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Uptake of Ca^{2+} mediated by the $(Ca^{2+} + Mg^{2+})$-ATPase in reconstituted vesicles

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The $(Ca^{2+} + Mg^{2+})$-ATPase was purified from skeletal muscle sarcoplasmic reticulum and reconstituted into sealed phospholipid vesicles by solution in cholate and deoxycholate followed by detergent removal on a column of Sephadex G-50. The level of Ca^{2+} accumulated by these vesicles, either in the presence or absence of phosphate within the vesicles, increased with increasing content of phosphatidylethanolamine in the phospholipid mixture used for the reconstitution. The levels of Ca^{2+} accumulated in the absence of phosphate were very low for vesicles reconstituted with egg yolk phosphatidylcholine alone at pH 7.4, but increased markedly with decreasing pH to 6.0. Uptake was also relatively low for vesicles reconstituted with dimyristoleoyl- or dinervonylphosphatidylcholine, and addition of cholesterol had little effect. The level of Ca^{2+} accumulated increased with increasing external K^+ concentration, and was also increased by the ionophores FCCP and valinomycin. Vesicle sizes changed little with changing phosphatidylethanolamine content, and the sidedness of insertion of the ATPase was close to random at all phosphatidylethanolamine contents. It is suggested that the effect of phosphatidylethanolamine on the level of Ca^{2+} accumulation follows from an effect on the rate of Ca^{2+} efflux mediated by the ATPase.

Introduction

The most detailed information about the nature of the interactions controlling membrane function has come from studies of simplified, reconstituted membrane systems. One of the best understood of these systems is the $(Ca^{2+} + Mg^{2+})$-ATPase purified from skeletal muscle sarcoplasmic reticulum and reconstituted into phospholipid bilayers of defined composition. Under conditions of reconstitution where sealed vesicles are not produced, ATPase activity is uncoupled from accumulation of Ca^{2+}, and the full ATPase activity of the ATPase is expressed. In such systems it has been shown that the phospholipid supporting maximal activity is dioleoylphosphatidylcholine (DOPC), with phospholipids containing either different fatty acyl chains or different headgroups supporting lower activities [1–11].

Experiments in which the ATPase has been reconstituted into sealed vesicles to study Ca^{2+}

Abbreviations: DMPC, dimyristoleoylphosphatidylcholine; DNPC, dinervonylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; MGDG, monogalactosyldiacylglycerol; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-4-piperazineethanesulfonic acid.

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transport as well as ATPase activity have given much less clear cut results. Early work on the sarcoplasmic reticulum by Martonosi et al. [12] showed that after phospholipase digestion of up to 60% of the total sarcoplasmic reticulum phospholipid, ATPase activity and Ca^{2+} uptake could be restored by addition of sonicated dispersions of phospholipid. Racker [13] demonstrated that reconstitution of the purified ATPase with mixed soyabean phospholipids gave vesicles that would accumulate Ca^{2+} if a Ca^{2+}-precipitating agent such as phosphate was trapped within the vesicles. Warren et al. [14] demonstrated that uptake was possible in reconstituted systems containing just the ATPase and DOPC if the vesicles contained oxalate, whereas reconstitution with a mixture of lipids extracted from SR membranes allowed uptake in the absence of a Ca^{2+}-precipitating agent. Racker and Eytan [15] reported that efficient uptake of Ca^{2+} was achieved only if the vesicles contained both the ATPase and a small proteolipid of molecular weight 12000. However, more recent studies [16,17] have confirmed the observations of Warren et al. [14] that the ATPase alone can function as a Ca^{2+} pump. Still unclear, however, is the role of phospholipid in Ca^{2+} uptake. Although Warren et al. [14] and Navarro et al. [18] observed Ca^{2+} uptake when the ATPase was reconstituted by cholate dialysis into vesicles of phosphatidylcholine if the vesicles contained trapped oxalate or phosphate, Knowles and Racker [19] and Zimniak and Racker [20] failed to observe uptake unless the phospholipids used to reconstitute the vesicles included phosphatidylethanolamine. Zimniak and Racker [20], however, did report uptake in vesicles of phosphatidylcholine alone in the presence of a Ca^{2+}-precipitating agent if reconstitution was carried out by the freeze-thaw-sonication method. What is not in doubt, however, is the considerably greater levels of Ca^{2+} uptake observed for vesicles reconstituted with mixtures of phosphatidylcholine and phosphatidylethanolamine compared to those reconstituted with phosphatidylcholine alone [1,18–21]. Navarro et al. [21] also showed that the effect of phosphatidylethanolamine could be mimicked by other phospholipids capable of forming hexagonal II phase structures, such as phosphatidylglycerols. Wakabayashi and Shigekawa [22] reported very high levels of Ca^{2+} uptake in vesicles reconstituted with crude soyabean phosphatidylcholine Type II-S obtained frm the Sigma Chemical Company, but this lipid preparation is, in fact, listed as containing only 10–20% phosphatidylcholine, so that the interpretation of these experiments is unclear.

