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Reconstitution experiments provide no evidence for a role for the 53-kDa glycoprotein in coupling Ca^{2+} transport to ATP hydrolysis by the (Ca^{2+}-Mg^{2+})-ATPase in sarcoplasmic reticulum

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The sarcoplasmic reticulum (SR) of skeletal muscle contains a 53 kDa glycoprotein of unknown function, as well as the (Ca^{2+}-Mg^{2+})-ATPase. It has been suggested that the glycoprotein couples the hydrolysis of ATP by the ATPase to the transport of calcium. It has been shown that if SR vesicles are solubilized in cholate in media containing low K^+ concentrations followed by reconstitution, then vesicles are formed containing the glycoprotein and with ATP hydrolysis coupled to Ca^{2+} accumulation, as shown by a large stimulation of ATPase activity by addition of A23187. In contrast, if SR vesicles are solubilized in media containing a high concentration of K^+, then the vesicles that are produced following reconstitution lack the glycoprotein and show low stimulation by A23187 (Leonards, K.S. and Kutchai, H. (1985) Biochemistry 24, 4876–4884). We show that the effect of K^+ on reconstitution does not follow from any changes in the amount of glycoprotein but rather from an effect of K^+ on the detergent properties of cholate. In low K^+ media, the cmc of cholate is high, cholate is a relatively poor detergent and incomplete solubilization results in 'reconstitution' of vesicles with the correct orientation of ATPase molecules. In high K^+ media, the cmc of cholate is reduced and more complete solubilization of the SR leads to a true reconstitution with the formation of vesicles with a random orientation of ATPase molecules. The experiments provide no evidence for an effect of the glycoprotein on the (Ca^{2+}-Mg^{2+})-ATPase.

Introduction

One approach to understanding the mechanism of ion transport is to purify the transporter and reconstitute it into phospholipid bilayer systems of defined composition. If the ion-transporter is a multi-subunit protein then it is obviously important in such studies that all subunits of the transporter be purified and reconstituted. A major difference between the (Na^+-K^+)-ATPase and the (Ca^{2+}-Mg^{2+})-ATPase of skeletal muscle sarcoplasmic reticulum (SR) is that whereas the former is purified from the membrane as an $\alpha\beta$ dimer, the latter is purified as a single subunit, corresponding to the α subunit of the (Na^+-K^+)-ATPase [1,2]. The question arises therefore whether a subunit of the (Ca^{2+}-Mg^{2+})-ATPase exists in the SR membrane analogous to the β subunit of the (Na^+-K^+)-ATPase but which is lost on purification. It has been suggested that the 53-kDa glycoprotein (53 kDa GP) found in the SR membrane could play this role [3] although the lack of sequence homology between the 53 kDa GP and the β subunit of the (Na^+-K^+)-ATPase [4] makes this less likely.

Potential effects of the 53 kDa GP on the (Ca^{2+}-Mg^{2+})-ATPase can be investigated by comparing the activity of the purified ATPase with that of the ATPase in the native SR membrane. The purified (Ca^{2+}-Mg^{2+})-ATPase shows an ATPase activity comparable to that of the ATPase in native SR vesicles in the presence of the Ca^{2+}-ionophore A23187 [5,6] so that the ATPase alone must be fully competent for Ca^{2+}-dependent ATP hydrolysis. Although Boyd et al. [7] have reported that the dependence of ATPase activity on the

Abbreviations: SR, sarcoplasmic reticulum; GP, glycoprotein; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; FITC, fluorescein isothiocyanate; EITC, eosin isothiocyanate; cmc, critical micelle concentration,. ANS, 1-anilino-naphthalene-8-sulphonate; GP, glycoprotein.

