

# Calcium Transport by Sarcoplasmic Reticulum of Skeletal Muscle Is Inhibited by Antibodies against the 53-Kilodalton Glycoprotein of the Sarcoplasmic Reticulum Membrane<sup>†</sup>

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**ABSTRACT:** The effects of an antiserum against the 53-kDa glycoprotein (GP-53) of the Sarcoplasmic reticulum (SR) and of monoclonal antibodies against GP-53 on  $\text{Ca}^{2+}$  transport and ATP hydrolysis by SR of rabbit skeletal muscle have been investigated. Preincubation of SR with an antiserum against GP-53 resulted in decreased ATP-driven  $\text{Ca}^{2+}$  transport by the SR but had no effect on  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis. Preincubation of SR with preimmune serum had no significant effect on either  $\text{Ca}^{2+}$  transport or  $\text{Ca}^{2+}$ -ATPase activity. The effect of anti-GP-53 serum was time and concentration dependent. Preincubation of SR with two monoclonal antibodies against GP-53 had no effect on  $\text{Ca}^{2+}$  transport or on  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis. However, preincubation of SR with either monoclonal antibody against GP-53 together with a monoclonal antibody against the  $\text{Ca}^{2+}$ -ATPase (at levels which had little effect alone) resulted in markedly decreased rates of  $\text{Ca}^{2+}$  uptake and ATP hydrolysis. Preincubation of SR with anti-GP-53-serum or with monoclonal antibodies, under the same conditions that inhibited  $\text{Ca}^{2+}$  uptake, did not increase the passive permeability of the SR membrane to  $\text{Ca}^{2+}$ , did not decrease the permeability of the SR to oxalate, and did not cause significant proteolysis of the  $\text{Ca}^{2+}$ -ATPase. Our results are consistent with the interpretation that GP-53 may modulate the function of the  $\text{Ca}^{2+}$ -ATPase of the SR membrane.

The sarcoplasmic reticulum (SR<sup>1</sup>) of skeletal and cardiac muscle plays a vital role in controlling the cytosolic concentration of  $\text{Ca}^{2+}$  (Hasselbach, 1964; Ebashi et al., 1969; MacLennan, 1970). The  $\text{Ca}^{2+}$ -ATPase is an integral protein of the SR membrane that actively transports  $\text{Ca}^{2+}$  from the cytosol to the lumen of the SR (Racker, 1972; Meissner & Fleischer, 1974; Warren et al., 1974).

The primary structure of the  $\text{Ca}^{2+}$ -ATPase of slow skeletal muscle appears to be identical with that of cardiac muscle but differs slightly from the primary structure of fast skeletal muscle  $\text{Ca}^{2+}$ -ATPase (MacLennan et al., 1985; Brandl et al., 1986). The  $\text{Ca}^{2+}$ -ATPase of cardiac muscle is regulated by phospholamban, a 22-kDa protein of the SR membrane [reviewed by Tada et al. 1982]. It has not been established that the  $\text{Ca}^{2+}$ -ATPase of skeletal muscle is subject to regulation by another protein, either cytosolic or membrane bound.

Michalak et al. (1980) identified GP-53 as an intrinsic protein of the membrane of the SR of skeletal muscle and showed it to be a major component of the SR membrane. Michalak et al. (1980) found GP-53 to be present in a constant molar ratio to the  $\text{Ca}^{2+}$ -ATPase: about two GP-53 molecules for every three  $\text{Ca}^{2+}$ -ATPase polypeptide chains. Campbell and MacLennan (1981) purified GP-53 and partially characterized it. They found GP-53 to span the SR membrane, with its carbohydrate chains in the SR lumen and much of its protein mass exposed on the cytoplasmic surface of the membrane.

Chiesi and Carafoli (1982) reported that trifluoperazine inhibits  $\text{Ca}^{2+}$ -ATPase activity of  $\text{Ca}^{2+}$  transport in skeletal SR. They correlated this effect of trifluoperazine with decreased affinity of the  $\text{Ca}^{2+}$ -ATPase for  $\text{Ca}^{2+}$  in the presence of the drug. Chiesi and Carafoli presented evidence that this effect of trifluoperazine was not due to inhibition of calmodulin but rather to the binding of trifluoperazine to GP-53. Chiesi and Carafoli suggested that GP-53 may modulate the function of the  $\text{Ca}^{2+}$ -ATPase.

Leonards and Kutchai (1985) studied reconstituted SR prepared by partially solubilizing SR in cholate, removing the cholate by dialysis, and then isolating the reconstituted SR vesicles on a sucrose gradient. Leonards and Kutchai found that increasing [KCl] in the solubilizing medium beyond 0.2 M led to a progressive decline in the amount of GP-53 that was present in the reconstituted membranes. The reconstituted SR, whether rich in GP-53 or depleted of GP-53, had a specific activity of Ca-stimulated ATP hydrolysis similar to native SR. However, the ability of the reconstituted SR to transport  $\text{Ca}^{2+}$  appeared to decline as GP-53 was extracted from the membrane. These results led Leonards and Kutchai to hypothesize that GP-53 can modulate the coupling between  $\text{Ca}^{2+}$  transport and ATP hydrolysis by the  $\text{Ca}^{2+}$ -ATPase.

<sup>1</sup> Abbreviations: SR, sarcoplasmic reticulum; GP-53, 53-kDa glycoprotein of the SR membrane; GP-160, 160-kDa glycoprotein of the SR membrane; ATP, adenosine 5'-triphosphate; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; Mops, 3-(*N*-morpholino)propanesulfonic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; TBS, Tris-buffered saline (0.2 M NaCl/20 mM Tris-HCl, pH 7.4); NADH, reduced nicotinamide adenine dinucleotide; SDS, sodium dodecyl sulfate; elon, *p*-(methylamino)phenol; bis(acrylamide), *N,N'*-methylenebis(acrylamide); Temed, *N,N,N',N'*-tetramethylethylenediamine; DTT, dithiothreitol.

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In this paper, we report studies using antibodies against GP-53. The evidence presented here is consistent with the interpretation that the 53-kDa glycoprotein (GP-53), an integral protein of the SR membrane, may modulate the function of the  $\text{Ca}^{2+}$ -ATPase of the SR of rabbit fast skeletal muscle.

#### EXPERIMENTAL PROCEDURES

**Materials.** Sucrose, Mops, Pipes, histidine, Tris, triethanolamine, PMSF,  $\text{Na}_2\text{ATP}$ , *p*-aminophenol, DTT, EGTA,  $\text{CaCl}_2$ , NADH, phospho(enol)pyruvate, L-lactate dehydrogenase, pyruvate kinase, hydrogen peroxide, and 4-chloro-1-naphthol were purchased from Sigma Chemical Corp. Acrylamide, bis(acrylamide), Temed, SDS, and ammonium persulfate were obtained from Bio-Rad. NaCl, KCl, potassium oxalate, and sulfuric acid were from J. T. Baker. Sodium azide was purchased from Mallinckrodt.  $^{45}\text{CaCl}_2$  was from New England Nuclear. Ammonium molybdate and  $\text{MgCl}_2$  were from Fisher. Nitrocellulose membrane was purchased from Whatman. Budget-solve scintillation cocktail was from Research Products International. Rabbit anti-sheep IgG and goat anti-mouse IgG were obtained from ICN Immunobiologicals or from Cappell. Secondary antibodies, coupled to horseradish peroxidase, were purchased from Cappell or from Tago. Millipore filters (HAWP, 0.45- $\mu\text{m}$  pore size) and Millititer plates were purchased from Millipore Corp. Lipoclean reagent was from Behring Diagnostics.

**Preparation of Sarcoplasmic Reticulum.** Light SR was isolated by using a combination of differential and sucrose density gradient centrifugations according to the method of Campbell et al. (1980). Preparation of SR membrane vesicles was carried out in the presence of protease inhibitors (aprotinin, benzamide, iodoacetamide, leupeptin, pepstatin A, and PMSF) as described in Campbell et al. (1987). Light SR fractions obtained by this method were suspended in 0.3 M sucrose/20 mM Tris-maleate (pH 7.0) and stored at  $-70^\circ\text{C}$ . Alternatively, light SR was prepared by the method of Eletr and Inesi (1972) and stored in  $-70^\circ\text{C}$  in 10 mM Mops (pH 7.0)/0.88 M sucrose.

**Preparation of Anti-GP-53 Serum.** The antiserum against the 53000-dalton glycoprotein (anti-GP-53 serum) had been previously produced by Campbell and MacLennan (1982) using the following methodology. GP-53 was purified by SDS column chromatography as described by Campell and MacLennan (1981) and injected in Freund's complete adjuvant into a mature sheep. The sheep received three intramuscular injections (1 mg of GP-53 each); the second and third injections (in Freund's incomplete adjuvant) were 3 and 5 weeks after the first. The animal was first bled 7 weeks after the first injection, and the second bleeding was 1 month after that. The anti-GP-53 serum was characterized by its ability to precipitate SR proteins (Zubrzycka-Gaarn et al., 1983). The anti-GP-53 serum did not react with purified SR  $\text{Ca}^{2+}$ -ATPase. The anti-GP-53 serum immunoprecipitated only GP-53 and the 160-kDa glycoprotein (GP-160). In immunoblots of polyacrylamide gels of SR, only GP-53 and GP-160 were found to bind anti-GP-53 serum (Campbell et al., 1983). The reactivity of the anti-GP-53 serum with GP-160 is considerably weaker than that with GP-53 (Figure 1).

