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A fast passive Ca^{2+} efflux mediated by the $(Ca^{2+} + Mg^{2+})$-ATPase in reconstituted vesicles

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The $(Ca^{2+} + Mg^{2+})$-ATPase from skeletal muscle sarcoplasmic reticulum was reconstituted into phospholipid bilayers. The permeability of lipid bilayers to Co^{2+} and glucose was increased slightly by incorporation of the ATPase, and the permeability of mixed bilayers of phosphatidylethanolamine and phosphatidylcholine increased with increasing content of phosphatidylethanolamine both in the presence and absence of the ATPase. The presence of the ATPase, however, resulted in a marked increase in permeability to Ca^{2+}, the permeability decreasing with increasing phosphatidylethanolamine content. Permeability to Ca^{2+} was found to be dependent on pH and the external concentrations of Mg^{2+} and Ca^{2+}, was stimulated by adenine nucleotides but was unaffected by inositol trisphosphate. A kinetic model is presented for Ca^{2+} efflux mediated by the ATPase. It is shown that the kinetic parameters that describe Ca^{2+} efflux from vesicles of sarcoplasmic reticulum also describe efflux from the vesicles reconstituted from the purified ATPase and phosphatidylcholine. It is shown that the effects of phosphatidylethanolamine on efflux can be simulated in terms of changes in the rates of the transitions linking conformations of the ATPase with inward- and outward-facing Ca^{2+}-binding sites, and that effects of phosphatidylethanolamine on the ATPase activity of the ATPase can also be simulated in terms of effects on the corresponding conformational transitions. We conclude that the ATPase can act as a specific pathway for Ca^{2+} efflux from sarcoplasmic reticulum.

Introduction

As described in the previous paper [1], the $(Ca^{2+} + Mg^{2+})$-ATPase can be purified from skeletal muscle sarcoplasmic reticulum and reconstituted into sealed vesicles of phospholipid. Uptake of Ca^{2+} by these vesicles is dependent on the chemical structures of the phospholipids used in the reconstitution and is low in the absence of phosphate ions within the vesicles. These observations suggest that the membranes of the reconstituted vesicles are relatively permeable to Ca^{2+}, and that the permeability is dependent on phospholipid structure. Since it is well established that the permeability of pure phospholipid bilayers to Ca^{2+} is very low [2], it is unlikely that the low levels of Ca^{2+} accumulation seen in the absence of a Ca^{2+}-precipitating agent are due to leak of Ca^{2+} across the bulk phospholipid bilayer. It could be

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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that the ATPase is not properly 'sealed' into the phospholipid bilayer, and that relatively rapid leak occurs at or near the lipid/protein interface. Such a leak pathway would be expected to be relatively nonspecific. An alternative possibility, however, is that the ATPase itself acts as a pathway for Ca^{2+} efflux from the vesicles, possibly involving the same Ca^{2+}-binding sites on the ATPase as are involved in uptake of Ca^{2+} driven by hydrolysis of ATP. It is the aim of this paper to explore the latter possibility.

In a number of recent papers we have suggested that the $(Ca^{2+} + Mg^{2+})$-ATPase can provide an efficient pathway for Ca^{2+} efflux from sarcoplasmic reticulum vesicles [3,4], as outlined in Scheme I. Studies of the ATPase activity of the $(Ca^{2+} + Mg^{2+})$-ATPase suggest that the ATPase can exist in one of two possible conformations, E1 or E2 (Scheme II). In the E1 conformation, the two Ca^{2+}-binding sites per ATPase molecule are of high affinity and are exposed on the outer (cytoplasmic) side of the sarcoplasmic reticulum membrane. After phosphorylation, the ATPase undergoes a conformation change in which the Ca^{2+}-binding sites become of low affinity and are exposed to the inside of the sarcoplasmic reticulum (Scheme II, Ref. 5). To explain Ca^{2+} efflux from sarcoplasmic reticulum vesicles mediated by the ATPase, we suggested that, after binding of Ca^{2+} to E1, the ATPase can undergo a slow conformational change in which the Ca^{2+}-binding sites transform to a low affinity state characteristic of E2 but remain exposed to the outside of the sarcoplasmic reticulum (Scheme I). Cycling of the Ca^{2+}-binding sites in this low-affinity state between being inward and outward facing then provides a pathway for Ca^{2+} efflux. If the ATPase can, in this way, provide an efficient pathway for Ca^{2+} efflux in native sarcoplasmic reticulum, then it would also be expected to do so in reconstituted systems. Further, it is known that the ATPase activity of the ATPase is sensitive to the structure of the surrounding phospholipids, with phosphatidylethanolamines supporting lower activities than the corresponding phosphatidylcholines [5] so that phospholipid structure could also have significant effects on Ca^{2+} efflux mediated by the ATPase.