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concentrations of Ca^{2+} and ATP are distinctly different for SR vesicles with and without the 53 kDa GP, we find identical dependencies for the ATPase in SR vesicles and for the purified ATPase in the absence of the 53 kDa GP [6]. Reconstituted vesicles containing the (Ca^{2+}-Mg^{2+})-ATPase as the only protein can accumulate Ca^{2+}, but the ratio of ATP hydrolysed to Ca^{2+} accumulated is less than that observed for SR vesicles [8,9]. Racker [9] has attributed this to defective coupling of ATP hydrolysis to Ca^{2+} transport on the ATPase and has suggested this to be due to the absence of a lipid or protein species which, in the native SR membrane, serves to prevent access of water to the phosphorylated residue on the ATPase thus preventing futile hydrolysis of ATP. However, we have shown that the low coupling ratio observed for reconstituted vesicles can, at least in part, be attributed to rapid efflux of Ca^{2+} from the reconstituted vesicles [10].

An alternative procedure for investigating the role of the 53 kDa GP has been reported by Leonards and Kutchai [3]. They reported that if SR vesicles were dissolved in cholate followed by removal of cholate by dialysis then reconstituted vesicles were formed whose content of 53 kDa GP varied with the concentration of KCl present during solubilization. If SR vesicles were solubilized in cholate in the presence of 1 M KCl then the resulting reconstituted vesicles contained no 53 kDa GP whereas vesicles reconstituted in the absence of added KCl showed a content of 53 kDa GP comparable to that in the original SR vesicles [3]. To test for any effect of the 53 kDa GP on the activity of the ATPase, Leonards and Kutchai [3] measured ATPase activity for the reconstituted vesicles in the absence and presence of A23187 and used the fractional stimulation caused by A23187 as a measure of the degree of coupling between ATP hydrolysis and Ca^{2+} transport. They reported that the degree of coupling was very low in the absence of 53 kDa GP [3]. In more recent studies Kutchai and Campbell [11] have reported changes in coupling ratio in the presence of antibodies to the 53 kDa GP, and attributed this to changes in interaction between the 53 kDa GP and the ATPase.

If the 53 kDa GP were to affect the degree of coupling between ATP hydrolysis and Ca^{2+} transport on the ATPase, then this would need to be accounted for in any explanation of the mechanism of the ATPase. However, in chemical cross-linking studies we failed to find any evidence for an interaction between the 53 kDa GP and the ATPase [6]. Here, we re-investigate the effect of KCl on the reconstitution of SR vesicles and argue that, in fact, the effects of KCl follow from effects on the critical micelle concentration of the detergent.

Materials and Methods

AnalaR reagents were obtained from BDH Chemicals and Hepes (Ultrol) was from Calbiochem. SR was prepared as described in McWhirter et al. [12]. ATPase activity was measured using the coupled enzyme assay described in [13]. Samples (12 μl, equivalent to 12 μg of ATPase) were added to a medium containing 40 mM Hepes-KOH (pH 7.2), 5 mM $MgSO_4$, 1.01 mM EGTA, 0.42 mM phospho*enol*pyruvate, 0.15 mM NADH, the required concentration of ATP, 7.5 IU pyruvate kinase and 18 IU lactate dehydrogenase in a total volume of 2.5 ml, with $CaCl_2$ added to give maximal ATPase activity. In experiments with A23187, 10 μl of a 1 mg/ml stock solution of A23187 in methanol was added to give a final concentration of 0.8 μM. This volume of methanol had no significant effect on the ATPase activity of SR vesicles and this concentration of A23187 had no significant effect on the ATPase activity of the purified ATPase (data not shown).

Potassium cholate and deoxycholate were purified by precipitation from methanol/ether. SR vesicles were reconstituted following the protocol described by Leonards and Kutchai [3]. Aliquots of SR vesicles were diluted to 2.5 mg protein/ml with RS1 buffer (250 mM sucrose, 100 mM NaCl, 50 mM KH_2PO_4 (pH 7.4)). The vesicles were solubilised by the addition of 5% (w/v) potassium cholate in RS1 to give a final cholate concentration of 2.5 mg/ml; the cholate was added in small aliquots with mixing. The samples were allowed to stand for 30 min before reconstitution in one of three ways:

(1) Dilution of the sample directly into the assay mixture.

(2) Removal of cholate using the centrifugation method of Penefsky [14]. 5 ml plastic syringes were filled with a boiled deaearated suspension of Sephadex G-50 which was then washed with RS1 buffer. The syringes were placed in plastic centrifuge tubes and spun at 200 × *g* in a bench-top centrifuge for 30 s. The sample was then applied to the column (100 μl per ml of Sephadex), 200 μl of buffer was added and the columns were spun at 200 × *g* for 20 s. The resulting column eluate was then applied to a second column in the same way.

