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THE PHOSPHOLIPID HEADGROUP SPECIFICITY OF AN ATP-DEPENDENT CALCIUM PUMP

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Summary

We have replaced the lipid associated with a purified calcium transport protein with a series of defined synthetic dioleoyl phospholipids in order to determine the effect of phospholipid headgroup structure on the ATPase activity of the protein. At 37°C the zwitterionic phospholipids (dioleoyl phosphatidylcholine and dioleoyl phosphatidylethanolamine) support the highest activity, while a phospholipid with two negative charges (dioleoyl phosphatidic acid) supports an activity which is at least twenty times lower. Dioleoyl phospholipids with a single net negative charge support at intermediate ATPase activity which is not affected by the precise chemical structure of the phospholipid headgroup. The protocol used to determine the phospholipid headgroup specificity of calcium transport protein is novel because it establishes the composition of the lipid in contact with the protein without the need to isolate defined lipid-protein complexes. This allows the lipid specificity to be determined using only very small quantities of test lipids.

We also determined the ability of the same phospholipids to support calcium accumulation in reconstituted membranes. Two requirements had to be met. The phospholipid had to support the ATPase activity of the pump protein and it had to form sealed vesicles as determined by electron microscopy. Since a number of phospholipids met those requirements it is clear that in vitro the lipid specificity of the calcium-accumulating system is rather broad.

Abbreviation: DOPC, dioleoyl phosphatidylcholine.

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Introduction

Skeletal muscle sarcoplasmic reticulum is isolated as vesicles whose protein composition reflects the highly specialised nature of this membrane: approximately 70% of the protein is a single membrane protein, the ATP-dependent calcium pump [1]. We have shown that the part of this protein that penetrates the lipid bilayer is surrounded by a single shell of about 30 lipid molecules which we have termed the "lipid annulus" [2]. In attempting to define the role of lipid in calcium uptake and accumulation we developed techniques to replace almost completely the endogenous lipids by synthetic phospholipids [1,3]. We then determined the response of ATPase activity to changes in the chemical and physical properties of the fatty acid chains. Using a series of synthetic phosphatidylcholines we were able to show that for highest ATPase activity the fatty acid chains in the lipid annulus had to be 16–18 carbon atoms in length, unsaturated and in a fluid state [3].

The effect of changes in phospholipid headgroup structure on ATPase activity is not so well-defined. We here examine the effect of a range of synthetic phospholipid headgroups, each attached to dioleoyl fatty acid chains, to determine in more detail the effect of the charge and the chemical structure of the headgroup on the ATPase activity of the protein. In order to do so we have used a protocol based on our "lipid titration" technique [3] to establish the composition of the lipid annulus without the need to isolate and analyse the lipid-protein complexes.

Although we find that ATPase activity correlates more closely with the charge structure of the phospolipid headgroup than with particular features of the chemical structure, Racker et al. [4–6] have suggested that certain chemical groupings on the phospholipid headgroup may be essential to facilitate the transport of calcium. We have, therefore, used our test lipids to prepare reconstituted membrane vesicles incorporating the calcium pump protein and measured the competence of these different lipids in supporting calcium transport and accumulation.

Materials and Methods

Calcium pump protein. This protein (ATPase) together with its associated lipid was purified by sucrose density gradient centrifugation of sarcoplasmic reticulum vesicles treated with potassium cholate as described previously [1]. In the isolated lipid-protein complexes each molecule of the purified ATPase was associated with about 30 molecules of endogenous lipid which were replaced by an equivalent number of dioleoyl phosphatidylcholine (DOPC) molecules using a lipid substitution technique described previously [1,7]. Analysis of the lipid composition of the resultant lipid-protein complexes (DOPC · ATPase) showed that more than 98% of the lipid was dioleoyl phosphatidylcholine.

Lipids. Dioleoyl phosphatidylcholine was prepared by the method of Robles and van den Berg [8]. Dioleoyl phosphatidylethanolamine, dioleoyl phosphatidylglycerol, *O*-methyl dioleoyl phosphatidic acid and dioleoyl phosphatidic acid were prepared by treating dioleoyl phosphatidylcholine with phospho-

lipase D in the presence of ethanolamine (5%), glycerol (10%), methanol (3%) and water respectively according to the method of Yang et al. [9]. Dioleoyl phosphatidylserine was prepared by treating DOPC with phospholipase D in the presence of carboxymethyl serine (20%) and removing the blocking methyl group by mild alkaline hydrolysis. *N*-Acetyl dioleoyl phosphatidylethanolamine was prepared by treating dioleoyl phosphatidylethanolamine with acetic anhydride. [^{3}H]DOPC was prepared as described [10]. All products were purified by silicic acid chromatography. Cardiolipin was purchased from Lipid Products. Cholesterol was purchased from Sigma.

