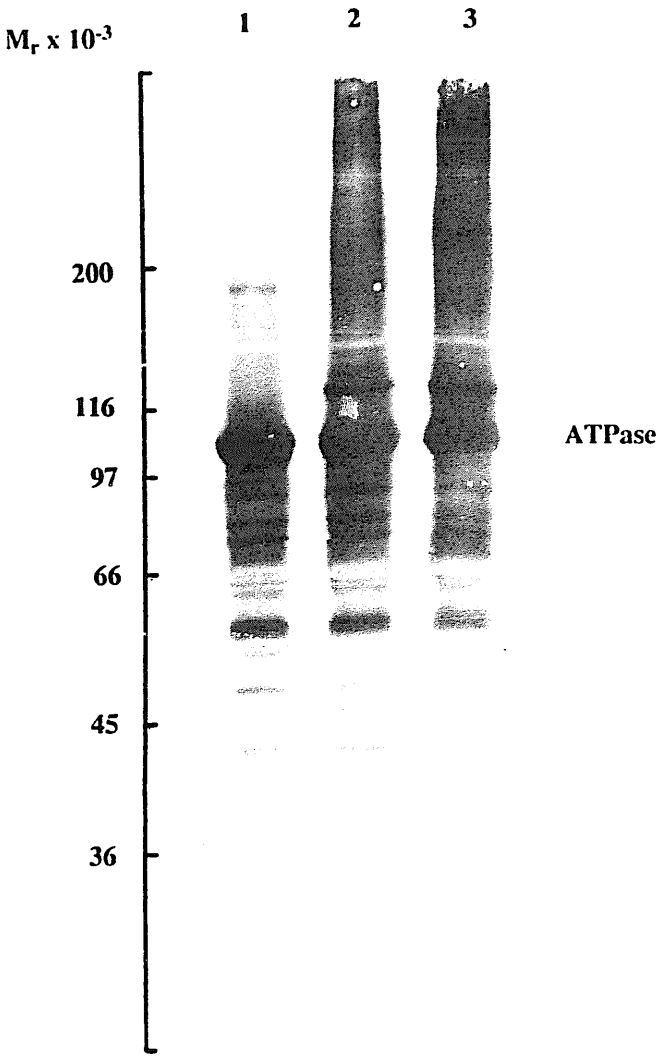


Fig. 3. Western blot of MBS cross-linked SR proteins probed with anti-ATPase mAb (1/2H7). SR vesicles (2 mg ml^{-1}) were cross-linked with MBS for 30 min. The cross-linked products were separated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was blocked overnight with low fat dried milk before sequential incubations with mAb 1/2H7, followed by rabbit anti-mouse Ig conjugated with horseradish peroxidase and finally substrate (H_2O_2 and 3,3′-diaminobenzidine). Lane 1, 0 mM MBS; lane 2, 0.1 mM MBS; lane 3, 1.0 mM MBS.

linking patterns have been achieved with the cross-linkers DST and DSP (Fig. 2B, C). In none was a band seen at 150–160 kDa.

Fig. 3 shows a Western blot of a gel, identical to that in Fig. 2A, probed with mAb 1/2H7 which is directed against the (Ca^{2+}-Mg^{2+})-ATPase. In the absence of cross-linker a large number of bands were apparent on the gel. The assay was performed with larger amounts of SR per track than for Fig. 1 and with a different substrate for the peroxidase in order to increase the sensitivity and, consequently, a large number of proteolysis products were apparent; dimers of the ATPase are also seen (200 kDa). Note that this antibody does not identify calsequestrin, the 53 kDa glycoprotein or the 160 kDa glycoprotein. Although a band around 50 kDa is revealed by mAb 1/2H7 comparison of Fig. 3 with Fig. 4, in which the gel is probed with mAb against the 53 kDa glycoprotein, shows that the band in Fig. 3 runs significantly ahead of the band corresponding to the 53 kDa glycoprotein. Further, antibodies against the 53 kDa glycoprotein also cross react with the 160 kDa glycoprotein (since the 53 kDa glycoprotein corresponds to the C-terminal portion of the 160 kDa glycoprotein [18]) and there is no band corresponding to 160 kDa on the blot (Fig. 3). Increasing the concentration of cross-linker resulted in the appearance of a number of

