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RESISTANCE OF Ca^{2+}-ATPase TO DILUTION BY EXCESS PHOSPHOLIPID IN RECONSTITUTED VESICLES

P.J. KONIGSBERG *

Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06511 (U.S.A.)

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Ca^{2+}-ATPase and other membrane proteins of the sarcoplasmic reticulum membrane from rabbit skeletal muscle have been reconstituted into lipid vesicles with increasing amounts of phosphatidylcholine. The protein composition and phospholipid concentration of these vesicles were analyzed by determining the density of the reconstituted membrane vesicles on linear H_2O–^2H_2O gradients, in a constant concentration of sucrose. In all combinations of the Ca^{2+}-ATPase with a weight excess of phosphatidylcholine, the reconstituted vesicles had a phospholipid-to-protein ratio similar to that of the native sarcoplasmic reticulum membrane, even though both solubilization and mixing had occurred. These vesicles of low phospholipid and high protein content exhibited all the original Ca^{2+}-ATPase activity and ATP-stimulated calcium transport. The Ca^{2+}-ATPase, and the calcium-binding proteins to a lesser extent, may order the lipid in such a manner so as to maintain the initial stoichiometry of lipid to protein observed in the native sarcoplasmic reticulum membrane.

Introduction

The reconstitution of purified membrane proteins and lipids into membrane vesicles is employed often for the following reasons: To separate them from a host of other proteins present in the native membrane; to study the effect of lipid on the function of a membrane protein; to study the function of a membrane protein in terms of the variables which control it; or to study the structure of the membrane protein. The assumption in this experimental approach has been that the phospholipids and lipid-requiring proteins mix in an ideal manner during detergent-mediated solubilization and that detergent removal will allow for the formation of a population of reconstituted vesicles which are homogenous in their phospholipid and protein composition.

The sarcoplasmic reticulum membrane has been studied extensively and the major protein, calcium -stimulated ATPase, often reconstituted into phospholipid vesicles. Ca^{2+}-ATPase (EC 3.6.1.3; M_r 100000) represents approximately 80% of the total membrane protein. The remainder is composed of high affinity, calcium-binding protein (M_r 55000), calsequestrin (M_r 44000) and several other proteins [1–3]. The physiological role of Ca^{2+}-ATPase is involved intimately in the process of muscular contraction, and structural studies of this protein are of extreme importance. In the presence of ATP, the Ca^{2+}-ATPase actively transports calcium across a phospholipid bilayer and into the lumen of the sarcoplasmic reticulum. It is a single protein and contains specific domains within its structure

* Present address: Physics Department, B-019 University of California at San Diego, La Jolla, CA 92093, U.S.A.

Abbreviations: PC, phosphatidylcholine; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid; SR, sarcoplasmic reticulum.

which hydrolyze ATP and also bind calcium. The actual physical mechanism of calcium transport is unknown.

In order to initiate studies on the structure of Ca^{2+}-ATPase, its high density in the sarcoplasmic reticulum membrane required that the proteins be reconstituted into lipid-rich vesicles to reduce lateral interactions between neighboring proteins. It was, therefore, necessary to characterize the reconstituted sarcoplasmic reticulum vesicles with respect to their protein and lipid concentration. In the present study a separation technique was developed to assess the precise composition of reconstituted membrane vesicles. The results of this analysis and the implications for the understanding of the structure of the Ca^{2+}-ATPase in a defined membrane system are discussed.

Materials and Methods

Materials

Na_2ATP, TES and PMSF were from Sigma. Cholic acid was purchased from Aldrich. Egg lecithin was purchased from Lipid Products; Nutfield, U.K. [^{14}C]Phosphatidylcholine was from Amersham, [^{3}H]cholic acid was from New England Nuclear, and Dr. J. Brunner kindly synthesized [^{3}H]phosphatidylcholine. *p*-Methylaminophenol sulfate was from Eastman Chemicals.

Preparation of sarcoplasmic reticulum membrane vesicles

Adult albino rabbits were killed by receiving an overdose of nembutal. The leg and back muscles were excised and frozen in liquid nitrogen until needed. Tissue was kept frozen for a 6 month period without any appreciable loss of ATP-dependent calcium-transport activity.

Sarcoplasmic reticulum membrane vesicles were prepared from 100 g of frozen muscle by homogenization and differential centrifugation according to the method of MacFarland and Inesi [4] except that 10 mM TES, pH 7.4, and 10 μM PMSF were used instead of Tris-HCl as buffer. The crude sarcoplasmic reticulum vesicles were purified further by centrifugation on a linear 60 ml gradient containing 26–45% (w/w) sucrose buffered with 10 mM TES, pH 7.4, and 10 μM PMSF. Centrifugation was carried out for 24 h at 4°C in a Beckman SW 25.2 rotor at 25000 rev./min. Fractions from the gradient were monitored for protein content, pooled, and frozen quickly in liquid nitrogen.

Reconstitution of sarcoplasmic reticulum-phosphatidylcholine membrane vesicles

PC-SR vesicles were reconstituted using a modification of the method of Brunner et al. [5]. An aliquot of a solution of PC containing 10^6 cpm of [^{3}H]PC in HCl_3/MeOH (2:1) was evaporated onto the walls of a large, screw-capped test tube and dried under vacuum over NaOH pellets to remove all traces of solvent. An aliquot of frozen sarcoplasmic reticulum membrane vesicles in TES-buffered sucrose was thawed and added to the dried film of lipid. The combination of the membrane vesicles and exogenous PC gave the estimated mean weight ratio of phospholipid to protein. A 10% solution of twice-recrystallized sodium cholate [5] in 0.1 TES, pH 7.5, was added to the opaque suspension to a final concentration of 1.3%. The suspension of mixed micelles was vortexed gently and incubated at 23°C for 30 min, at which point clearing had occurred. The mixed micelles were layered onto a Sephadex G-50 column (1.5 × 26 cm) and eluted with 0.01 M TES, pH 7.3, 0.24 M KCl, 0.1 mM EDTA and 10 μM PMSF. The buffer also contained sucrose, the concentration of which will be discussed in the next section. The column was eluted under pressure at a flow rate of 8 ml/h at 4°C. Reconstituted PC-SR membrane vesicles were found in the void volume (15–18 ml) and the detergent eluted with a peak at 60 ml. Trace amounts of sodium cholate were found in the void volume but amounted to only one molecule of cholate per 6500 molecules of PC as measured by the distribution of [^{3}H]cholate passed down the same column. All ATPase activity was found in the void volume and the vesicles were concentrated with Amicon ultrafiltration membrane cones (CF25).