**Preparation of Monoclonal Antibodies against SR Proteins.** The procedure for preparing monoclonal antibodies against SR proteins was as described by Campbell et al. (1987). Five- to six-week-old female BALB/c mice were immunized intraperitoneally with 0.5 mg of purified SR (MacLennan, 1970) emulsified in Freund's complete adjuvant. After 4 weeks, the immunization was repeated 3 or 4 times at 2-week intervals with the same amount of SR in Freund's incomplete adjuvant.

Hybridoma cells were produced by fusion of spleen cells with NS-1 myeloma cells (Kennett, 1980). Hybridoma supernatants were screened against SR vesicles by using an immunodot assay (Hawkes et al., 1982; Campbell et al., 1987) with Millititer plates (Millipore Corp.). Positive hybridomas were grown, dilution-cloned, and passaged in RPMI 1640 medium supplemented with 10% fetal bovine serum. The specificities of monoclonal antibodies produced by the clones were determined on Western blots of SDS-polyacrylamide gels of SR (Figure 1). Hybridoma cells were grown in ascites fluid by injecting  $5 \times 10^6$  hybridoma cells intraperitoneally into Pristane-primed BALB/c mice. The ascites fluid was delipidated with Lipoclean reagent (Sieber, 1978).

**Determination of  $^{45}\text{C}$  Uptake by Sarcoplasmic Reticulum.**  $^{45}\text{Ca}$  uptake was measured by a modification of the method by Jones et al. (1977). Light SR (30-35  $\mu\text{g}$  of protein) was mixed with 2.925 mL of 100 mM KCl, 50 mM histidine hydrochloride, 6 mM potassium oxalate, 3 mM  $\text{MgCl}_2$ , and 50  $\mu\text{M}$   $^{45}\text{CaCl}_2$  (2.5-4  $\mu\text{Ci}/\mu\text{mol}$ ) at room temperature. Alternatively, the uptake medium contained in addition 0.5 mM EGTA and 0.55 mM added  $\text{CaCl}_2$ . The final pH of this mixture was adjusted to 6.8. An aliquot of this mixture (0.5 mL) was taken, and two 100- $\mu\text{L}$  samples were pipetted onto Millipore filters (HA Millipore filters, 0.45- $\mu\text{m}$  pore size, 25-mm diameter) and filtered under vacuum. Active uptake was initiated by adding 75  $\mu\text{L}$  of 0.1 M  $\text{Na}_2\text{ATP}$ . Samples (100  $\mu\text{L}$  each) were taken at known time intervals after addition of ATP and filtered on Millipore filters as just described. The filters were washed twice with 5 mL of ice-cold Tris-buffered saline, then each filter was added to 5 mL of scintillation fluid (Beckman EP or RPI Budget-Solve), and the samples were counted in a liquid scintillation spectrometer. Aliquots of the uptake mixture were counted to estimate the specific activity of  $^{45}\text{Ca}$ .

**Determination of ATP Hydrolysis by Sarcoplasmic Reticulum.** ATP hydrolysis was determined by a modification of the method of Jones et al. (1977). SR (50-100  $\mu\text{g}$  of protein) was added to 5 mL of buffer with similar composition as the solution described above for  $\text{Ca}^{2+}$  uptake and containing, in addition, 1 mM  $\text{NaN}_3$  and 0.5 mM EGTA. Background ATPase activities were determined in the absence of added  $\text{Ca}^{2+}$ .  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis was determined in the presence of 0.55 mM added  $\text{CaCl}_2$ . At 1-min time intervals, 250- $\mu\text{L}$  samples were taken, and the amount of inorganic phosphate present was determined as the phosphomolybdate complex by a modification of the Fiske-SubbaRow procedure using *p*-(methylamino)phenol (elton) as the reducing agent (Harris & Popat, 1954). In some of our experiments, we added  $^{45}\text{CaCl}_2$  (7-10  $\mu\text{Ci}/\mu\text{mol}$ ) to the reaction mixture and took samples for determination of  $^{45}\text{Ca}$  uptake from the same incubation mixture used to estimate ATPase activity. In some other cases, the rate of ATP hydrolysis by the  $\text{Ca}^{2+}$ -ATPase was determined by an enzyme-coupled assay (Madden et al., 1979). The decrease in absorbance at 340 nm (due to NADH oxidation) was followed in a reaction mixture containing 100 mM triethanolamine (pH 7.4), 100 mM KCl, 5 mM  $\text{MgCl}_2$ , 0.5 mM EGTA, 3 mM  $\text{MgATP}$ , 1.25 mM phospho(enol)pyruvate, 0.2 mM NADH, and 20 IU each of pyruvate kinase and L-lactic acid dehydrogenase. SR preparations were incubated for 5 min without  $\text{Ca}^{2+}$  to determine background activity. Then  $\text{CaCl}_2$  was injected to give a total  $[\text{Ca}^{2+}]$  of 0.55 mM and the rate of  $\text{Ca}^{2+}$ -stimulated ATP hydrolysis determined.

**Estimation of the Passive Permeability of the SR Membrane to  $\text{Ca}^{2+}$ .** SR vesicles (0.5 mg of protein/mL) were

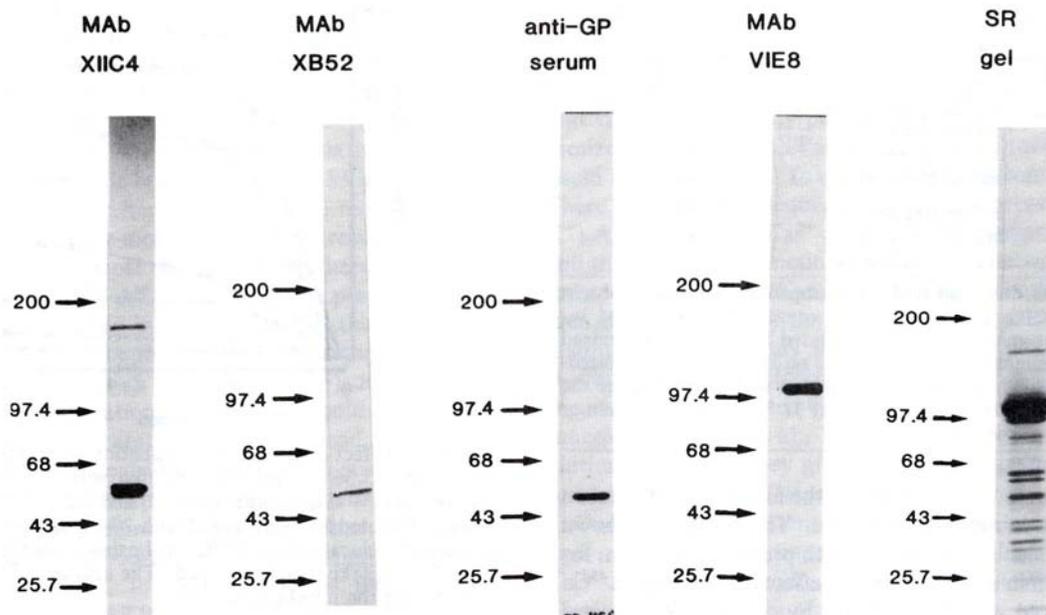


FIGURE 1: Specificity of the anti-GP-53 serum and monoclonal antibodies used in this study. Western blots are shown of SDS-polyacrylamide gels of SR. A Coomassie blue stained gel of SR is shown for reference. Arrows indicate the positions of prestained molecular weight markers for each blot or gel. The blots were probed with anti-GP-53 serum or with MAbs VIES, XIIC4, or XB52, as indicated. Antibody reactivity was visualized with the appropriate anti-IgG coupled to horseradish peroxidase as described under Experimental Procedures. The amounts of SR protein loaded on each gel lane shown are as follows: Mab XIIC4, 5  $\mu$ g; Mab XB52, 25  $\mu$ g; anti-GP serum, 25  $\mu$ g; Mab VIE8, 1  $\mu$ g; SR gel, 30  $\mu$ g.

preincubated for 2.5 h at 37 °C in 30 or 40  $\mu$ L of 0.1 M KCl, 10 mM Pipes (pH 6.8), and 5 mM  $^{45}\text{CaCl}_2$  (about mCi/mmol) along with normal serum, anti-GP-53 serum, or monoclonal antibodies. The vesicles were then diluted into 227 volumes of room temperature efflux solution [0.1 M KCl, 10 mM Pipes (pH 6.8), 5 mM  $\text{MgCl}_2$ , and 1 mM EGTA (Meissner, 1984)] in order to initiate  $^{45}\text{Ca}$  efflux. Various times after dilution, 0.5-mL samples were removed and filtered on HA Millipore filters. The filters were washed with two 5-mL volumes of efflux solution, and the  $^{45}\text{Ca}$  retained on the filters was determined by liquid scintillation counting.