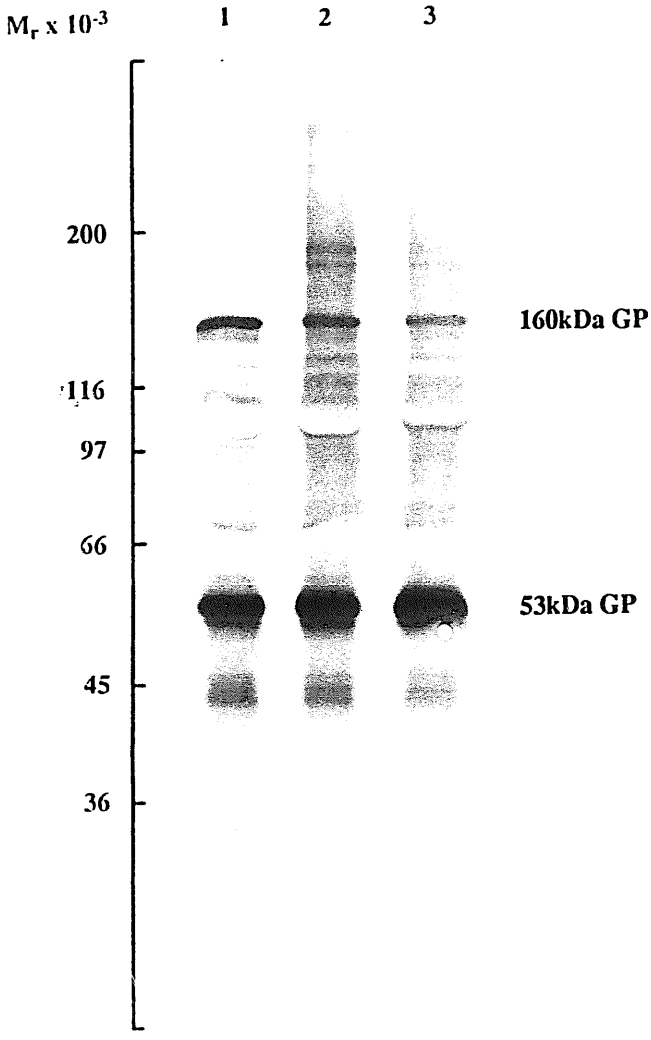


Fig. 4. Western blot of MBS cross-linked SR proteins probed with anti-53 kDa glycoprotein mAb (L/5F8). SR vesicles (2 mg ml^{-1}) were cross-linked with MBS for 30 min. The cross-linked products were separated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was blocked overnight with low fat dried milk before sequential incubations with mAb L/5F8, followed by rabbit anti-mouse Ig conjugated with horseradish peroxidase and finally substrate (H_2O_2 and 3,3′-diaminobenzidine). Lane 1, 0 mM MBS; lane 2, 0.1 mM MBS; lane 3, 1.0 mM MBS. GP = glycoprotein.

new bands. A distinct band is apparent at 130 kDa together with a number of broader less distinct bands corresponding to material of higher molecular weight. Again, although it is difficult to assign molecular masses to these bands, one of them appears to correspond to the high molecular mass band (300 kDa and above) seen on the Coomassie stained gel (Fig. 2A). The other is a broader band of molecular weight 200–260 kDa.

Fig. 4 is an identical blot to that performed in Fig. 3 except that it was probed with a mAb against the 53 kDa glycoprotein (L/5F8). Two major bands are apparent; the 53 kDa glycoprotein against which the antibody is directed and the 160 kDa glycoprotein which is known to share part of the sequence of the 53 kDa glycoprotein [18]. As before, the blot was performed to give maximum sensitivity and so other bands are also seen, which probably correspond to proteolysis products. However, there is no cross reaction with the ATPase or calsequestrin. Cross-linking (tracks 2 and 3) did not produce any higher molecular mass aggregates which were identifiable on the blot using the anti-53 kDa glycoprotein antibody.

