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Rapid reconstitution and characterization of highly-efficient sarcoplasmic reticulum Ca pump

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The Ca pump was reconstituted from the purified sarcoplasmic reticulum ATPase and excess soybean phospholipids by the freeze-thaw sonication procedure in the presence of cholate. In the absence of Ca precipitating agents, the reconstituted proteoliposomes accumulated Ca^{2+} at an initial rate of up to 0.7 μmol/mg per min at 25°C, and a value of 1.54 was obtained for the coupling ratio between Ca uptake and Ca^{2+}-dependent ATPase activities. The proteoliposomes were mainly unilamellar vesicles but were heterogeneous with respect to their size. When reconstituted at a lipid/protein ratio of 40, proteoliposomes had a buoyant density of about 1.04 and their average internal volume was 1.4–1.6 μl/mg of phospholipids. More than 95% of the ATPase was incorporated randomly into these proteoliposomes and the fraction of proteoliposomes that represented about 50% of the total intravesicular isotope space contained right-side-out oriented enzyme. ^{86}Rb efflux from the ^{86}Rb-loaded proteoliposomes was found to be slow even at 25°C. Therefore, the proteoliposomes prepared by the present simple method should be useful for the study of the side-specific interaction of ions such as alkali metal cations with the sarcoplasmic reticulum Ca pump.

Introduction

Reconstitution of a membrane transport system into artificial liposomes is an effective method to study its catalytic and transport mechanism. This technique has been applied to the sarcoplasmic reticulum Ca pump, providing direct evidence that the ATPase protein is responsible for active Ca transport and that the Ca pump is electrogenic [1–4]. It has also provided a good deal of information about the structural and functional role of phospholipids for proper functioning of the Ca pump [5]. Although the sarcoplasmic reticulum Ca pump is considered to be electrogenic, it is not clear at present whether the pump transports Ca^{2+} independently of other ions. It has been suggested that H^+ and/or K^+ fluxes may be obligatorily coupled to Ca^{2+} movement during the pump activity (see Ref. 6 for review). The side-specific interaction of the Ca-pump ATPase with various ions and possible movements of these ions can be studied with reconstituted vesicles because the ion composition at the inside and outside surfaces of these vesicles can readily be controlled. To perform this type of study, however, the reconstituted membrane should have relatively low passive permeability to the relevant ions. In addition, if reconstitution is to be performed in the absence of alkali metal ions such as K^+, Na^+ or Rb^+, it

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mops, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid.

should be done in as short a time as possible because the Ca-pump ATPase is unstable without these ions, especially in the presence of detergents (Wakabayashi, S. and Shigekawa, M., unpublished observation, see also Ref. 2).

The previous reconstitution studies have not been very successful in producing preparations that meet these requirements. The previous preparations appear to have relatively high passive permeability to Ca^{2+} because Ca^{2+} was taken up efficiently only when vesicles were preloaded with very high concentrations of oxalate or phosphate or when Ca uptake was measured with oxalate in the reaction medium [1–4,7–12]. In most of the previous studies [1,2,4,7–9,11,12], reconstitution took a relatively long time because vesicles were formed by removing detergents, using dialysis or column chromatography.

In an effort to obtain a preparation suitable for the type of study described above, we employed the freeze-thaw sonication procedure [13,14] for reconstitution of the Ca pump. In the present report, we describe the rapid reconstitution and characterization of an active preparation which exhibits a high coupling ratio between Ca uptake and ATP hydrolysis even in the complete absence of Ca-precipitating anions. It was found that the reconstituted membranes were relatively impermeable to $^{86}Rb^{+}$.

Materials and Methods

Materials. Native sarcoplasmic reticulum vesicles and the purified ATPase protein were prepared from rabbit skeletal muscle as described previously [15]. Crude soybean phospholipid (L-α-phosphatidylcholine type II-S) was purchased from Sigma. Cholic acid (recrystallized), obtained from Nakarai Chemicals, was further crystallized from ethanol. Bovine serum albumin (crystallized and Fraction V) was bought from Miles Laboratories. Cation-exchange resins (AG 50W-X8, 20 –50 mesh and 50–100 mesh) were purchased from Bio-Rad. ^{45}Ca and ^{86}Rb were obtained from New England Nuclear, and ^{42}K and $^{32}P_i$ from Japan Atomic Research Institute. Tris-ATP and [γ-^{32}P]ATP were prepared as described previously [15]. Other reagents used were of analytical grade.

Reconstitution of the sarcoplasmic reticulum Ca pump. Reconstitution of the Ca pump into phospholipid vesicles was carried out essentially with the procedure of Karlish and Pick [14] which is a modification of the freeze-thaw sonication method of Kasahara and Hinkle [13]. Crude soybean phosphatidylcholine (50–100 mg/ml) in 40 mM imidazole-HCl, (pH 7.2) was sonicated to clarity at 0°C under N_2 with a probe-type sonicator (Branson sonifier, Model 200) and then suspended in an appropriate salt medium. The ATPase protein (0.79 mg) was solubilized at 0°C with cholate at various ratios (w/w) of cholate to protein in 0.393 ml of a medium containing 20 mM imidazole-HCl (pH 7.2) and 0.12–0.3 M monovalent salt. An aliquot of the phospholipid suspension was added immediately to this cholate/protein mixture to give an appropriate lipid/protein ratio. The final reconstitution mixture was 1.18 ml, which contained 0.67 mg/ml of ATPase protein, 28–40 mM imidazole-HCl, (pH 7.2), 0 or 0.15 M oxalate, 0.12–0.3 M of radioactive or nonradioactive monovalent salt, 0 or 50 mM of radioactive glucose and various concentrations of phospholipids. The mixture then was frozen in liquid N_2, thawed at room temperature and sonicated in an ice bath with the Branson sonifier for 60 to 180 s at a rate of one pulse per 0.5 s and at 20% of the maximum output. Sonication was usually done for 60 s. For exchange of the external medium, as well as for removal of free cholate, the resultant proteoliposome suspension (approx. 0.4 ml) was passed through a plastic column containing 5 ml of Sephadex G-50 which had been equilibrated with a salt medium of choice, according to the Sephadex column centrifugation method of Penefsky [16].