Materials and Methods

Sources of phospholipids and other chemicals were as described in the previous paper [1]. The $(Ca^{2+} + Mg^{2+})$-ATPase was purified and reconstituted as described in Ref. 1. Ca^{2+} efflux from reconstituted vesicles was determined using $^{45}Ca^{2+}$. Vesicles were loaded with $^{45}Ca^{2+}$ by including $^{45}Ca^{2+}$ in the buffer used for reconstitution. Efflux was initiated by diluting the loaded vesicles into buffer not containing Ca^{2+}. Aliquots were taken at the appropriate time intervals and layered onto 4 ml columns of Bio-Rad 50W-X8 resin (Tris form), previously equilibrated with 10 ml of 40 mM Hepes-KOH, 0.25 M sucrose and 10 mg/ml bovine serum albumin (pH 7.2); to produce fast flow rates, 75% of 20–50 mesh resin was mixed with 25% 50–100 mesh resin [6]. Vesicles were washed through the column with 9 ml of the bovine serum albumin-containing buffer. Aliquots were counted in Optiphase RIA scintillant (LKB-Wallac). The above procedure was limited to about one aliquot per 90 s. For samples where this rate of sampling was too slow, aliquots (100–500 μl) were taken and mixed with 150 μl of 40 mM Hepes-KOH/100 mM KCl (pH 7.2)/0.2 mM $MgSO_4$ before applying to a column up to 10 min later; as shown below, this concentration of Mg^{2+} effectively stops efflux. The initial level of trapped Ca^{2+} was determined by passing an aliquot of undiluted vesicles through a column. The level of Ca^{2+} untrapped within the vesicles which passed through the column was determined by disrupting vesicles with 2% deoxycholate before application to the column: this value was always less than 5% of the level of the initially trapped Ca^{2+}, and was subtracted from all values. It was confirmed that all the vesicles applied to the column were recovered by reconstituting vesicles with phosphatidyl[*N-methyl*-^{3}H]choline, and comparing the levels of labelled phospholipid applied to, and recovered from, the column. All efflux measurements were performed at 22° C.

Efflux of glucose from the reconstituted vesicles was determined by reconstituting vesicles in buffer (40 mM Hepes-KOH/100 mM KCl/0.1 mM EGTA (pH 7.2)) containing 12.5 mM glucose, at a molar ratio of lipid to protein of 3000:1. Phospholipid vesicles were reconstituted following an

exactly analogous procedure but in the absence of ATPase. After formation of the vesicles, the external untrapped glucose was removed by passage through a Sephadex G-50 column equilibrated with 40 mM Hepes-KOH/100 mM KCl (pH 7.2). 30 μl of the vesicle suspension was then added to 3 ml of buffer at 30 °C containing MgATP (3 mM), phospho*enol*pyruvate (0.6 mM), NADH (0.3 mM), pyruvate kinase (15 units), lactate dehydrogenase (36 units) and yeast hexokinase (51 units). Leak of glucose from the vesicles was monitored measuring the oxidation of NADH at 340 nm; under these conditions (purified ATPase and zero Ca^{2+}), the rate of hydrolysis of ATP by the ATPase was insignificant. Measurements were continued for about 5 min, and the level of trapped glucose was then established by addition of Triton X-100 to a final concentration of 0.5% (v/v).

The permeability of the reconstituted vesicles to Co^{2+} was determined by a fluorescence quenching method based on that of Oku et al. [7]. Vesicles were reconstituted in buffer (40 mM Hepes-KOH (pH 7.2)) containing 100 μM calcein (Sigma). Phospholipid vesicles were reconstituted following an exactly analogous procedure. An aliquot (40 μl) of the reconstituted vesicles was diluted into a cuvette containing buffer (3 ml) at 22 °C and the fluorescence intensity was determined at 520 nm, exciting fluorescence at 490 nm. A solution of cobalt chloride was then added to give a final concentration of 25 μM, and the fluorescence intensity was recorded at 5 min intervals for up to 40 min. Since it was found that calcein undergoes photobleaching upon prolonged exposure to light, the excitation shutters on the fluorimeter were closed between readings. A blank fluorescence level was determined by addition of an aliquot of a 10% (v/v) solution of Triton X-100 to give a final concentration of 0.5% (v/v). Permeabilities to Co^{2+} were compared in terms of $t_{1/2}$ values, defined as the time for the fluorescence intensities to fall to one-half of the value observed immediately after addition of Co^{2+}. It was confirmed that the observed decreases in fluorescence intensities were due to influx of Co^{2+} into the vesicles, and not due to efflux of calcein out of the vesicles, by reconstituting vesicles in buffer containing 50 mM calcein. Calcein at this concentration shows extensive self-quenching of fluorescence. Untrapped dye was removed by two passes through columns of Sephadex G-50, and the fluorescence intensity was measured after dilution into buffer as before, but in the absence of Co^{2+}. No increase in fluorescence intensity was observed over a 3 h time period, confirming that the reconstituted vesicles were impermeable to calcein. Fluorescence measurements were made using a Spex Fluorolog fluorimeter.

For measurements of ATPase activity, lipid substitutions were carried out by mixing purified ATPase and excess phospholipid in cholate, followed by dilution into buffer as described in Froud et al. [8]. ATPase activities were determined using the coupled enzyme assay described in Froud et al. [8]. When used, ionophores were added as solutions in methanol.

Equations for simulation of the steady-state kinetics of the ATPase were derived by using a version of the program Kinal of Cornish-Bowden [9] modified to run on a microcomputer, the simulations also being performed on a microcomputer. Simulations of Ca^{2+} efflux were carried out by using the FACSIMILE program [10] running on an ICL 2976 computer.