**Estimation of Passive Permeability of SR Vesicles to Oxalate.** SR vesicles (1.2  $\mu$ g of protein/mL) were preincubated for 2 h at 37 °C in the presence of normal serum, anti-GP-53 serum, or monoclonal antibodies in 0.1 M KCl, 10 mM Pipes (pH 6.8), 0.5 mM  $\text{CaCl}_2$ , and 12.5 mM [ $^{14}\text{C}$ ]oxalate (about 2.2 mCi/mmol). After the preincubation period, the mixture was diluted into 3 mL of room temperature efflux medium [0.1 M KCl, 10 mM Pipes (pH 6.8), 5 mM  $\text{MgCl}_2$ , and 1 mM EGTA]. After various times, 0.2-mL samples were removed and filtered on HA Millipore filters, and the filters were washed twice with 5 mL of ice-cold efflux medium.  $^{14}\text{C}$  retained on the filters was determined by scintillation counting.

**Gel Electrophoresis.** SDS-polyacrylamide slab gel electrophoresis was performed as described by Laemmli (1970) using 8% acrylamide in the separating gels or a 3-12% acrylamide gradient.

**Determination of Specificities of Anti-GP-53 Serum and of MAbs against GP-53 and  $\text{Ca}^{2+}$ -ATPase.** A preparation of sarcoplasmic reticulum was subjected to SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). The protein bands were electrophoretically transferred to nitrocellulose paper (0.2- $\mu$ m pore size) using the Transphor TE-50 apparatus (Hoefer Scientific Instruments) with a tank buffer composed of 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. The nitrocellulose paper was soaked for 1 h in blocking solution [0.5% nonfat dry milk in 200 mM NaCl, 10 mM Tris (pH 8.0), and 0.5% Tween-20]. The nitrocellulose was then in-

cubated for 1 h at room temperature with a 1:1000 dilution in blocking solution of anti-GP-53 serum or one of three MAbs (VIES, XIIC4, or XB52). After being washed with blocking solution (three washes, 10 min each), the nitrocellulose was incubated for 1 h with a 1:1000 dilution of the appropriate anti-IgG (anti-sheep or anti-mouse) coupled to horseradish peroxidase. Horseradish peroxidase was visualized by incubating the nitrocellulose with 100 mL of Tris-buffered saline with 0.018% hydrogen peroxide to which 20 mL of ice-cold 0.3% 4-chloro-1-naphthol in methanol was added.

**Determination of Calcium Content of Sera.** The total calcium contents of sera were determined by atomic absorption spectrophotometry (Cali et al., 1973). The calcium content of the anti-GP-53 serum was 1.97 mM, and that of the pre-immune serum was 2.25 mM.

## RESULTS

**Specificity of Anti-GP-53 Serum and of MAbs against GP-53 and Fast Skeletal SR  $\text{Ca}^{2+}$ -ATPase.** Figure 1 shows immunoblots of SR vesicles probed with anti-GP-53 serum or with MAbs VIES, XB52, or XIIC4. Antibody reactivity was visualized with the appropriate anti-IgG coupled to horseradish peroxidase. The immunoblots show that the specificities of the anti-GP-53 serum and the MAbs are as previously reported (Campbell et al., 1983; Zubrzycka-Gaarn et al., 1983; Jorgensen et al., 1988). The anti-GP-53 serum and Mab XIIC4 were found to react with GP-53 and, to a lesser extent, with GP-160. Mab XB52 was found to react only with GP-53. Mab VIE8 was specific for the  $\text{Ca}^{2+}$ -ATPase. Mab IID8, specific for the  $\text{Ca}^{2+}$ -ATPase of cardiac muscle, failed to bind to fast skeletal SR, as reported by Jorgensen et al. (1988).

**Effect of Anti-GP-53 Serum on  $\text{Ca}^{2+}$  Uptake.** In an initial experiment, light SR (13.9 mg/mL) was diluted 2-fold with 0.3 M sucrose/20 mM Tris-maleate (pH 7.0). Diluted SR (4.4  $\mu$ L) was mixed with 20  $\mu$ L of preimmune serum, anti-GP-53 serum from the first bleeding of the sheep, or anti-GP-53 serum from the second bleeding and preincubated at 37 °C for either 15 or 30 min. The mixtures for each

Table I: Amount of  $^{45}\text{Ca}$  Taken Up by Samples in 10 min at Room Temperature<sup>a</sup>

preincubation time (min)	preincubation with	$^{45}\text{Ca}$ uptake (nmol/ $\mu\text{g}$ of protein)
15	preimmune serum	1.92
15	first bleed serum	2.01
15	second bleed serum	0.68
30	preimmune serum	1.71
30	first bleed serum	1.48
30	second bleed serum	0.11

<sup>a</sup> Determined as described under Experimental Procedures. Light SR (13.9 mg/mL) was diluted with an equal volume of 0.3 M sucrose/20 mM Tris-maleate (pH 7.0). Diluted SR (4.4  $\mu\text{L}$ ) was mixed with 20  $\mu\text{L}$  of preimmune serum, immune serum from the first bleeding of the sheep, or immune serum from the second bleeding of the sheep. The mixtures were preincubated at 37 °C for either 15 min or 30 min and then put on ice.

preincubation were then assayed for the amount of  $^{45}\text{Ca}$  taken up after 10 min at room temperature. The results are shown in Table I. Preincubation of SR with preimmune serum, for either 15 or 30 min at 37 °C, had no effect on subsequent  $^{45}\text{Ca}$  uptake compared with  $^{45}\text{Ca}$  uptake by control SR that was preincubated either for 15 min or for 30 min at 37 °C in the absence of serum or was kept on ice during the preincubation. Preincubation with anti-GP-53 serum from the first bleeding of the sheep had at most a small effect on  $^{45}\text{Ca}$  uptake. By contrast, preincubation of SR with anti-GP-53 serum from the second bleeding of the sheep, either for 15 or for 30 min at 37 °C, resulted in a marked decrease in  $^{45}\text{Ca}$  uptake. In all subsequent experiments, anti-GP-53 serum from the second bleeding was used exclusively. Preincubation for 30 min led to a greater decrease in  $\text{Ca}^{2+}$  uptake than did 15-min preincubation. The effect of anti-GP-53 serum was not altered by including a protease inhibitor (0.2 mM PMSF) in the preincubation mixture, but this precaution was taken in subsequent experiments.

**Effect of Duration of Preincubation of SR with Anti-GP-53 Serum.** Light SR (13.9 mg/mL) was diluted with 2 volumes of 0.3 M sucrose, 20 mM Tris-maleate (pH 7.0), and 1.2 mM PMSF. Anti-GP-53 serum (20  $\mu\text{L}$ ) was mixed with 6  $\mu\text{L}$  of the diluted SR in a 1.5-mL microcentrifuge tube, incubated in a 37 °C water bath for different time periods, and then kept on ice until  $\text{Ca}^{2+}$  uptake was measured by the method described above. As shown in Figure 2, increasing the time of preincubation with anti-GP-53 serum led to a progressively greater inhibition of  $^{45}\text{Ca}$  uptake by the SR. Preincubation with anti-GP-53 serum for 90 min yielded almost complete inhibition of  $^{45}\text{Ca}$  uptake.

**Effect of Concentration of Anti-GP-53 Serum.** Light SR was diluted as described in the previous section. Diluted SR (6  $\mu\text{L}$ ) was mixed with 0, 5, 10, or 20  $\mu\text{L}$  of anti-GP-53 serum. The total volume of the preincubation was kept constant by adding the appropriate volume of Tris-buffered saline (0.2 M NaCl/20 mM Tris-HCl, pH 7.5). The microcentrifuge tubes were preincubated in a water bath at 37 °C for 60 min. Decreasing the concentration of anti-GP-53 serum in the preincubation resulted in a diminished degree of inhibition of  $^{45}\text{Ca}$  transport (Figure 3). In other experiments, the volume of anti-GP-53 serum was held constant, and the amount of SR protein added was varied. If more than 20  $\mu\text{g}$  of SR protein was added to 20  $\mu\text{L}$  of anti-GP-53 serum, there was a marked reduction in the inhibitor effect of preincubation with anti-GP-53 serum (results not shown).