Measurements of fluxes of ^{45}Ca, ^{86}Rb and ^{42}K. Ca uptake by the reconstituted proteoliposomes was usually measured at 25°C in a medium containing 30 mM imidazole-HCl (pH 7.0 or 7.4)/0.07 mM $^{45}CaCl_2$/5 mM $MgCl_2$/2.2 mM Tris-ATP/0.2 M KCl/proteoliposomes (10 to 20 μg/ml of protein) unless otherwise stated. In the experiments of Figs. 1–5 and Fig. 7, ^{45}Ca uptake was measured by a membrane filtration method using a GS 0.22-μm Millipore filter as described previously [15]. In the experiment of Table I, Ca uptake was measured by the cation-exchange resin method: 0.1 ml of an aliquot was taken from the reaction mixture and then applied to an approx.

9.3-cm column of Bio-Rad AG 50W-× 8 resins (Tris form) which had been formed in a Pasteur pipette and washed with 2 ml of ice-cold solution containing 0.25 M sucrose, 5 mM imidazole-HCl (pH 7.2) and 10 mg/ml of bovine serum albumin [17]. Approx. three fourths of the column volume was filled with 20–50 mesh resins to increase the flow rate, while the remainder was filled with 50–100 mesh resins. The proteoliposomes were eluted with 2 ml of the above sucrose-imidazole-albumin solution and the radioactivity of the eluate was counted. Although the total elution time was 35–40 s, the actual elution time for vesicles would be less than 30 s because the void volume of the column was about 1.1 ml. Comparison of ^{45}Ca uptake measured by these two methods showed that the estimate was 6 to 10% greater with the cation-exchange resin method than with the membrane filtration method. The ^{86}Rb or ^{42}K releases from the reconstituted vesicles were measured using the same cation-exchange resin columns as those used for ^{45}Ca uptake. When the maximum amounts of either ^{45}Ca, ^{86}Rb or ^{42}K used in the present study, namely, 0.1 ml of 0.07 mM $^{45}CaCl_2$ or 0.15 ml of 150 mM $^{86}RbCl$ or ^{42}KCl, were applied to the column, less than 0.01% of the applied radioactivity was recovered in the column eluate.

Measurement of intravesicular aqueous volume. Proteoliposomes were reconstituted in 0.15 M potassium gluconate/14 mM Tris-Mops (pH 7.1)/0 or 10 mM $CaCl_2$/50 mM [^{3}H]glucose/24 μM $^{86}RbCl$. After thawing and sonication, a mixture of ATPase protein, cholate and lipid was passed through a Sephadex G-50 column equilibrated with the above radioactive reconstitution solution so that free cholate was removed but the specific radioactivities of glucose and Rb were maintained. To estimate the size of the intravesicular isotope spaces for [^{3}H]glucose and ^{86}Rb, 200 μl of the radioactive, reconstituted proteoliposomes (0.12 mg of protein) were subsequently centrifuged at 0°C three times on Sephadex G-50 columns equilibrated with the nonradioactive reconstitution solution which was free of cholate. Aliquots were then taken from the column eluate for determination of protein and radioactivities of [^{3}H]glucose and ^{86}Rb. This centrifugation procedure took about 10 min. Leakage of [^{3}H]glucose from the proteoliposomes during this procedure was insignificant because less than 3% of the radioactivity was found to be lost during a 10-min incubation at 0°C when it was measured under the same experimental conditions using membrane filtration method. Leakage of ^{86}Rb during this procedure was also insignificant because values of the intravesicular isotope space for ^{86}Rb determined by this procedure differed by less than 3% from those obtained by passing 40 μl of the same radioactive reconstituted proteoliposomes directly through a cation-exchange resin column as described above.

Measurement of ATP hydrolysis and phosphoenzyme formation. Phosphorylation of the Ca pump ATPase was carried out at 0°C for 10 s in 30 mM imidazole-HCl (pH 7.0)/0.15 M KCl/5 mM $MgCl_2$/95 μM $CaCl_2$/10–51 μM [γ-^{32}P]ATP. Phosphoenzyme levels and the amount of $^{32}P_i$ liberated were measured as described previously [15].

Gel filtration. Gel filtration of the reconstituted proteoliposomes was performed at 4°C at a flow rate of 4 ml/h on a Sepharose 2B column (1.5 × 90 cm) equilibrated with 0.15 M KCl, 30 mM imidazole-HCl (pH 7.4).

Sucrose density gradient. The reconstituted proteoliposomes in 0.3 M KCl/10 mM imidazole-HCl (pH 7.2) were placed on a linear sucrose gradient formed using 0.1 M sucrose containing 0.25 M KCl/10 mM imidazole-HCl (pH 7.4), and 0.5 M sucrose containing 0.05 M KCl/10 mM imidazole-HCl (pH 7.4) with 0.3 ml of cushion of 1.42 M sucrose containing 10 mM imidazole-HCl (pH 7.4) at the bottom of the 5-ml centrifuge tube. The tubes were centrifuged at 50000 rpm for 1 h at 4°C in a Sorval vertical rotor (Model TV 865) and fractions (0.2 ml each) were collected after centrifugation. Sucrose density gradient was also carried out using a steeper sucrose gradient (0.1–0.7 M) with sample size and the volumes of gradient and cushion of 1.42 M sucrose increased 8-fold. The tubes were centrifuged at 50000 rpm for 1.5 h at 4°C in a Hitachi vertical rotor (Model RPV 50T) and 1.5 ml of fractions were collected after centrifugation.