It is the aim of this paper to explore the effects of phospholipids on Ca^{2+} uptake in reconstituted systems in more detail, and to compare effects on uptake with previously reported effects on ATPase activity.

Materials and Methods

Egg yolk phosphatidylcholine (egg PC), egg yolk phosphatidylethanolamine (egg PE) and dioleoylphosphatidylcholine (DOPC) were obtained from Lipid Products, dimyristoleoylphosphatidylcholine (DMPC) and dinervonylphosphatidylcholine (DNPC) were obtained from Avanti Polar Lipids and monogalactosyldiacylglycerol was obtained from Sigma. Arsenazo III was obtained from Sigma. Cholic acid and deoxycholic acid were also obtained from Sigma and were recrystallised as potassium salts from methanol/diethyl ether before use. Ionophore A23187 was from Calbiochem, valinomycin and carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) were from Sigma and alamethicin was from Upjohn.

$(Ca^{2+} + Mg^{2+})$-ATPase was purified from hind leg muscle of rabbit as described by East and Lee [5], and was more than 97% pure by Commassie blue staining [23]. The final preparation contained 30 phospholipid molecules per ATPase, assuming a protein molecular weight of 110000. Reconstituted vesicles containing the ATPase were prepared using methods analogous to those of Karlish and Pick [24] and Penefsky [25]. Phospholipid (10 mg) was dried onto the sides of a 3 ml glass test tube and then dispersed into 600 μl of the buffer of choice (usually 0.4 M potassium phosphate (pH 7.4)) by vortex mixing for 15–30 s. An aliquot of 10% (w/v) potassium cholate in buffer (40 mM Hepes-KOH/100 mM KCl (pH 7.2)) was then added to give the required cholate : lipid ratio, usually 1 mg cholate to 1 mg lipid. The suspension was sonicated to clarity under nitrogen using a Branson bath sonicator. An aliquot (3–10 μl) of

10% (w/v) deoxycholate in buffer (40 mM Hepes-KOH (pH 8.0)) was added to an aliquot of the purified ATPase (0.3–1.0 mg protein in 15–50 μl buffer) to give a final deoxycholate to ATPase ratio of 0.6 : 1.0 (mg : mg). The mixture was vortexed for 5 s and then spun at 10000 × *g* in a microfuge for 5 min to remove any unsolubilised aggregates. The lipid and protein samples were then mixed to give the required lipid to protein ratio. The detergent was removed using the centrifugation method of Penefsky [25]. 5 ml plastic syringes were filled with a boiled deaerated suspension of Sephadex G-50, which was then washed with the buffer of choice (usually 0.4 M potassium phosphate (pH 7.4)). The syringes were placed in plastic centrifuge tubes and spun at 200 × *g* in a bench-top centrifuge for 30 s. The lipid/protein/detergent mixture was 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. The final eluate was a cloudy suspension of reconstituted vesicles, containing 70–90% of the initial protein. Samples were kept on ice for no more than 4 h before use. Samples were also reconstituted removing detergent by dialysis for 24 h.

Ca^{2+} uptake by the reconstituted vesicles was followed by dual-wavelength spectrophotometry using arsenazo III to monitor the external Ca^{2+} concentration. Reconstituted vesicles were added to buffer (usually 40 mM Hepes-KOH/100 mM KCl/5 mM $MgSO_4$) containing 50 μM calcium chloride and 50 μM arsenazo III, to give a final protein concentration of 20–40 μg/ml. Ca^{2+} uptake was initiated by addition of an aliquot of ATP in buffer at pH 7.2, to give a final concentration of 0.5 mM. Uptake of Ca^{2+} was followed by measuring the change in absorbance at 675–685 nm, using a Shimadzu UV3000 dual-wavelength spectrophotometer. Samples were stirred with a 'Cell-spinbar' magnet (Bel-Art Products) in the sample cuvette, and ATP was injected directly into the cuvette using a Hamilton syringe. All uptake experiments were performed at 30 °C. A calibration curve was established by addition of aliquots of known Ca^{2+} concentration to the sample prior to initiation of uptake. At the Mg^{2+} concentration used here, binding of Ca^{2+} to ATP is negligible, so that the decrease in external Ca^{2+} concentration observed following addition of ATP can be attributed to uptake of Ca^{2+} by the vesicles.