(3) Dialysis overnight against 11 of RS1 buffer at 4°C.

Light scatter was measured in a Perkin-Elmer fluorimeter using excitation and emission wavelengths of 400 nm. Measurements of the ability of cholate to solubilise ANS were made by exciting fluorescence at 367 nm and measuring the fluorescence intensity at 475 nm.

For electron microscopy, samples of SR were solubilised and reconstituted by dialysis as described above, followed by pelleting at 93000 × *g* for 1 h. Samples were then treated as described by Saito et al. [15]. They were block stained with 0.5% uranyl acetate for 2 h and dehydrated in a series of increasing ethanol concentration before being embedded in Epon resin, thin sec-

TABLE I

Effect of A23187 on ATPase activities of vesicles reconstituted from high and low KCl

ATPase activities were measured at 25°C, 2.1 mM ATP in the presence or absence of A23187 (0.8 μM) as described in Materials and Methods. The fractional stimulation was calculated as ([Activity + A23187] − [Activity − A23187])/[Activity − A23187].

System	ATPase activity (IU/mg)		Fractional stimulation
	− A23187	+ A23187	
Control SR	0.4	4.4	10.1
Low K^+-dilution [a]	1.1	4.1	2.8
-Sephadex [a]	0.8	3.0	2.8
-dialysis [a]	1.1	4.6	3.2
High K^+-dilution [a]	1.9	1.8	0
-Sephadex [a]	1.0	1.5	0.5
-dialysis [a]	1.6	1.9	0.2

[a] The three techniques for reconstitution as described in Materials and Methods.

tioned and stained with 1% uranyl acetate in 50% ethanol for 10 min, counterstained with lead citrate and examined under a Hitachi HU-12 electron microscope.

Protein was estimated using the extinction coefficient given by Hardwicke and Green [16] or using BCA (Pierce) with bovine serum albumin as standard.

Results

We have confirmed the observations of Leonards and Kutchai [3] that if SR vesicles (2.5 mg protein/ml) are solubilised in RS1 buffer containing cholate (2.5 mg/ml) and no added KCl (low K^+ buffer), followed by reconstitution and fractionation on a sucrose gradient, then the resulting vesicles contain the 53 kDa GP at a level comparable to that in the original SR vesicles (typically 10% in our preparations). We have also found, in agreement with Leonards and Kutchai [3], that if the same procedure is repeated but with solubilisation performed in buffer containing 1 M KCl (high K^+ buffer) then the resulting vesicles contain undetectable levels of the 53 kDa GP (data not shown).

Leonards and Kutchai [3] have also reported that the ratio of ATPase activities of reconstituted vesicles measured in the presence and absence of A23187 differ for vesicles solubilised in low and high K^+ buffers. This result is confirmed in Table I. For vesicles reconstituted from high K^+ fractional stimulation by A23187 is very low, as reported by Leonards and Kutchai [3]. However, this cannot be simply attributed to the presence of a large proportion of vesicles in which hydrolysis of ATP has been 'uncoupled' from Ca^{2+} transport since the ATPase activity measured in the absence of A23187 is about half that of the full ATPase activity shown by the original SR vesicles in the presence of A23187 (Table I). Also inconsistent with the idea of uncoupled vesicles is

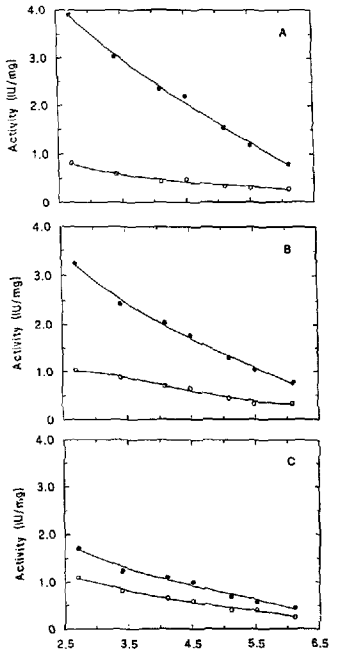

Fig. 1. ATPase activities for: (A) intact SR, (B) preparations reconstituted from low K^+ medium, and (C) preparations reconstituted from high K^+ medium in the absence (○) and in the presence (●) of A23187 (0.8 μM). SR (2.5 mg protein/ml) was solubilised in cholate (2.5 mg/ml) and reconstituted by dilution into the assay medium (see Materials and Methods).