Other procedures. Electron microscopy was performed in a Hitachi H-600 electron microscope. Protein concentration was determined by the method of Bensadoun and Weinstein [18] using bovine serum albumin as a standard except that

1% sodium dodecyl sulfate was included in Lowry reagent C. The soybean phospholipids used in the present study contained 3.0 μg protein per mg of lipid. This contaminating protein was taken into account when the amount of the ATPase protein in the reconstituted proteoliposomes was estimated. The amount of phospholipid was determined by measuring the total lipid phosphorus by the method of Bartlett [19]. The ionized Ca^{2+} concentration was calculated according to Fabiato and Fabiato [20]. The calcium contaminating the reconstituted proteoliposomes was not taken into account for the calculation of ionized Ca^{2+} in the reaction medium.

Results

Optimal conditions for reconstitution.

Fig. 1 shows the time-courses of ATP-dependent Ca uptake by the proteoliposomes formed under optimal conditions (see below) in the presence and absence of Ca precipitating anions. Without the use of Ca precipitating anions (Fig. 1, ○), Ca accumulation was fairly rapid and reached a level of up to 1.75 μmol/mg of protein in 10 min. This level of Ca accumulation is approximately 10-fold that obtained with native sarcoplasmic reticulum vesicles under similar conditions. Inclusion of oxalate within the proteoliposomes markedly increased the extent of Ca accumulation but minimally affected the initial rate of Ca uptake (Fig. 1, △). Ca uptake leveled off earlier in the proteoliposomes formed at a lipid/protein ratio (w/w) of 40 (Fig. 1A) than in those formed at a lipid/protein ratio (w/w) of 80 (Fig. 1B), which probably reflects high density of the Ca-pumping unit per vesicle in the former preparation.

Successful reconstitution of an active preparation depended upon the ratio of the amounts of cholate, the ATPase protein and soybean phospholipids. In the experiment of Fig. 2, the amount of added cholate in the initial solubilization step was varied while the amount of the ATPase protein was kept constant (0.79 mg). Proteoliposomes were then formed at the lipid/protein ratios of 40 and 80 (see Materials and Methods). As shown in the figure, cholate/protein ratios (w/w) of 4.5 and 6–12 were found to be optimal for the lipid/protein ratios of 40 and 80, respectively. When the amounts of cholate and protein were fixed at a ratio of 4.5 and 8, lipid/protein ratios of 40–80 and 60–80 were found to be optimal for the re-

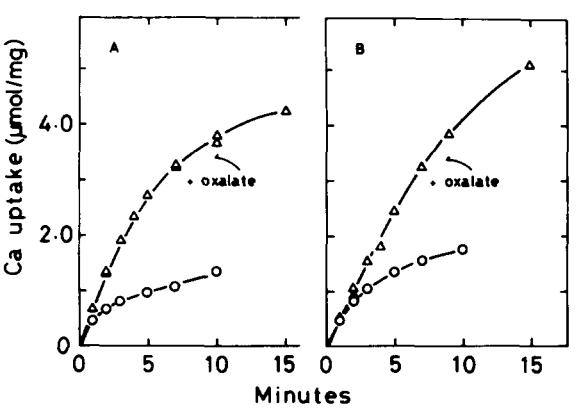

Fig. 1. Time-courses of Ca uptake by the proteoliposomes reconstituted under optimal conditions. Reconstitution was carried out in 28 mM imidazole-HCl (pH 7.2) and either 0.23 M KCl (○) or 0.15 M dipotassium oxalate (△) with cholate/protein/lipid ratios of 4.5 : 1 : 40 (A) or 8 : 1 : 80 (B). Proteoliposomes were made free from cholate by passage through a Sephadex G-50 column equilibrated with 39 mM imidazole-HCl (pH 7.2) and 0.23 M KCl. Ca uptake was measured at pH 7.0 in 0.23 M KCl/201 μM $^{45}CaCl_2$. Other conditions were standard.

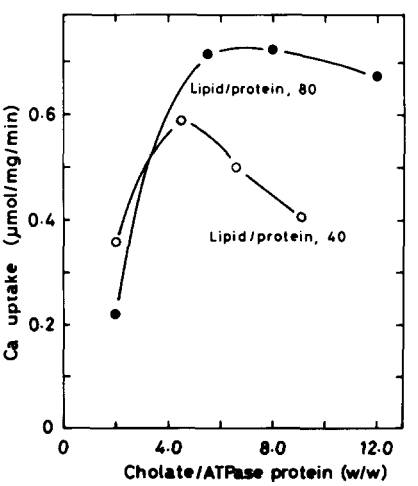

Fig. 2. Effect of cholate/protein ratios on Ca uptake by the reconstituted proteoliposomes. Reconstitution was carried out in 28 mM imidazole/HCl (pH 7.2) and 0.2 M KCl with various cholate/protein ratios. The lipid/protein ratio was fixed at either 40 (○) or 80 (●). Ca uptake was measured for 1 min at pH 7.4 in 3 mM $MgCl_2$/50 μM $^{45}CaCl_2$. Other conditions were standard.

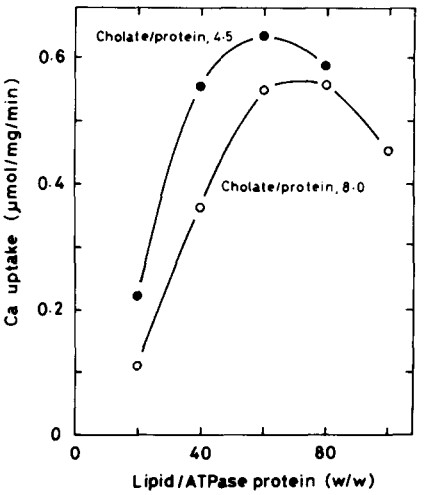

Fig. 3. Effect of lipid/protein ratios on Ca uptake by the reconstituted proteoliposomes. Lipid/protein ratios were varied as indicated while the cholate/protein ratio was fixed at 4.5 (●) or 8 (○). All the other conditions were the same as those described in the legend to Fig. 2.