The data of Figures 2 and 3 show that preincubation of SR with anti-GP-53 serum resulted in a decrease in the plateau value of  $\text{Ca}^{2+}$  accumulation, as well as a decrease in the initial

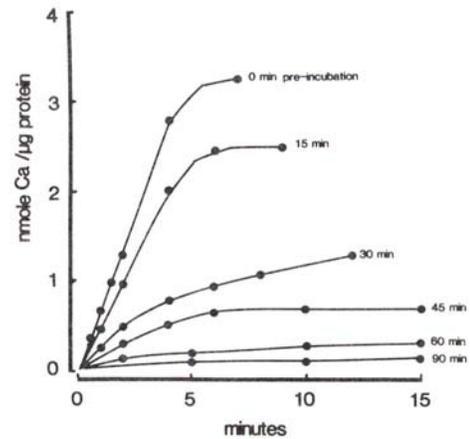


FIGURE 2: Effect of duration of incubation with anti-GP-53 serum. Light SR (13.56 mg/mL) was diluted with 2 volumes of 0.3 M sucrose/20 mM Tris-maleate (pH 7.0) and 1.2 mM PMSF. Aliquots (6  $\mu\text{L}$ ) of diluted SR were mixed with 20- $\mu\text{L}$  aliquots of anti-GP-53 serum and preincubated at 37 °C for the times indicated on the curves. Then  $^{45}\text{Ca}$  uptake was determined. The uptake of  $^{45}\text{Ca}$  is plotted vs time during the uptake assay.

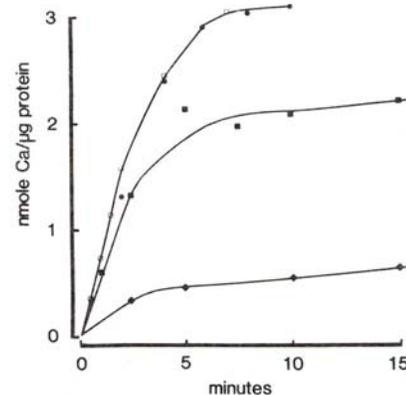


FIGURE 3: Effect of concentration of anti-GP-53 serum. Light SR was diluted as described in the legend to Figure 1. Aliquots (7  $\mu\text{L}$ ) of diluted SR were mixed with 0 ( $\square$ ), 5 ( $\bullet$ ), 10 ( $\blacksquare$ ), or 20 ( $\blacklozenge$ )  $\mu\text{L}$  of anti-GP-53 serum. The total volume was kept constant by adding the appropriate volume of Tris-buffered saline. The mixtures were preincubated at 37 °C for 60 min. Then  $^{45}\text{Ca}$  uptake was determined. Uptake of  $^{45}\text{Ca}$  is plotted vs time during the transport assay.

rate of  $\text{Ca}^{2+}$  uptake. We believe that at the plateau,  $\text{Ca}^{2+}$  is leaking out of the SR vesicles at the same rate at which it is being accumulated. A decrease in the rate of  $\text{Ca}^{2+}$  uptake or an increase in the rate of  $\text{Ca}^{2+}$  efflux from the vesicles would be expected to decrease the amount of  $\text{Ca}^{2+}$  accumulated at the plateau. Experiments described below show that preincubation with anti-GP-53 serum did not increase the passive efflux of  $\text{Ca}^{2+}$  from the SR.

**Effect of Anti-GP-53 Serum on  $\text{Ca}^{2+}$ -ATPase Activity and on the Coupling Ratio.** Light SR (13.56 mg/mL) was diluted 3-fold in Tris/sucrose as described above. Diluted sarcoplasmic reticulum (7  $\mu\text{L}$ ) was mixed with 20  $\mu\text{L}$  of either preimmune serum or anti-GP-53 serum and preincubated for 45 min in a water bath at 37 °C. Each preincubation mixture was then added to the solution used for measuring  $^{45}\text{Ca}$  uptake, which also contained 1 mM Na<sub>3</sub>N. A sample was removed for blanks, and active uptake was initiated by adding ATP. At measured time intervals, samples were taken for determining  $\text{Ca}^{2+}$  uptake and for estimating the hydrolysis of ATP. In this experiment, preincubation with either preimmune serum or anti-GP-53 serum led to a 20% reduction in the rate of  $\text{Ca}^{2+}$ -stimulated hydrolysis of ATP (Figure 4, inset) compared to the  $\text{Ca}^{2+}$ -ATPase activity of SR that was not incubated with

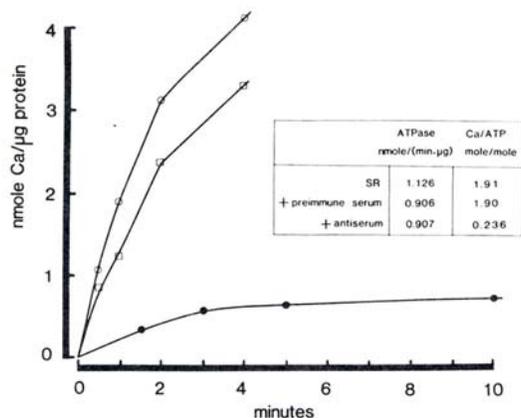


FIGURE 4: Effect of preincubation with anti-GP-53 serum on  $^{45}\text{Ca}$  uptake and ATP hydrolysis. SR ( $7\ \mu\text{L}$  diluted as described in the legend to Figure 2) was mixed with  $20\ \mu\text{L}$  of either preimmune serum ( $\square$ ) or anti-GP-53 serum ( $\bullet$ ) and preincubated for 45 min at  $37^\circ\text{C}$ . Then  $^{45}\text{Ca}$  transport was assayed, and samples were taken from the same mixture for estimation of  $\text{Ca}^{2+}$ -stimulated ATPase activity from the rate of appearance of inorganic phosphate. The curves show  $^{45}\text{Ca}$  uptake as a function of time. The inset shows values of  $\text{Ca}^{2+}$ -ATPase activity and of Ca/ATP ratios computed by dividing the initial rates of  $^{45}\text{Ca}$  uptake by the initial rates of ATP hydrolysis. The values denoted by ( $\circ$ ) are for samples of diluted SR that were kept on ice during the preincubation period.

serum and was kept on ice during the preincubation period. Preincubation with anti-GP-53 serum thus appears to have no greater effect on  $\text{Ca}^{2+}$ -ATPase activity than preincubation with preimmune serum. In this particular experiment, preincubation for 45 min with preimmune serum resulted in a 20% decline in the initial rate of  $^{45}\text{Ca}$  uptake relative to the control of SR kept on ice. In some of our experiments, preincubation with preimmune serum had no effect on ATPase activity and  $^{45}\text{Ca}$  uptake of SR (compared with SR that was not preincubated). Preincubation with anti-GP-53 serum led to a marked inhibition of  $^{45}\text{Ca}$  uptake: 90.1% inhibition relative to control SR and 87.6% inhibition compared to SR preincubated with preimmune serum. The ratio of the number of moles of  $\text{Ca}^{2+}$  ions taken up to the number of moles of ATP hydrolyzed was estimated by dividing the initial rate of  $^{45}\text{Ca}$  uptake by the initial rate of ATP hydrolysis. Incubation with preimmune serum yielded a Ca/ATP ratio of 1.90, which was not significantly different from the Ca/ATP ratio of control SR (Figure 4, inset). Preincubation with anti-GP-53 serum resulted in a decrease in the Ca/ATP ratio of 0.236, a reduction of 87.6% from the control value.

*Influence of Monoclonal Antibodies against GP-53 on the Function of the  $\text{Ca}^{2+}$ -ATPase.* Two different MABs against GP-53, produced in mouse ascites fluid, were used. These MABs are designated XB52 and XIIC4. XB52 is specific for GP-53. XIIC4 reacts with GP-53 and to a lesser extent with the 160-kDa glycoprotein of the SR membrane (Figure 1).