Results

As described in the previous paper [1], the $(Ca^{2+} + Mg^{2+})$-ATPase can be purified from sarcoplasmic reticulum and reconstituted into sealed phospholipid vesicles by dissolving the ATPase in detergent with a 3000:1 molar ratio of phospholipid to ATPase, followed by removal of detergent on a Sephadex G-50 column. To test the nonspecific permeability properties of these vesicles, the rate of efflux of glucose trapped within the vesicles was measured as described above, with the results listed in Table I. It is clear that, although the presence of ATPase increases the rate of glucose leak from the vesicles, the effect was relatively small. The permeability to Co^{2+} was also measured, measuring the rate of Co^{2+} influx into the vesicles from the resulting quenching of the fluorescence of calcein trapped within the vesicles. As shown in Table I, the presence of the ATPase also increases the permeability of the vesicles to Co^{2+}, but again the effects are small.

The rate of efflux of Ca^{2+} from reconstituted

TABLE I

PERMEABILITIES OF RECONSTITUTED VESICLES AT 30 °C

Molar ratio egg PE : egg PC	Time (min) for 50% release ($t_{1/2}$)	
	Co^{2+}	glucose
Pure lipid vesicles:		
0:1	75	18
0.2:1	36	–
1:1	25	13
4:1	24	9
3000:1 lipid:protein:		
0:1	60	9
0.2:1	26	–
1:1	23	6
4:1	23	5

vesicles was measured by addition of hexokinase and glucose to remove ATP from reconstituted vesicles actively accumulating Ca^{2+} in the presence of Mg^{2+} and ATP. As shown in Fig. 1, release of Ca^{2+} is very slow under these conditions. However, in previous papers it has been suggested that efflux of Ca^{2+} mediated by the ATPase is inhibited by Mg^{2+}, so that Ca^{2+} efflux under the conditions of Fig. 1 would be expected to be slow [3,4]. Ca^{2+} efflux from reconstituted vesicles was therefore also followed using vesicles passively loaded with $^{45}Ca^{2+}$.

We found that reconstituted vesicles prepared by the column method were too small to allow reliable retention on Millipore filters, and so reconstituted vesicles were separated from external Ca^{2+} by passage through columns of Bio-Rad 50W-X8 resin. Unfortunately, the use of such columns prevented the use of EGTA to control the external Ca^{2+} concentration, and the capacity of the columns for divalent metal ions limited the useable range of external concentrations of Ca^{2+} and Mg^{2+}. Fig. 2 shows that, if reconstituted vesicles are passively loaded with $^{45}Ca^{2+}$ at the reconstitution stage and Ca^{2+} efflux is initiated by dilution into a medium free of $^{45}Ca^{2+}$, then Ca^{2+} efflux from the vesicles is observed, and that the rate of efflux decreases as the egg PE content of the reconstituted vesicles increases. Fig. 3 shows that the rate of efflux decreases with increasing Ca^{2+} concentration in the external medium, and

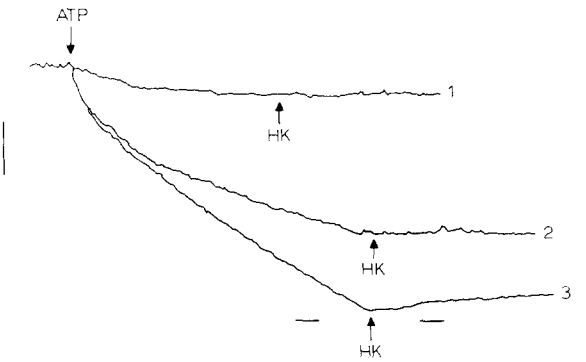


Fig. 1. Ca^{2+} leak from actively loaded vesicles. Vesicles were reconstituted with molar ratios of (egg) PE:PC of (1) 0:1, (2) 1:1 and (3) 4:1, in the presence of 0.4 M potassium phosphate (pH 7.4) at a molar ratio, lipid to ATPase, of 3000:1. Calcium accumulation was measured in a medium containing 40 mM Hepes-KOH, 100 mM KCl, 5 mM $MgSO_4$, 50 μM $CaCl_2$, 50 μM arsenazo III (pH 7.4), 30 °C. Accumulation of Ca^{2+} was initiated by addition of ATP to a concentration of 0.5 mM. At the times marked by the arrows, hexokinase and glucose (HK) were added to rapidly deplete the ATP. Shown are the observed changes in absorbance of arsenazo III recorded at 675–685 nm, where the vertical bar corresponds to a 0.003 *A* change in absorbance (corresponding to a 5 μM change in external Ca^{2+} concentration), and the horizontal bars correspond to time-scales of 0.5 and 2 min, respectively, before and after addition of HK.