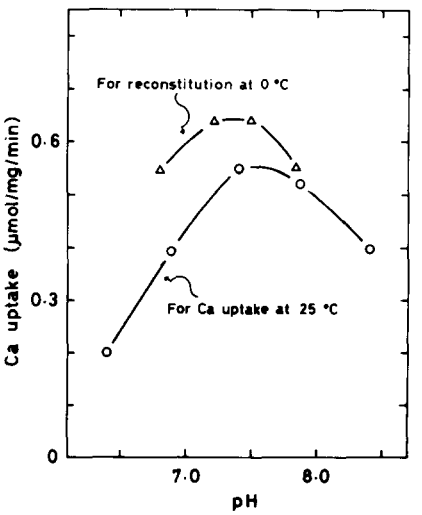

Fig. 4. Optimal pH for reconstitution and Ca uptake. Optimal pH for reconstitution was determined first by reconstituting proteoliposomes at pH indicated (△) and then by measuring Ca uptake at pH 7.4 and 25°C under the standard conditions. Reconstitution was carried out in 21 mM imidazole/Mes (pH adjusted by Tris) at a cholate/protein/lipid ratio of 8:1:80. For determination of optimal pH for Ca uptake, proteoliposomes were reconstituted at the same cholate/protein/lipid ratio but in 28 mM imidazole-HCl (pH 7.2)/0.2 M KCl. Ca uptake was measured at pH indicated (○) using 20 mM Tris/imidazole-HCl. Other conditions were standard.

spective cholate/protein ratios (Fig. 3). In the subsequent experiments, therefore, proteoliposomes were reconstituted using mixtures of cholate, the ATPase protein and phospholipids at weight ratios of 4.5 : 1 : 40 or 8 : 1 : 80. The optimal sonication time for these mixtures after thawing was 60–120 s (cf. Table I). When the sonication time was increased from 60 to 180 s, the initial rate of Ca uptake decreased by 14%. The optimum pH range for the proteoliposome reconstitution was found to be 7.2–7.5 (Fig. 4, △).

Functional properties of reconstituted Ca pump

The rate of ATP-dependent Ca uptake by the reconstituted proteoliposomes was stimulated by ionized Ca^{2+} in the reaction medium at a concentration range 0.01–1 μM with a half-maximum activation occurring at about 0.05 μM (Fig. 5, ○). A slightly higher optimum range was observed for Ca uptake by native sarcoplasmic reticulum vesicles (Fig. 5, △). The optimum pH for ATP-dependent Ca uptake was found to be 7.4–7.8 (Fig. 4, ○).

Reconstituted proteoliposomes exhibited high efficiency of coupling between Ca uptake and Ca^{2+}-dependent ATPase activities (Table I). When both activities were measured during the initial

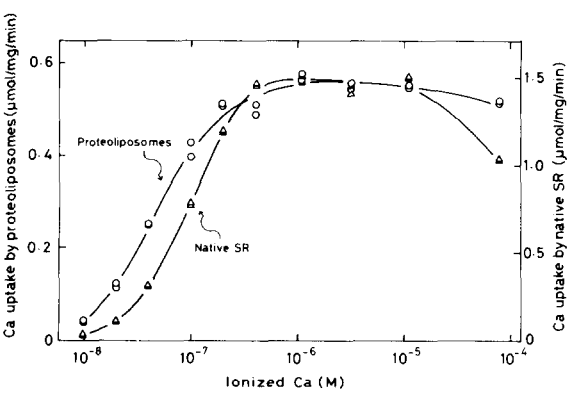

Fig. 5. Ca^{2+}-dependence of Ca uptake by reconstituted proteoliposomes (○) and native sarcoplasmic reticulum (SR) vesicles (△). Proteoliposomes were reconstituted in 28 mM imidazole-HCl (pH 7.2)/0.15 M KCl at a cholate/protein/lipid ratio of 4.5 : 1 : 40. Ca uptake was measured for 40 s at pH 7.0 in 0.15 M KCl/100 μM $^{45}CaCl_2$/0–4.64 mM EGTA. Other conditions were standard. Ca uptake medium for native sarcoplasmic reticulum (SR) vesicles contained additionally 5.0 mM K_2 oxalate.

TABLE I

COUPLING RATIO BETWEEN Ca UPTAKE AND ATP HYDROLYSIS

Proteoliposomes were reconstituted in 28 mM imidazole-HCl (pH 7.2)/0.15 M KCl at a cholate/protein/lipid ratio of 4.5:1:40. Rates of Ca uptake and ATP hydrolysis were measured at 25°C for the initial 40 s in 32 mM imidazole-HCl (pH 7.0), 0.15 M KCl, 5.0 mM $MgCl_2$, 70.6 μM $^{45}CaCl_2$ or $CaCl_2$, and 0.501 mM ATP or [γ-^{32}P]ATP. Results are expressed as means ± S.D. ($n = 4$) in μmol/mg per min.

Sonication time after thawing (min)	Ca uptake rate	ATP hydrolysis rate	Ca/P_i ratio
1	0.448 ± 0.009	0.313 ± 0.009	1.43
2	0.448 ± 0.026	0.291 ± 0.008	1.54

40-s incubation at 0.5 mM ATP but in the absence of a Ca precipitating anion, values of up to 1.54 were obtained for the number of Ca transported per mole of ATP hydrolyzed (Table I). These values are the highest among those obtained without using Ca precipitating anions in the reconstitution studies reported to date [8,11,21], indicating that our preparation is much less permeable to Ca^{2+} than those used previously.