To prepare lipids for the lipid titration and reconstitution procedures, the phospholipid (25 mg) or cholesterol (12.5 mg) was dissolved in chloroform and the lipid was dried down as a thin film in the bottom of a glass vial by removing the chloroform under a stream of nitrogen and then under a vacuum. The dried lipid film was treated with 1.0 ml of 250 mM sucrose/1.0 M potassium chloride/50 mM potassium phosphate buffer, pH 8.0, (sucrose buffer) containing 12.3 mg potassium cholate. The mixture was then sonicated to homogeneity by suspending the vial in a Shuco ultrasonic cleaning bath.

Forward titration procedure. An aliquot of DOPC · ATPase (81 μg; 2.5 μl of 32.5 mg protein/ml in sucrose buffer) was added at 0°C to a mixture comprising 20 μl test lipid (25 mg/ml in sucrose buffer containing 12.5 mg/ml potassium cholate), 11.5 μl of 10 mg/ml potassium cholate (in sucrose buffer) and sucrose buffer containing 1.0 mM $MgATP^{2-}$ to a final volume of 0.52 ml. The test lipid comprised 96.5% of the total lipid in the incubation mixture, the remainder being dioleoyl phosphatidylcholine. After 30–60 min incubation at 0°C, 25 μl were assayed for ATPase activity at 37°C.

Back titration procedure. DOPC · ATPase (81 μg) was added at 0°C to 12.5 mg/ml cholesterol/12.5 mg/ml potassium cholate in sucrose buffer containing 5.0 mM MgATP in a final volume of 100 μl. After 1 min incubation at 0°C, 5 μl were assayed for ATPase activity at 37°C and after a further 1 min incubation, reactivation of cholesterol/ATPase was effected by addition of 400 μl sucrose buffer followed by 20 μl of 25 mg/ml test lipid/12.5 mg/ml potassium cholate in sucrose buffer. After 30–60 min incubation at 0°C, 25 μl were assayed for ATPase activity at 37°C.

Reconstitution procedure. Sarcoplasmic reticulum vesicles (1.0 mg protein; 50 μl of 20 mg protein/ml in sucrose buffer) were mixed with potassium cholate (1.5 mg; 15 μl of 100 mg/ml in sucrose buffer) with vigorous mixing. Aliquots (5 μl) of the mixture were added immediately to a mixture comprising 0.40 ml of 25 mg/ml test lipid/12.5 mg/ml potassium cholate in sucrose buffer/0.1 ml 1.0 M potassium oxalate at 0°C. This incubation was dialysed for 16 h at 4°C against 500 ml 0.20 M potassium oxalate, pH 7.4. Assays of calcium accumulation in the presence and absence of ATP, and ATPase assays, were carried out using 50-μl samples. In order to correct for sample dilution during dialysis, a small amount of [^{3}H]DOPC ($2 \cdot 10^4$ cpm/sample; less than 10^{-3} mg DOPC) was incorporated with the lipid, and 50-μl aliquots taken for liquid scintillation counting immediately before and after dialysis.

ATPase activity. An aliquot of the lipid titration mixture (less than 0.05 mg protein) was diluted rapidly into 2.0 ml of 2.0 mM ATP/5.0 mM $MgSO_4$/100 μM $CaCl_2$/50 μm EGTA/0.5 mM phospho*enol*pyruvate/0.15 mM NADH/20

I.U./ml lactate dehydrogenase/5 I.U./ml pyruvate kinase/100 mM triethanolamine hydrochloride-KOH buffer, pH 7.2, at 37°C and the decrease in absorbance at 340 nm was observed.

In order to measure ATPase activity in reconstitution experiments under exactly the same conditions as calcium uptake, the coupled assay above could not be used since lactate dehydrogenase is inhibited by potassium oxalate, and instead a stopped radiochemical assay was used. To 1.0 ml calcium uptake cocktail (see below) at 25°C was added 50 μl 4 mM $CaCl_2$, 20 μl 100 mM γ-^{32}P-labelled Mg · ATP, pH 6.8, (at approx. 10^6 cpm/μmol) and 50 μl sample to start the reaction. Aliquots (100 μl) were removed over a 1 h time course and mixed rapidly with 0.30 ml 10 mM EGTA/100 mM triethanolamine hydrochloride-KOH buffer, pH 7.2, containing 10% (w/v) activated charcoal (which binds ATP). The charcoal was pelleted in a microcentrifuge at 15000 × *g* for 5 min and 200 μl of the supernatant was sampled for liquid scintillation counting.