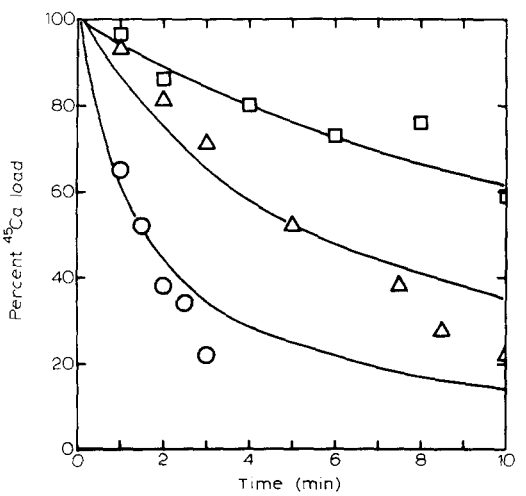


Fig. 2. Efflux of $^{45}Ca^{2+}$ from passively loaded vesicles reconstituted in the presence of 150 μM $^{45}Ca^{2+}$ with a molar ratio of (egg) PE:PC of (□) 4:1; (△) 1:1 and (○) 0:1, measured after a 15-fold dilution into a medium comprising 40 mM Hepes-KOH/100 mM KCl (pH 7.2), 22 °C. The initial level of $^{45}Ca^{2+}$ corresponds to an initial internal Ca^{2+} concentration of 100 μM. Solid lines are simulations with the parameters in Table II.

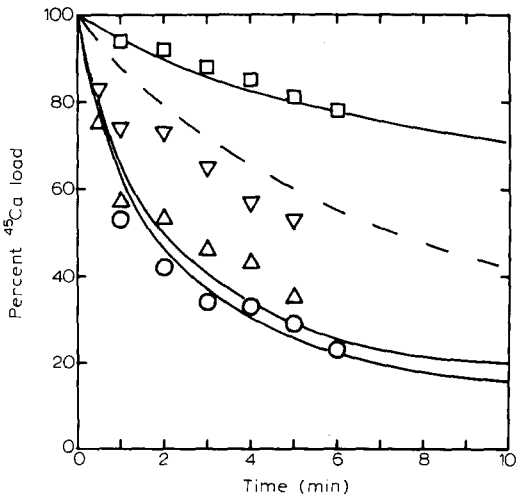


Fig. 3. Efflux of $^{45}Ca^{2+}$ from passively loaded vesicles reconstituted with egg PC following dilution at pH 7.2 to give an external Ca^{2+} concentration of (□) 50 μM, (△) 10 μM, and (○) 7.5 μM, with an initial internal Ca^{2+} concentration of 80 μM, and (▽) following dilution at pH 6.0 to give an external Ca^{2+} concentration of 10 μM. Solid and broken lines are simulations of the data at pH 7.2 and pH 6.0, respectively, with the parameters in Table II.

also decreases with decreasing pH. Fig. 4 shows that the presence of Mg^{2+} in the external medium decreases the rate of efflux. Fig. 5 shows that addition of the nonhydrolysable analogue of ATP,

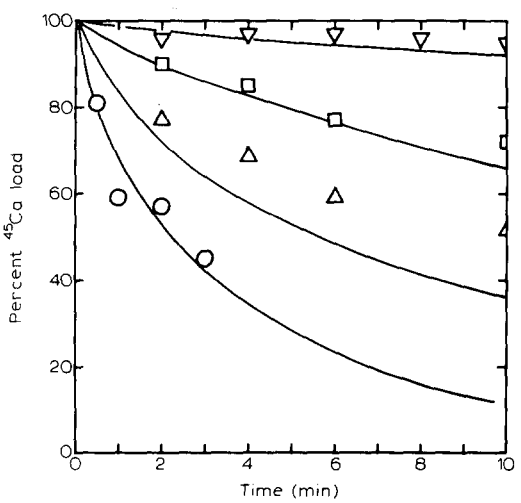


Fig. 4. Efflux of $^{45}Ca^{2+}$ from passively loaded vesicles reconstituted with egg PC following dilution at pH 7.2 into a medium containing 10 μM Ca^{2+} and either (○) no Mg^{2+} or Mg^{2+} at (△) 10 μM, (□) 50 μM, and (▽) 200 μM. Initial internal Ca^{2+} concentration 70 μM. Solid lines are simulations with the parameters in Table II.

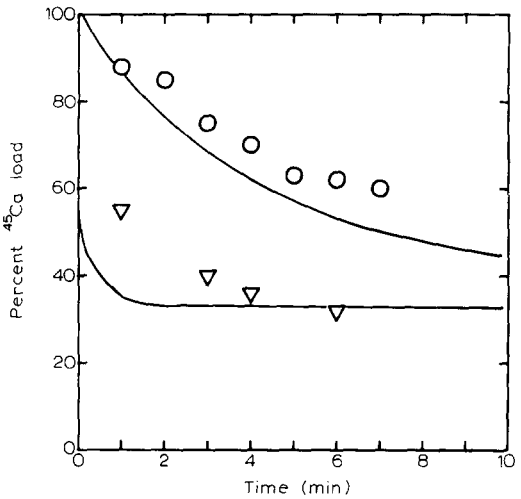


Fig. 5. Efflux of $^{45}Ca^{2+}$ from passively loaded vesicles reconstituted with egg PC following dilution at pH 7.2 into a medium containing 30 μM Ca^{2+} in the presence (▽) or absence (○) of 3.0 mM AMP-PCP. Initial internal Ca^{2+} concentration 90 μM. Solid lines are simulations with the parameters in Table II.

adenosine 5′-γ-methylenetriphosphate (AMP-PCP) causes a large increase in the efflux rate. Efflux of Ca^{2+} from vesicles reconstituted in the presence of 100 mM KCl is unaffected by the addition of valinomycin, and addition of up to 10 μM inositol trisphosphate had no effect on the rate of Ca^{2+} efflux, either in the presence or absence of external Mg^{2+} (data not shown).