Ca transport and ATP hydrolysis by the Ca pump involve turnover of the acid-stable phosphoenzyme intermediate(s) [22–24]. Table II shows steady-state levels of phosphoenzyme formed in the reconstituted proteoliposomes in the presence and absence of cholate. The phosphoenzyme level was relatively low in the absence of cholate but increased up to 1.8-fold in the presence of the

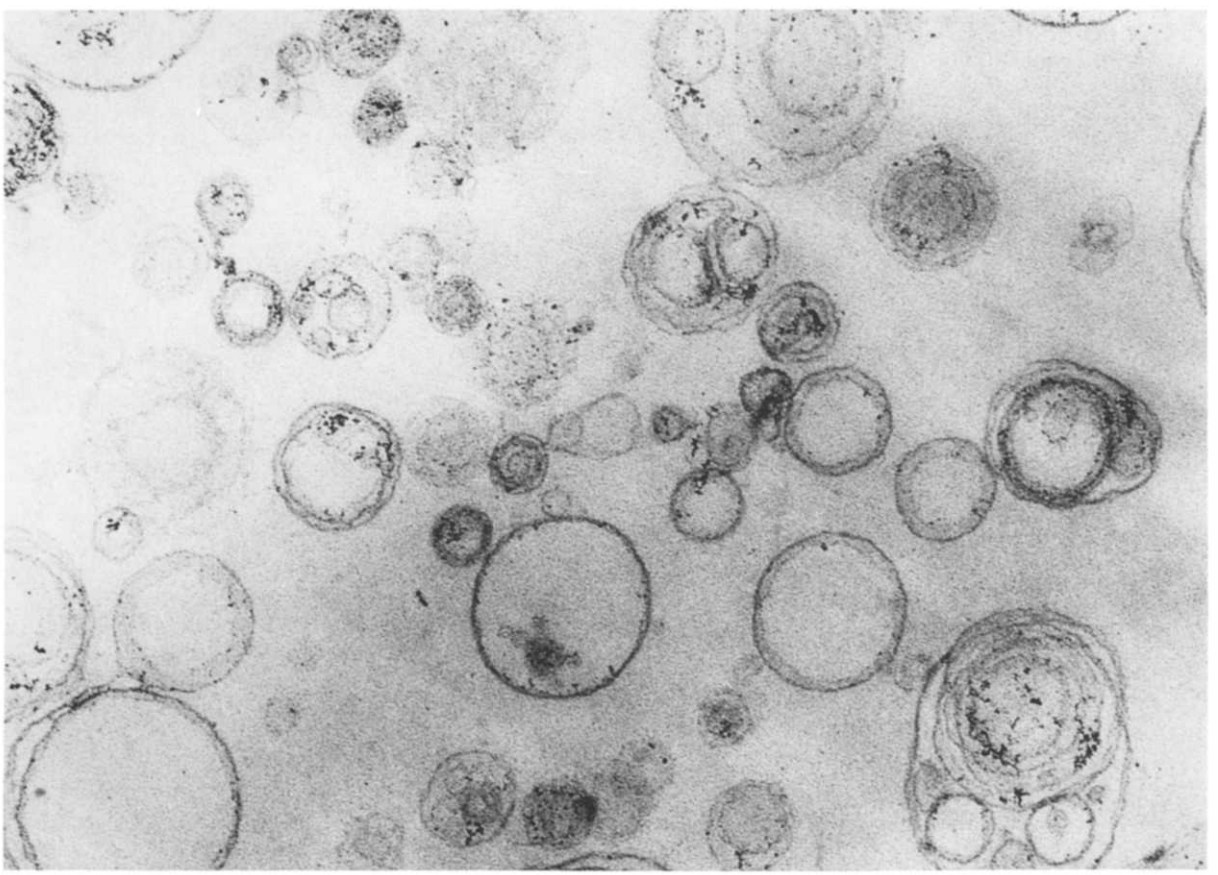

Fig. 6. Electron micrograph of reconstituted proteoliposomes. Proteoliposomes were formed in 28 mM imidazole-HCl (pH 7.2)/0.15 M KCl at a cholate/protein/lipid ratio of 4.5:1:40 and sonicated for 60 s after thawing. The sample was fixed with 2% glutaraldehyde and 0.2 M cacodylate (pH 7.2) for 30 min at 4°C, postfixed with 2% OsO_4 at 4°C, and embedded. ×65000.

TABLE II

EFFECT OF CHOLATE ON PHOSPHOENZYME LEVELS

Proteoliposomes were reconstituted in 28 mM imidazole-HCl (pH 7.2)/0.15 M KCl at a cholate/protein/lipid ratio of 4.5 : 1 : 40. Proteoliposomes (0.175 mg/ml of protein) were pretreated with various concentrations of cholate for 30 s at 0°C in the phosphorylation medium and then enzyme phosphorylation was started by the addition of [γ-^{32}P]ATP (final concentration, 51 μM) under the conditions described in Materials and Methods. The values for the phosphoenzyme level are the average of at least two determinations.

Cholate (%, w/v)	EP (nmol/mg)	% of control
0	2.35	
0.16	2.28	97
0.33	3.99	170
0.65	4.17	177
1.10	2.85	121

detergent at 0.33 and 0.65% (w/v). Since this concentration of cholate could be inhibitory to the enzyme activity, the result may be interpreted as indicating that only half of the active sites of the ATPase protein in the reconstituted membranes can be phosphorylated by [γ-^{32}P]ATP in the absence of cholate.

Characterization of reconstituted proteoliposomes

Electron microscopy revealed that the reconstituted proteoliposomes consisted mostly of unilamellar vesicles (Fig. 6). The vesicles were highly heterogeneous with respect to their size, which ranged from 50 to 600 nm in diameter (Fig. 6). Gel filtration on Sepharose 2B confirmed the size heterogeneity of the reconstituted proteoliposomes (Fig. 7). However, distributions of the ATPase protein as revealed by determination of total pro-