The blot was also probed with anti-calsequestrin mAb (C/1F9) (Fig. 5). The major band appears at 66 kDa as a doublet, with a number of other bands of lesser intensity, the most obvious being around 50 kDa. The antibody does not cross react with the ATPase, the 53 kDa or the 160 kDa glycoproteins. Cross-linking did not result in the appearance of any higher molecular mass products recognised by the anti-calsequestrin antibodies.

Fig. 6 shows a Western blot of a two-dimensional SDS-polyacrylamide gel in which the SR was cross-linked with the cleavable cross-linker DSP, resolved in the first dimension and then the cross-linker cleaved with 2-mercaptoethanol and a strip of the sample run in the second dimension. The gel was then probed sequentially with mAbs against the 53 kDa glycoprotein, calsequestrin and ATPase. Uncross-linked products should lie on a diagonal line and any deviation from the diagonal is evidence of cross-linking. Clearly the ATPase has been cross-linked as shown by the bands on the gel corresponding to a molecular mass of around 100 kDa (these band were identified as ATPase by 1/2H7) but shifted to the right of the position of the uncross-linked ATPase. In marked contrast, calsequestrin and the 53 kDa glycoprotein appear mainly as single spots (although there was always some streaking in these experiments) which lie on the diagonal along with the uncross-linked ATPase. There is no evidence for a cross-linked product at 150–160 kDa corresponding to ATPase cross-linked to calsequestrin or the 53 kDa glycoprotein.

If the activity of the (Ca^{2+}-Mg^{2+})-ATPase were to be modulated by interaction with the 53 kDa glycoprotein or calsequestrin, then the ATPase activity of the ATPase in intact SR would be expected to be signifi-

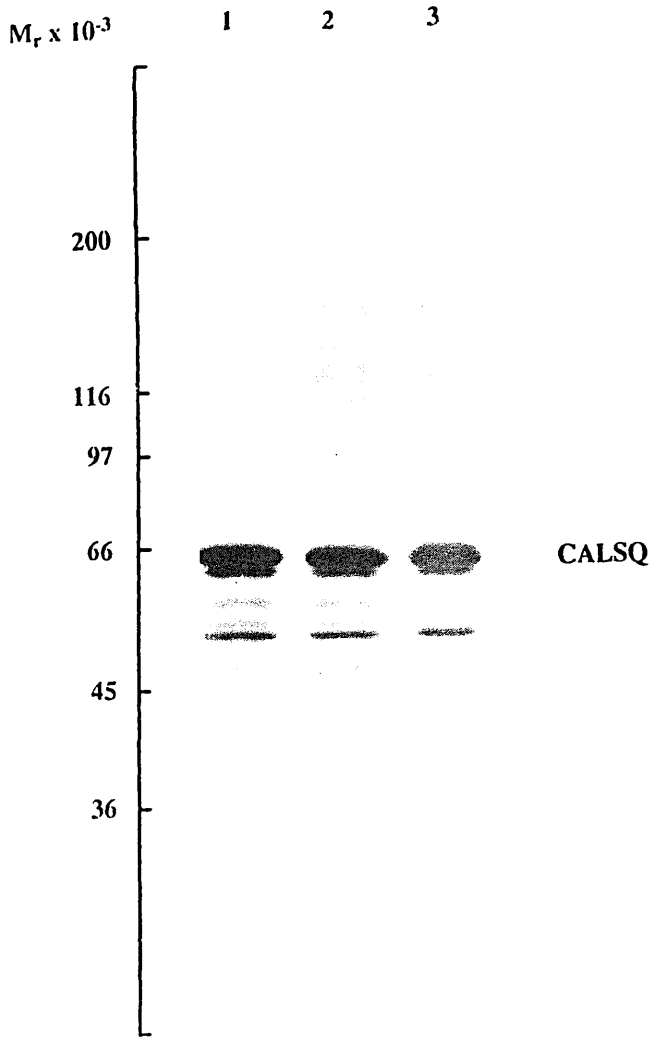


Fig. 5. Western blot of MBS cross-linked SR proteins probed with anti-calsequestrin mAb (C/1F9). SR vesicles (2 mg ml^{-1}) were cross-linked with MBS for 30 min. The cross-linked products were separated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was blocked overnight with low fat dried milk before sequential incubations with mAb C/1F9, followed by rabbit anti-mouse Ig conjugated with horseradish peroxidase and finally substrate (H_2O_2 and 3,3′-diaminobenzidine). Lane 1, 0 mM MBS; lane 2, 0.1 mM MBS; lane 3, 1.0 mM MBS. CALSQ = calsequestrin.

