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Coupling of Ca^{2+} Transport to ATP Hydrolysis by the Ca^{2+}-ATPase of Sarcoplasmic Reticulum: Potential Role of the 53-Kilodalton Glycoprotein†

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Received December 5, 1984

ABSTRACT: An essential feature of the function of the Ca^{2+}-ATPase of sarcoplasmic reticulum (SR) is the close coupling between the hydrolysis of ATP and the active transport of Ca^{2+}. The purpose of this study is to investigate the role of other components of the SR membrane in regulating the coupling of Ca^{2+}-ATPase in SR isolated from rabbit skeletal muscle, reconstituted SR, and purified Ca^{2+}-ATPase/phospholipid complexes. Our results suggest that (1) it is possible to systematically alter the degree of coupling obtained in reconstituted SR preparations by varying the [KCl] present during cholate solubilization, (2) the variation in coupling is not due to differences in the permeability of the reconstituted SR vesicles to Ca^{2+}, and (3) vesicles reconstituted with purified Ca^{2+}-ATPase are extensively uncoupled under our experimental conditions regardless of the lipid/protein ratio or phospholipid composition. In reconstituted SR preparations prepared by varying the [KCl] present during cholate treatment, we find a direct correlation between the relative degree of coupling between ATP hydrolysis and Ca^{2+} transport and the level of the 53-kilodalton (53-kDa) glycoprotein of the SR membrane. These results suggest that the 53-kDa glycoprotein may be involved in regulating the coupling between ATP hydrolysis and Ca^{2+} transport in the SR.

A major goal of membrane biology is to understand how interactions between membrane components (both lipids and proteins) may be involved in regulating the function of membrane transport proteins. The Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum (SR),[1] which mediates the active uptake of Ca^{2+} into the SR at the expense of ATP, has proven to be a useful model system for examining such membrane interactions. From these studies, a wealth of detailed information has become available on Ca^{2+} binding and the ATPase hydrolysis reaction cycle (Yamada & Ikemoto, 1978; Ikemoto, 1982) and on lipid composition (Swoboda et al., 1979; Bennett et al., 1978, 1980; Knowles et al., 1976), membrane fluidity (Warren et al., 1974; Hesketh et al., 1976; Hidalgo et al., 1976; Hidalgo, 1985), and membrane thickness effects (Caffrey & Feigenson, 1981; Johannson et al., 1981) on ATPase activity. However, very little is known about the mechanism by which

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[1] Abbreviations: SR, sarcoplasmic reticulum; R-SR, reconstituted sarcoplasmic reticulum; GP-53, 53-kDa glycoprotein; kDa, kilodalton(s); ATP, adenosine 5′-triphosphate; NADH, nicotinamide adenine dinucleotide, reduced form; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TFP, trifluoperazine.

0006-2960/85/0424-4876$01.50/0

Ca^{2+} is actually translocated across the SR membrane by the Ca^{2+}-ATPase or the roles that other membrane components may play in regulating this process.

Understanding this regulation is important because an essential feature of the normal function of the Ca^{2+}-ATPase is the close coupling between Ca^{2+} transport and ATP hydrolysis. Present evidence indicates that the two Ca^{2+} ions are transported for each ATP hydrolyzed in fragmented rabbit SR (Andersen et al., 1983; Ikemoto et al., 1982; Inesi, 1979; Meltzer & Berman, 1984). However, it has not yet been possible to reconstitute detergent-purified Ca^{2+}-ATPase molecules into lipid vesicles which maintain this level of coupling. Instead, typical ratios of Ca^{2+} ions transported to ATP molecules hydrolyzed obtained for such reconstituted preparations range from 0.2 to 0.9 (Caffrey & Feigenson, 1981; Racker, 1972). Moreover, this degree of coupling required the use of high concentrations of phosphate or oxalate to "trap" Ca^{2+} in the intravesicular space. The failure to achieve good coupling has sometimes been attributed to an increase in the Ca^{2+} permeability of the reconstituted preparations. However, low coupling ratios have also been obtained with reconstitued Ca^{2+}-ATPase/lipid vesicles that are highly impermeable to Ca^{2+} (Caffrey & Feigenson, 1981). Consequently, a number of researchers have recently investigated whether other SR membrane proteins or specific membrane lipids may be involved in regulating the coupling of ATP hydrolysis to Ca^{2+} transport. Knowles et al. (1976) reported that phosphatidylethanolamine is required to obtain optimal Ca^{2+} uptake in reconstituted Ca^{2+}-ATPase/lipid vesicles. Hidalgo et al. (1982) have shown that it is possible to inhibit Ca^{2+} transport without affecting ATPase activity by modifying the phosphatidylethanolamine head groups in isolated SR vesicles. On the basis of these, and similar, results, it was proposed (Navarro et al., 1984) that the ability of membrane lipids, such as phosphatidylethanolamine, to form nonbilayer structures (i.e., hexagonal II phases) may be important in regulating Ca^{2+} transport/ATP hydrolysis coupling.

Other proteins of the SR might also be involved in regulating the coupling of the Ca^{2+}-ATPase. Repke et al. (1976) reported that they obtained higher Ca^{2+} transport activities in reconstituted SR preparations which contained some, but not all, of the smaller SR protein components in addition to the Ca^{2+}-ATPase. More recently, Chiesi & Carafoli (1982) found that trifluoperazine (TFP) binds tightly to a 53-kDa glycoprotein (Campbell & MacLennan, 1981; Michalak et al., 1980) in the SR membrane and presented evidence that this binding is responsible for the inhibition, under certain conditions, by TFP of Ca^{2+}-ATPase activity and Ca^{2+} transport in skeletal muscle SR. This led them (Chiesi & Carafoli, 1982, 1983) to speculate that the 53-kDa glycoprotein might be involved in regulation of Ca^{2+} transport by the Ca^{2+}-ATPase.

In the present study, we have investigated the potential roles of various membrane lipid and protein components in regulating the coupling between Ca^{2+} transport and ATP hydrolysis in skeletal muscle sarcoplasmic reticulum vesicles. Specifically, we have studied the relationships between the composition of isolated SR, reconstituted SR, and purified Ca^{2+}-ATPase/phospholipid preparations, the experimental procedures used to generate such preparations, and the qualitative degree of coupling obtained for these vesicles. Our results demonstrate (1) that it is possible to systematically alter the relative degree of coupling obtained for reconstituted SR vesicles, (2) that the variation in this coupling is not due to differences in the permeability of the vesicles to Ca^{2+}, and (3) that vesicles reconstituted with the purified Ca^{2+}-ATPase are extensively uncoupled under our experimental conditions irrespective of lipid composition or lipid/protein ratio. In addition, our results indicate that there is a direct correlation between the ability to obtain a highly coupled reconstituted vesicle preparation and the degree to which the 53-kDa glycoprotein of the SR membrane is retained in the preparation, suggesting that this glycoprotein may be intimately involved in regulating the coupling between Ca^{2+} transport and ATP hydrolysis by the Ca^{2+}-ATPase in the native SR. Abstracts of this research have been presented previously (Leonards & Kutchai, 1984a,b).