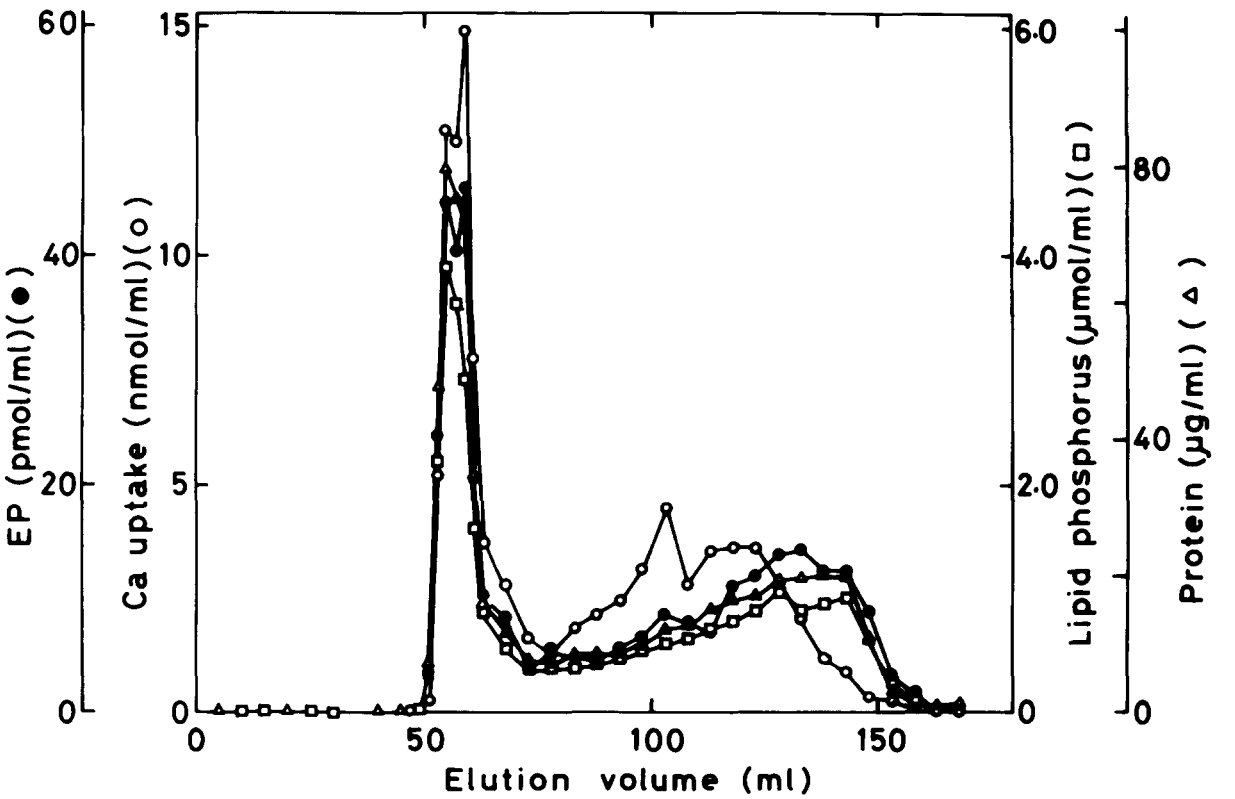

Fig. 7. Sepharose 2B chromatography of reconstituted proteoliposomes. Proteoliposomes were reconstituted in 30 mM imidazole-HCl (pH 7.2)/0.15 M KCl at a cholate/protein/lipid ratio of 8 : 1 : 80. Sepharose 2B chromatography was carried out as described under Materials and Methods and aliquots from each fraction (2 or 5 ml) were monitored for Ca uptake activity (○), phosphoenzyme formation (●), and the concentrations of lipid phosphorus (□) and protein (△). Ca uptake was measured for 1 min at pH 7.0 in 0.15 M KCl/48 μM $^{45}CaCl_2$ otherwise under the standard conditions. Phosphorylation of proteoliposomes was carried out in 20 μM [γ-^{32}P]ATP under conditions described in Materials and Methods.

tein or the ^{32}P-labeled phosphoenzyme level, and of phospholipids as detected by determination of total lipid phosphorus were found to be parallel among dimensionally heterogeneous vesicle fractions. This parallel distribution of the ATPase protein and phospholipids observed at lipid/protein ratios of 40 (data not shown) or 80 (Fig. 7), indicates that the density of the ATPase protein per unit area of the reconstituted membrane was apparently the same among the vesicles with different size. It should be noted that in fractions whose elution volumes were more than 130 ml, Ca uptake activity was significantly low as compared with corresponding levels of phosphoenzyme, lipid or protein, although its distribution was almost parallel to other markers in fractions with a smaller elution volume. The data thus indicate that small vesicles are incapable of accumulating Ca efficiently. Our preliminary result obtained via quasi-elastic light scattering at a scattering angle of 90° (Coulter Model N-4) showed that the fractions which eluted at 55 and 138 ml contained vesicles whose mean diameter were 230 and 64 nm, respectively.

In the experiment of Fig. 8, the proteoliposomes reconstituted at a lipid/protein ratio of 40 were subjected to isopycnic sucrose (0.1–0.5 M) density-gradient centrifugation. The proteoliposomes were resolved into two fractions, the higher density fraction being approx. one third of the lower density one when the ATPase protein-cholate-lipid mixture was sonicated for 60 s after thawing (Fig. 8). The size of the former fraction decreased to about one sixth of the latter when the sonication time was increased to 180 s. At any rate, distribution of ^{32}P-labeled phosphoenzyme roughly paralleled that of phospholipids except for the very light fractions (Fig. 8). When a similar density-gradient centrifugation was carried out using a steeper sucrose gradient (0.1–0.7 M) with sample size and the volumes of gradient and sucrose cushion increased 8-fold in order to measure protein distribution (see Materials and Methods), it was found that less than 5% of the total protein appeared at a position just above 1.42 M sucrose cushion, to which the purified ATPase protein was usually sedimented. As ^{32}P-label was not incorporated from [γ-^{32}P]ATP into this small protein fraction, it appears that the fraction represents the denatured ATPase protein. The result, therefore, indicates that most of the added ATPase protein was incorporated into proteoliposomes.