cantly different from that of the ATPase purified from the SR membrane. The steady state ATPase activity of intact SR vesicles is low, because of the build up of high concentrations of Ca^{2+} within the vesicles and, indeed, it has been suggested that the ATPase activity measured for SR vesicles in fact corresponds to a small fraction of unsealed vesicles present in any preparation of SR [23]. However, the presence of the Ca^{2+} ionophore A23187 allows the full steady state ATPase activity of the SR vesicles to be expressed. Fig. 7A and B show the steady state ATPase activity of purified ATPase and SR vesicles permiabilised to Ca^{2+} with the ionophore A23187, as a function of the concentrations of Ca^{2+} and ATP. The

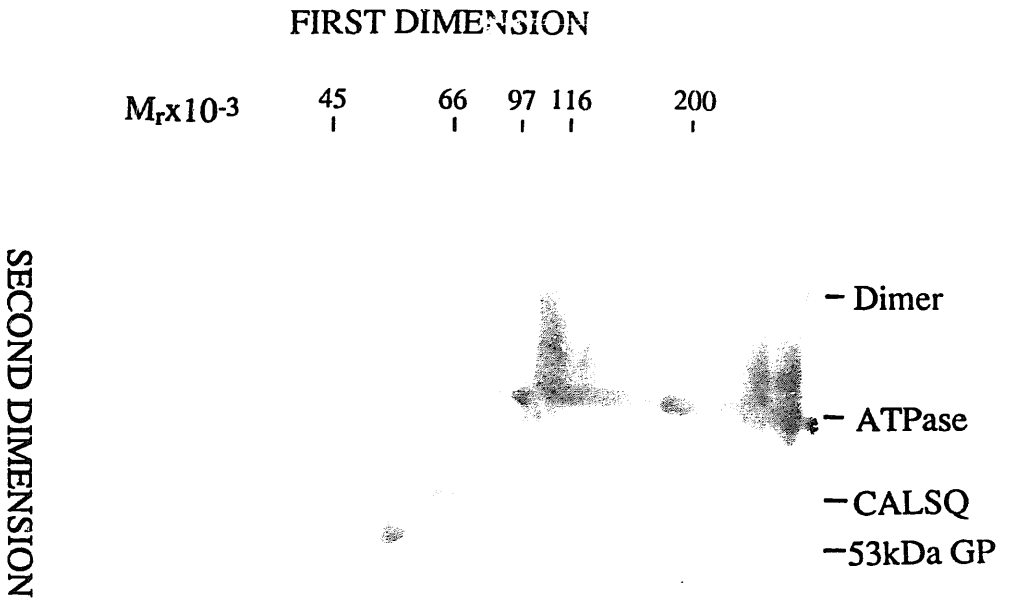


Fig. 6. Western blot of a two dimensional polyacrylamde gel of SR cross-linked with DSP. SR vesicles were cross-linked with DSP and separated by SDS-PAGE in the first dimension. A track of the gel was then excised and treated with 3% 2-mercaptoethanol. After extensive washing in sample buffer the gel slice was cast into the stacking gel of an identical polyacrylamide gel. Following electrophoresis in the second dimension the proteins were transferred to a sheet of nitrocellulose which was blocked overnight with low fat dried milk before sequential incubations with mAbs L/5F8, C/1F9, and 1/2H7, followed on each occasion by rabbit anti-mouse Ig conjugated with horseradish peroxidase and finally substrate (H_2O_2 and diaminobenzidine). CALSQ = calsequestrin, GP = glycoprotein.