The effect of eggPE on the ATPase activity of the ATPase is shown as a function of the concentration of ATP in Fig. 6.

Discussion

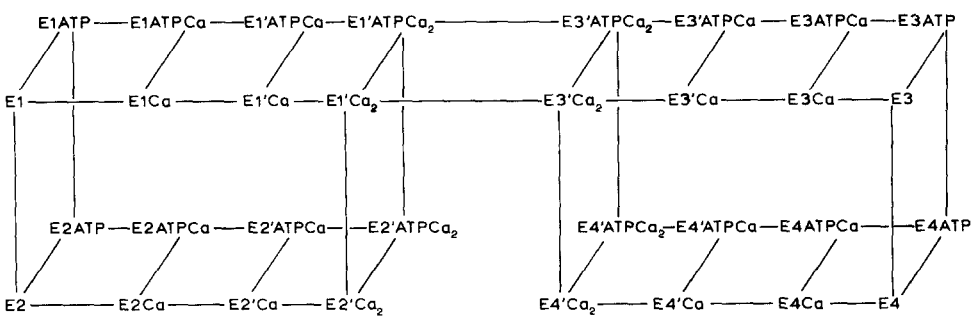
One of the most powerful techniques for studying the function of a particular membrane protein is to study the properties of that protein in a simplified, reconstituted system. Homogenisation of skeletal muscle gives a preparation of sealed vesicles derived from the sarcoplasmic reticulum which can take up Ca^{2+} from the external medium in the presence of ATP. Studies with reconstituted systems have established that this uptake of Ca^{2+} is mediated by the $(Ca^{2+} + Mg^{2+})$-ATPase which is the major protein component of the membrane [11]. Following uptake of Ca^{2+} by sarcoplasmic reticulum vesicles, Ca^{2+} is released spontaneously

in two phases, a relatively slow phase whilst ATP is still present in the system, and a faster phase which starts when all the ATP in the system has been exhausted [3,4]. Ca^{2+} efflux from sarcoplasmic reticulum vesicles passively loaded with Ca^{2+} has also been demonstrated, with the rate of efflux being sensitive to the concentrations of Ca^{2+} and Mg^{2+} in the external medium and being stimulated by nucleotides [3,12,13]. Although it has been suggested that this efflux of Ca^{2+} is mediated by Ca^{2+} channels within the sarcoplasmic reticulum membrane, we have shown that the experimental data can, in fact, be simulated quantitatively assuming that Ca^{2+} efflux is mediated by the $(Ca^{2+} + Mg^{2+})$-ATPase [3,4].

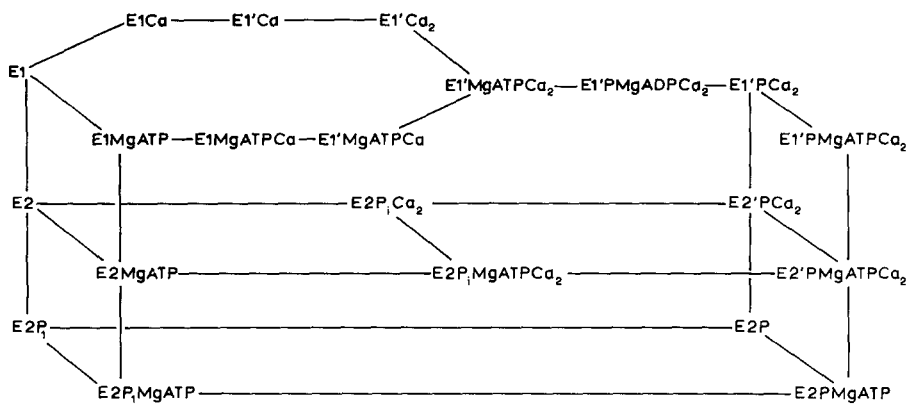
Of course, if the ATPase can indeed act as pathway for rapid efflux of Ca^{2+} from sarcoplasmic reticulum vesicles, then it should also be able to do so in reconstituted vesicles containing the ATPase as the sole protein species. As shown in Gould et al. [18], the $(Ca^{2+} + Mg^{2+})$-ATPase can be purified from sarcoplasmic reticulum in a state of high purity. Here we have shown that incorporation of the ATPase into a lipid bilayer results in only a small increase in the permeability of the membrane to glucose and Co^{2+} (Table I) but a large increase in permeability to Ca^{2+} with a sensitivity to external Ca^{2+} and Mg^{2+} concentration, to pH and to adenine nucleotides comparable to that observed for Ca^{2+} efflux from sarcoplasmic reticulum vesicles (Figs. 2–5).