Vesicle populations were analysed by gel permeation chromatography. Vesicles were reconstituted with egg yolk phosphatidylcholine containing phosphatidyl[*N-methyl*-^{3}H]choline at a molar ratio of lipid to protein of 3000 : 1 as described above, except that the buffer was 40 mM Hepes-KOH/100 mM NaCl (pH 7.4). Samples were run at 5 ml/h at 4 °C on a column of Sepharose 2B-300 (60–200 μm pore size), equilibrated and eluted with the buffer used for reconstitution. Fractions were assayed for lipid content by scintillation counting in Tritoscint, and for protein content by measuring intrinsic protein fluorescence (excitation and emission wavelengths 280 and 340 nm, respectively) using a Spex Fluorolog fluorimeter. The same column was used to determine the diameters of reconstituted vesicles. The column was equilibrated with 40 mM Hepes-KOH, 100 mM NaCl, 2% (w/v) Triton-X100 and 1 mg/ml bovine serum albumin (pH 7.2), and calibrated using polystyrene beads of known diameter (Polysciences). The column was subsequently re-equilibrated with 40 mM Hepes-KOH, 100 mM NaCl and 1 mg/ml bovine serum albumin (pH 7.2), and vesicle fractions were applied to the column in volumes less than 1% of the column volume. A column flow rate of 5 ml/h was used and fractions were assayed for phospholipid as described above. Void and column volumes were determined with blue dextran and potassium dichromate respectively.

Vesicle diameters were also determined using electron microscopy. Vesicles prepared as described above were applied to Formvar-coated grids and stained with 1% (w/v) uranyl acetate. Vesicle diameters were determined using a transect sampling procedure to minimise subjective errors on an IBASS computer image analysis system.

ATPase activities were determined using the coupled enzyme assay described in Froud et al. [11]. When used, ionophores were added as solutions in methanol.

Results

The $(Ca^{2+} + Mg^{2+})$-ATPase can be reconstituted into sealed vesicles by mixing the ATPase in deoxycholate with excess phospholipid in cholate followed by removal of detergent on a column of Sephadex G-50. We have found that the most convenient procedure to follow Ca^{2+} uptake is using arsenazo III as a Ca^{2+}-sensitive dye; comparable results were also obtained using murexide as dye or following changes in the fluorescence of chlortetracycline (Stefanova and Lee, unpublished observations). Where comparisons can be made, our measurements of accumulation of Ca^{2+} are very similar to those made by Navarro et al. [21] using $^{45}Ca^{2+}$.

As shown in Fig. 1, if vesicles are reconstituted in the presence of 0.4 M potassium phosphate and then diluted into a medium free of phosphate so that the vesicles contain a high internal concentration of phosphate, a high level of accumulation of Ca^{2+} is observed. Further, as reported by Navarro et al. [21], the level of Ca^{2+} accumulated increases with increasing content of phosphatidylethanolamine in the phospholipid mixture used in the reconstitution (Fig. 1).