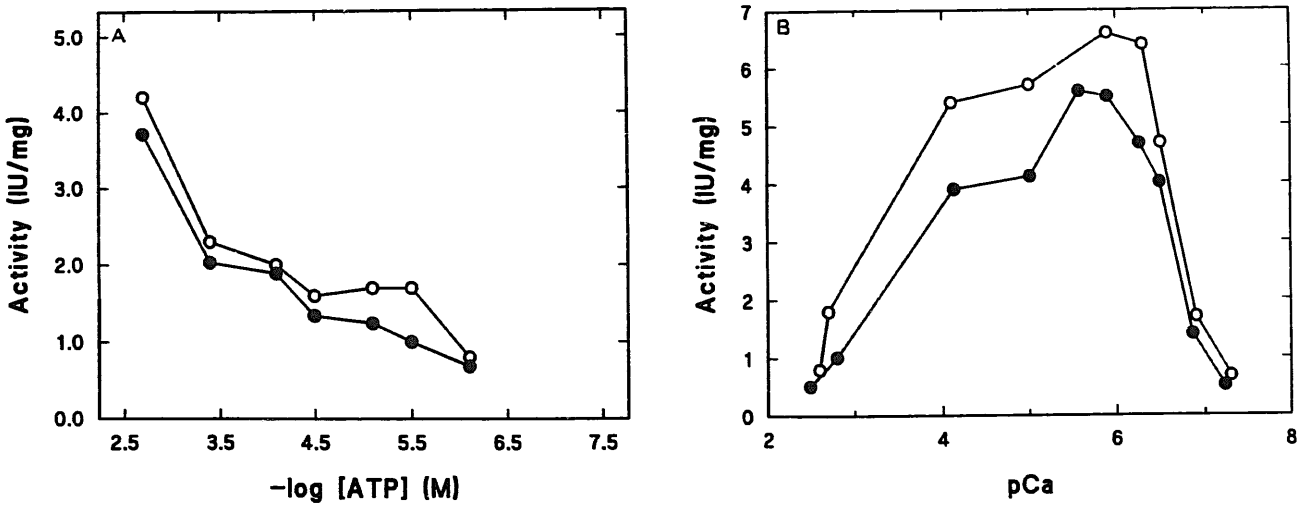


Fig. 7. The effect of varying (A) ATP concentration and (B) Ca^{2+} concentration on the steady state ATPase activity of the purified ATPase (○) and sarcoplasmic reticulum permeabilised to Ca^{2+} with A23187 (●).

dependence of ATPase activity on the concentrations of Ca^{2+} and ATP are indistinguishable for the two systems.

Discussion

The three major protein components of SR are the (Ca^{2+}-Mg^{2+})-ATPase, calsequestrin and the 53 kDa glycoprotein. The (Ca^{2+}-Mg^{2+})-ATPase is responsible for the active transport of Ca^{2+} across the membrane of the SR, and calsequestrin, a Ca^{2+}-binding protein, acts to lower the free concentration of Ca^{2+} within the lumen of the SR. The role of the 53 kDa glycoprotein is unknown, but it has been suggested that the 53 kDa glycoprotein could be analogous to the β subunit of the (Na^{+}-K^{+})-ATPase, and could serve to control the coupling of Ca^{2+} transport to the hydrolysis of ATP [19–21,35]. Here, we have investigated any possible interaction between the (Ca^{2+}-Mg^{2+})-ATPase and the 53 kDa glycoprotein using chemical cross-linking and comparative kinetics.

Chemical cross-linking has been used extensively to probe interactions between membrane proteins, the major problem in such studies being to distinguish between cross-linking of pre-existing aggregates and cross-linking that follows from diffusional collisions between initially separated molecules in or on the membrane [22]. Despite the potential problems, the association between the α and β subunits of the (Na^{+}-K^{+})-ATPase has been clearly demonstrated using this approach [38].