Assay of total lipid phosphorous

The samples, 10–20 μl, were digested by heating in the presence of 500 μl of 8 N H_2SO_4 for 3 h at 150°C in 1.5 × 18.5 cm Pyrex tubes, decolorized by the addition of two drops of 30% H_2O_2 to the

cooled tubes, and again heated at 200°C for 1 h.

Samples were analyzed for total lipid phosphorus according to the method of Bartlett [6] with the following modifications: Solution A, *p*-methylaminophenol sulfate was used as the reducing reagent and was prepared as a solution containing 5 g *p*-methylaminophenol sulfate, and 50 g sodium bisulfate made up to 500 ml with glass-distilled water; solution B, 50 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 400 ml of 10 N H_2SO_4 were mixed and made 1 l with glass-distilled H_2O. The decolorized samples were mixed with 2 ml H_2O and 0.5 ml of solutions A and B, heated at 37°C for 1 h and the absorbance at 660 nm was determined against an appropriate blank.

Assay of ATPase activity

Vesicles were incubated in 1.0 ml of assay mix which contained 0.12 M KCl/0.01 M TES, pH 7.3/5 mM $MgCl_2$/50 μM $CaCl_2$. ATP at a final concentration of 5 mM was added to start the reaction, and the enzyme concentration was adjusted to give about 10% hydrolysis of MgATP. Samples were incubated at 37°C for 10 min. The phosphate liberated from enzymatic hydrolysis was assayed by addition of 0.5 ml of solutions A and B used in the total phosphate assay, incubated at 23°C for 30 min and the absorbance of the samples measured at 660 nm.

The Ca^{2+} uptake capacity of sarcoplasmic reticulum membrane vesicles was measured by following the uptake of 2.5 mM $^{45}CaCl_2$ ($4 \cdot 10^{10}$ cpm/mol) after incubation of the vesicles in the above medium used in the ATPase assay. Incubations were carried out for 30 s and samples were then filtered on 0.22 μm membrane filters, and the filters counted.

Polyacrylamide gel electrophoresis

Samples were monitored for protein content by polyacrylamide gel electrophoresis according to the method of Laemmli [7]. SDS slab gels containing 7.5 and 3% polyacrylamide in separating and stacking gels, respectively, were used. Samples were dissolved in an equal volume of 10% SDS/2 mM EDTA, and incubated for 3 h at 37°C, prior to electrophoresis. electrophoresis was performed at a current of 10–15 mA per slab.

Sepharose 4B column chromatography

Sarcoplasmic reticulum membrane vesicles were solubilized in sodium cholate at a final concentration of 1% and incubated at 23°C for 30 min. An aliquot was taken for determination of Ca^{2+}-stimulated ATPase activity. The solubilized membrane was layered onto a Sepharose 4B column (1.5 × 90 cm) and eluted with 10 mM cholate/10 mM TES, pH 7.3/10% sucrose/0.1 M KCl/0.1 mM EDTA/10 μM PMSF at 4°C with a flow rate of 6 ml/h.

Electron microscopy

20–50 μl of membrane vesicles in sucrose-buffer were air-dried on 200 mesh formvar-coated carbon shadowed copper grids. The samples were allowed to sit on the grids for 1 min and were then stained nehatively with a freshly prepared solution of 0.2% uranyl acetate. The grids were examined in a Phillips 300 transmission electron microscope at 60 KeV.

Protein determination

Protein assays were done according to the method of Hartree [8].

Determination of equilibrium buoyant density in linear H_2O–^2H_2O gradients

Determination of the equilibrium buoyant density of reconstituted membrane vesicles on a density gradient can give a direct measure of the protein fractional content of the membrane if the internal and external aqueous compartments have the same density. It is possible, therefore, to follow the distribution of both lipid and protein. The density of pure phospholipid is 1.02 g/cm^3 [9], corresponding to a specific volume of 0.98 cm^3/g. The lipid-free protein is assumed to have a specific volume of 0.75 cm^3/g, a value characteristic of most proteins in acqueous solution. (However, the appropriate value for the transmembrane portion of a protein is uncertain at this time.) The density of the membrane of a given vesicle fraction is governed by the weight fraction of protein (X_P) in the bilayer.

Let: ρ = density, g/cm^3; V = specific volume, cm^3/g = $1/\rho$. Subscripts M, P and L refer to membrane, protein and lipid, respectively.

$$V_M = X_P V_P + X_L V_L \quad (1)$$

$$X_P + X_L = 1 \quad (2)$$

$$X_P = V_L - V_M / V_L - V_P \quad (3)$$

or

$$X_P = (0.98 - V_M)/0.23 \quad (4)$$

Separation techniques currently used to follow lipid and protein distribution in vesicles routinely use sucrose gradients [10]. The sarcoplasmic reticulum membrane is permeable to sucrose [11] and the position of such vesicles on a gradient reflects their protein content. In contrast, liposomes are much less permeable to sucrose, and equilibrium sucrose gradients, therefore, do not necessarily provide an accurate separation of vesicles with different protein contents. Failure of the gradient component (sucrose) to equilibrate on both sides of the phospholipid bilayer would result in an accurate apparent buoyant density of the composition of the reconstituted vesicle.