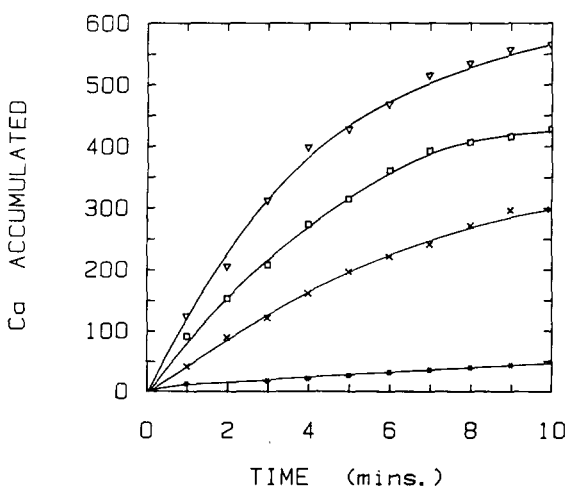

Fig. 1. The effect of egg PE on Ca^{2+} accumulation (nmol/mg protein) by vesicles reconstituted with the $(Ca^{2+} + Mg^{2+})$-ATPase in the presence of 0.4 M potassium phosphate (pH 7.4) at a molar ratio of lipid to ATPase of 3000:1. Calcium accumulation measured in a medium containing 40 mM Hepes-KOH/100 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 0.4 mM ATP. Molar ratios PE:PC from egg yolk: *, 0:1; ×, 0.5:1; □, 1:1; and ▽, 4:1.

We studied the degree of insertion of the ATPase into reconstituted vesicles using gel filtration. As shown in Fig. 2, the gel filtration profile indicates quite a wide range of sizes for the reconstituted vesicles, but the identical distribution of lipid and protein suggests complete mixing in the reconstitution process. Fig. 2 also demonstrated that essentially all of the ATPase is reconstituted into the lipid vesicles. Similar profiles were obtained for vesicles reconstituted with various mixtures of egg yolk phosphatidylcholine (egg PC) and egg yolk phosphatidylethanolamine (egg PE), but shifted to smaller elution volumes with increased egg PE content.

Columns were calibrated with polystyrene beads of known diameter and used to calculate the vesicle diameters given in Table I. Negatively stained vesicles were also sized by electron microscopy, giving comparable results (Table I).

The level of Ca^{2+} accumulated decreases with decreasing molar ratios of lipid to protein (data not shown), presumably because of smaller vesicular volumes per mg of protein. Navarro et al. [21] reported that, for vesicles reconstituted by dialysis, effects of monogalactosyldiacylglycerol (MGDG) were comparable to those of phosphatidylethanolamine. We also find that levels of Ca^{2+} accumulation are greater for vesicles reconstituted by the column method with mixtures of MGDG and egg PC than for egg PC alone (data not shown). Fig. 3 shows that levels of Ca^{2+} accumulated are comparable for vesicles reconstituted with egg PC or dioleoylphosphatidylcholine (DOPC), but that phosphatidylcholines with shorter (C14, DMPC) or longer (C24, DNPC) fatty acyl chains support lower levels of accumula-

TABLE I

DIAMETERS OF RECONSTITUTED VESICLES AT A LIPID:PROTEIN MOLAR RATIO OF 3000:1

Molar ratio PE:PC (from egg yolk)	Diameter (nm)	
	gel chromatography	electron microscopy
0:1	53.0±15.0	56.0±28.0
1:1	60.0±12.8	72.0±38.4
4:1	69.0±17.2	71.2±28.4

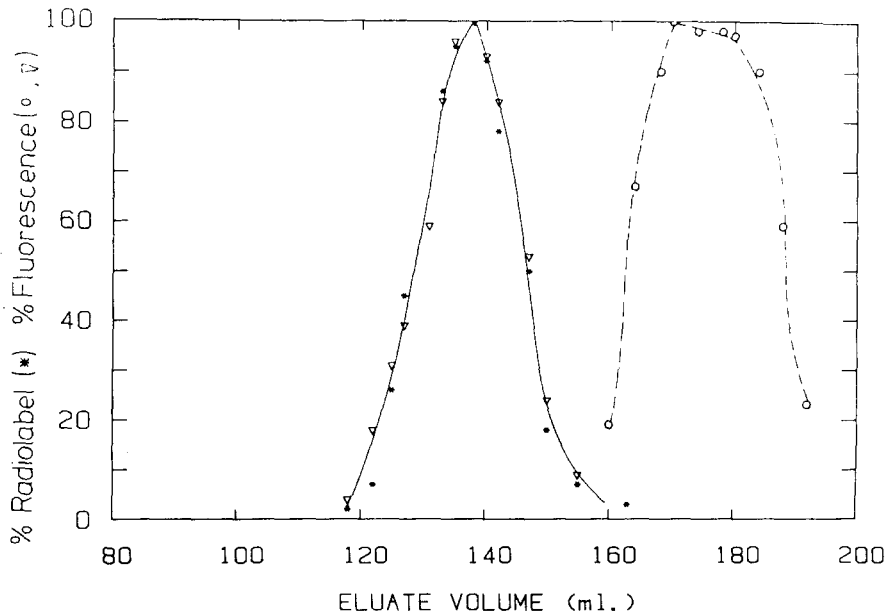

Fig. 2. Elution profile from a Sepharose 2B column of the (Ca^{2+} + Mg^{2+})-ATPase reconstituted with a molar ratio of 3000 : 1 of egg PC to ATPase (solid line). Protein (▽) was detected by intrinsic fluorescence and lipid (*) using egg [^{3}H]PC. Also shown (broken line) is the elution profile for the ATPase solubilised in the absence of added phospholipid.

tion: these phospholipids have also been shown to support lower ATPase activities than DOPC [11].