Experimental Procedures

Materials. Phospho(enol)pyruvate (monopotassium salt), pyruvate kinase (type III), L-lactic acid dehydrogenase (type XI), MgATP (vanadium free), β-NADH (from yeast, grade III, disodium salt), triethanolamine hydrochloride, MOPS, histidine, EGTA, DTT, PMSF, cholic acid, and chloride salts were obtained from Sigma Chemical Corp. SDS, acrylamide, *N,N'*-methylenebis(acrylamide), 2-mercaptoethanol, *N,N,N',N'*-tetramethylenediamine, glycine, and Tris-HCl were purchased from Bio-Rad Laboratories. Egg PC (>99%), egg PE [made by transphosphatidylation of egg PC (>99%)], and soybean total phosphatide extract were obtained from Avanti Polar Lipids, Inc. Sepharose 4B was from Pharmacia. Sodium azide was from Mallinckrodt Chemical Co. $^{45}CaCl_2$ was obtained from New England Nuclear. Dialysis tubing (Spectrapore 6, M_r 1000 cutoff) was from Fisher Scientific. The calcium ionophore A23187 was purchased from Calbiochem. Cholic acid was recrystallized at 4 °C from 50% (v/v) ethanol/water after a hot solution (5% w/v) in 100% ethanol was filtered through activated charcoal. Organic solvents were redistilled before use.

Isolation of Sarcoplasmic Reticulum (SR) Vesicles. Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle by a modification of published procedures (East & Lee, 1982; Robinson et al., 1972). All procedures were conducted at 4 °C or with ice-cold buffers. Briefly, freshly dissected rabbit hind limb and back muscle (450–500 g) was minced, passed through a meat grinder, and then twice homogenized by using a Willems FT-20 Polytron (Brinkmann Instruments) in 4–6 volumes of buffer A [0.3 M sucrose, 20 mM histidine (or MOPS), 1 mM DTT, and 200 μM PMSF (pH 7.3)]. The homogenate was centrifuged at 8000*g* for 15 min, and the supernatants were centrifuged at 11000*g* overnight. The pellets were resuspended in 160 mL of buffer B [10% sucrose (w/v), 10 mM histidine (pH 7.3), 0.6 M KCl, 1.0 mM DTT, and 200 μM PMSF], homogenized with a Potter–Elvehjem homogenizer, and left on ice for at least 60 min. This preparation was centrifuged at 10000*g* for 15 min and the resulting supernatant centrifuged again at 37500*g* for 4 h. The white layer of the pellet (avoiding the lower brown layer of the pellet) was resuspended in 6–10 mL of buffer C [0.25 M sucrose, 1.0 M KCl, 2.5 mM DTT, 200 μM PMSF, and 50 mM potassium phosphate (pH 8.0)], dialyzed overnight against 1 L of buffer C, and then centrifuged at 10000*g* for 15 min. The supernatant was layered onto a discontinuous sucrose gradient [60%, 30%, and 20% (w/w) based on buffer C] and centrifuged overnight at 95000*g*. High-base-line ATPase activity, but almost no Ca^{2+}-stimulated ATPase activity, was found in the 20% sucrose layer. The 30% layer was enriched in Ca^{2+}-stimulated ATPase activity. The 30% sucrose layer was removed, diluted with 3 volumes of buffer D [100 mM NaCl, 1.0 mM NaN_3, 1.0 mM DTT, and 50 mM potassium phosphate (pH 7.4)], layered onto a 60% sucrose cushion (w/v) in buffer D, and centrifuged at 95000*g* for 8 h. The material (SR vesicles) that accumulated at the in-

terface between the 60% sucrose cushion and the sample solution was collected, dialyzed overnight against 1 L of solution 1 [0.25 M sucrose, 100 mM NaCl, 1.0 mM DTT, 1.0 mM NaN_3, and 50 mM potassium phosphate (pH 7.4)], frozen in small aliquots in liquid N_2, and stored at −20 °C.

Purification of Ca^{2+}-ATPase/Lipid Complexes. Ca^{2+}-ATPase/lipid complexes were purified [according to the method of East & Lee (1982)] by branching off the SR vesicle preparation procedure described above. After overnight dialysis of the sample against buffer C, the protein concentration was determined and the sample treated with a 10% (w/v) cholate solution in buffer C to give a final ratio of 0.4 mg of cholate/mg of protein. The preparation was kept on ice for 60–90 min and centrifuged at 32000*g* for 1 h at 4 °C, and the supernatant was layered onto a 60/40/30/20% (w/w) sucrose gradient in buffer C as described above. Subsequent steps were as described by East & Lee (1982). The purified Ca^{2+}-ATPase/lipid complexes were frozen in small aliquots in liquid N_2 and stored at −20 °C. The purity of the Ca^{2+}-ATPase preparation was assessed by SDS gel electrophoresis.

Reconstitution of Membrane Vesicles Containing Ca^{2+}-ATPase. (*A*) *SR Vesicles Reconstituted in the Absence of Added Lipids* (*R-SR*). Aliquots of isolated SR vesicle preparations in RS1 buffer [250 mM sucrose, 100 mM NaCl, 1.0 mM DTT, 1.0 mM NaN_3, and 50 mM potassium phosphate (pH 7.4)] were thawed, diluted as required with RS1, and then solubilized at 25 °C by injecting aliquots of 5% (w/v) cholate in RS1 and mixing by hand. The volumes of these solutions were manipulated so that the final protein and cholate concentrations were each 2.5 mg/mL (1/1 protein/cholate). Control experiments using various cholate/protein ratios and cholate concentrations verified that the 1/1 ratio and 2.5 mg/mL cholate concentration chosen did not inactivate the Ca^{2+}-stimulated ATPase under these conditions. The samples were incubated for 30 min at room temperature and then dialyzed overnight (Spectrapore 6, M_r 1000 cutoff dialysis tubing) against 1 L of RS1 at room temperature with one or more buffer changes. The dialysis procedure yields reconstituted vesicles (Racker, 1972). The sequence of additions in this procedure is important (SR vesicles + RS1 and then cholate) because cholate is in dynamic equilibrium between that free in solution (dimers), that present as cholate micelles, and that which is partitioning into the membrane causing its solubilization. Mixing the cholate solution with the SR vesicle preparation before it is diluted with RS1 alters this equilibrium, resulting in a more extensive disruption of the SR membrane, and in some cases inactivating the Ca^{2+}-ATPase.