To circumvent this problem ^2H_2O was chosen, which is freely permeable to phospholipid bilayer as the variable component of a density gradient. A density gradient was made of H_2O–^2H_2O in a constant concentration of sucrose to adjust the density range. The vesicles were reconstituted in the presence of the same concentration of sucrose subsequently used in the density gradient. Table I gives a list of sucrose concentrations used in the Sephadex G-50 column buffer for a given density range to be studied in the ^2H_2O gradients. Gradient solutions were prepared by dissolving the desired weight of sucrose in either H_2O or ^2H_2O (99.97%) and adding a stock solution of 2.0 M TES, pH (or p^{2}H) 7.3 to a buffer concentration of 0.01 M. Gradients were made in a Buchler gradient maker attached to a peristaltic pump. Density ranges were chosen to either include or exclude the density of the sarcoplasmic reticulum membrane (1.16–1.19 g/cm^3).

The gradients were centrifuged in a Beckman SW 50.1 rotor or SW 41 rotor for 48 h at 250000 $\times g$. Fractions were collected from the bottom of the tube by inserting a capillary and pumping out the contents. The fractions were stoppered immediately to prevent H_2O–^2H_2O exchange from water vapor in the air. The density of the fractions was determined as described by Low and Richards [12] using a gradient column of bromobenzene and xylene. The column was calibrated using the solutions of H_2O-sucrose and ^2H_2O-sucrose used to make the desired gradient. The present of radioactively labeled PC in the PC-SR gradients was determined by counting aliquots from all gradient fractions.

TABLE I

CONCENTRATION AND DENSITY OF SUCROSE SOLUTIONS USED IN BOTH VESICLE RECONSTITUTION AND H_2O–^2H_2O DENSITY GRADIENTS

Table I gives representative samples of sucrose concentrations used to load PC-SR membrane vesicles internally during reconstitution and the range of density achieved by making H_2O–^2H_2O gradients in the same concentration of sucrose.

Percent sucrose (w/v)	Density of sucrose (g/cm^3)	Density (g/cm^3) range of H_2O–^2H_2O sucrose-containing gradients
5	1.0179	1.0179–1.1179
10	1.0381	1.0381–1.1381
15	1.0592	1.0592–1.1592
20	1.0810	1.0810–1.1810

Results

Reconstitution of PC-SR membrane vesicles

The addition of sarcoplasmic reticulum membrane vesicles to a dried film of phosphatidylcholine produced a turbid suspension. Addition of sodium cholate to the suspension resulted in clearing within 30 min when a sample was incubated at 23°C. Sarcoplasmic reticulum membranes solubilized under these conditions did not pellet when centrifuged at 100000 $\times g$ for 60 min, indicating that the detergent had disrupted the vesicular structure of the native membranes, reducing it presumably to a micellar state.

Reconstitution by the method of Brunner et al. [5], produced small, unilamellar vesicles ranging in size between 400 and 800 Å in diameter, as shown in Fig. 1. The reconstituted PC-SR vesicles retained all calcium-stimulated ATPase activity. Their small size prevented determination of calcium transport by a standard filter assay method.

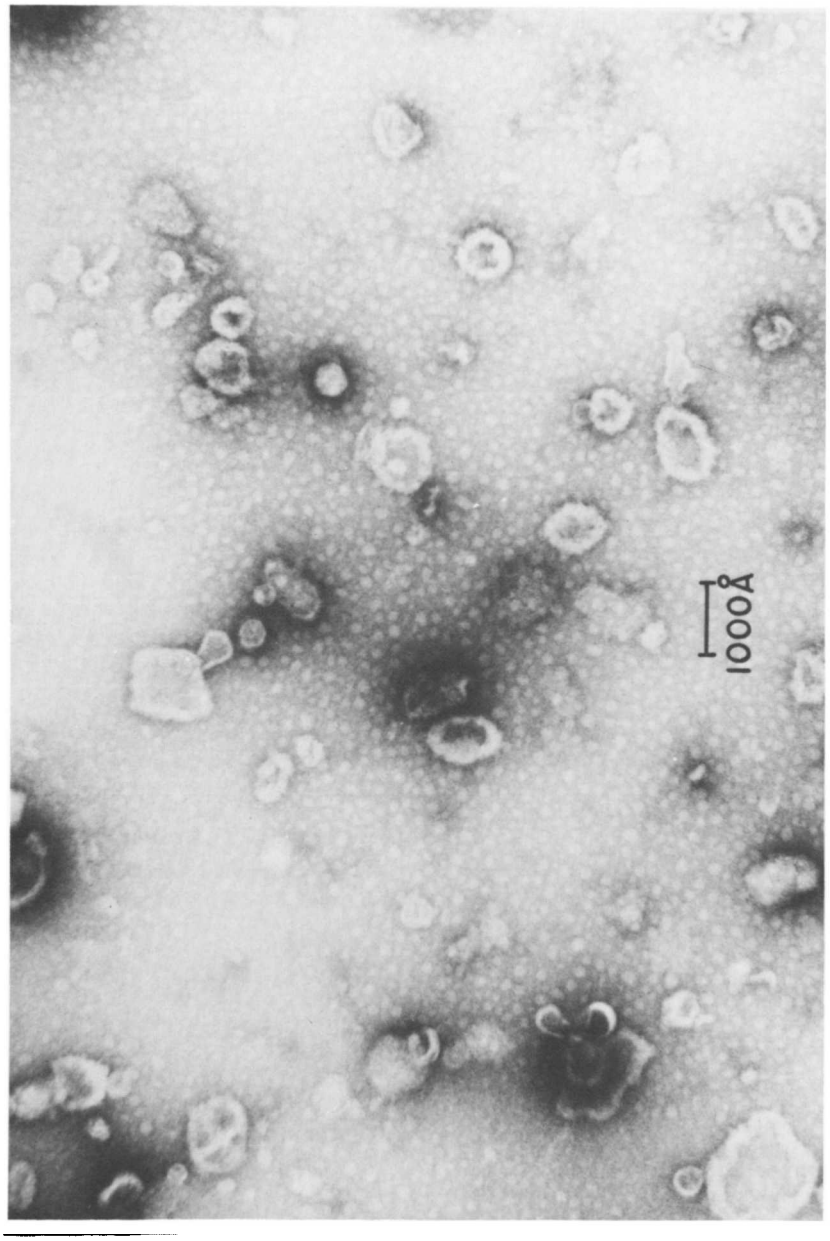

Fig. 1. PC-SR membrane vesicles reconstituted with a 10-fold excess (w/w) of PC. Negatively stained with 0.5% uranyl acetate, and photographed at 66420×. The bar indicates 1000 Å.