The importance of the concentration of phosphate trapped within the vesicles is shown in Fig. 4. Reducing the level of phosphate present during the reconstitution considerable reduces the level of Ca^{2+} accumulated. That this effect is due to phosphate and not due to potassium (which is known to have significant effects on the ATPase [26]) was shown by the comparable levels of Ca^{2+} accumulated by vesicles reconstituted in the presence of either 0.4 M potassium phosphate or 0.4 M choline phosphate: 0.4 M choline chloride had no effect on the level of Ca^{2+} accumulation (Fig. 5). Fig. 5 does show, however, that the concentration of potassium in the external medium has a large effect on the level of Ca^{2+} accumulation. Fig. 6 shows that accumulation of Ca^{2+} is markedly dependent on pH, and that comparatively high levels of Ca^{2+} accumulation are observed at pH 6.0 for vesicles reconstituted in the absence of phosphate.

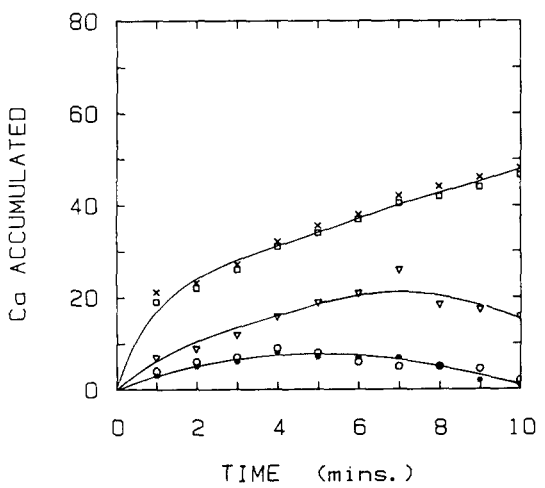

Fig. 3. The effect of phosphatidylcholine structure on Ca^{2+} accumulation (nmol/mg protein). Vesicles were reconstituted in the presence of 0.4 M potassium phosphate and Ca^{2+} accumulation was measured as in the legend to Fig. 1, with: ○, dimyristoleoylphosphatidylcholine; *, dinervonylphosphatidylcholine; □, dioleoylphosphatidylcholine; ×, egg PC; ▽, 1 : 10 molar ratio of cholesterol to egg PC.

To investigate the possible importance of membrane potentials built up across the membrane as a result of Ca^{2+} transport, the effects of valinomycin and FCCP were studied. As shown in Fig. 7, addition of valinomycin and FCCP to vesicles reconstituted in the presence of 0.4 M potassium phosphate with either egg PC or a 1 : 1