(*B*) *Reconstitution of SR or Purified Ca^{2+}-ATPase with Exogenous Lipids.* Isolated SR vesicles or Ca^{2+}-ATPase/lipid vesicles were reconstituted with exogenous lipids by solubilizing the lipid- and protein-containing samples separately and then mixing them together. Purified lipid stock solutions in chloroform (e.g., egg PC, egg PE, total soybean phosphatide extract) were mixed as necessary in chloroform to give the final desired composition and taken to dryness first under N_2 and then under vacuum. The lipid samples were resuspended by adding aliquots of 5% (w/v) cholate in RS1 buffer to give a final lipid/cholate ratio of 2/1 (w/w), mixed, and diluted to 0.5–1 mL with RS1 buffer. The samples were mixed on a vortex mixer for 5 min under N_2 and then sonicated to clarity in a bath-type sonicator (Branson Model B-12) under N_2 at 25 °C. The lipid samples were then left standing at room temperature under N_2 for 20–30 min. In some cases, the K^+ concentration of the RS1 buffer was modified with KCl. SR vesicles were solubilized as described above (section A) with two modifications. First, the total volume of the SR vesicle sample was slightly reduced so that the final concentration of SR protein, after mixing with the exogenous lipid/cholate mixture, was maintained at 2.5 mg/mL (final total volume after mixing was 3 mL). Second, the cholate concentration of the solubilized SR sample was kept constant at 2.5 mg/mL. Consequently, the cholate/protein ratio was also slightly reduced in the solubilized SR sample [usually to 0.85/1 (w/w)]. The samples were then incubated for 30 min at room temperature. After the cholate/lipid and solubilized SR preparations were incubated separately, the samples were mixed, incubated for 30 min at 25 °C, and then dialyzed as previously described for vesicle reconstitution. Purified Ca^{2+}-ATPase/lipid complexes were also reconstituted with exogenous lipids as described above with one further modification: after the Ca^{2+}-ATPase/lipid complexes were solubilized with cholate, the preparation was mixed with the desired lipid/cholate sample after only a few minutes to avoid inactivation of the Ca^{2+}-ATPase.

(*C*) *Fractionation of Reconstituted Preparations.* Preparations were fractionated by using three methods. In the first, reconstituted vesicles were separated from unincorporated material by density gradient centrifugation. After dialysis, the preparations were layered onto 60/40/30/20% (w/w) sucrose gradients in RS1 buffer and centrifuged overnight at 95000*g* at 4 °C. The reconstituted vesicles, which accumulated in the 30% sucrose layer, were removed with a Pasteur pipet and used for subsequent experients. Unincorporated material, accumulating at the RS1/20% sucrose interface, was also removed and assayed as a control preparation. In some experiments, the isolated SR vesicles were solubilized with cholate and fractionated on sucrose gradients as above (but at 20 °C), and the individual sucrose layers were then dialyzed against RS1 buffer overnight at 25 °C to reconstitute vesicles. In the third method, R-SR preparations were fractionated on a Sepharose 4B column (1.9 × 45 cm) using RS1 as elution buffer (flow rate 6 mL/h) at 25 °C. One-milliliter fractions were collected.

Assay of ATPase Activity. The ATPase activities of isolated SR, reconstituted SR (R-SR), and reconstituted Ca^{2+}-ATPase/lipid vesicles were determined spectrophotometrically at 25 °C by a coupled enzyme assay (Madden et al., 1979). The decrease in absorbance at 340 nm (NADH oxidation) was followed in a reaction mixture containing 100 mM triethanolamine (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 3 mM MgATP, 1.25 mM phospho(enol)pyruvate, 0.2 mM NADH, and 20 IU each of pyruvate kinase and L-lactic acid dehydrogenase. Vesicle preparations (2–10 μg of protein/mL) were preincubated for 10 min in the reaction mixture without Ca^{2+} to determine the base-line ATPase activity. Background activity was undetectable in R-SR and Ca^{2+}-ATPase/lipid samples. The reaction was started by injecting $CaCl_2$ to give a final total calcium concentration of 0.55 mM.

Estimation of Coupling between Ca^{2+} Transport and ATP Hydrolysis. In these studies, the degree of coupling between Ca^{2+} transport and ATP hydrolysis is operationally defined to be proportional to stimulation of the Ca^{2+}-ATPase activity when the calcium ionophore A23187 (6.5 μM) is added. The difference between the specific activity in the presence and absence of A23187 divided by the specific activity in the absence of ionophore is used as an index of coupling. The higher the degree of coupling between Ca^{2+} transport and ATP hydrolysis, the greater this quantity. A portion of the isolated SR preparation used to generate R-SR or Ca^{2+}-ATPase/lipid vesicles was assayed ±A23187 concomitantly with the re-

constituted preparations. The coupling of isolated SR was used as a reference point for determining the relative degree of coupling of various reconstituted preparations. This method is a qualitative, but internally consistent, measure of the coupling between Ca^{2+} transport and ATP hydrolysis. It cannot be used to determine the absolute stoichiometry of Ca^{2+} transported to ATP hydrolyzed.

Estimating the Rate of Passive Ca^{2+} Efflux from Reconstituted SR. Aliquots (2.5 mg of protein) of a sarcoplasmic reticulum preparation were solubilized in cholate in RS1 in a total volume of 1 mL as described above in the method for preparing R-SR. The RS1 contained 0.0, 0.25, 0.5, or 1.0 M added KCl. This gave total K^+ concentrations of 0.094, 0.344, 0.594, and 1.094 M, respectively. After standing for 30 min at room temperature, the preparations were dialyzed overnight against RS1 with no added KCl at 25 °C. The R-SR preparations were assayed for Ca^{2+}-stimulated ATPase activity as described above in the presence and absence of A23187.

To load the R-SR vesicles with $^{45}Ca^{2+}$, 300 μL of each preparation was put in a vial, and 14 nmol of $CaCl_2$ and 2.8 nmol of $^{45}CaCl_2$ (2.5 μCi/μg) were added. The vials were incubated overnight on ice and then at 37 °C for 1 h. Efflux was initiated by adding enough EGTA to chelate all extravesicular Ca^{2+} [700 μL of 0.5 mM EGTA in 100 μM triethanolamine (pH 7.4), 100 mM KCl, and 5 mM $MgCl_2$ was added to each vial]. This solution was chosen to mimic the solution used in our ATPase assays.

For estimation of Ca^{2+} efflux, 100-μL samples were taken after 2.5, 7.5, 12.5, 22.5, 32.5, and 62.5 min of incubation at room temperature. Then A23187 (0.4 μg/mL) was added, and further samples were taken at 5-min intervals. Samples were immediately filtered on HA Millipore filters (0.45-μm pore size) and washed with 2 mL of the ATPase buffer. The filters were dried and counted in Beckman HP liquid scintillation cocktail. To estimate the rate of $^{45}Ca^{2+}$ leakage from the R-SR, the natural logarithm of the counts on the filter was plotted vs. time. The plots were linear, and their slopes were taken to be the apparent first-order rate constants (k) for Ca^{2+} efflux. The half-time for Ca^{2+} efflux was computed as $(\ln 2)/k$.

Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970) using 10% acrylamide separating gels. Gels were stained with Coomassie blue or dansylhydrazine. Protein bands were identified on the basis of their apparent molecular weights estimated by using molecular weight marker proteins (Boehringer-Mannheim GMBH-Biochemica) and comparisons with the designations in the literature (Campbell & MacLennan, 1981). Glycoproteins were identified by their fluorescence after dansylhydrazine treatment (Chiesi & Carafoli, 1982; Eckhardt et al., 1976; Lutz et al., 1979). The band identified as the 53-kDa glycoprotein shifted to an apparent molecular weight of about 49 kDa after treatment with endoglycosidase H (Campbell & MacLennan, 1981). The relative concentrations of the Ca^{2+}-ATPase, calsequestrin, and 53-kDa glycoprotein present in the R-SR vesicle samples were determined by cutting out the Coomassie blue stained bands from the SDS gels and measuring the concentration of extracted dye spectrophotometrically at 590 nm as described by Michalak et al. (1980). Protein concentrations were estimated by the method of Lowry (1951), as modified for membrane proteins by Wang & Smith (1975), using bovine serum albumin as standard.

Results

Purified Ca^{2+}-ATPase Reconstituted with Exogenous Lipids. Previous studies have suggested that lipid composition may be an important regulator of Ca^{2+} transport/ATP hydrolysis coupling of the Ca^{2+}-ATPase. To examine this possibility, the Ca^{2+}-ATPase was isolated, purified, and reconstituted into vesicles of known phospholipid composition. Freeze–fracture electron microscopy of such preparations confirmed the presence of large unilamellar vesicles with a random distribution of the Ca^{2+}-ATPase molecules in the vesicle membrane. In the first series of experiments, the Ca^{2+}-ATPase was reconstituted with egg PC at an exogenous lipid to protein ratio of 1/1 (w/w). The ATPase activities of these preparations were then determined in the presence and absence of the ionophore A23187 to estimate the degree of coupling between ATP hydrolysis and Ca^{2+} transport present in the samples. The results (Table IA) show that although the ATPase activities of such preparations were high, there was no significant difference between their activities in the presence and absence of A23187, indicating that these preparations were completely uncoupled. When these experiments were repeated at an egg PC/Ca^{2+}-ATPase ratio of 20/1 (w/w), similar results (not shown) were obtained. In contrast, isolated SR vesicles, assayed under the same conditions, displayed a marked increase in ATPase activity in the presence of A23187 (Table IA), demonstrating that these preparations were highly coupled.

Since PE has been reported to be required for optimal Ca^{2+} uptake in reconstituted Ca^{2+}-ATPase vesicles, another set of experiments was conducted using purified Ca^{2+}-ATPase and mixtures of egg PC and egg PE. The egg PE employed was prepared by transphosphatidylation of the egg PC head group, thus keeping the fatty acyl chain composition constant in these experiments. The results obtained for vesicles reconstituted with equimolar amounts of egg PC and egg PE, at a ratio of total lipid added to protein of 1/1 (w/w), are also shown in Table IA. The ATPase activities in the presence and absence of A23187 obtained for the preparations reconstituted with egg PC and with egg PC/egg PE mixtures are essentially the same as those observed for egg PC/Ca^{2+}-ATPase vesicles under the same conditions. Related experiments conducted by using an egg PE to egg PC ratio of 1/4 (w/w) at a ratio of total lipid added to protein of 20/1 (w/w) also gave com-

Table I: Ca^{2+}-ATPase Activities of Reconstituted Preparations

prepn	Ca^{2+}-ATPase without A23187 (IU)	Ca^{2+}-ATPase with A23187 (IU)	fractional stimulation by A23187
(A) Purified Ca^{2+}-ATPase Reconstituted with Egg Phospholipids			
control SR	0.56 ± 0.004	2.90 ± 0.04	4.15
PC/ATPase (1/1)	2.18 ± 0.03	2.20 ± 0.11	NS[a]
PC/PE/ATPase (1/1/2)	2.14 ± 0.07	1.84 ± 0.07	NS
PC/ATPase/other SR components (1/2/1)	1.47 ± 0.06	1.47 ± 0.01	NS
(B) Reconstituted SR Prepared with Low or High KCl at 4 or 25 °C with No Added Lipids			
control SR	0.56 ± 0.004	2.90 ± 0.04	4.15
high KCl, 4 °C	1.61 ± 0.05	2.09 ± 0.05	0.30
high KCl, 25 °C	0.67 ± 0.01	1.16 ± 0.02	0.73
low KCl, 4 °C	1.95 ± 0.03	3.23 ± 0.07	0.65
low KCl, 25 °C	0.67 ± 0.01	3.04 ± 0.10	3.51
(C) Reconstituted SR Prepared with Low-KCl Solution at 25 °C with Variable Amounts of Egg PC Added			
control SR	0.56 ± 0.004	2.90 ± 0.04	4.15
R-SR, no egg PC	0.67 ± 0.01	3.04 ± 0.10	3.51
egg PC/SR (1/1)	0.57 ± 0.04	2.12 ± 0.02	2.71
egg PC/SR (5/1)	0.74 ± 0.03	1.52 ± 0.11	1.05
egg PC/SR (10/1)	2.02 ± 0.05	1.84 ± 0.01	NS

[a] NS, no significant stimulation of Ca^{2+}-ATPase activity by A23187.

pletely uncoupled preparations (data not shown).

The possibility that essential lipids, such as plasmalogens, were being removed during the purification procedure was examined in a third series of experiments. The Ca^{2+}-ATPase was purified from isolated SR vesicles as described above, except that all the fractions obtained from the sucrose gradient layers were recombined in the presence of cholate to regenerate the original sample mixture. These samples were then dialyzed, as described above, to obtain reconstituted vesicles and their ATPase activities measured with and without A23187. The results (Table IA) show that these vesicles remained completely uncoupled, indicating that the decrease in coupling is not reversible under these experimental conditions, even in the presence of all components of the SR.

Fractionation of the reconstituted lipid/Ca^{2+}-ATPase vesicles by sucrose gradient centrifugation did not affect these results; neither did using HEPES or MOPS buffers rather than potassium phosphate.

[K^+] and Temperature Effects on Reconstituted SR Vesicles. The inability to obtain reconstituted lipid/Ca^{2+}-ATPase vesicles that were coupled led us to reexamine how the methods used to isolate, purify, and reconstitute the Ca^{2+}-ATPase might be modifying the coupling between Ca^{2+} transport and ATP hydrolysis that is present in the isolated SR vesicles. A major difference between the isolated SR vesicles and the reconstituted lipid/Ca^{2+}-ATPase preparations was the exposure of the latter to cholate and high [KCl] (1.0 M). SR vesicles had been washed with 1.0 M KCl but were not exposed simultaneously to cholate and high [KCl]. To investigate the effects of K^+ concentration and temperature on our preparations, we determined the effects of solubilizing and reconstituting SR in high and low [KCl] at 4 and 25 °C.