To compare the observed rates of Ca^{2+} efflux from reconstituted vesicles to those from sarcoplasmic reticulum vesicles in detail, a kinetic model for the efflux is required. The scheme for efflux mediated by the ATPase that we proposed (Scheme I) involves the same Ca^{2+}-binding sites on the ATPase as are involved in Ca^{2+} uptake. In ATP-driven uptake, a concerted change in the Ca^{2+}-binding sites occurs following phosphorylation in which the Ca^{2+}-binding sites change from being outward facing and high affinity in the E1 conformation of the ATPase, to being inward facing and low affinity in the E2 conformation (Scheme II). Calcium efflux is proposed to follow from a slow conformation change in the presence of Ca^{2+} to a conformation, E3, in which the Ca^{2+}-binding sites change from high to low affinity without changing their orientation. Cycling between the E3 conformation with outward-facing Ca^{2+}-binding sites and an E4 conformation with inward-facing Ca^{2+}-binding sites then provides a pathway for Ca^{2+} efflux. The suggested conformation change is somewhat analogous to the process of desensitization observed for the acetylcholine receptor in the presence of acetylcholine [14]. We stress that at this stage the suggested kinetic model must be very preliminary and it is introduced simply to demonstrate that, with only a few new postulates, it is possible to show how the ATPase could act as a pathway for release, and that in terms of such a model it is possible to interpret, quantitatively, the available data on Ca^{2+} efflux both from native sarcoplasmic reticulum vesicles and from reconstituted systems.

In McWhirter et al. [3] we derived rate parameters for the steps in Scheme I based on data on the rate of efflux of Ca^{2+} from sarcoplasmic reticulum



Scheme I. Proposed scheme for the Ca^{2+} release pathway. In the E1 conformation, the Ca^{2+}-binding sites are of high affinity and outward facing. In the E2, E3 and E4 conformations the Ca^{2+} binding sites are of low affinity and outward facing in E3, but inward facing in E2 and E4.



Scheme II. Simplified scheme for ATPase activity [5].

vesicles. To simulate Ca^{2+} efflux from the reconstituted systems using these same rate parameters it is necessary to known the internal volumes of the reconstituted vesicles per mg of protein, the internal concentrations of Ca^{2+} achieved following reconstitution and the percentage of ATPase molecules oriented each way across the membrane. As described in the previous paper [1], volumes calculated from gel-chromatography data, assuming a membrane thickness of 6 nm, for the ATPase reconstituted with a 3000:1 molar ratio of lipid to protein, were 39, 44 and 52 μl/mg protein for molar ratios of egg PE to egg PC of 0:1, 1:1 and 4:1 respectively. Using these internal volumes, the levels of $^{45}Ca^{2+}$ generally found trapped in the vesicles after reconstitution of the vesicles in the presence of $^{45}Ca^{2+}$ correspond to internal Ca^{2+} concentrations about 75% of those in the reconstitution media (see legend to Fig. 2). An alternative explanation for the lower than expected internal concentrations of Ca^{2+} could be, of course, that the internal Ca^{2+} concentrations were equal to those in the reconstitution medium but that the available internal volumes were only 75% of those estimated from measurements of vesicle diameter. Similar simulations of efflux are obtained making either assumption. As described in the previous paper [1], the orientation of ATPase molecules in the reconstituted vesicles is close to random.

We have found that the rate of Ca^{2+} release from actively loaded vesicles of sarcoplasmic reticulum is highly variable between preparations and have shown that this variability can be simulated assuming differences in the rates of the conformational transitions $E2'Ca_2$–$E1'Ca_2$ and $E4'Ca_2$–$E3'Ca_2$ [3]. We have also found that the ATPase activity of the purified ATPase is also highly variable between preparations and have shown that this variability can also be simulated in terms of differences in the rates of the conformational transitions involving changes in the orientation of the Ca^{2+}-binding sites, that is, by changes in the rates of the $E1'PCa_2$–$E2'PCa_2$ and $E1'PMgATPCa_2$–$E2'PMgATPCa_2$ transitions [5]. It is known that the properties of the ATPase and sarcoplasmic reticulum are very sensitive to oxidation of -SH groups, and we have suggested that oxidation of these groups could account for the observed variability of ATPase activity and rate of Ca^{2+} efflux. In the simulations of Ca^{2+} efflux from reconstituted vesicles we have, therefore, treated the rates of the $E2'Ca_2$–$E1'Ca_2$ and $E4'Ca_2$–$E3'Ca_2$ transitions as variables. The data on Ca^{2+} efflux from reconstituted vesicles passively loaded with $^{45}Ca^{2+}$ can be fitted to Scheme I with rates for the $E2'Ca_2$–$E1'Ca_2$ and $E4'Ca_2$–$E3'Ca_2$ transitions as given in Table II, with all the other parameters fixed at the values determined previously from studies of sarcoplasmic reticulum vesicles [3]. Simulations show that only those ATPase molecules with the correct orientation (as found in native sarcoplasmic reticulum vesicles) will mediate Ca^{2+} release at a significant rate and that ATPase molecules in the membrane with the opposite orientation can be ignored in the simulations. The rates of the transi-

TABLE II

KINETIC PARAMETERS FOR THE EFFECT OF PHOSPHOLIPID ON Ca^{2+} EFFLUX MEDIATED BY THE ATPase AT 22°C

All other values as given in McWhirter et al. [3].

Phospholipid mixture (molar ratio PE:PC, from egg yolk)	Rate constant (s^{-1})	
	$E2'Ca_2$–$E1'Ca_2$	$E4'Ca_2$–$E3'Ca_2$
0:1	$1.2 \cdot 10^{-3}$	35.2
1:1	$3.1 \cdot 10^{-4}$	9.4
4:1	$1.0 \cdot 10^{-4}$	3.0

tions $E2'Ca_2$–$E1'Ca_2$ and $E4'Ca_2$–$E3'Ca_2$ for the ATPase reconstituted with egg PC are within the range of values that fit the experimental data for Ca^{2+} efflux from sarcoplasmic reticulum vesicles [3,4]. This result then suggests that the phospholipid shell or annulus surrounding the ATPase in the native sarcoplasmic reticulum membrane is composed predominantly of phosphatidylcholine and, since the majority of the phospholipid in the native sarcoplasmic reticulum membrane is phosphatidylcholine [4], this result is consistent with the nonspecific phospholipid–protein interaction reported by East and Lee [16].