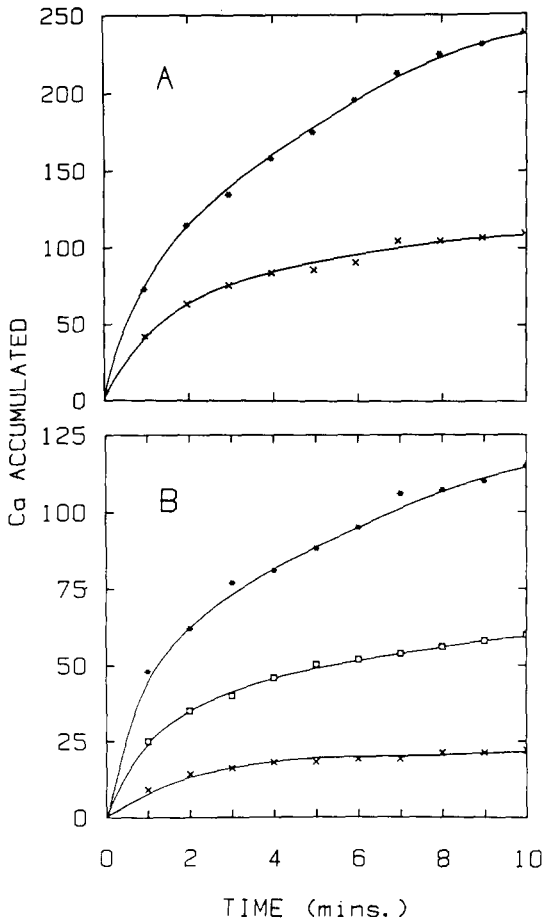

Fig. 4. The effect of phosphate on Ca^{2+} accumulation (nmol/mg protein). The ATPase was reconstituted in the presence (A) or absence (B) of potassium phosphate (50 mM) with molar ratios of PE:PC from egg yolk of: ×, 0:1; □, 1:1; and *, 4:1. Ca^{2+} accumulation was measured in the medium described in the legend to Fig. 1, all other conditions also being as given in that legend.

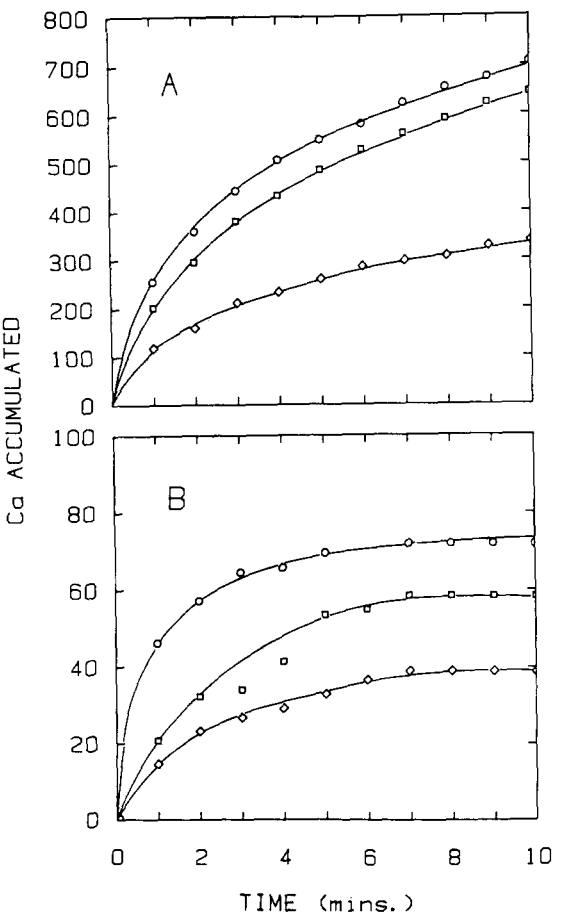

Fig. 5. The effect of external K^+ on Ca^{2+} accumulation (nmol/mg protein). In (A) vesicles were reconstituted with an (egg yolk) PE:PC molar ratio of 4:1 in the presence of 0.4 M choline phosphate and in (B) with an (egg yolk) PE:PC molar ratio of 1:1 in the presence of 0.4 M choline chloride, under the conditions described in Fig. 1 except that the external K^+ concentration in the uptake medium was; ◇, 50 mM; □, 100 mM; and ○, 200 mM.

mixture of egg PC and egg PE results in increased Ca^{2+} accumulation, the effect being particularly marked for the system containing no egg PE. Effects of FCCP and valinomycin alone are comparable to those of a mixture of the two (data not shown).

Discussion

A variety of procedures have been published for the reconstitution of the $(Ca^{2+} + Mg^{2+})$-ATPase into sealed phospholipid vesicles [1,6,3, 17–22]. The procedure we have presented here is based on solubilisation of phospholipid and ATPase in mixtures of cholate and deoxycholate followed by passage through a column of Sephadex G-50 to remove detergent, a procedure that we have found to be fast and reproducible. The gel-filtration results (Fig. 2) are consistent with complete mixing of the purified ATPase and phospholipid in the reconstitution process, in agreement with the results of Andersen et al. [27] and Wakabayashi and Shigekawa [22].