In the first series of experiments, isolated SR vesicles were solubilized and reconstituted in either RS1 (low K^+) or RS1 with 1.0 M added KCl (high K^+) buffers. In each case, the reconstitution mixture contained the complete SR vesicle sample, and identical volumes and protein and detergent concentrations were used One set of experiments was carried out at 4 °C and another at 25 °C. The Ca^{2+}-ATPase activities of these R-SR preparations were determined at 25 °C in the presence and absence of A23187 to determine the extent of coupling. As shown in Table IB, R-SR vesicles solubilized and reconstituted in high-K^+ buffer had lower maximal (+A23187) ATPase activities and were less coupled than R-SR prepared in low-K^+ buffer. The low-K^+ R-SR vesicles generated at 25 °C were significantly more coupled than the 4 °C preparations. The maximal ATPase activities (+A23187) of R-SR vesicles prepared in low K^+ at both 4 and 25 °C were similar to those observed for isolated SR vesicles.

To determine whether the uncoupling observed in high-K^+ R-SR vesicles occurred during the solubilization or the reconstitution phase of the procedure, the following experiments were performed. Isolated SR vesicles were solubilized with cholate in RS1 (low K^+) or RS1 with 1.0 M KCl added (high K^+). All samples were then reconstituted with RS1 (low K^+) buffers. The Ca^{2+}-ATPase activities obtained for these preparations were similar to those shown in Table IB, indicating that uncoupling occurs during SR vesicle solubilization, rather than during reconstitution.

Effect of pH on Reconstitution. To ascertain if pH had any influence on these results, the experiments just described were repeated at pHs 7.4, 7.6, 7.8, and 8.0, using both high- and low-K^+ buffers at 25 °C. No differences in coupling were found for the high-K^+ R-SR preparations over this pH range. A very small decrease in coupling was observed for the low-K^+ R-SR samples as the pH was increased from 7.4 to 8.0. These results indicate that the uncoupling observed was not due to pH effects.

Passive Ca^{2+} Efflux from Reconstituted SR. An increased permeability to Ca^{2+} of R-SR vesicles prepared in high-K^+ buffers could account for the uncoupling we observed under these conditions. To investigate this possibility, we estimated the passive permeability of R-SR preparations prepared in RS1 with 0.0, 0.25, 0.5, and 1.0 M added KCl. The ATPase activities and degrees of coupling of these R-SR preparations were similar to those shown in Table IB. The 0.0 and 0.25 M KCl preparations showed good coupling; the 0.5 and 1.0 M KCl preparations were progressively less coupled. The half-times for Ca^{2+} efflux were 56.4, 48.1, 42.8, and 48.1 min for 0.0, 0.25, 0.5, and 1.0 M KCl preparations, respectively. Addition of A23187 (0.4 μg/mL) caused a 10-fold increase in the Ca^{2+} efflux rate. There was no significant correlation between the degree of coupling and the half-time of Ca^{2+} leak. The 0.25 M KCl R-SR preparations, which had 71% of the coupling of the 0.0 M KCl R-SR, had the same Ca^{2+} efflux rate as the 1.0 M KCl R-SR that was only 3.5% as coupled as the 0.0 M KCl R-SR. These data demonstrate that the decreased coupling we observed in R-SR prepared in high KCl was not due to an increase in Ca^{2+} permeability of the reconstituted vesicles.

Effects of Increasing Lipid/Protein Ratio in Reconstituted SR Vesicles. A possible explanation for the uncoupling observed in R-SR vesicles prepared in high-K^+ buffers is that the Ca^{2+}-ATPase itself is altered in the presence of cholate and high K^+. Another possibility is that there is another SR membrane component that is involved in regulating coupling and that it is this component, or its interaction with the Ca^{2+}-ATPase, that is being modified during solubilization of the SR in high-K^+ buffers. To help distinguish between these two possibilities, isolated SR vesicles were solubilized with cholate in RS1 (low K^+) buffer, mixed with increasing concentrations of exogenous egg PC to alter the lipid/protein ratio, and then reconstituted in RS1 buffer (low K^+). If the uncoupling previously observed is due to a direct effect of high K^+ concentration on the Ca^{2+}-ATPase, increasing the lipid/protein ratio in low-K^+ buffer would be expected to have no major effect on coupling. However, if interaction of the Ca^{2+}-ATPase with another component of the SR membrane is involved in coupling, it might be possible to progressively reduce coupling by "diluting out" this component by increasing the egg PC/protein ratio in the reconstituted vesicles.

The results of these experiments (Table IC) demonstrate that the R-SR vesicles became more uncoupled as the ratio of egg PC added to SR protein was increased from 1/1 to 5/1 (w/w). Further increasing the egg PC/protein ratio to 10/1 (w/w) results in R-SR vesicles that were completely uncoupled (as observed for vesicles reconstituted with purified Ca^{2+}-ATPase and egg PC), even though all SR components were present in the reconstitution mixture. Experiments using soybean total phosphatide extract instead of egg PC gave similar results (not shown).

Effects of Increasing [K^+] on Coupling of Reconstituted SR. The results presented above are consistent with the possibility that another component of the SR membrane is involved in regulating the coupling of ATP hydrolysis to Ca^{2+} transport by the Ca^{2+}-ATPase. Two candidates for such a hypothetical coupling regulator are (1) a specific lipid component of the SR membrane or (2) another SR protein that interacts with the Ca^{2+}-ATPase to regulate coupling. Our results with R-SR preparations having the same SR lipid