As shown in Table II, the rates of the $E2'Ca_2$–$E1'Ca_2$ and $E4'Ca_2$–$E3'Ca_2$ transitions decrease by a factor of about 10 as the content of egg PE used to reconstitute the ATPase increases to 80%. The observation that high molar ratios of egg PE are required before significant effects on transition rates are observed is also consistent with a relatively non-specific phospholipid–protein interaction for the ATPase and suggests that the phosphatidylethanolamine content of the native sarcoplasmic reticulum membrane (about 20%, Ref. 4) is too small to have any significant effect on the ATPase.

The effect of egg PE on the rate of Ca^{2+} efflux can be compared to its effect on ATPase activity (Fig. 6, Table III). As shown in Fig. 6 and as previously reported by East and Lee [16], effects of mixtures of egg PC and egg PE on ATPase activity are complex, with activity first increasing and then decreasing with increasing egg PE content. As shown in Fig. 6, these effects can be simulated in terms of Scheme II. As reported for a

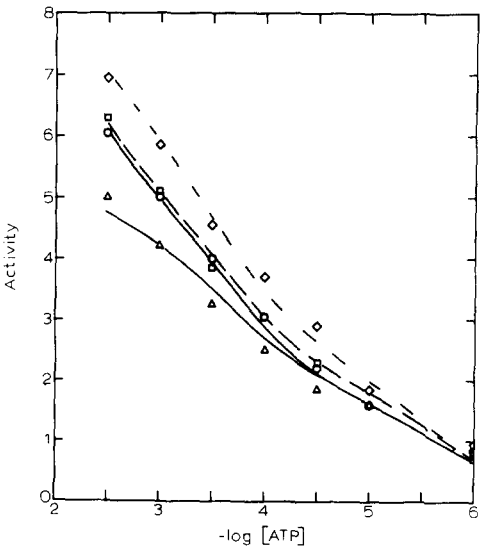


Fig. 6. ATP concentration dependence of the activity (IU/mg) of the ATPase at 25°C after reconstitution with molar ratios of (egg) PE:PC of (○) 0:1; (□) 1:1; (◇) 4:1; and (△) 1:0. Lines are simulations with the parameters in Table III.

variety of other phospholipids, the structures of the phospholipids surrounding the ATPase determine the rates of the conformational transitions between the various E1 and E2 forms of the ATPase and the rate of dephosphorylation [8].

TABLE III

KINETIC PARAMETERS OBTAINED BY SIMULATION FOR THE ATPase ACTIVITY OF THE RECONSTITUTED ATPase AT 25°C

All other parameters as given in Gould et al. [5] and Stefanova et al. [17] with an off-rate constant and K_d for binding of MgATP to E1 of 37 s^{-1} and $1.1 \cdot 10^{-4}$, respectively, and an off-rate constant and K_d for binding of MgATP to $E1'PCa_2$ of 2.0 s^{-1} and $3.1 \cdot 10^{-6}$, respectively.

Reaction	Rate constant (s^{-1}) [a]			
Molar ratio (egg)PE:PC:	0:1	1:1	4:1	1:0
E2–E1	38.0	38.0	68.4	76.0
$E1'PCa_2$–$E2'PCa_2$	5.3	6.2	7.8	8.9
$E1'PMgATPCa_2$– $E2'PMgATPCa_2$	26.6	31.1	38.9	88.8
E2P dephosphorylation	71.4	53.6	35.7	10.7

[a] Composite rate constants for pH 7.2, $K^+ = 116$ mM, $Mg^{2+} = 5.0$ mM.

Assuming that egg PE supports a higher rate for the conformational transitions than does egg PC, but a lower rate of dephosphorylation, it is possible to simulate the effects of mixtures of PE and PC (from egg yolk) on the ATP dependence of ATPase activity (Fig. 6, Table III). Particularly noticeable is that this scheme can account for the observation that mixtures of PE and PC from egg yolk support higher ATPase activites than either egg PC or egg PE alone (Fig. 6). The model presented here therefore suggests that the presence of egg PE affects the rates of the conformational transitions involving changes in the orientation of the Ca^{2+}-binding sites, both for ATPase activity and for Ca^{2+} efflux.