When XB52 and XIIC4, either one at a time or in combination, were preincubated at  $37^\circ\text{C}$  with SR, the MABs had no effect on  $\text{Ca}^{2+}$  uptake or ATP hydrolysis by the SR. However, when XB52 or XIIC4 was preincubated with SR in the presence of an MAB, designated VIE8, against the  $\text{Ca}^{2+}$ -ATPase, the result was a marked inhibition of  $\text{Ca}^{2+}$  uptake (Figure 5A,B). The inhibition of  $\text{Ca}^{2+}$  uptake occurred at levels of the MABs at which each MAB alone had no significant effect on  $\text{Ca}^{2+}$  uptake. Higher levels of VIE8 alone inhibited  $\text{Ca}^{2+}$  uptake and ATP hydrolysis. Preincubation with higher levels of XB52 and XIIC4, alone or in combination, in the absence of VIE8, was without effect in a large number of experiments (data not shown). Since XB52 (specific for

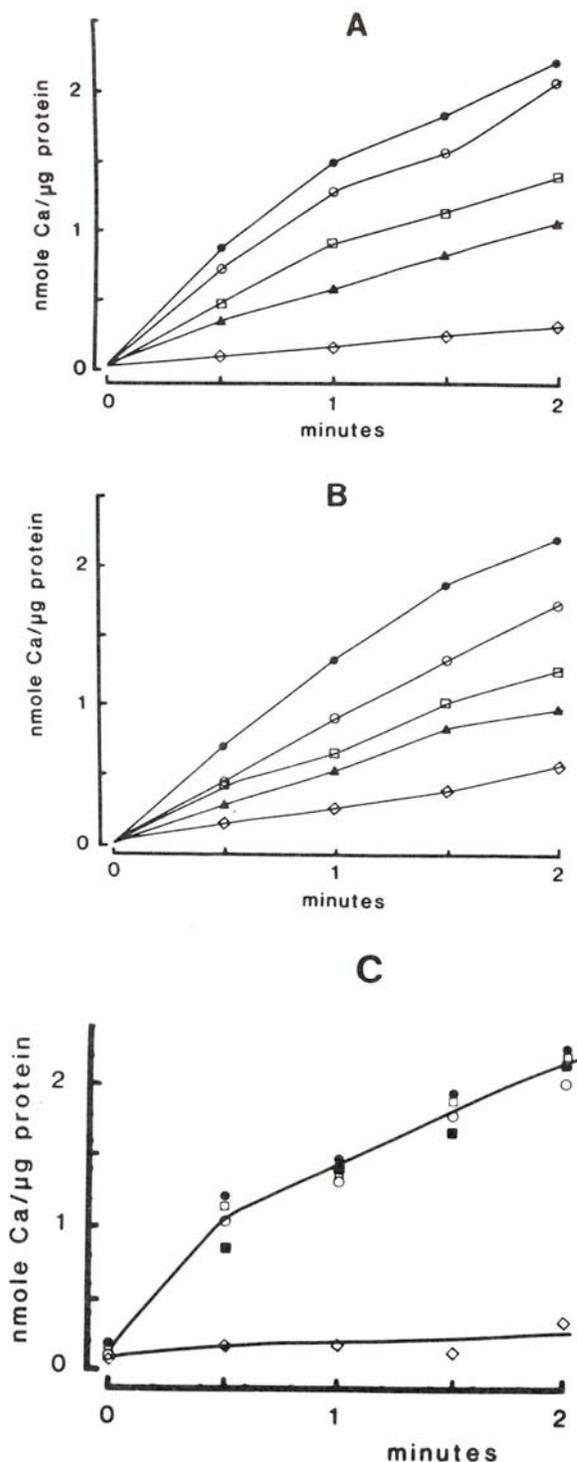


FIGURE 5: Effects of preincubating SR with monoclonal antibodies on  $\text{Ca}^{2+}$  uptake by the SR. SR ( $10.8\ \text{mg}$  of protein/mL) was diluted with 2 volumes of  $0.3\ \text{M}$  sucrose,  $20\ \text{mM}$  Tris-HCl ( $\text{pH}\ 7.0$ ), and  $1.2\ \text{mM}$  PMSF. (Panel A) Five microliters of the diluted SR was preincubated for 2 h at  $37^\circ\text{C}$  with  $10\ \mu\text{L}$  of normal serum ( $\bullet$ ) or with  $5\ \mu\text{L}$  of MAB VIE8 (specific for Ca-ATPase) plus  $0$  ( $\circ$ ),  $2$  ( $\square$ ),  $5$  ( $\blacktriangle$ ), or  $10$  ( $\diamond$ )  $\mu\text{L}$  of MAB XIIC4 (cross-reacts with 53- and 160-kDa glycoproteins). Following the preincubation,  $\text{Ca}^{2+}$  uptake was determined for each preincubation mixture. (Panel B) Similar to panel A, but MAB XB52 (specific for 53-kDa glycoprotein) was used in place of XIIC4 in the volumes  $0$  ( $\circ$ ),  $5$  ( $\square$ ),  $10$  ( $\blacktriangle$ ), or  $15\ \mu\text{L}$  ( $\diamond$ ). (Panel C) Similar to panels A and B, except that monoclonal IID8 (specific for  $\text{Ca}^{2+}$ -ATPase of cardiac SR) was included where indicated. Preincubations were carried out in the presence of normal serum ( $\bullet$ ),  $3\ \mu\text{L}$  of MAB VIE8 ( $\square$ ),  $3\ \mu\text{L}$  of VIE8 plus  $15\ \mu\text{L}$  of IID8 ( $\circ$ ),  $15\ \mu\text{L}$  of IID8 plus  $15\ \mu\text{L}$  of XIIC4 ( $\blacksquare$ ), or  $3\ \mu\text{L}$  of VIE8 plus  $15\ \mu\text{L}$  of XIIC4 ( $\diamond$ ).

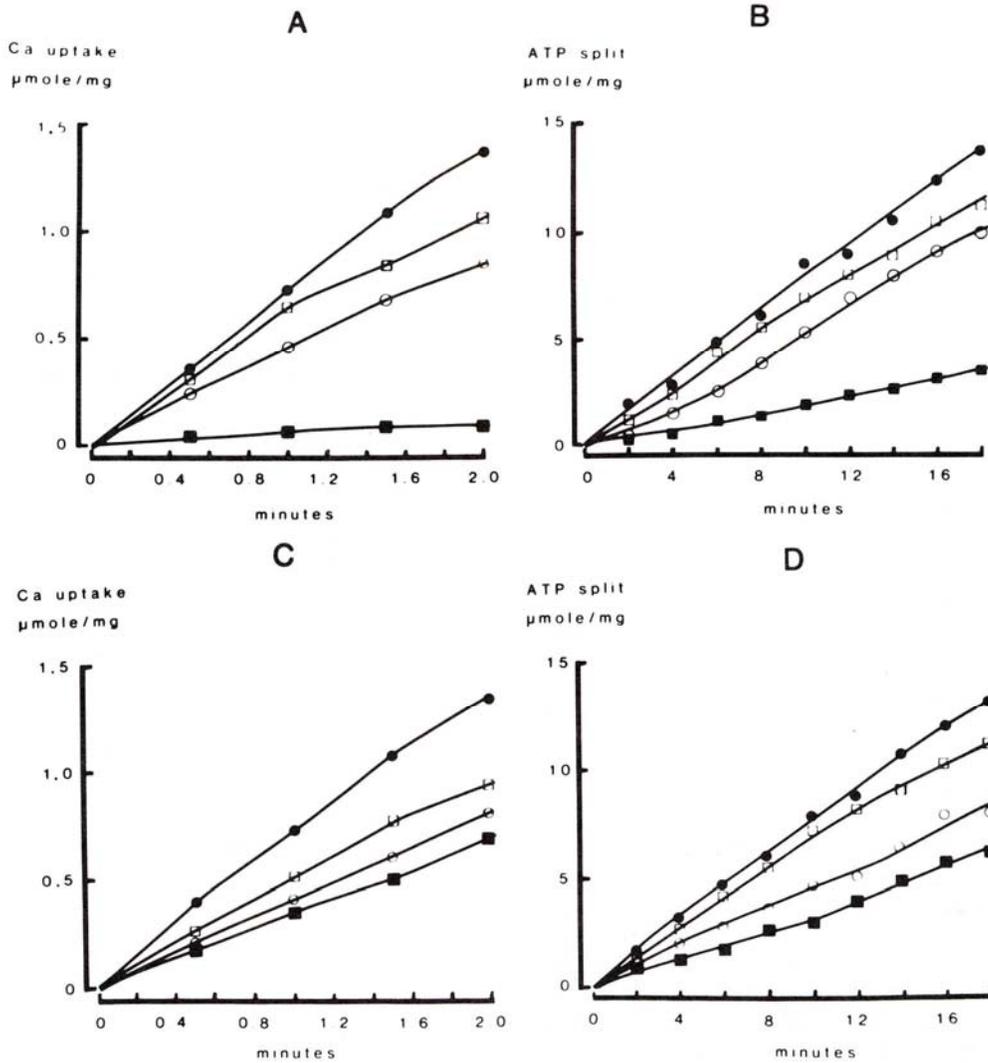


FIGURE 6:  $^{45}\text{Ca}^{2+}$  uptake (panels A and C) and ATP hydrolysis (panels B and D) by SR preincubated with MAb VIES (specific for  $\text{Ca}^{2+}$ -ATPase) and either XIIC4 (cross-reacts with GP-53 and the 160-kDa glycoprotein) or XB52 (specific for GP-53). (Panels A and B) VIES with XIIC4. (Panels C and D) VIES with XB52. SR (12.1 mg/mL) was diluted with 2 volumes of 0.3 M sucrose, 20 mM Tris-HCl (pH 7.0), and 1.2 mM PMSF. (Panels A and B) Diluted SR (45 µL) was preincubated for 2 h at 37 °C with 30 µL of normal sheep serum (●), with 30 µL of VIES alone (○), with 60 µL of XIIC4 alone (□), or with 30 µL of VIES plus 60 µL of XIIC4 (■). (Panels C and D) Diluted SR (45 µL) was preincubated for 2 h at 37 °C with 30 µL of normal serum (●), with 18 µL of VIES alone (□), with 18 µL of VIES plus 30 µL of XB52 (○) or with 18 µL of VIES plus 90 µL of XB52 (■). Following preincubation, the mixtures were kept on ice and samples taken for determination of  $\text{Ca}^{2+}$  uptake and ATP hydrolysis as described under Experimental Procedures.