TABLE II

Effect of $C_{12}E_8$ on ATPase activities of vesicles reconstituted from high and low KCl

ATPase activities were measured at 25°C, 2.1 mM ATP as described in Materials and Methods.

Additions	ATPase activity (IU/mg)		
	native SR	low K^+ reconstituted	high K^+ reconstituted
None	1.0	1.8	1.7
A23187 (0.8 μM)	8.6	7.4	3.4
$C_{12}E_8$ (1 mg/ml)	6.3	5.1	6.2

the observation that the ATPase activity measured in the presence of A23187 is about half that observed for vesicles reconstituted from low K^+ (Table I); a similar observation was made by Leonards and Kutchai [3] but not commented on. As shown in Table I, comparable results are obtained if solubilised SR is reconstituted by dialysis, by removal of detergent on a Sephadex column, or by simple dilution into assay buffer. Fig. 1 shows ATPase activites for native SR vesicles and for vesicles reconstituted from low and high K^+ medium, as a function of ATP concentration.

Table II shows the results of experiments in which

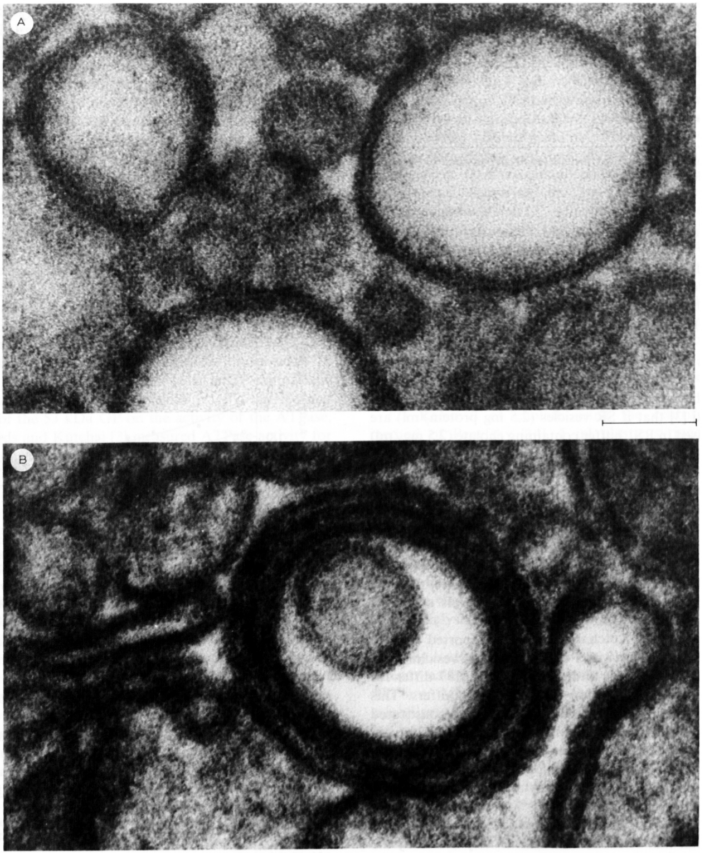

Fig. 2. Electron micrographs of SR samples reconstituted by dialysis after solubilisation in low K^+ (A) or high K^+ (B) buffers. The bar represents 50 nm.

vesicles were made permeable to both Ca^{2+} and ATP by addition of 1 mg/ml of the detergent $C_{12}E_8$; we have shown that the ATPase is fully active at this concentration of detergent [18]. For intact SR and for SR reconstituted from low K^+, comparable ATPase activities are seen in the presence of either A23187 or $C_{12}E_8$, confirming that in these cases the low activities observed for sealed vesicles can be attributed to the build-up of a high concentration of Ca^{2+} within the vesicles and not to any problems in accessibility of the ATP to its binding site. In contrast, for vesicles reconstituted from high K^+, a very much larger stimulation of activity is observed on addition of $C_{12}E_8$ than on addition of A23187, confirming the formation of structures under these conditions in which the ATP binding site on the ATPase is inaccessible to the outside medium.