Centrifugation of reconstituted PC-SR membrane vesicles on H_2O–^2H_2O gradients

The expected density of membrane vesicles reconstituted with a given weight fraction of PC is shown in Fig. 2. Pure phospholipid vesicles have a density of 1.02 g/cm^3 and would be found at position 28; we have found that native sarcoplasmic reticulum membrane vesicles have densities ranging from 1.16 to 1.19 g/cm^3, corresponding to a phospholipid/protein ratio of 1.0–0.67, respectively, Vesicles reconstituted with a 1–20-fold excess of phospholipid by weight would be expected

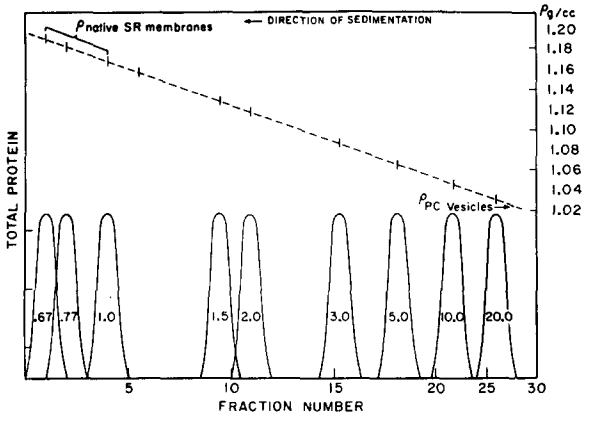

Fig. 2. The calculated equilibrium position of vesicles in a H_2O–^2H_2O gradient reconstituted with increasing amounts of PC (w/w). The phospholipid/protein ratio is indicated under each curve. The densities of native sarcoplasmic reticulum membranes and PC liposomes are located at either end of the dashed line.

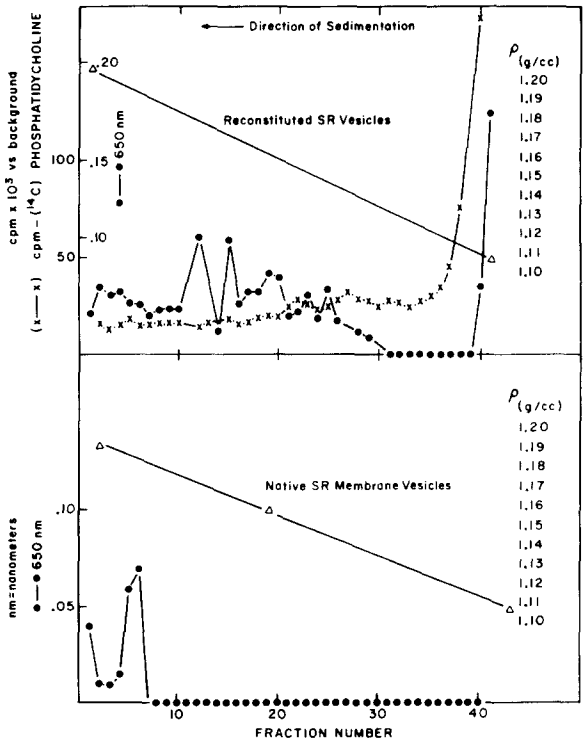

Fig. 3. H_2O–^2H_2O density gradient profile of vesicles reconstituted with 20-fold weight excess of PC. 194 μg of protein and 3.88 mg of [^{3}H]PC ($3 \cdot 10^4$ cpm/mg) were reconstituted as described. The PC-SR vesicles were layered on a H_2O–^2H_2O gradient ($\rho = 1.10$–1.20) and centrifuged at 38000 rev./min in a Beckman SW41 rotor for 45 h at 4°C. Fractions were collected by pumping out through a capillary lowered to the bottom of the tube, and analyzed for total protein as described in Materials and Methods. Aliquots of each fraction were counted to locate [^{3}H]PC.

to distribute between 1.03 and 1.19 g/cm^3, as indicated. PC-SR vesicles reconstituted with a 20-fold weight excess of ^{14}C-labeled egg lecithin were centrifuged to equilibrium on a ^2H_2O gradient containing 25% sucrose (density range, 1.10–1.20 g/cm^3). This density range allows native sarcoplasmic reticulum membrane vesicles to be resolved as a peak by the gradient.

As shown in Fig. 3, the observed distribution of sarcoplasmic reticulum protein and phospholipid in PC-SR vesicles did not correspond to the density calculated from the composition of the original mixture, indicating that the sarcoplasmic reticulum membrane proteins, sarcoplasmic reticulum phospholipid and exogenous PC did not mix homogenously after removal of sodium cholate. The reconstituted vesicles would be expected to have a density of 1.025 g/cm^3 (cf. Fig. 2). All the membrane protein was located in vesicles having densities in the range 1.13–1.20 g/cm^3, with the most protein-rich vesicles having densities in the range 1.16–1.19 g/cm^3. The protein-rich vesicles retained the original calcium-stimulated ATPase activity. The appearance of proteins in vesicles of this density was observed regardless of the initial amount of PC used in the reconstitution procedure. The increase in absorbance at 650 nm in fractions 39–40 represents interference in the color development of the protein assay by the very high concentration of PC present.