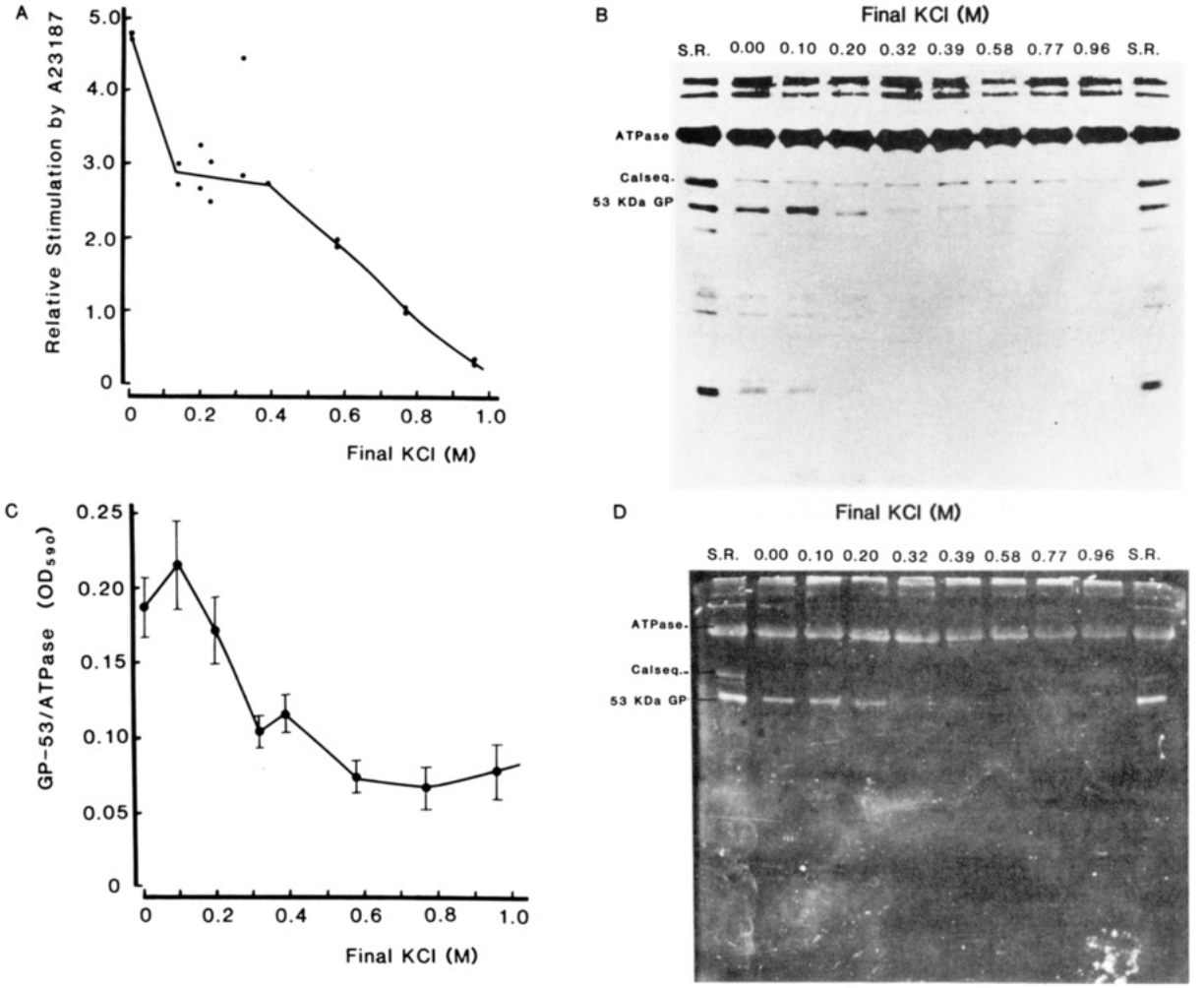

FIGURE 1: Effect of [KCl] present during solubilization on coupling and protein composition of reconstituted Ca^{2+}-ATPase preparations. (A) Degree of coupling of reconstituted SR preparations as a function of [KCl] present during solubilization. SR vesicles were solubilized in cholate in RS1 with different amounts of added KCl at 25 °C and reconstituted by dialysis at 25 °C against RS1 with no added KCl. R-SR vesicles were then isolated by sucrose density gradient centrifugation. Concentrations of SR protein and detergent are the same for each [KCl]. As an index of coupling, the difference between the specific Ca^{2+}-ATPase activity in the presence and absence of A23187 divided by the specific activity in the absence of ionophore is plotted on the ordinate. (B) Gel electrophoresis of the samples in (A). Proteins are stained with Coomassie blue. The positions of several proteins are indicated: the Ca^{2+}-ATPase (ATPase), calsequestrin (Calseq), and the 53-kDa glycoprotein (53-kDa GP). (C) Ratios of the 53-kDa glycoprotein to the Ca^{2+}-ATPase for R-SR solubilized in different [KCl] determined by cutting out the bands and extracting the Coomassie blue. (D) Gels of the samples shown in (B) but stained with dansylhydrazine to label glycoproteins.

composition, but solubilized in low- vs. high-K^+-containing buffers, argue against the difference we observed in coupling being due to differences in the level of a specific membrane lipid. We thus examined the possibility that another SR membrane protein is involved in regulating coupling.

To determine whether there was a correlation between the effects of different K^+ concentrations on the protein composition of R-SR vesicle preparations and the effects of different K^+ levels on the relative degree of coupling, SR vesicles were solubilized at 25 °C in RS1 buffers as previously described, but containing concentrations of KCl ranging from 0.0 to 1.0 M. Identical volumes and protein and detergent concentrations were used. All samples were reconstituted in RS1 buffer (low K^+), and the resultant R-SR vesicles were isolated by sucrose gradient centrifugation. The Ca^{2+}-ATPase activities of the R-SR vesicle preparations were determined in the presence and absence of A23187 to estimate the extent of coupling. The protein composition of these preparations (at constant total protein concentration) was analyzed by SDS slab gel electrophoresis. The results of these experiments are shown in Figure 1. In Figure 1A, the fractional stimulation of Ca^{2+}-ATPase activity by A23187 (a quantity proportional to coupling) present in the R-SR vesicle samples is plotted vs. KCl concentration. A progressive decrease in relative coupling was observed as the KCl concentration was increased, with the values obtained for the RS1 + 1.0 M KCl preparations being only about 3% of those found for the RS1 with no KCl added samples. Figure 1B illustrates the gel electrophoresis patterns obtained with Coomassie blue staining for such R-SR samples. The relative amounts of Ca^{2+}-ATPase and calsequestrin present in each sample remain fairly constant with increasing [KCl]. However, there is a marked and progressive decrease in the relative amount of the 53-kDa glycoprotein present as the [KCl] in the solubilizing buffer was increased. The change in the 53-kDa glycoprotein concentration is shown semiquantitatively in Figure 1C, where the relative ratio of the 53-kDa glycoprotein/Ca^{2+}-ATPase is plotted as a function of KCl concentration. These ratios were determined by cutting out the Coomassie blue stained spots, extracting the dye, and determining the optical density at 590 nm (Campbell & MacLennan, 1981). The decrease in the 53-kDa glycoprotein content of the preparations is also apparent in Figure 1D, where the gels were stained with dansylhydrazine to label SR glycoproteins. The dansylhydrazine-stained gels of SR show bands whose positions correspond to bands we identified in Coomassie blue stained gels as Ca^{2+}-ATPase, calsequestrin,

and 53-kDa glycoprotein. There is a higher molecular weight band that stains with dansylhydrazine that apparently corresponds to the 160-kDa glycoprotein (Campbell & MacLennan, 1981). There is also diffuse staining at the top of the gel. In control gels, in which the oxidation with periodic acid was omitted, only the Ca-ATPase and the diffuse band at the top of the gel were visible, suggesting that these two bands were stained nonspecifically (Eckhardt et al., 1976). The R-SR preparations show barely detectable levels of calsequestrin (which is poor in carbohydrate), a fairly constant level of Ca^{2+}-ATPase, and levels of the 53- and 160-kDa glycoproteins that diminish as the [KCl] used in solubilizing the SR increases.

Fate of the 53-kDa Glycoprotein during Solubilization in High [K^+]. What is the fate of the 53-kDa glycoprotein when SR vesicles are solubilized with cholate in the presence of high-K^+ buffers? SR vesicles were solubilized as above in high-K^+ buffers (RS1 + 1.0 M KCl) and reconstituted in low-K^+ buffers (RS1 with no added KCl), both at 25 °C. The entire sample was then layered onto a Sepharose 4B column, made in low-K^+ buffer, and eluted with low-K^+ buffer at 25 °C. Fractions (1 mL) were collected and analyzed by SDS gel electrophoresis. The 53-kDa glycoprotein eluted at the very front of the void volume, whereas the vesicles containing the bulk of the Ca^{2+}-ATPase emerged significantly later, indicating that the 53-kDa glycoprotein formed large aggregates after solubilization (Campbell & MacLennan, 1981) which remained dissociated from the R-SR vesicles under these experimental conditions.