The formation of very different structures after reconstitution from low and high K^+ is confirmed by electron microscopy (Fig. 2). After reconstitution from low K^+, sealed vesicular structures are observed, of fairly uniform size, comparable to those observed for native SR. In contrast, after reconstitution from high K^+, a very heterogeneous preparation is obtained, containing membrane fragments and multi-lamellar structures.

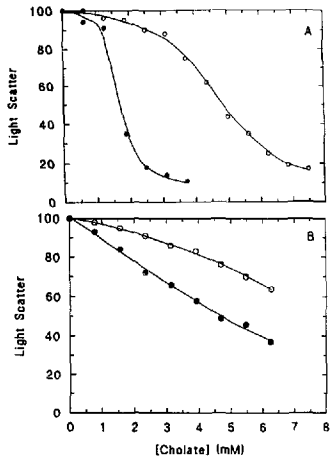

Fig. 3. Solubilisation of (A) egg yolk phosphatidylcholine (0.12 mg/ml) and (B) SR vesicles (2.5 mg protein/ml) by cholate measured by Rayleigh scattering in: ○, low K^+ buffer; ●, high K^+ buffer. The buffer was 50 mM potassium phosphate (pH 7.4), 250 mM sucrose, 100 mM NaCl.

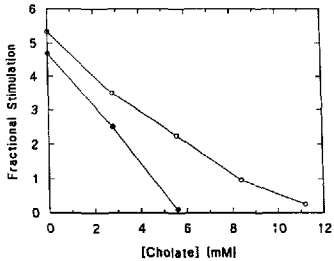

Fig. 4. Fractional stimulation of the ATPase activity of reconstituted vesicles by A23187 (0.8 μM) as a function of cholate concentration used in the solubilisation medium: ○, low K^+ medium; ●, high K^+ medium. Reconstitution was by dilution into the assay medium and ATPase activities were measured at 25°C, 2.1 mM ATP.

To determine whether the different results obtained at low and high concentrations of K^+ could be attributed to different degrees of solubilisation of the vesicles, we investigated the effects of cholate on Rayleigh scattering by the vesicles. As shown in Fig. 3B, K^+ concentration has a large effect on solubilisation; at 5.6 mM cholate (2.5 mg/ml) the lower light scatter in high K^+ medium than in low K^+ medium indicates a greater degree of solubilisation. A similar effect of K^+ is seen with liposomes (Fig. 3A) although here the more marked decrease in Rayleigh scattering suggests a greater degree of solubilisation at any given cholate concentration compared to that seen with SR vesicles.

The effect of cholate concentration on coupling efficiency is shown in Fig. 4; as expected from the results reported above, comparable levels of stimulation by

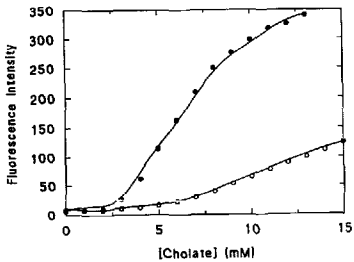

Fig. 5. The effect of KCl on the critical micelle concentration of cholate, determined by the ability to solubilise ANS. The fluorescence intensity of ANS (5 μM) in 50 mM potassium phosphate (pH 7.4), 250 mM sucrose, 100 mM NaCl is plotted against cholate concentration for low K^+ medium (○) or high K^+ medium (●).

A23187 are observed, for example, in 3 mM cholate in high K^+ medium and 6 mM cholate in low K^+ medium.