Table II gives the concentration by weight of PC used during reconstitution and compares it to the expected density of reconstituted vesicles, assuming complete mixing during solubilization. In every dilution mixture examined, protein was only found in vesicles of densities equal to, or greater than, 1.13 g/cm^3, with significant amounts in vesicles having densities of 1.16–1.19 g/cm^3. This corresponds to a maximal phospholipid/protein ratio of 1.5 : 1 (w/w), whereas native sarcoplasmic reticulum vesicles ad a phospholipid/protein ratio of 0.67. The appearance of [^{14}C]- or [^{3}H]PC in these fractions (Fig. 3) indicated that exogenous

TABLE II

PROTEIN CONTENT AND EQUILIBRIUM BOUYANT DENSITY OF RECONSTITUTED VESICLES

The observed densities of PC-SR vesicles reconstituted from the indicated amounts (w/w) of PC and sarcoplasmic reticulum protein are compared with the calculated density of such mixtures, assuming complete mixing of phospholipid and protein. The mol% protein was calculated from the measured density of each fraction, as described in Materials and Methods.

Initial ratio of lipid (PC) protein (w/w)	Expected mean density (g/cm^3) [a]	Density classes of containing protein (g/cm^3)	X_P
1 : 1	1.16	1.174	0.65
		1.152	0.48
		1.143	0.48
		1.13	0.43
5 : 1	1.07	1.174	0.65
		1.157	0.52
		1.147	0.48
		1.134	0.43
10 : 1	1.05	1.190	0.70
		1.180	0.57
		1.148	0.48
20 : 1	1.03	1.170	0.57
		1.153	0.48
		1.143	0.48
		1.130	0.43

[a] cf. Fig. 2.

phospholipid exchanged with sarcoplasmic reticulum phospholipid during reconstitution and was incorporated into newly formed vesicles. In every case, the bulk of the added phospholipid was found as phospholipid vesicles at the top of the gradients containing sarcoplasmic reticulum membrane proteins.

Equilibrium density profiles of PC-SR vesicle prepared by cholate-dialysis

The following experiment was performed to see if reconstitution by column chromatography, which yielded relatively small vesicles, prevented dilution of the sarcoplasmic reticulum membrane protein on reconstitution with added PC. PC-SR vesicles were prepared according to the method of Warren et al. [13] in which sodium cholate is removed by dialysis, a method expected to yield larger vesicles [22]. Vesicles reconstituted by cholate-dialysis in the presence of 20% sucrose ($\rho = 1.0810$ g/cm^3) and a 10-fold weight excess of phosphatidylcholine were subsequently centrifuged to equilibrium. In order to extend the density range of this experiment a parallel sample was reconstituted in the presence of 5% sucrose ($\rho = 1.017$ g/cm^3) and centrifuged to equilibrium in a ^2H_2O gradient made up in 5% sucrose.

The profile of the sample centrifuged in the higher density gradient is shown in Fig. 4. Protein could be found from fractions 1–23; however, the majority of the sarcoplasmic reticulum proteins was found in fractions 1–6 ($\rho = 1.16$–1.18 g/cm^3). Radioactive phosphatidylcholine was also found in these fractions, yet the majority of the lipid was in the form of liposomes on top of the gradient. The most unique feature of the gradient was that almost all of the ATP-dependent calcium transport activity was found in fractions 1–6, in those vesicles having the highest density. Polyacrylamide gel electrophoresis (Fig. 5) showed that proteins corresponding to Ca^{2+}-ATPase, the high-affinity, calcium-binding protein and calsequestrin could be found in all pooled fractions from the gradient; however, there were differences in the distribution of the lower molecular weight proteins in these fractions. Table III gives the phospholipid/protein ratio, (on a molar basis) of the pooled fraction from the gradient shown in Fig. 4 as well as the amount of [^{3}H]PC in these fractions. This repre-

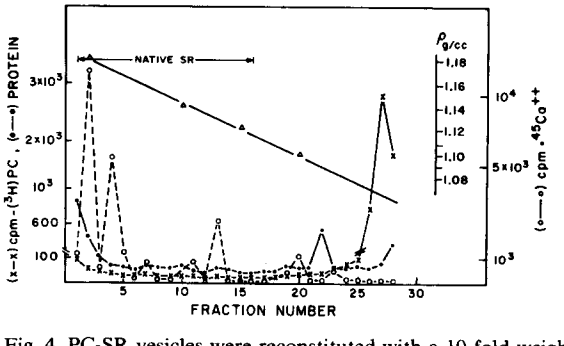

Fig. 4. PC-SR vesicles were reconstituted with a 10-fold weight excess of [^{3}H]PC according to the method of Warren et al. [14] and loaded with 25% sucrose. Vesicles were centrifuged on H_2O–^2H_2O gradients ($\rho = 1.1019$–1.2019 g/cm^3) at 42800 rev./min in a Beckman SW 50.1 rotor for 41 h at 4°C. Fractions were collected and aliquots monitored for $^{45}Ca^{2+}$ uptake.

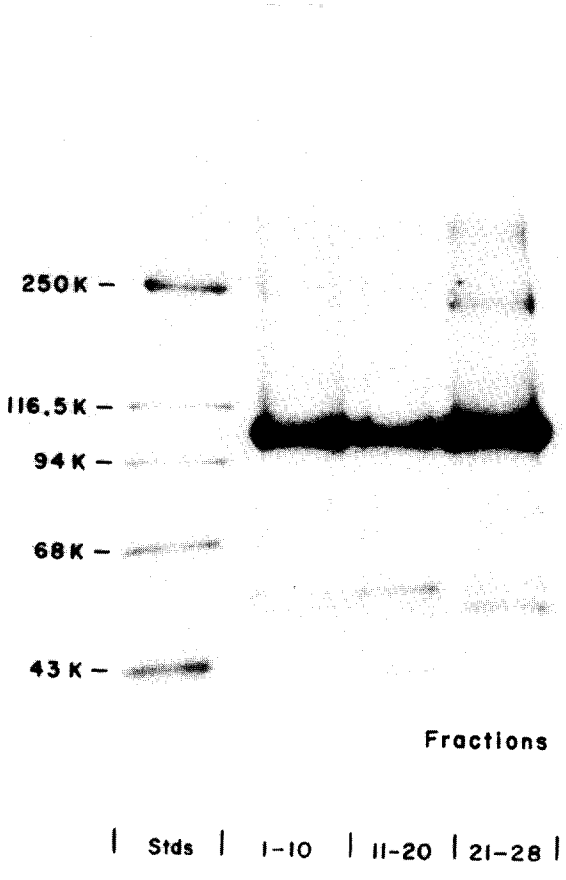

Fig. 5. SDS-polyacrylamide gel electrophoresis of the pooled fractions from the gradient describd in Fig. 4 and Table III. 7.5% gels were prepared according to the method of Laemmli [7].

sents PC that exchanged with endogenous sarcoplasmic reticulum lipid during solubilization and reconstitution.