In this study a number of cross-linked products with molecular masses of 130 kDa, 200–260 kDa and approx. 300 kDa have been identified (Fig. 2A) as containing the (Ca^{2+}-Mg^{2+})-ATPase using the mAb 1/2H7 (Fig. 3). Cross-linking of the (Ca^{2+}-Mg^{2+})-ATPase to produce high molecular mass aggregates has been reported previously [31–33] but, in agreement with previous work, cross-linking of the ATPase does not produce a large increase in the amount of dimeric species, but rather produces aggregates too large to enter the gels [33]. The cross-linked product with an apparent molecular mass of 130 kDa could correspond to an internally cross-linked monomeric form of the ATPase; such a cross-linked product has been reported previously using glutaraldehyde to cross-link the ATPase [31].

The major observation of the studies reported here is that none of the cross-linked products seen on SDS-polyacrylamide gels (Fig. 2A) were identified by Western blotting using the mAb directed against the 53 kDa and 160 kDa glycoproteins (Fig. 4). In particular, if interaction between the 53 kDa glycoprotein and the ATPase were to be important, a cross-linked product of ca 150 kDa would have been expected, by analogy to experiments cross-linking the α and β subunits of the (Na^{+}-K^{+})-ATPase [38]. The reason for the lack of any such cross-linked product here is not simply that the cross-linker MBS fails to react with the 53 kDa glycoprotein, since it is clear from the reduction in intensity of the band corresponding to monomeric 53 kDa glycoprotein that the 53 kDa glycoprotein is being cross-linked (Fig. 2A). The failure to detect any cross-linked products of the 53 kDa glycoprotein and ATPase using a variety of cross-linkers argues against any significant interaction between these two components in the membrane (Figs. 2–5). We also find no evidence for cross-linking of calsequestrin to the ATPase or the 53 kDa glycoprotein (Fig. 5).

If interaction between the 53 kDa glycoprotein and the ATPase were to be important in controlling the coupling between transport of Ca^{2+} and hydrolysis of ATP, then the interaction might be expected to have a significant effect on the activity of the ATPase. Indeed, Kutchai and Campbell [21] reported that the dependence of the steady state ATPase activity of SR vesicles on the concentrations of Ca^{2+} and ATP were different for preparations of SR vesicles with and without the 53 kDa glycoprotein. The dependence of ATPase activity on the concentration of ATP is complex, with high concentrations of ATP causing a stimulation of ATPase activity; it has been suggested that this stimulation of activity follows from binding of ATP to the empty nucleotide binding site on the ATPase made available after phosphorylation of the ATPase by bound ATP and the dissociation of the formed ADP (see references in Ref. 4). The dependence of ATPase activity on the concentration of Ca^{2+} is also complex, with increasing concentrations of Ca^{2+} increasing activity at low (micromolar) concentrations but decreasing activity at higher (millimolar) concentrations. Inhibition of activity at higher concentrations of Ca^{2+} has been attributed both to the binding of Ca^{2+} to the phosphorylated ATPase, decreasing the rate of dephosphorylation and to the formation of CaATP, which binds to the ATPase in competition with the normal substrate, MgATP (see Ref. 4).

We have chosen to compare the steady-state ATPase activity of SR vesicles with that of the purified ATPase (which contains no 53 kDa glycoprotein, see Ref. 39). The ATPase activity of intact SR vesicles is low because of the build up of high, inhibitory, concentrations of Ca^{2+} within the vesicles, although the presence of calcium binding proteins (mainly calsequestrin) does allow SR to accumulate more calcium than would otherwise be possible by reducing the internal free calcium concentration. We therefore measured activities of the SR vesicles in the presence of the Ca^{2+} ionophore A23187, when the full ATPase activity of the vesicles will be expressed. As shown in Fig. 6A and B, the dependence of ATPase activities on the concentrations of ATP and Ca^{2+} are indistinguishable for the two systems. In particular, the stimulatory effects of

high concentrations of ATP (Fig. 7A) and the inhibitory effects of high concentrations of Ca^{2+} (Fig. 7B) are comparable. There is no evidence for any significant change in the affinity of the ATPase for Ca^{2+} (Fig. 7B). Under these conditions, therefore, we find no evidence for a role of the 53 kDa glycoprotein in modulating the ATPase activity of the ATPase.

These data support the growing concensus that the 53 kDa glycoprotein is not transmembranous and indicate that in fast twitch skeletal muscle SR the ATPase is not subject to the influence of other SR proteins.

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