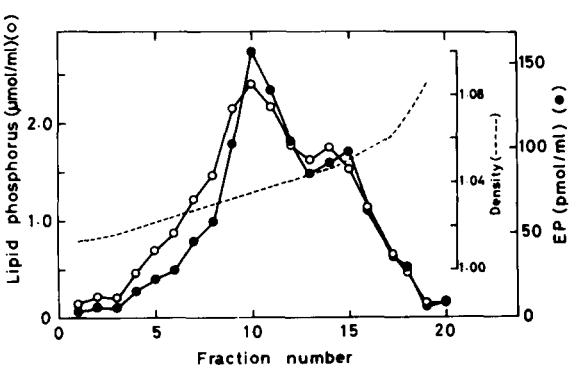

Fig. 8. Sucrose gradient centrifugation of reconstituted proteoliposomes. Proteoliposomes which were reconstituted in 28 mM imidazole-HCl (pH 7.2)/0.3 M KCl at a cholate/protein/lipid ratio of 4.5 : 1 : 40, were subjected to sucrose-gradient centrifugation as described in Materials and Methods. Aliquots from each fraction (0.2 ml) were monitored for the Phospholipid phosphorus (○) and phosphoenzyme formation (●). Phosphoenzyme formation was measured in 0.26 M KCl/68 mM glucose/10 μM [γ-^{32}P]ATP/4.3 mM $MgCl_2$. Other conditions are those described in the legend to Fig. 7.

Fig. 9A shows an efflux time-course for ^{42}K initially incorporated into the proteoliposomes reconstituted at a lipid/protein ratio of 40. At 25°C, ^{42}K efflux exhibited a biphasic time-course which can be simulated using the equation

$$F = 0.69\, e^{-0.047t} + 0.72\, e^{-0.002t}$$

(solid line in Fig. 9A), where F is the intravesicular space for ^{42}K (μl/mg of lipid) and t is the time in minutes. This indicates that approx. 50% of the trapped ^{42}K was released at a rate more than 20-times greater than that for the remainder. When ^{42}K efflux was measured with plain lipid vesicles prepared under the same conditions but without the ATPase protein, it proceeded almost monoexponentially with a rate constant of 0.0016 min^{-1} which corresponded to the smaller rate constant used in the above equation. When ^{86}Rb instead of ^{42}K was used, the result was almost the same (data not shown). In the experiment of Fig. 9B, the time-course of ^{86}Rb efflux was monitored over a much shorter time range using gluconate as a co-ion for Rb^+. ^{86}Rb efflux from the proteoliposomes reconstituted in 0.15 M potassium gluco-

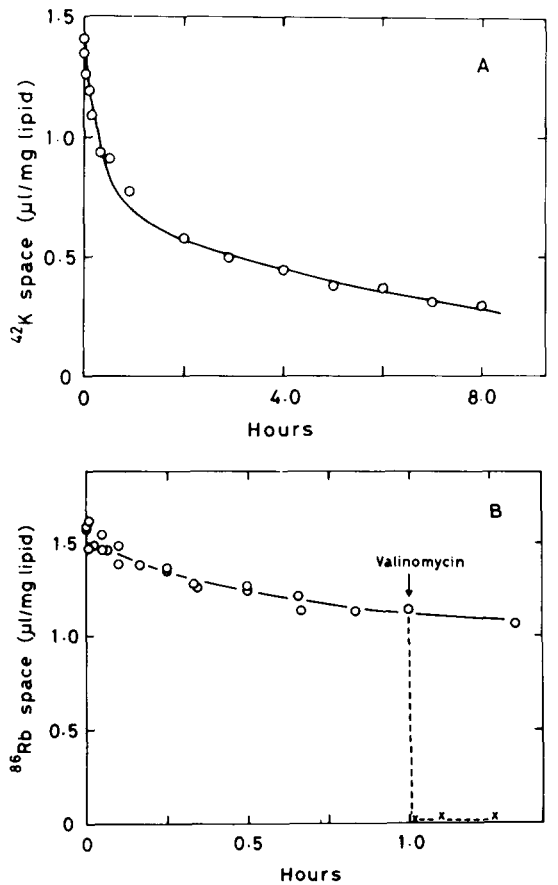

Fig. 9. ^{42}K or ^{86}Rb effluxes from reconstituted proteoliposomes. Proteoliposomes were reconstituted at a cholate/protein/lipid ratio of 4.5 : 1 : 40 in 28 mM imidazole-HCl (pH 7.2)/0.15 M ^{42}KCl (A) or 14 mM Tris-Mops (pH 7.1)/0.15 M K gluconate/24 μM ^{86}RbCl/50 mM [^{3}H]glucose (B). To remove free cholate without changing the intravesicular contents of ^{42}K or ^{86}Rb and [^{3}H]glucose, samples were passed through Sephadex columns equilibrated with the radioactive, cholate-free reconstitution solution. At zero time, samples were diluted 33-fold into nonradioactive media of identical composition and ^{42}K or ^{86}Rb effluxes were followed at 25°C as described in Materials and Methods. In B, valinomycin (final concentration, 12 μM) was added 1 h after the start of the efflux and ^{86}Rb release was followed (×). In the experiment shown in B, [^{3}H]glucose was included in the reconstitution medium to examine whether the intravesicular isotope spaces for ^{86}Rb and [^{3}H]glucose agreed with each other (see text and also Materials and Methods).

nate and 24 μM ^{86}RbCl was found to be relatively slow even at 25°C. Valiomycin caused complete release of ^{86}Rb (Fig. 9B). This result showed that all the trapped ^{86}Rb was exchangeable.

The intravesicular aqueous volume of the proteoliposomes reconstituted at a lipid/protein ratio of 40 was estimated by trapping both [^{3}H]glucose and ^{86}Rb in the same reconstituted proteoliposomes as described in Materials and Methods. Although estimated values of the internal volume varied from preparation to preparation and ranged from 1.4 to 1.6 μl/mg phospholipids, the values obtained for [^{3}H]glucose and ^{86}Rb in the same preparation differed by less than 4%. These equal isotope spaces for glucose and Rb can be interpreted as indicating that there is almost no detectable fraction of proteoliposomes from which Rb selectively and rapidly leaks out within the time range used for the isotope flux measurement (see Materials and Methods).