The results reported here (Figs. 1 and 4) confirm earlier reports [1,19–21] that increasing the

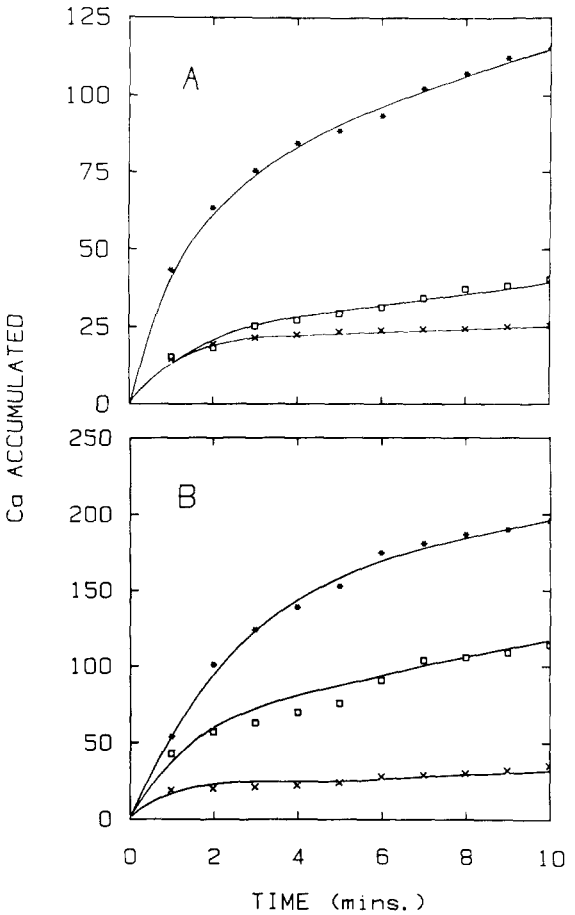

Fig. 6. The effect of pH on Ca^{2+} accumulation (nmol/mg protein) in the absence of phosphate. Vesicles were reconstituted at pH 7.2 in the absence of phosphate with either (A) egg PC or (B) a molar ratio of (egg) PE:PC of 4:1. Ca^{2+} accumulation was measured in the medium given in the legend to Fig. 1, all other conditions also being as given in that legend. Uptake was mesured at pH values of: *, 6.0; □, 6.5; and ×, 7.4.

proportion of phosphatidylethanolamine in the phospholipid mixture used to reconstitute the vesicles up to a mole fraction of 0.8 phosphatidylethanolamine leads to increased uptake of Ca^{2+}: attempts to reconstitute the ATPase into vesicles of pure phosphatidylethanolamine failed. As reported by Navarro et al. [21], the effect is not specific for phosphatidylethanolamine but is also shown with phosphatidylglycerols. The effect of phosphatidylethanolamine is surprising because it has been shown that phosphatidylcholines support higher ATPase activities than the corresponding phosphatidylethanolamines [5].

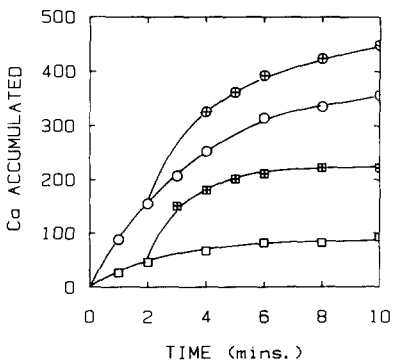

Fig. 7. The effect of simultaneous addition of FCCP and valinomycin on Ca^{2+} accumulation (nmol/mg protein). Vesicles were reconstituted in the presence of 0.4 M potassium phosphate with a molar ratio of (egg yolk) PE:PC of either 1:1 (○, ⊕) or 0:1 (□, ⊞), and accumulation was measured in the medium given in the legend to Fig. 1, all other conditions also being as given in the legend to Fig. 1. In parallel samples, accumulation of Ca^{2+} was measured for 10 min either in the absence of any ionophore (○, □) or following addition of FCCP and valinomycin 2 min after the addition of ATP, to give final concentrations of 30 μg/ml (⊕, ⊞).