GP-53) and XIIC4 (binds to GP-53 and GP-160) have similar effects in the presence of VIES, it appears that the inhibitor effects of these MABs are due to their binding to GP-53. IID8, a MAb that reacts with the cardiac, but not the fast skeletal, isoform of  $\text{Ca}^{2+}$ -ATPase (Jorgensen et al, 1988), either alone or in combination with VIES, XIIC4, or XB52, had no effect on  $\text{Ca}^{2+}$  transport by SR (Figure 5C). The data of Figure 5C suggest that the effects we observed with the MABs against skeletal  $\text{Ca}^{2+}$ -ATPase and GP-53 are specific.

In additional experiments, SR was preincubated with VIE8 and either XIIC4 or XB52, and the aliquots of the same preincubation mixtures were used to estimate rates of  $^{45}\text{Ca}$  uptake and rates of ATP hydrolysis (Figure 6). Table II lists initial rates of  $\text{Ca}^{2+}$  uptake,  $\text{Ca}^{2+}$ -ATPase activities, and apparent coupling ratios for the experiments of Figure 6.

Following preincubation with the MABs, similar decreases in  $\text{Ca}^{2+}$  transport and ATP hydrolysis were observed, so that the coupling ratio does not change much in the face of larger decreases in  $\text{Ca}^{2+}$  uptake rate and  $\text{Ca}^{2+}$ -ATPase activity. The data of Table II are not sufficient to determine whether the MABs have a statistically significant effect on the coupling

Table II:  $\text{Ca}^{2+}$  Uptake and  $\text{Ca}^{2+}$ -ATPase Activities for the Experiments Shown in Figure 6<sup>a</sup>

SR preincubated with	$\text{Ca}^{2+}$ uptake	$\text{Ca}^{2+}$ -ATPase activity	Ca/ATP coupling ratio
normal serum	0.702	0.775	0.906
VIE8	0.515	0.683	0.754
VIE8 + 30 µL of XB52	0.385	0.452	0.852
VIE8 + 90 µL of XB52	0.335	0.317	1.06
normal serum	0.700	0.761	0.920
XIIC4	0.610	0.667	0.915
VIE8	0.460	0.539	0.853
VIE8 + XIIC4	0.098	0.126	0.778

<sup>a</sup>  $\text{Ca}^{2+}$  uptake and  $\text{Ca}^{2+}$ -ATPase activities (both in micromoles per minute per milligram of protein) were calculated from the initial slopes of the curves in Figure 6. The coupling ratio was computed as the ratio of the  $\text{Ca}^{2+}$  uptake rate and the  $\text{Ca}^{2+}$ -ATPase activity.

ratio, but these data support the contention that the effect of the MABs on the coupling ratio is quite small compared to the dramatic uncoupling observed following preincubation with anti-GP-53 serum (Figure 4). The coupling ratios listed in Table II for normal serum controls are about 50% of those

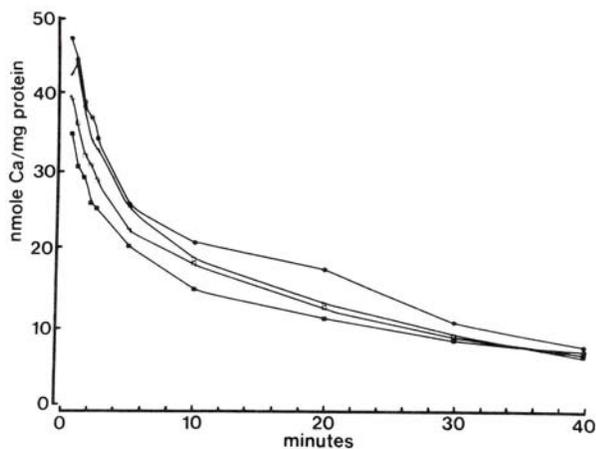


FIGURE 7:  $\text{Ca}^{2+}$  permeability of SR preincubated with anti-GP-53 serum or with monoclonal antibodies under conditions that lead to greatly diminished ATP-driven  $\text{Ca}^{2+}$  uptake. SR (10.8 mg/mL) was diluted with 2 volumes of 0.3 M sucrose, 20 mM Tris-HCl (pH 7.0), and 1.2 mM PMSF. Diluted SR (2  $\mu\text{L}$ ) was mixed with 8  $\mu\text{L}$  of normal serum (■), 8  $\mu\text{L}$  of anti-GP-53 serum (□), 3  $\mu\text{L}$  of VIES plus 10  $\mu\text{L}$  of XB52 (●), or 3 nL of VIES + 10  $\mu\text{L}$  of XIIC4 (○). The mixture for each preincubation for 2 h at 37 °C contained, in addition to the SR and antibodies or sera, 0.1 M KCl, 10 mM Pipes (pH 6.8), and 5 mM  $^{45}\text{CaCl}_2$  (20 mCi/mmol) in a total volume of 40  $\mu\text{L}$ . Following the preincubation period, each preincubation mixture was diluted to 9.1 mL with 0.1 M KCl, 10 mM Pipes (pH 6.8), 5 mM  $\text{MgCl}_2$ , and 1 mM EGTA ("efflux medium"). At the times indicated, 0.5-mL aliquots were removed and filtered on HA Millipore filters. The filters were washed with ice-cold efflux medium, and the amount of  $^{45}\text{Ca}$  retained in the SR vesicle was determined by liquid scintillation counting.

shown in Figure 4. This is a result of the longer preincubation period employed with the MAbs (2 h) in order to enhance the effect of the MAbs, compared to the 45-min preincubation used in Figure 4. With the longer preincubations, we consistently observed decreases in  $\text{Ca}^{2+}$  uptake,  $\text{Ca}^{2+}$ -ATPase activity, and coupling ratio of control SR.

**Effect of Preincubation with Anti-GP-53 Serum or with Monoclonal Antibodies on the Permeability of SR Membrane to  $\text{Ca}^{2+}$ .** SR vesicles were preincubated with anti-GP-53 serum or monoclonal antibodies under conditions previously shown to produce a marked inhibition of ATP-dependent accumulation of  $\text{Ca}^{2+}$ .  $^{45}\text{CaCl}_2$  was included in the preincubation mixture, so that the SR vesicles were passively loaded with  $^{45}\text{Ca}^{2+}$  during the preincubation period. The SR was then diluted into efflux solution and the efflux of  $^{45}\text{Ca}$  followed for 45 min (Figure 7). The rate of  $^{45}\text{Ca}$  efflux from SR preincubated with normal sheep serum is similar to that observed by other investigators (Meissner, 1984). Preincubation of SR with anti-GP-53 serum, with VIES + XIIC4, or with VIES + XB52, under conditions that led to dramatic inhibition of active  $\text{Ca}^{2+}$  accumulation by the SR, had no effect on the passive  $\text{Ca}^{2+}$  permeability of the SR. Thus, the inhibition of  $\text{Ca}^{2+}$  uptake caused by preincubation of SR with anti-GP-53 serum or with monoclonal antibodies does not appear to be due to the preincubation rendering the SR vesicles leaky to  $\text{Ca}^{2+}$ .

In other experiments, after preincubation with normal serum or with anti-GP-53 serum, SR vesicles were actively loaded with  $^{45}\text{Ca}^{2+}$  in the presence of oxalate. The SR that had been preincubated with antiserum took up less  $^{45}\text{Ca}^{2+}$  than SR preincubated with normal serum, but the rate constants for  $^{45}\text{Ca}$  efflux were similar in the two cases. The rates of  $^{45}\text{Ca}^{2+}$  efflux from the actively loaded vesicles were smaller than those of passively loaded SR. We believe this difference may be due to the rate of  $\text{Ca}^{2+}$  efflux from the actively loaded vesicles