We measured the critical micelle concentration (cmc) of cholate from the ability to solubilise the fluorescent probe 1-anilino-naphthalene-8-sulphonate (ANS). The fluorescence emission intensity of ANS is higher in a hydrophobic environment than in water and, as shown in Fig. 5, increases on addition of cholate to a solution of ANS in water. The concentration of detergent at which significant solubilisation of the probe occurs, as shown by the increase in fluorescence intensity, is usually taken as a measure of the cmc [19] and is approx. 3 mM for cholate in the high K^+ medium and 6 mM in the low K^+ medium (Fig. 5).

The results presented above therefore suggest that the effects of K^+ follow from differences in the extent of solubilisation of the SR vesicles. This has been demonstrated directly by measurements of the fraction of the vesicles that can be sedimented by centrifugation following solubilisation (Fig. 6). As shown, in the low K^+ medium, less than half of the SR remains in the supernatant after centrifugation even at 11 mM cholate, whereas in the high K^+ medium, essentially all the SR is solubilised at 11 mM cholate, although only half is solubilised at 6 mM cholate; these results are in good agreement with the measurements of Rayleigh scattering (Fig. 3). The similar results seen with Cl^- or methanesulphonate as anion (Fig. 3) confirm that, as expected, it is the cation concentration that affects solubilisation. ATPase activities of preparations reconstituted by dilution from the supernatant and pellet fractions are shown in Table III. It is clear that preparations reconstituted from solubilised material show a low fractional stimulation by A23187, irrespective of whether they were solubilised in low or high K^+ medium. Correspondingly, fractional stimulations of samples reconstituted from the pelleted material were high and again independent of whether the SR was solubilised in low or high K^+ medium.

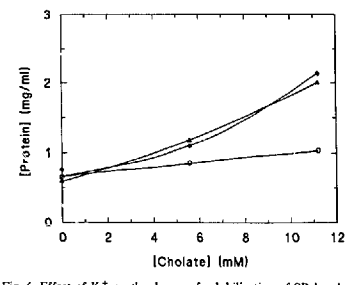

Fig. 6. Effect of K^+ on the degree of solubilisation of SR by cholate. SR (2.5 mg/ml) was solubilised at the given concentration of cholate in low K^+ (○) or high K^+ (▲) medium or medium containing 1 M potassium methanesulphonate (◆). Following centrifugation of the samples at 100000 × g in a Beckman airfuge, the concentration of protein in the supernatant was determined.

TABLE III

Effect of A23187 on ATPase activities of vesicles reconstituted from supernatant and pelletable fractions of SR solubilised in high and low K^+ medium

The samples were reconstituted by dilution. ATPase activities were measured at 25°C, 2.1 mM ATP in the presence or absence of A23187 (0.8 μM) as described in Materials and Methods.

System	ATPase activity (IU/mg)		Fractional stimulation
	−A23187	+A23187	
Control SR	1.1	8.6	6.7
Low K^+-supernatant	0.4	0.7	0.7
-pellet	2.7	8.4	2.1
High K^+-supernatant	0.7	1.1	0.6
-pellet	2.8	9.1	2.3

Discussion

The major protein found in the SR of skeletal muscle is the (Ca^{2+}-Mg^{2+})-ATPase. Other protein components include two Ca^{2+} binding proteins (calsequestrin [20,21] and the 55 kDa high-affinity Ca^{2+} binding protein [22]), the ryanodine-sensitive Ca^{2+} release channel [23] and the 53 and 160 kDa glycoproteins [4,24,25] of unknown function. Based on reconstitution studies in which the amount of the 53 kDa GP was varied, Leonards and Kutchai [3] have suggested that the 53 kDa GP could regulate the coupling of Ca^{2+} transport to ATP hydrolysis by the (Ca^{2+}-Mg^{2+})-ATPase. Chiesi and Carafoli [26] found that trifluoperazine bound to the 53 kDa GP, and attributed the inhibitory effect of trifluoperazine on Ca^{2+} uptake by SR to an effect on the 53 kDa GP, modifying its presumed interaction with the ATPase. Kutchai and Campbell [11] have reported that binding of antiserum against the 53 kDa GP to SR vesicles uncouples Ca^{2+} transport from ATP hydrolysis. These results have been interpreted as meaning that the 53 kDa GP functions in SR analogously to the β subunit of the (Na^+K^+)-ATPase although the 53 kDa GP shows no significant homology with the β subunit [4].