The parallel sample of vesicles reconstituted in the presence of 5% sucrose and centrifuged equilibrium ($\rho = 1.0175-1.175$) was found as a pellet on the bottom of the tube. This pellet contained calsequestrin, high-affinity, calcium-binding protein and Ca^{2+}-ATPase. The buoyant density of the vesicles exceeded that of the gradient, even though a density region was chosen to accommodate vesicles whose expected buoyant density would be 1.05–1.06 g/cm^3 if all the phospholipid in the 10-fold dilution were found in the reconstituted vesicles.

Intermediates in reconstitutions

Detergent solubilization. The appearance of Ca^{2+}-ATPase, calsequestrin and high-affinity, calcium-binding protein in protein-rich vesicles, in spite of an excess of phospholipid, could be explained by incomplete solubilization [23] of the native sarcoplasmic reticulum membrane by sodium cholate, a detergent widely used in studying the sarcoplasmic reticulum membrane [2,3,13,14] or perhaps by the structure of the resultant mixed micelles of sarcoplasmic reticulum proteins, lipids and added PC [17,18,24]. To see if incomplete solubilization took place and fragments of membranes were incorporated into vesicles, native sarcoplasmic reticulum membranes were solubilized with 1.3% 30 mM cholate and chromatographed on a Sepharose 4B column [15],

TABLE III

CHARACTERIZATION OF SARCOPLASMIC RETICULUM MEMBRANE VESICLES RECONSTITUTED WITH 10% (w/w) EGG LECITHIN

Protein determinations and $^{45}Ca^{2+}$ uptake assays were done on the indicated pooled fractions from the gradient described in Fig. 4. Aliquots were taken from the pooled fractions for quantitation of [^{3}H]PC.

Fractions	Equilibrium buoyant density range (g/cm^3)	cpm [^{3}H]PC/mg protein	mol PC/mol Ca^{2+}-ATPase [a]	$^{45}Ca^{2+}$ uptake (μmol/mg per min)
1–10	1.140–1.182	25720	70.25	2
11–20	1.100–1.138	18500	121.70	0.3
21–38	1.062–1.094	624400	647.00	–

[a] Calculated on basis that 80% of the total protein is Ca^{2+}-ATPase.

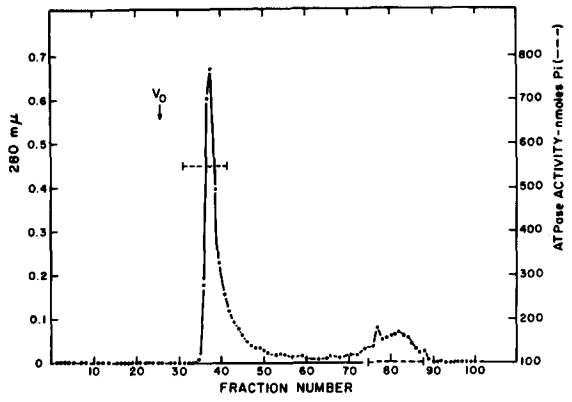

Fig. 6. Sepharose 4B chromatography of detergent-solubilized sarcoplasmic reticulum membrane vesicles. Native membrane vesicles were solubilized with 30 mM sodium cholate and eluted as described. The dashed lines across the main peak indicates the level of calcium-specific ATPase activity found in the pooled fractions.

as shown in Fig. 6. The elution profile showed that the front-running peak contained all of the Ca^{2+}-ATPase protein and the Ca^{2+}-stimulated ATPase activity originally present [16]. In addition, there were 400 mol of lipid per mol of Ca^{2+}-ATPase present in this peak. The calcium-binding proteins, another 100000 dalton protein and the remaining sarcoplasmic reticulum phospholipids were found in fractions 70–90.

This distribution of the sarcoplasmic reticulum proteins indicated that membrane fragments were not present during reconstitution and the sarcoplasmic reticulum membrane did appear to be well solubilized. When native sarcoplasmic reticulum membrane vesicles were solubilized in 1.3% (w/w) sodium cholate and centrifuged for 1 h at 100000 $\times g$, no sedimentable material was found.

A second detergent, β-octyl glucoside, was used to solubilize the sarcoplasmic reticulum membrane prior to chromatography. However, solubilization with this detergent caused immediate and irreversible inhibition of Ca^{2+}-stimulated ATPase activity. In contrast, Ca^{2+}-stimulated ATPase activity could be measured readily in sodium cholate-solubilized samples.

Protein and lipid composition of mixed micelles. The particular distribution of protein found in the reconstituted vesicles, if not the result of incomplete solubilization, could be the expression of specific protein-protein or protein-lipid interactions occurring during the generation of mixed micelles prior to detergent removal. If this were the case, phospholipid requirements and protein association would be expressed gradually during detergent removal. The following experiment addressed this question. Native sarcoplasmic reticulum membranes and a 10-fold excess of [^{3}H]PC were solubilized with sodium cholate under conditions identical to those used for reconstitution by chromatography or dialysis. The micelles were centrifuged to equilibrium in 18–50% (w/w) sucrose gradients containing either a final concentration of 30 or 10 mM sodium cholate, detergent concentrations that would exist transiently during reconstitution. Fig. 7 shows the protein and lipid distribution of micelles in the two gradients.