It has been shown that passive efflux of Ca^{2+} from sarcoplasmic reticulum vesicles is inhibited by Mg^{2+}, H^+, and high external Ca^{2+} concentrations, but is activated by a variety of nucleotides [12,13]; we have shown that these effects can be simulated in terms of Scheme I [3]. As shown in Figs. 3–5, Ca^{2+} efflux from reconstituted vesicles is also inhibited by Mg^{2+}, H^+ and high external Ca^{2+} concentrations and is also activated by the non-hydrolysable ATP analogue AMP-PCP. Importantly, these effects on efflux can be simulated quantitatively using the parameters determined previously from studies on sarcoplasmic reticulum vesicles (Figs. 3–5). The marked inhibition of efflux by Mg^{2+} means that efflux of Ca^{2+} from sarcoplasmic reticulum vesicles in the presence of Mg^{2+} and ATP or other nucleotides would be expected to be slow [3], as observed experimentally [12,13]. Efflux of Ca^{2+} from reconstituted vesicles containing phosphate, actively loaded in the presence of Mg^{2+} and ATP would, therefore, also be expected to be slow when ATP is removed from the system by addition of hexokinase and glucose, as observed (Fig. 1). Efflux from reconstituted vesicles containing no internal phosphate is considerably faster under these same conditions, with 50% release of the accumulated Ca^{2+} in 30–40 min (data not shown): in the absence of internal phosphate, the internal free Ca^{2+} concentration will be higher, leading to a faster rate of Ca^{2+} efflux.

Comparison of measurements of Ca^{2+} efflux with the measurements of Ca^{2+} accumulation reported in the previous paper [1] show that conditions which lead to slow Ca^{2+} efflux also lead to increased levels of accumulation. Thus, the rate of Ca^{2+} efflux from reconstituted vesicles decreases with increasing content of egg PE (Fig. 2) and the level of Ca^{2+} accumulation increases (Fig. 1 of Ref. 1); the rate of Ca^{2+} efflux decreases with decreasing pH (Fig. 3) and the level of Ca^{2+} accumulation increases (Fig. 6 of Ref. 1); and the presence of a Ca^{2+}-precipitating agent within the vesicles which will lead to a decrease in the rate of Ca^{2+} efflux by decreasing the internal free concentration of Ca^{2+} leads to an increase in the level of Ca^{2+} accumulation (Figs. 4, 5 of Ref. 1). These observations therefore suggest that the level of Ca^{2+} accumulated by the reconstituted vesicles is determined by a balance between the rates of Ca^{2+} influx driven by hydrolysis of ATP and the rate of efflux. Unfortunately, as described in the previous paper [1], insufficient information is available about counter-ion movement in these reconstituted systems to allow any simulation of Ca^{2+} accumulation in the reconstituted systems.

The observation of fast and specific efflux of Ca^{2+} from reconstituted vesicles containing the $(Ca^{2+} + Mg^{2+})$-ATPase as the only protein species shows that the ATPase alone can mediate Ca^{2+} efflux. The observation that this efflux can be described by the same kinetic parameters as described Ca^{2+} efflux from sarcoplasmic reticulum vesicles suggests that Ca^{2+} efflux from sarcoplasmic reticulum vesicles could also be mediated by the ATPase. The relationship between this efflux and the efflux of Ca^{2+} from sarcoplasmic reticulum in muscle leading to muscle contraction must remain uncertain at the present time. However, it would be surprising if a pathway for Ca^{2+} efflux from sarcoplasmic reticulum were to exist which was not exploited physiologically.

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References

1 Gould, G.W., McWhirter, J.M., East, J.M. and Lee, A.G. (1987) Biochim. Biophys. Acta 904, 36–44

2 Jilka, R.L., Martonosi, A.N. and Tillack, T.W. (1975) J. Biol. Chem. 250, 7511–7524
3 McWhirter, J.M., Gould, G.W., East, J.M. and Lee, A.G. (1987) Biochem. J. 245, 713–722
4 Gould G.W., McWhirter, J.M., East, J.M. and Lee, A.G. (1987) Biochem. J. 245, 739–749
5 Gould, G.W., East, J.M., Froud, R.J., McWhirter, J.M., Stefanova, H.I. and Lee, A.G. (1986) Biochem. J. 237, 217–227
6 Gasko, O.D., Knowles, A.F., Shertzer, H.G., Sholinna, E.M. and Racker, E. (1976) Anal. Biochem. 72, 57–65
7 Oku, N., Kendall, D.A. and Macdonald, R.C. (1982) Biochim. Biophys. Acta 691, 332–340
8 Froud, R.J., East, J.M., Jones, O.T. and Lee, A.G. (1986) Biochemistry 25, 7544–7552
9 Cornish-Bowden, A. (1977) Biochem. J. 165, 55–59
10 Chance, E.M., Curtis, A.R., Jones, I.P. and Kirby, C.R. (1977) FACSIMILE: A Computer Program for Flow and Chemistry Simulation, H.M.S.O., London
11 De Meis, L. (1981) The Sarcoplasmic Reticulum, John Wiley & Sons, New York
12 Meissner, G. (1984) J. Biol. Chem. 259, 2365–2374
13 Meissner, G., Darling, E. and Eveleth, J. (1986) Biochemistry 25, 236–244
14 Anholt, R., Lindstrom, J. and Montal, M. (1982) in The Enzymes of Biological Membranes (Martonosi, A.N., ed.), Vol. 3, 2nd Edn., pp. 335–402, Plenum Press, New York
15 Froud, R.J. and Lee, A.G. (1986) Biochem. J. 237, 197–206
16 East, J.M. and Lee, A.G. (1982) Biochemistry 21, 4144–4151
17 Stefanova, H.I., Napier, R.M., East, J.M. and Lee, A.G. (1987) Biochem. J. 245, 723–730
18 Gould, G.W., Colyer, J., East, J.M. and Lee, A.G. (1987) J. Biol. Chem. 262, 7676–7679