In the experiment of Fig. 10, ^{45}Ca efflux from the proteoliposomes reconstituted in 10 mM $^{45}CaCl_2$ was measured in the presence of either EGTA plus P_i or a mixture of EGTA, P_i, and excess ADP. In the presence of the former (Fig. 10, ○), ^{45}Ca efflux proceeded at a rate slower than that for the fast component of ^{42}K efflux (cf. Fig.

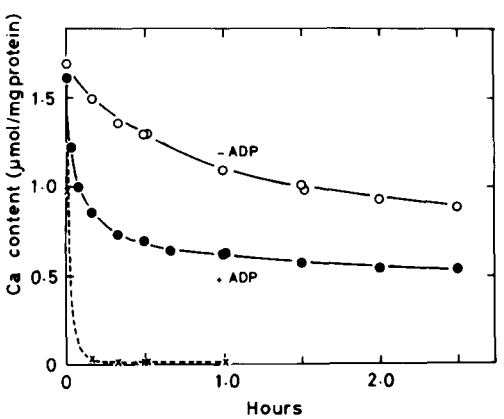

Fig. 10. ADP-dependent ^{45}Ca efflux from reconstituted proteoliposomes. Proteoliposomes were reconstituted in 28 mM imidazole-HCl (pH 7.2)/10 mM $^{45}CaCl_2$/0.15 M KCl at a cholate/protein/lipid ratio of 4.5 : 1 : 40. To remove free cholate without changing the specific radioactivity of ^{45}Ca, the sample was passed through a Sephadex column equilibrated with the radioactive reconstitution solution. At zero time, the sample was diluted 22-fold into a solution containing 30 mM imidazole-HCl (pH 7.0), 29 mM KCl, 100 mM Tris-HCl, 20 mM potassium phosphate, 10 mM EGTA, 10 mM $MgCl_2$ and either 0 (○) or 2.0 mM ADP (●), and ^{45}Ca efflux was followed at 25°C. In one experiment (×), 13 μM A23187 was included in the efflux medium. The ^{45}Ca trapped in the proteoliposomes was measured by a membrane filtration method [15].

9A). When ADP was added together with EGTA and P_i, ^{45}Ca efflux exhibited a clear biphasic time-course, its fast component accounting for about 50% of the initial level of trapped ^{45}Ca (Fig. 10, ●). It should be pointed out that the intravesicular isotope space for ^{45}Ca estimated in this experiment was 3.8 μl/mg of lipid, a value more than twice as great as those obtained with ^{86}Rb and [^{3}H]glucose. As the intravesicular isotope space for ^{86}Rb did not change by inclusion of 10 mM $CaCl_2$ in the reconstitution medium, the large isotope space probably means that a significant portion of ^{45}Ca was bound to phospholipids on the inner surface of the vesicle membrane. The bound ^{45}Ca was, however, in rapid equilibrium with ^{45}Ca present in the intravesicular water space because A23187 caused complete release of the radioactive Ca associated with proteoliposomes (Fig. 10, ×).

Discussion

The data presented in this paper showed that a highly active and efficient Ca pump can be reconstituted from the purified sarcoplasmic reticulum ATPase protein and excess soybean phospholipids employing the freeze-thaw sonication procedure [13,14]. The reconstituted proteoliposomes were mainly unilamellar vesicles but were heterogeneous with respect to their size (Figs. 6 and 7). When vesicles were formed at a lipid/protein ratio of 40, their buoyant densities were about 1.04 (Fig. 8) and their average intravesicular aqueous volume was 1.4–1.6 μl/mg of phospholipids (Fig. 9). More than 95% of the ATPase protein was incorporated into the reconstituted vesicles (see Results) and distributions of the ATPase protein and phospholipids among these dimensionally heterogeneous vesicles were apparently parallel (Fig. 7). These results strongly support the idea that distribution of the ATPase protein is random in the reconstituted vesicles, at least in terms of the number of enzyme molecules per area unit of the vesicle membrane.

The fractional size of the reconstituted vesicles that contained the ATPase protein was estimated in the isotope efflux experiments. ^{45}Ca efflux from the ^{45}Ca-loaded vesicles showed that the fast component of ^{45}Ca efflux, which was ADP-dependent, accounted for up to 50% of the total internal ^{45}Ca (Fig. 10). As ADP- and P_i-dependent Ca efflux from the Ca-loaded vesicles in the presence of excess EGTA presumably arises from reversal of the Ca pump [22–25], it was concluded that up to 50% of the total isotope space was represented by the vesicles containing the ATPase molecules of right-side-out orientation. The ^{42}K efflux experiment (Fig. 9A) also showed that vesicles can be grouped into two types of fraction: the fraction which represented approx. 50% of the total intravesicular isotope space was more than 20-times more permeable to ^{42}K than the rest of the vesicles. As the permeability property of the latter fraction was similar to that of plain lipid vesicles (see Results), we concluded that the more permeable fraction of the reconstituted vesicles contained the ATPase protein (cf. Ref. 26).

It should be noted that ^{86}Rb efflux from the vesicles loaded with 150 mM potassium gluconate and 24 μM ^{86}Rb appeared to be slower than ^{42}K efflux from the vesicles loaded with 150 mM ^{42}KCl (Figs. 9A and B). In these experiments, the salt composition of the extravesicular medium was identical to that of the intravesicular medium, except that low ^{86}Rb was included only in the latter. As Rb^+ and K^+ behaved similarly in terms of their passive permeabilities (see Results) and as the same high concentration of K^+ was present both inside and outside the vesicles, the inside-negative diffusion potential that may be created by the Rb^+ movement cannot be responsible for the slow ^{86}Rb efflux observed in the presence of gluconate anion. We consider that the conditions used in the experiment of Fig. 9B may be optimal for reconstitution of membranes that exhibit apparently low passive permeability to alkali metal ion such as Rb^+. At any rate, because a highly efficient Ca pump activity can be reconstituted by the present method which is simple, rapid and highly reproducible, this method would be useful for the study of the role of ions in the catalytic and transport mechanism of sarcoplasmic reticulum Ca pump.

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