In both gradients there are multiple peaks of protein in the cholate micelles. Exogenous [^{3}H]PC is distributed across the gradient, with the bulk found in a zone where high-affinity, calcium-binding protein was located. No [^{3}H]PC could be found associated with 100 μg of aldolase subjected to the same conditions of centrifugation (data not shown). Aldolase was chosen as a control as it did not bind PC.

The distribution of Ca^{2+}-ATPase in micelles of two different cholate concentrations revealed that, as expected, the phospholipid concentration in Ca^{2+}-ATPase-containing micelles varied with detergent concentration but was never higher than that observed in the reconstituted vesicles. Ca^{2+}-ATPase was also found in the same zones of the two gradients with the two calcium-binding proteins, and all three proteins could have been present in the same micelles. In Fig. 7A the highest concentrations of Ca^{2+}-ATPase are in fractions 16–25 and the phospholipid/protein ratios of these fractions range from 30 to 80 mol of PC per mol of Ca^{2+}-ATPase with fraction 25 having 300 mol/mol. The high-affinity, calcium-binding protein is found in a more lipid-rich environment (fractions 23–33), with the highest concentrations in fractions 24–29. In contrast, Fig. 7B shows that Ca^{2+}-ATPase is only found in fractions 1–24 and these fractions contain 100–900 mol of PC/mol of protein. Calsequestrin is found in fractions 16–24, with the highest amounts in fractions 19–22. These fractions contain 200–500 moles of PC/mol of protein. The most lipid-rich micelles contain only

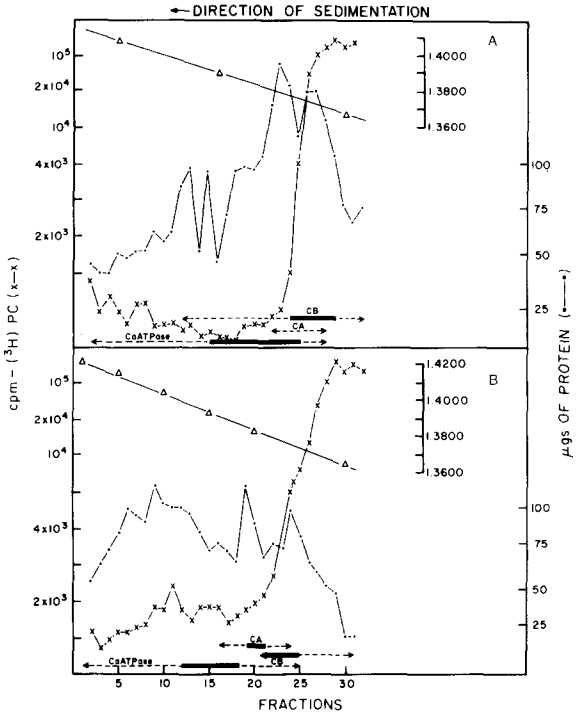

Fig. 7. (A) 6 mg sarcoplasmic reticulum membrane vesicles were added to a dry film of 60 mg [^{3}H]PC, and solubilized with 1.3% (30 mM) sodium cholate. The micelles were incubated for 30 min at 23°C and layered on top of an 18–50% sucrose gradient containing 10 mM TES, pH 7.5, 0.15 M NaCl and 30 mM sodium cholate. The gradient was centrifuged at 45000 rev./min in a Beckman SW 50.1 rotor for 24 h at 4°C. Fractions were collected from the bottom and monitored for the presence of protein and [^{3}H]PC. The horizontal bars at the bottom of the graph indicate the distribution range and relative amount of the major sarcoplasmic reticulum proteins present in micelles, as determined by SDS-polyacrylamide gel electrophoresis of each gradient fraction. (B) 6 mg of sarcoplasmic reticulum membrane vesicles and 60 mg [^{3}H]PC were solubilized as described in (A) and layered on top of a 18–50% sucrose gradient containing 10 mM TES, pH 7.5, 0.15 NaCl and 10 mM sodium cholate, and treated as described in (A). CA, calsequestrin; CB, high-affinity, calcium-binding protein.

the high-affinity, calcium-binding protein.

Detergent removal results in more PC in association with the ATPase, and the calcium-binding proteins. Even though the association of detergent molecules with lipid is an equilibrium phenomenon, and lipid will exchange between micelles [19,20,24], the micelles may have a unique phospholipid-protein composition mediated by Ca^{2+}-ATPase and the calcium-binding proteins, which would order the phospholipid around it and exclude the bulk of the added lipid.

Discussion

Ca^{2+}-ATPase of the sarcoplasmic reticulum has been reconstituted by detergent solubilization [6,31,21] and removal using molecular sieve chromatography or dialysis [2,3,19–22]. Several points regarding the reconstitution of the sarcoplasmic reticulum membrane proteins in this study should be stressed: First of all, the PC (egg lecithin) used for reconstitution supplied both the major head group as well as the 16:0, 18:0, 18:1 and 18:2 fatty acids required for ATP hydrolysis and calcium transport [22]. These studies were carried out with Ca^{2+}-ATPase which was never first purified or totally delipidated, in order to minimize any changes which could occur potentially in the structure of the protein. It would be of interest to characterize this phenomenon further by examining the composition of vesicles reconstituted with delipidated, purified Ca^{2+}-ATPase to see if Ca^{2+}-ATPase could be found in lipid-rich vesicles.

Equivalent levels of calcium-stimulated ATPase activity were observed in the native and reconstituted membrane vesicles; and in vesicles of diameter greater than 1000 Å, it was feasible to measure all ATP-stimulated calcium uptake activity when monitored by a membrane filter assay (Table III). Ca^{2+}-ATPase must, therefore, be asymetrically disposed across the bilayer with the ATPase domain exposed to the external medium. Ca^{2+}-ATPase appeared only in vesicles of density greater than 1.14 g/cm^3, the high-affinity, calcium-binding protein and calsequestrin were also found in reconstituted vesicles of density greater than 1.14 g/cm^3; therefore, these PC-SR vesicles do possess properties of a functional, reconstituted membrane system.