Before the effects of phosphatidylethanolamine observed in these systems can be assigned some interesting and biological significance, it is important to establish that they are not attributable to an artifact of the reconstitution procedure. First, it is necessary to establish that increasing the phosphatidylethanolamine content of the reconstituted vesicles does not lead to a large increase in the internal volume of the vesicles per ATPase molecule, since this would result in increased accumulation of Ca^{2+}. Reconstituted vesicles were sized both by electron microscopy and gel filtration, and both techniques gave comparable results (Table I). Internal volumes were calculated from the vesicle diameters given in Table I, assuming a membrane thickness of 6 nm. Volumes calculated from the gel chromatography data for the ATPase reconstituted with a 3000:1 molar ratio of lipid to protein are 39, 44 and 52 μl/mg protein for molar ratios of egg PE to PC of 0:1, 1:1, and 4:1, respectively. Thus although internal volumes do increase with increasing content of phosphatidylethanolamine, the increase in internal volume is small compared to the increase in Ca^{2+} accumulation (Fig. 1). A second reconstitution artifact that could potentially be important is the sidedness of the ATPase molecules in the reconstituted vesicles;

a greater proportion of ATPase molecules oriented with their ATP binding sites facing outward in vesicles containing phosphatidylethanolamine could explain the increased Ca^{2+} accumulation in the presence of phosphatidylethanolamine. We have estimated the percentages of ATPase molecules oriented with their ATP binding sites facing outward and inward by measuring ATPase activities in the presence of A23187, which makes the membrane permeable to Ca^{2+}, and in the presence of alamethicin which makes the membrane permeable to both ATP and Ca^{2+} [28]. Activities measured in the presence of A23187 are 40–50% of those measured in the presence of alamethicin for the ATPase reconstituted with either egg PC or mixtures of egg PC and egg PE, consistent with a close to random incorporation of the ATPase across the membrane. We conclude, therefore, that simple reconstitution artifacts cannot account for the observed effects of phosphatidylethanolamine.

Navarro et al. [21] suggested that phosphatidylethanolamines could affect directly the ratio of ATP hydrolysed to Ca^{2+} transported by the ATPase. We believe, however, that the effect of phosphatidylethanolamine on Ca^{2+} accumulation follows from an effect on Ca^{2+} leak from the reconstituted vesicles. This is suggested by the observation that the levels of Ca^{2+} accumulated by the reconstituted vesicles are low unless the vesicles contain a Ca^{2+}-precipitating agent such as phosphate (Fig. 4): the observation of equal stimulation of uptake by potassium phosphate and choline phosphate, with choline chloride having no effect, confirm that the effect is attributable to the phosphate anion (Fig. 5). The possibility that the $(Ca^{2+} + Mg^{2+})$-ATPase can provide a specific pathway for Ca^{2+} efflux is demonstrated directly in the following paper [29].

The effect of pH on the level of Ca^{2+} accumulated is particularly noticable (Fig. 6). If accumulation of Ca^{2+} were being limited by a rapid, non-specific leak of Ca^{2+}, then decreasing pH from 7.4 to 6.0 would be expected to lead to reduced accumulation, since ATPase activity decreases with decreasing pH in this range [26]: in fact, accumulation of Ca^{2+} is observed to increase with decreasing pH. These effects of pH are more consistent with Ca^{2+} leak being mediated by some specific protein carrier, with protons and Ca^{2+} competing for binding sites on the carrier. In the following paper [29] we will argue that this is indeed the case, with Ca^{2+} leak being mediated by the $(Ca^{2+} + Mg^{2+})$-ATPase.

In previous papers we have presented a detailed kinetic model for the ATPase [26] and in the following paper [29] we present a kinetic model for Ca^{2+} efflux mediated by the ATPase. Unfortunately, it is not possible at present to combine these two models to account for the level of Ca^{2+} accumulation seen in the reconstituted system, largely because of the lack of data concerning the effects of membrane potentials generated by Ca^{2+} fluxes. Zimniak and Racker [20] and Navarro and Essig [18] have reported that the generation of membrane potentials can have important effects on Ca^{2+} accumulation in these reconstituted systems. We have confirmed these observations and have found, for example, that addition of valinomycin and FCCP results in a considerable increase in the level of Ca^{2+} accumulated (Fig. 7). In native sarcoplasmic reticulum vesicles addition of either FCCP or valinomycin has no effect on uptake of Ca^{2+}, presumably because of the presence of ion channels which can maintain electrical neutrality across the membrane. Membrane potentials generated as a result of Ca^{2+} fluxes in the reconstituted systems will have very marked effects on the rates and extent of Ca^{2+} uptake. However, the observation of Ca^{2+} accumulation in the absence of added ionophore shows that some counter-ion movement must occur during Ca^{2+} uptake, presumably mediated by the ATPase itself – the nature of this ion movement is unknown.

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