Selective Removal of Other SR Proteins. While the results illustrated in Figure 1 suggest a possible relationship between the reduction in coupling observed and a decrease in the relative concentration of the 53-kDa glycoprotein present in the R-SR vesicles, the SDS gels of the samples also indicate the presence of a number of other proteins, such as calsequestrin and several low molecular weight proteins, in these preparations. To clarify any potential role of the 53-kDa glycoprotein, or any other protein component, in coupling, it is useful to simplify the protein composition of the R-SR vesicles used for the Ca^{2+}-ATPase assays. Because of the tendency of the 53-kDa glycoprotein to irreversibly aggregate after solubilization, we have not yet succeeded in purifying both the Ca^{2+}-ATPase and the 53-kDa glycoprotein and then reconstituting both components into lipid vesicles. We therefore attempted to selectively eliminate other proteins from the R-SR preparations, without solubilizing the 53-kDa glycoprotein.

In the first series of experiments, SR vesicles were solubilized with cholate in *low*-K^+ buffer at 25 °C. The samples were then divided into two portions. The first was reconstituted in low-K^+ buffer at 25 °C, and the R-SR vesicles were separated from nonincorporated material by sucrose gradient centrifugation. The second was fractionated by sucrose gradient centrifugation first (at 20 °C) and then reconstituted in low-K^+ buffer. The Ca^{2+}-ATPase activities of both samples were measured in the presence and absence of A23187 to estimate the extent of coupling present and their protein compositions examined by SDS gel electrophoresis. The results are shown in lanes 1 and 2 of Figure 2A,B. The SDS gel electrophoresis patterns are quite similar for both preparations (both contain the 53-kDa glycoprotein and Ca^{2+}-ATPase), except for the marked reduction in the amount of calsequestrin present in the samples which were first fractionated and then reconstituted. The Ca^{2+}-ATPase activities ±A23187 of these samples show that both preparations were extensively and similarly

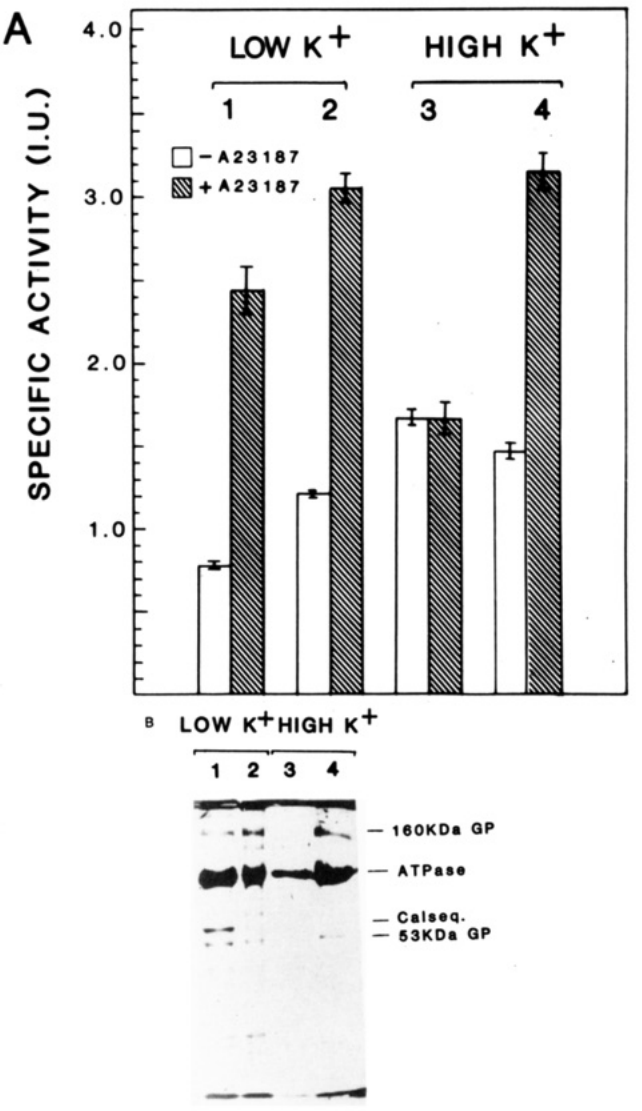

FIGURE 2: Selective removal of other SR membrane proteins. (A) Ca^{2+}-ATPase activities in the presence and absence of A23187. (1) SR vesicles were solubilized in cholate in *low* K^+ at 25 °C, reconstituted by dialysis at 25 °C against low-K^+ buffers, and then fractionated on a sucrose gradient. (2) Similar to (1) but sucrose gradient fractionation preceded reconstitution by dialysis. (3) SR vesicles were solubilized in cholate in *high* K^+, incubated for 30 min at room temperature, and then fractionated by sucrose density gradient centrifugation. (4) Similar to (3) but the 30-min incubation was omitted, and the sample was immediately fractionated by sucrose gradient centrifugation. (B) SDS gel electrophoresis patterns of the preparations in (A). The positions of certain proteins are indicated: Ca^{2+}-ATPase (ATPase), calsequestrin (Calseq.), and the 53000- and 160000-dalton glycoproteins (53-kDa GP and 160-kDa GP, respectively).

coupled, indicating that the degree of coupling is independent of the calsequestrin content of the R-SR vesicles.

A second series of experiments was conducted with *high*-K^+ buffers (RS1 + 1.0 M KCl). In one group of samples, SR vesicles were solubilized with cholate in high-K^+ buffer, and the mixture was incubated for 30 min at 25 °C and then fractionated by sucrose gradient centrifugation (at 20 °C). R-SR vesicles were subsequently reconstituted in low-K^+ buffers at 25 °C. In other samples, SR vesicles were solubilized with cholate in high-K^+ buffer as above, but *immediately* fractionated by sucrose gradient centrifugation and then reconstituted in low-K^+ buffers. The resultant protein composition and Ca^{2+}-ATPase activities ±A23187 of these preparations are shown in lanes 3 and 4 of Figure 2A,B. The S-SR preparations, incubated for 30 min after solubilization

and then centrifuged, were found to be completely uncoupled. Ca^{2+}-ATPase was the only protein detected in these preparations. In contrast, the R-SR preparations that were solubilized and immediately fractionated were found to be significantly coupled under the same conditions. An analysis of the protein composition of the latter preparations indicated the presence of only four proteins: the Ca^{2+}-ATPase, the 53-kDa glycoprotein, a protein we believe is the 160-kDa glycoprotein, and a minor unidentified low molecular weight protein. These results are consistent with the interpretation that the 53-kDa glycoprotein is involved in regulating the coupling of the Ca^{2+}-ATPase in these preparations.