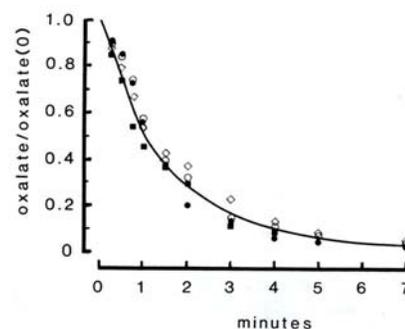


FIGURE 8: Influence of preincubation of SR with anti-GP-53 serum or with monoclonal antibodies on the permeability of the SR membrane to oxalate. SR vesicles were preincubated for 2 h at 37 °C with normal sheep serum, with anti-GP-53 serum, or with MAbs against GP-53 and  $\text{Ca}^{2+}$ -ATPase in 0.1 M KCl, 10 mM Pipes (pH 6.8), 0.5 mM  $\text{CaCl}_2$ , and 12.5 mM [ $^{14}\text{C}$ ]oxalate (about 4754 dpm/nmol). After the preincubation period, the mixture was diluted into 3 mL of efflux medium [0.1 M KCl, 10 mM Pipes (pH 6.8), 5 mM  $\text{MgCl}_2$ , and 1 mM EGTA] at room temperature. After the times shown, 0.2-mL samples were filtered on HA Millipore filters and washed twice with 5 mL of ice-cold efflux medium, and the  $^{14}\text{C}$  retained was determined by liquid scintillation counting. Each preincubation mixture had a volume of 40  $\mu\text{L}$  and included 2  $\mu\text{L}$  of SR (24 mg of protein/mL) and 10  $\mu\text{L}$  of 50 mM [ $^{14}\text{C}$ ]oxalate, as well as 8  $\mu\text{L}$  of normal sheep serum (□), 8  $\mu\text{L}$  of anti-GP-53 serum (○), 3  $\mu\text{L}$  of VIES (Mab against  $\text{Ca}^{2+}$ -ATPase) plus 10 pL of XIIC4 (Mab against GP-53 and GP-160) (◇), or 3 fL of VIES plus 10  $\mu\text{L}$  of XB52 (Mab specific for GP-53) (●).

being limited by the rate of dissolution of calcium oxalate precipitated in the SR lumen. For this reason, we believe that the rates of  $\text{Ca}^{2+}$  efflux from the passively loaded SR more accurately reflect the  $\text{Ca}^{2+}$  permeability of the SR membrane.

**Effect of Preincubation with Anti-GP-53 Serum or Monoclonal Antibodies on the Permeability of the SR to Oxalate.** SR vesicles were preincubated with [ $^{14}\text{C}$ ] oxalate and with anti-GP-53 serum or with MAbs against GP-53 and  $\text{Ca}^{2+}$ -ATPase under conditions previously shown to inhibit ATP-driven, oxalate-supported  $\text{Ca}^{2+}$  uptake. Following the preincubation, the mixture was diluted into efflux medium, and samples were taken at various times for estimation of the rate of efflux of [ $^{14}\text{C}$ ]oxalate from the SR (Figure 8). The rate of oxalate efflux was not appreciably altered by preincubation with anti-GP-53 serum or with the MAbs. It thus appears unlikely that the inhibition of  $\text{Ca}^{2+}$  uptake by the SR following preincubation with anti-GP-53 serum or with MAbs is due to the antibodies rendering the SR impermeable to oxalate. In addition, when GP-53 is selectively removed from the SR by treatment with cholate with 1 M KCl (Leonards & Kutchai, 1985), the oxalate permeability of the GP-53-depleted SR is similar to that of cholate-treated SR that retains its content of GP-53 (Weis and Kutchai, unpublished results). These results suggest that GP-53 is not involved in the permeation of oxalate through the SR membrane.

**Does Proteolysis Result from Preincubation with Anti-GP-53 Serum?** Light SR (13.9 mg/mL) was diluted 3-fold with sucrose/Tris. To 135  $\mu\text{L}$  of diluted SR was added 405  $\mu\text{L}$  of anti-GP-53 serum. Half of this mixture was kept on ice; the other half was incubated in a water bath at 37 °C for 60 min. Both samples were then diluted 5-fold with cold Tris-buffered saline and centrifuged at 50000g at 4 °C for 90 min. The supernatants were discarded. Each pellet was taken up in 60  $\mu\text{L}$  of Laemmli (1970) sample buffer and analyzed by gel electrophoresis in slab gels of 8% polyacrylamide. The gel (Figure 9) contains bands from sarcoplasmic reticulum and from anti-GP-53 serum. The  $\text{Ca}^{2+}$ -ATPase bands are identified by virtue of their apparent molecular weight. The  $\text{Ca}^{2+}$ -ATPase band was absent in gels of anti-GP-53 serum

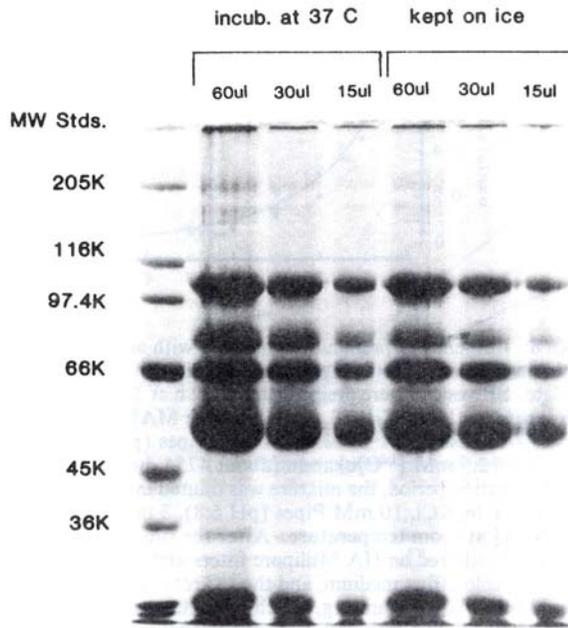


FIGURE 9: SDS-polyacrylamide gel of light SR following incubation with anti-GP-53 serum. 135  $\mu$ L of diluted SR was mixed with anti-GP-53 serum (405  $\mu$ L). Half of this mixture was kept on ice; the other half was incubated at 37  $^{\circ}$ C for 60 min. The mixtures were then diluted with ice-cold Tris-buffered saline and centrifuged at 50000g for 90 min at 4  $^{\circ}$ C. The pellets were taken up in 60  $\mu$ L of Laemmli sample buffer and analyzed by gel electrophoresis (Laemmli, 1970). Molecular weight standards are as indicated. The gel pattern contains bands from both antiserum and from light SR.  $\text{Ca}^{2+}$ -ATPase is the band between the 97.4K and 116K molecular weight standards. This band was absent in gels of anti-GP-53 serum alone.

alone (not shown). The amounts of the ATPase bands are not markedly different in samples of the mixture kept on ice and the mixture incubated at 37  $^{\circ}$ C for 60 min.

When we deliberately exposed the SR to trypsin for brief periods at room temperature, we observed (results not shown) progressive diminution of the 110-kDa band with the appearance of bands at approximately 45 and 55 kDa and, with longer digestion, complete disappearance of the 110-kDa bands and appearance of bands smaller than 45 kDa (Inesi & Scales, 1974; Scott & Shamoo, 1982).

Scott and Shamoo (1982) reported that the second tryptic cleavage of the  $\text{Ca}^{2+}$ -ATPase resulted in uncoupling of the pump. For proteolysis to account for the inhibition of  $\text{Ca}^{2+}$  uptake caused by preincubation with anti-GP-53 serum, about 90% of the  $\text{Ca}^{2+}$ -ATPase would have had to be cleaved to peptides of 45 kDa and smaller. It is clear from Figure 9 that cleavage to this extent did not occur.

Shoshan-Barmatz et al. (1987) reported that tryptic cleavage renders SR vesicles leaky to  $\text{Ca}^{2+}$  before appreciable proteolysis of the  $\text{Ca}^{2+}$ -ATPase occurs. The data of Figure 7 suggest that an effect of this sort was not occurring in our SR after preincubation with anti-GP-53 serum or with monoclonal antibodies.

## DISCUSSION

The results described above show that preincubation of SR with an antiserum directed against the 53-kDa glycoprotein (GP-53) of the SR membrane results in uncoupling of  $\text{Ca}^{2+}$  transport from ATP hydrolysis. Decreased  $\text{Ca}^{2+}$  transport and ATP hydrolysis by the  $\text{Ca}^{2+}$ -ATPase occur following preincubation of SR with either of two MABs against GP-53 in the presence of an MAB against the  $\text{Ca}^{2+}$ -ATPase. The effects of the anti-GP-53 serum and the MABs were not due to

changes in the permeability of the SR membrane to  $\text{Ca}^{2+}$  or oxalate or to proteolysis of the  $\text{Ca}^{2+}$ -ATPase. These results are consistent with the notion that the 53-kDa glycoprotein may be required for coupling or may be involved in regulating coupling of the  $\text{Ca}^{2+}$ -ATPase of the SR (Chiesi & Carafoli, 1982; Leonards & Kutchai, 1985). One interpretation of our results is that the anti-GP-53 serum uncouples  $\text{Ca}^{2+}$  transport from ATP hydrolysis by disrupting the interaction between GP-53 and the  $\text{Ca}^{2+}$ -ATPase that is required for optimal coupling. Further research is required to determine the validity of this interpretation.

The function of the 53-kDa glycoprotein remains to be elucidated. Campbell and MacLennan (1983) found that 8- $\text{N}_3$ -ATP binds specifically to the 53- and 160-kDa glycoproteins, suggesting that the two glycoproteins have high-affinity binding sites for ATP. They speculate that the glycoproteins might be membrane-bound kinases. Alternatively, the binding of ATP might serve to regulate the functions of the glycoproteins.