Leberer et al. [4] have co-transfected COS-1 cells with cDNA coding for both the 53 kDa GP and the (Ca^{2+}-Mg^{2+})-ATPase and shown that the resulting microsomes have an ability to pump Ca^{2+} identical to those derived from COS-1 cells transfected with cDNA encoding the (Ca^{2+}-Mg^{2+})-ATPase alone, arguing against any regulatory role for the 53 kDa GP. Kutchai and Campbell [11] and Boyd et al. [7] have reported that removal of the 53 kDa GP from SR vesicles produces significant changes in the dependence of ATPase

activity on the concentrations of Ca^{2+} and ATP although we have found identical dependences for SR vesicles containing the 53 kDa GP and the purified ATPase [6]. Further, in cross-linking studies, we have failed to find any evidence for significant interaction between the 53 kDa GP and the (Ca^{2+}-Mg^{2+})-ATPase [6]. We have therefore reinvestigated the reconstitution experiments of Leonards and Kutchai [3] which were claimed to provide evidence for a regulatory role of the 53 kDa GP.

In the experiments of Leonards and Kutchai [3] SR vesicles (2.5 mg protein/ml) were solubilised in cholate solution (2.5 mg/ml) in media containing no KCl (low K^+) or 1 M KCl (high K^+) and reconstituted by dialysis. It was found that samples reconstituted from the high K^+ medium no longer contained the 53 kDa GP whereas samples reconstituted from the low K^+ medium had a 53 kDa GP content similar to that in native SR vesicles; we confirmed these results (data not shown). To determine the degree of coupling between ATP hydrolysis and Ca^{2+} accumulation by the reconstituted systems, Leonards and Kutchai [3] measured ATPase activity in the presence and absence of the Ca^{2+}-ionophore A23187. In fully coupled vesicles, accumulation of Ca^{2+} will lead to high, inhibitory, concentrations of Ca^{2+} within the vesicles and a low steady state ATPase activity; addition of A23187 to such vesicles will therefore lead to a very marked increase in ATPase activity. For vesicles in which ATP hydrolysis is uncoupled from Ca^{2+} transport, large internal concentrations of Ca^{2+} will not be accumulated, so that steady state ATPase activities will be high and, consequently, addition of A23187 will have little effect on activity. Leonards and Kutchai [3] reported that addition of A23187 to samples reconstituted from high K^+ media had little effect on ATPase activity, and so were uncoupled, whereas addition of A23187 to samples reconstituted from low K^+ media resulted in a large increase in ATPase activity, so that such vesicles were coupled. The uncoupling observed for samples reconstituted from high K^+ was not simply due to the formation of vesicles leaky to Ca^{2+}, since the rate of efflux of Ca^{2+} from passively loaded vesicles varied little with solubilisation conditions [3]. Leonards and Kutchai [3] correlated the loss of coupling with the loss of the 53 kDa GP, and suggested that the 53 kDa GP was intimately involved in regulating the coupling between Ca^{2+} transport and ATP hydrolysis by the (Ca^{2+}-Mg^{2+})-ATPase in SR. As shown in Table I, our results confirm the measurements of Leonards and Kutchai [3] although we disagree with their interpretation.

If the low degree of coupling observed after reconstitution of SR solubilised in high K^+ were due to uncoupling of ATP hydrolysis from Ca^{2+} accumulation, then the measured ATPase activity should be the same as that for coupled vesicles (either native SR or vesicles reconstituted from low K^+) in the presence of A23187; in fact, as shown in Table I, the ATPase activity is about half that expected. A similar observation was made by Leonards and Kutchai [3] but not commented on. Fig. 1 shows ATPase activities for native SR vesicles and for vesicles reconstituted from low and high K^+ medium, as a function of ATP concentration. As described previously, the dependence of ATPase activity on the concentration of ATP is complex [17], but, at all ATP concentrations, ATPase activites are similar for all preparations in the absence of A23187, but are much lower in the presence of A23187 for preparations reconstituted from high K^+ media than from low K^+ media (Fig. 1). The observed low activity for prenarations reconstituted from high K^+ media in the presence of A23187 suggests the formation of a proportion of sealed vesicles in which the ATP binding site is not available to ATP in the external medium. This would be the result if, for example, on reconstitution under these conditions the ATPase molecules were randomly oriented across the membrane or if multilamellar vesicles were formed.