Discussion

The results obtained in this study can be divided into two phases. In the first phase, attempts were made to reconstitute purified Ca^{2+}-ATPase into vesicles of defined lipid composition in order to examine the roles of lipids in regulating the coupling between Ca^{2+} transport and ATP hydrolysis by the Ca^{2+}-ATPase. The failure to obtain coupled preparations from such samples led us to the second phase, in which we reexamined the methods used to isolate, purify, and reconstitute the Ca^{2+}-ATPase to determine how these procedures were modifying coupling and to investigate the possibility that other membrane proteins are involved in regulating coupling.

Our results indicate that ATPase activity and Ca^{2+} transport are readily separable in reconstituted SR and that vesicles reconstituted by using the purified Ca^{2+}-ATPase are extensively uncoupled under our experimental conditions, irrespective of phospholipid composition or lipid/protein ratio. Significant literature exists on the effects of SR membrane lipid composition on the transport and enzymatic activities of the Ca^{2+}-ATPase [reviewed by Bennett et al. (1980) and Hidalgo (1985)]. Hidalgo et al. (1982) and Racker's group (Knowles & Racker, 1975; Knowles et al., 1975) proposed that PE is essential for coupling of the Ca^{2+}-ATPase. More recently, Navarro et al. (1984) proposed that Ca^{2+} transport/ATP hydrolysis coupling is regulated by PE and other phospholipids capable of adopting nonbilayer structures (i.e., hexagonal II phases). However, PE to PC ratios greater than 10 to 1 (mol/mol) were required to obtain Ca^{2+} transport/ATP hydrolysis coupling ratios approaching 1 in these studies. Such PE to PC ratios are far beyond physiological levels (20–30% of the phospholipid in SR is PE). When the PE to PC ratios employed were similar to the levels used in our study (1 to 1 mol/mol), the coupling observed by Navarro et al. (1984) was reduced to less than 0.1 Ca^{2+} transported per ATP hydrolyzed, even in the presence of internally trapped 0.4 M phosphate. Hidalgo et al. (1982) have shown that it is possible to inhibit Ca^{2+} transport without affecting ATPase activity by modifying the phosphatidylethanolamine head groups of isolated SR vesicle membranes with fluorescamine. While these results are consistent with the idea that PE plays an important role in coupling, the possibility remains that the uncoupling observed results from interaction of the fluorescamine moieties bound to PE with the Ca^{2+}-ATPase. We feel that the influence of PE on coupling, particularly at physiological levels of PE, remains to be demonstrated.

Our results suggest that the degree of coupling obtained in reconstituted SR can be varied by varying the temperature and the concentration of K^+ present during cholate solubilization. It has previously been reported that SR vesicles (R-SR) reconstituted at 25 °C were more coupled than those generated at 4 °C (Meissner & Fleischer, 1974; Repke et al., 1976). While our results also show a similar influence of temperature to that previously observed, they indicate that the KCl concentration present during cholate solubilization is a more critical variable affecting the degree of coupling in the R-SR vesicle preparations (Repke et al., 1976). The idea that uncoupling may be obtained in reconstituted SR preparations in which important properties of the Ca^{2+}-ATPase itself are normal is supported by the results of Inesi et al. (1983), who found that the uncoupling present in their R-SR preparations could not be attributed to an alteration in the oligomeric structure of the Ca^{2+}-ATPase or to changes in the kinetics of E–P formation and decomposition.

The difficulty of obtaining reconstituted SR preparations in which ATP hydrolysis is tightly coupled to Ca^{2+} transport is consistent with the possibility that a component of the SR membrane, that is frequently lost when SR is treated with detergent, is required for optimal coupling. Our results suggest that the 53-kDa glycoprotein (GP-53) of the SR membrane may play a role in regulating the coupling of ATP hydrolysis to Ca^{2+} transport by the Ca^{2+}-ATPase. The correlation between the apparent degree of coupling obtained in reconstituted SR and the amount of GP-53 present (Figure 1) and the results obtained for R-SR vesicles having markedly simplified protein compositions (Figure 2) are particularly supportive of a possible role of GP-53 in coupling. The 53-kDa glycoprotein has certain characteristics that might be required of a regulator of Ca^{2+} transport/ATP hydrolysis coupling: GP-53 is an integral, transmembrane protein; GP-53 is present in amounts of sufficient concentration to interact with much of the Ca^{2+}-ATPase in the SR membrane; GP-53 maintains a constant stoichiometry with the Ca^{2+}-ATPase in light, medium, and heavy SR vesicle fractions (about 3 Ca^{2+}-ATPase/2 GP-53) (Michalak et al., 1980; Campbell & MacLennan, 1981). MacLennan & DeLeon (1983) found that the Ca^{2+}-ATPase and GP-53 are synthesized and intercalated into the SR membrane at the same time in cell culture, in constrast to the other SR proteins which appear earlier.

In our study, the coupling of ATP hydrolysis to Ca^{2+} transport has been estimated from the extent to which the Ca^{2+} ionophore A23187 stimulates the rate of ATP hyrolysis by SR and reconstituted preparations. The case for the involvement of GP-53 in regulating coupling would be strengthened by studies in which the rate of Ca^{2+} transport is determined directly. Such studies are currently under way in our laboratory.

The notion that the coupling of ATP hydrolysis by Ca^{2+}-ATPase to Ca^{2+} transport may be influenced significantly by other proteins of the SR membrane is supported by studies of the SR of cardiac muscle [reviewed by Tada et al. (1982)]. Phospholamban, an M_r 22 000 protein of the membrane of cardiac SR, when phosphorylated, stimulates both the hydrolysis of ATP and the transport of Ca^{2+} by the Ca^{2+}-ATPase. Campbell et al. (1983) have demonstrated that a protein identical (or very similar) with GP-53 of skeletal muscle SR is present in cardiac SR. The function of the GP-53 analogue in cardiac muscle has not been investigated.

Our hypothesis that GP-53 is involved in coupling of the Ca-ATPase may have significance for plasma membrane transport ATPases in general. Kyte (1981) stressed the similarities in the reaction cycles of the (Na^+,K^+)- and Ca^{2+}-ATPases, similar molecular weights of the α subunit of the (Na^+,K^+)-ATPase and the Ca^{2+}-ATPase, the same amino acid sequences around the aspartates that are phosphorylated during the reaction cycles, and similar locations of the aspartates in the catalytic subunits. The (Na^+,K^+)-ATPase has a β subunit, a 55-kDa glycoprotein, that may be required for ion pumping. The 53-kDa glycoprotein of the SR membrane

may function for the Ca^{2+}-ATPase in a way analogous to the function of the β subunit of the (Na^+,K^+)-ATPase. Further studies will be required to unequivocally define the role of the 53-kDa glycoprotein of the SR membrane.

ACKNOWLEDGMENTS

We thank Lisa M. Geddis and Jan Tokarz for their skillful technical assistance and the laboratory of Dr. Michael Peach for supplying rabbit muscle.

Registry No. ATPase, 9000-83-3; Ca, 7440-70-2; K, 7440-09-7.

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