MacLennan and his colleagues have studied the biosynthesis and degradation of SR proteins. Zubrzycka-Gaarn et al. (1983) found that three intrinsic proteins of the SR membrane [ $\text{Ca}^{2+}$ -ATPase, GP-53, and the 160-kDa glycoprotein (GP-160)] are synthesized and degraded in coordinate fashion in myogenic L6 cells in culture. The synthesis of all three intrinsic membrane proteins was turned on at the beginning of cell fusion to form myotubes, and synthesis increased dramatically during cell fusion.

Campbell and associates have used monoclonal antibodies to localize  $\text{Ca}^{2+}$ -ATPase, GP-53, and GP-160 in various preparations. In microsomal vesicles from red and white skeletal muscle, cardiac muscle, and smooth muscle from esophagus, stomach, intestine, and uterus, GP-53 and GP-160 always co-localized with  $\text{Ca}^{2+}$ -ATPase (Campbell et al., 1983; Pepper et al., 1985). Membranes that lack the  $\text{Ca}^{2+}$ -ATPase of SR type also lack GP-53 and GP-160 (Pepper et al., 1985). On the basis of cross-reactivities of monoclonal antibodies, Pepper et al. (1985) argue that GP-160 is not a trimer of GP-53, as had previously been suggested, but rather that GP-160 contains a region with substantial sequence homology to GP-53. Timms and Campbell (1985) used immunoelectron microscopic techniques to show that GP-53 is distributed throughout the free (longitudinal) SR of rabbit skeletal muscle but is absent from the junctional SR and the transverse tubule membranes.

Reconstituted SR may be prepared by solubilizing SR in detergent and then removing the detergent (Racker, 1972; Meissner & Fleischer, 1974; Warren et al., 1974; Repke et al., 1976; Chiesi et al., 1978). Reconstituted SR usually possesses  $\text{Ca}^{2+}$ -stimulated ATPase activity but may lack the ability to perform ATP-driven  $\text{Ca}^{2+}$  transport. Repke et al. (1976) reconstituted SR by solubilizing in deoxycholate, centrifuging at high speed, and dialyzing away the detergent. They found that increasing the concentration of KCl in the solubilization mixture led to an increased degree of solubilization but yielded reconstituted SR that was deficient in the ATP-driven transport of  $\text{Ca}^{2+}$ . Meissner and Fleischer (1974) found that reconstituted SR prepared by solubilization in deoxycholate followed by dialysis has the  $\text{Ca}^{2+}$ -ATPase as essentially its only protein component and is fully capable of ATP-driven  $\text{Ca}^{2+}$  transport. [Campbell and MacLennan (1981) reported that reconstituted SR made by a similar method contained GP-53 as well as the  $\text{Ca}^{2+}$ -ATPase.]

Chu et al. (1987) reported that a highly purified fraction of longitudinal SR, that contained only 0.07 equiv of GP-53

per mole of  $\text{Ca}^{2+}$ -ATPase, was quite effective in active  $\text{Ca}^{2+}$  accumulation. The estimate by Chu et al. of the relative amounts of  $\text{Ca}^{2+}$ -ATPase and GP-53 was based on the relative intensity of staining of the two proteins with Coomassie blue in SDS-polyacrylamide gels. Michalak et al. (1980) and Leonards and Kutchai (1985) also used the relative intensity of staining with Coomassie blue to quantify GP-53 in light SR. Michalak et al. estimated here are two GP-53 molecules for every three  $\text{Ca}^{2+}$ -ATPase molecules, and the results of Leonards and Kutchai are most consistent with there being one GP-53 for each two  $\text{Ca}^{2+}$ -ATPases. Since the preparation of Chu et al. is more purified in longitudinal SR than previous preparations and since the longitudinal SR is the major location of GP-53 (Timms & Campbell, 1985), it might be expected that the preparation of Chu et al. would have more GP-53 than previous preparations. When we load 2.5 or 5  $\mu\text{g}$  of SR protein per lane, the loading used by Chu et al., we have difficulty quantifying the faint band that appears at 53 kDa. When we load 20  $\mu\text{g}$ , or more SR protein per lane, we obtain values for the ratio of GP-53 to  $\text{Ca}^{2+}$ -ATPase similar to those of Leonards and Kutchai. In silver-stained gels, the apparent ratio of GP-53 to  $\text{Ca}^{2+}$ -ATPase is about twice as high as in Coomassie blue stained gels.

It is well-established that pure, monomeric  $\text{Ca}^{2+}$ -ATPase can hydrolyze ATP (Martin, 1983) and can undergo most of the partial reactions of the ATPase/Ca transport cycle (Kosk-Kosicka et al., 1983). Demonstrably monomeric  $\text{Ca}^{2+}$ -ATPase can occlude  $\text{Ca}^{2+}$  and release  $\text{Ca}^{2+}$  under appropriate conditions (Andersen et al., 1985; Klemens et al., 1986; Vilsen et al., 1986). There is also evidence that pure, monomeric Ca-ATPase in membrane vesicles can actively pump  $\text{Ca}^{2+}$  (Andersen et al., 1983). On the basis of this evidence, some investigators believe that  $\text{Ca}^{2+}$  transport and ATP hydrolysis are inseparable manifestations of the same cycle of chemical and conformational changes. This evidence argues against a requirement for GP-53 in  $\text{Ca}^{2+}$  transport but does not exclude a modulatory role for GP-53.

There is evidence that the  $\text{Ca}^{2+}$ -ATPase in the SR membrane functions as a dimer [reviewed by Ikemoto (1982) and Inesi (1985)]. Kinetic evidence (Ikemoto et al., 1981; Froehlich & Heller, 1985) is consistent with the interpretation that the active  $\text{Ca}^{2+}$  pumping unit is a dimer. The proposed dimeric nature of the  $\text{Ca}^{2+}$ -ATPase suggests the possibility that GP-53 may mediate or modulate cooperative interactions between the two monomers of a dimeric complex. Preliminary experiments in our laboratory are consistent with this interpretation. Boyd and Kutchai (unpublished data) find that extracting GP-53 from the SR membrane results in the negative cooperativity of the dependence of  $\text{Ca}^{2+}$ -ATPase activity on ATP concentration becoming less negative. Xu, Weis, and Kutchai (unpublished data) find that the positive cooperativity of the dependence of  $\text{Ca}^{2+}$ -ATPase activity on the free  $\text{Ca}^{2+}$  concentration is diminished when GP-53 is extracted from the membrane. Preincubation of SR with anti-GP-53 serum decreases the negative cooperativity with which  $\text{Ca}^{2+}$ -ATPase activity depends on [ATP] and decreases the positive cooperativity of the dependence of  $\text{Ca}^{2+}$ -ATPase activity on free [ $\text{Ca}^{2+}$ ] (Boyd and Kutchai, unpublished results).

It may be that GP-53 is homologous in function to the  $\beta$ -subunit of the ( $\text{Na}^+$ ,  $\text{K}^+$ )-ATPase (Chiesi & Carafoli, 1982; Kutchai & Leonards, 1985), which is also a glycoprotein and has an apparent molecular weight near 50 000. The amino acid sequence of the  $\beta$ -subunit has been deduced from the cDNA sequence (Noguchi et al., 1986) and gives a molecular weight of 34671 for the protein portion. The carbohydrate

part of the  $\beta$ -subunit contributes about 5000 to its molecular weight (Kyte, 1972). The function of the  $\beta$ -subunit is not known, but it may be required for activity of the catalytic  $\alpha$ -subunit of the ( $\text{Na}^+$ ,  $\text{K}^+$ )-ATPase. Recent evidence suggests that the  $\beta$ -subunit may play a role in directing newly synthesized ( $\text{Na}^+$ ,  $\text{K}^+$ )-ATPase to the plasma membrane (Noguchi et al., 1987; K. Takeyasu, personal communication). There are important sequence homologies between the ( $\text{Na}^+$ ,  $\text{K}^+$ )-ATPase and the  $\text{Ca}^{2+}$ -ATPase (MacLennan et al., 1985), and there are clear functional homologies between these ion-transporting ATPase (Kyte, 1981). This is consistent with the idea that a protein of the SR membrane, GP-53 being the leading candidate, may have functional homologies to the  $\beta$ -subunit. A glycoprotein of about 55 kDa has also been reported to be associated with the ( $\text{K}^+$ ,  $\text{H}^+$ )-ATPase of gastric mucosa (Karpilow et al., 1987). It is possible that the association of a regulatory glycoprotein is a general feature of ion-transporting ATPases of the E1-E2 type.

Our data are consistent with the interpretation that GP-53 can modulate  $\text{Ca}^{2+}$  transport and ATP hydrolysis by the  $\text{Ca}^{2+}$ -ATPase of the SR. It remains for further research to elucidate the function of GP-53.

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**Registry No.** ATPase, 9000-83-3; Ca, 7440-70-2.

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