Other reports on membrane reconstitution characterize the final product by a numerical average of the lipid and total protein concentration, e.g., mol of phospholipid per mol or mg of protein. However, a homogenous distribution of a membrane protein has not necessarily taken place. Analysis of reconstituted vesicles by thin section or freeze-fracture electron microscopy cannot characterize membrane structure adequately in

terms of composition or types of protein present. It is by establishing a method of analysis which eliminates the differential effects of sucrose permeability across the phospholipid bilayer that a clear picture of membrane protein/distribution in this system can be obtained.

These points do not suggest why Ca^{2+}-ATPase in particular, and to a lesser extent the calcium-binding proteins, could not be diluted by reconstitution in the plane of the bilayer beyond a phospholipid/protein ration (w/w) of 1.5. The results clearly indicate that inclusion of a weight excess of phospholipid during reconstitution yielded protein-rich vesicles and a large percentage of liposomes. The range of phospholipid/protein ratios observed were 0.67–2.0, where 0.67 was that of the native sarcoplasmic reticulum membrane. These observations are at variance with the assumption that a multicomponent system of micelles containing various membrane proteins, phospholipids and a detergent will always mix homogenously, and upon detergent removal become spherical membrane vesicles having a relatively constant composition.

This is illustrated in Fig. 8 where the observed density and weight ratio of PC/protein of the PC-SR vesicles in the ^2H_2O gradients is plotted against the molar equivalents of PC/Ca^{2+}-ATPase. The arrows indicate the range of densities of reconstituted vesicles containing Ca^{2+}-ATPase and having maximal ATP-mediated calcium transport. On occasion, traces of low molecular weight proteins could be found in vesicles whose density is less than 1.10 g/cm^3. What is unique is that 83% of all calcium transport activity is found in vesicles whose equilibrium bouyant density is 1.14–1.19 g/cm^3 (Fig. 4, Table III). Each Ca^{2+}-ATPase molecule would have, therefore, from 85 to 140 molecules of PC per molecule of protein or approximately 43–70 molecules of phospholipid per monolayer. The same phospholipid/protein ratios were observed with vesicles a mean diameter of 400 or 1000 Å. This would also suggest that the radius of curvature of the final vesicular structure was not a determinant in the failure of Ca^{2+}-ATPase to be diluted in the plane of the bilayer.

The appearance of lipid-poor and lipid-rich vesicles implies that the bulk of the added PC is excluded totally. The reason for this is unknown

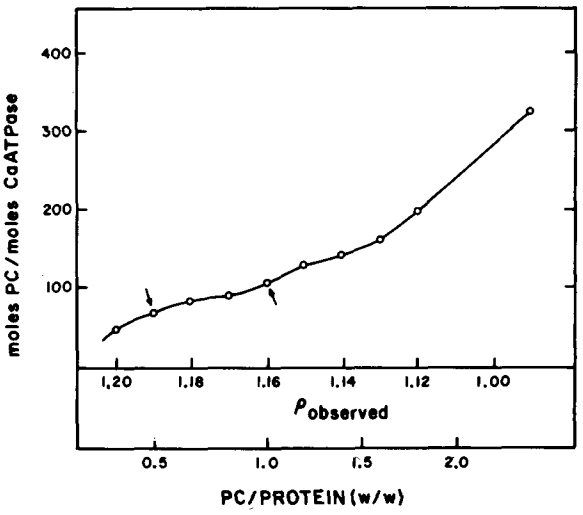

Fig. 8. The density of PC-SR vesicles obtained from equilibrium centrifugation in H_2O–^2H_2O gradients and the calculated phospholipid/protein ratio is plotted against the calculated molar ratio of PC/Ca^{2+}-ATPase. The arrows indicate the density range of reconstituted vesicles which possess ATPase activity, and actively transport calcium.

but could be explained by considering the lateral array and characteristic shape of the phospholipids in a phospholipid-protein-cholate micelle [17,32], as well as protein-protein interactions taking place during [25] vesicle formation.

During solubilization and subsequent detergent removal not only is the lipid adjacent to the protein present with a certain affinity but the close association of the first 'shell' of lipid may effect the packing and structure of lipids for an unknown distance away from the proteins [26–28]. This specificity could begin during solubilization, as suggested by the experiments described in Fig. 7 where conditions simulating detergent removal generate mixed micelles with a progressively defined protein composition. These micelles have phospholipid/protein composition which could reflect the fact that the lipid is being highly ordered by Ca^{2+}-ATPase, a transmembrane protein, and to a lesser extent by the high-affinity, calcium-binding protein and calsequestrin [29].

Reconstituted membrane vesicles that are protein-rich have been observed in other membrane systems, when excess lipid was used during reconstitution. Goldin and Rhodin [30] reported that

reconstitution of the human erythrocyte glucose transport system resulted in incorporation of the protein into only 15% of the phospholipid vesicles. Papahadjopoulos et al. [31], in studying the reconstitution of the N-2 protein isolated from the myelin sheath membrane observed a decrease in the phospholipid/protein ratio of their reconstituted material from the initial ratio used. No claim is being made that resistance to dilution is a general feature of membrane protein reconstitution.

The data of Wang et al. [22] which describes the reconstitution of the sarcoplasmic reticulum membrane proteins with increasing amounts of the sarcoplasmic reticulum membrane phospholipids is entirely consistent with the results which have been described here. Their highest lipid concentration used during reconstitution was equivalent to approximately 2400 mol of lipid per mol of protein. The vesicles that they recovered from this mixture containing only 129 mol of lipid per mol of protein (cf. Fig. 8). They also would appear to have reconstituted membrane vesicles that are protein-rich, and a population of liposomes. It would seem that a more thorough characterization of reconstituted memrane systems is required before conclusions can be made about the distribution, function and structure of reconstituted membrane proteins in phospholipid vesicles, particularly those responsible for the active transport of biologically important ions.

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