That the low activities of vesicles reconstituted from high K^+ can, indeed, be attributed to the formation of structures in which the ATP binding site on the ATPase is inaccessible to ATP in the external medium is confirmed by the experiments in Table II; vesicles reconstituted from high K^+ show a full ATPase activity if vesicles are made permeable to both Ca^{2+} and ATP by addition of the detergent $C_{12}E_8$ (Table II). These observations therefore suggest that after reconstitution from high K^+, vesicles are formed in which either the ATPase molecules are randomly oriented across the membrane or that multi-lamellar vesicles are formed, where only ATPase molecules with the correct orientation in the outer membrane will be able to hydrolyse ATP. In previous studies, we have shown that reconstitution of the purified ATPase with phospholipids gives rise to sealed vesicles with a random orientation of ATPase molecules [5]. The electron micrographs in Fig. 2 show that indeed multi-lamellar structures are formed from high K^+, unlike the simple unilamellar vesicles formed from low K^+. We have found that ATPase activities and degrees of coupling are much more variable between experiments for preparations reconstituted from high K^+ than from low K^+, which probably reflects small differences in the extent of formation of multilamellar structures in the former case, reflected in relatively large effects on ATPase activities.

A variety of experiments suggest that the formation of highly coupled vesicles with a normal orientation of ATPase molecules following reconstitution from low K^+ can be largely attributed to incomplete solubilisation of the SR under these conditions. It is well established that increasing the concentration of positively

charged ions will decrease the cmc of negatively charged detergents such as cholate and that the concentration of detergent required to solubilise a membrane decreases with decreasing cmc [19]. As shown in Fig. 5, the cmc of cholate in RSI buffer decreases from ca 6 mM to 3 mM on changing from low K^+ to high K^+ medium. This is correlated with a decrease in the concentration of cholate required to solubilise phosphatidylcholine, as measured by Rayleigh scattering (Fig. 3). Cholate is also better able to solubilise SR in high K^+ medium than in low K^+ medium, as shown by the two fold lower concentration of cholate required to produce any given decrease in Rayleigh scatter (Fig. 3). Fig. 4 shows that the degree of coupling observed with cholate at 2.5 mg/ml (5.6 mM) in high K^+ can be reproduced in low K^+ buffer using a cholate concentration of 5.0 mg/ml (11.2 mM). Deoxycholate has a lower cmc than cholate [19], and deoxycholate at 1.25 mg/ml in low K^+ medium gives the same fractional stimulation by A23187 as observed for cholate at 2.5 mg/ml in high K^+ medium (data not shown). A direct demonstration of incomplete solubilisation of the SR vesicles under these conditions is shown in Fig. 6, which shows that, in low K^+ medium, a large proportion of the SR can be pelleted by centrifugation, consistent with the incomplete clarification of the sample shown in Fig. 3. At high K^+, a greater proportion of the SR is solubilised at 5.6 mM cholate, but even under these conditions solubilisation is incomplete (Fig. 6). Finally, Table III compares ATPase activities and degrees of coupling for samples reconstituted from supernatant and pellet fractions of SR solubilised at low and high K^+. It is clear that reconstitution of non-pelletable material gives low coupling ratios, for material solubilised in either low or high K^+. Similarly, reconstitution of pelletable material gives high coupling ratios, again independent of whether solubilisation was performed in low or high K^+.

We conclude that at 2.5 mg/ml cholate in low K^+ medium, a high proportion of the SR remains unsolubilised and that the presence of this high proportion of unsolubilised material throughout the experimental procedure accounts for the high fractional stimulation by A23187 observed in the final sample. In contrast, in high K^+ medium, a greater proportion of the SR is solubilised, attributable to the lower cmc of the detergent under these conditions; reconstitution of this truly solubilised material then gives rise to vesicles with a random orientation of ATPase molecules and to multilammelar vesicles, and this explains the low fractional stimulation by A23187.

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