In either of the above systems, addition of EGTA to a final concentration of 1.0 mM abolished 98% of the ATPase activity.

Calcium uptake activity. To 1.0 ml of 2 mM $MgSO_4$/0.1 mM EGTA/0.5 mM phospho*enol*pyruvate/5 I.U./ml pyruvate kinase/100 mM triethanolamine hydrochloride-KOH buffer, pH 7.2, (calcium uptake cocktail) at 25°C was added 20 μl 100 mM Mg · ATP, pH 6.8, and 50 μl 4 mM $^{45}CaCl_2$ (at approx. $4 \cdot 10^6$ cpm/μmol). 50 μl of a reconstitution sample in 200 mM potassium oxalate (final concentration in assay 8.9 mM) was added to start the reaction and 100 μl aliquots were sampled over a 10 min time course by filtering through pre-wetted 0.45 μm Millipore filters and washed with 5.0 ml 1.0 mM EGTA/100 mM triethanolamine hydrochloride-KOH buffer, pH 7.2. The filters were dispersed in a Triton-toluene scintillation fluid and counted. A control time-course in the absence of Mg · ATP was performed for each sample; non-specific calcium binding was normally less than 10% of ATP-dependent calcium uptake but rose to 20% for some negatively charged lipids (notably dioleoyl phosphatidic acid).

Electron microscopy. Reconstituted samples after dialysis were negatively stained using 2% phosphotungstate, pH 7.0, and dried down on 200 mesh colloidin- and carbon-coated copper grids. These were examined at 40 000× magnification on an AEI electron microscope type 8B.

Results and Discussion

Lipid titration methodology

The phospholipid headgroup dependence of the Ca^{2+}-dependent ATPase activity of the sarcoplasmic reticulum calcium pump was determined using the lipid titration technique described previously [3]. The procedure involves treating DOPC · ATPase with an excess of the test lipid in the presence of the detergent potassium cholate. The detergent facilitates random exchange of lipids between the DOPC · ATPase and test-lipid pools so that the composition of the lipids in the annulus surrounding the penetrant part of the ATPase protein eventually becomes the same as the total lipid pool. A small aliquot of this mixture is then diluted into assay medium and the ATPase activity determined.

This procedure is simple and rapid, but depends on the action of cholate in catalysing equilibration of the lipid pools. We determined peviously the fatty acid chain requirements of the ATPase using a series of phosphatidylcholines, and randomisation of the lipid pools was shown to have occurred by isolating the ATPase protein together with the annulus of lipids and analysing their composition. In the present study insufficient quantities of many of the test lipids were available for isolation and analysis. However, we cannot assume that cholate will exchange the dioleoyl phosphatidylcholine in DOPC · ATPase with a test lipid just because it has been shown to exchange dioleoyl phosphatidylcholine with a number of synthetic phosphatidylcholines. For example, we have observed that cholate will not exchange dioleoyl phosphatidylcholine for cerebrosides, and detergents such as the Triton series will not exchange lipids at all.

To allow for the possibility that equilibration of lipid pools is not, or is only partially, occurring we have developed a protocol whereby DOPC · ATPase is first incubated with an approximately 70-fold molar excess of cholesterol in the presence of cholate; this results in a loss of ATPase activity to less than 2% of that of the DOPC · ATPase. The ATPase activity is completely restored when an excess of dioleoyl phosphatidylcholine is added back to an aliquot of this mixture (Table I) indicating that no irreversible inactivation of the ATPase has occurred. If a similar "back-titration" of a test-lipid with the cholesterol/ATPase mixture elevates the ATPase activity to the same level found for a "forward titration" of the lipid with DOPC · ATPase, then cholate must be facilitating a random exchange of lipids.

For example, if DOPC · ATPase is treated with a test lipid in cholate and no change in ATPase activity occurs than we must conclude either that equilibration of the lipid pools has occurred and the test lipid supports the same activity as does dioleoyl phosphatidylcholine or that the cholate has not exchanged the lipids between the two pools. If equilibration does not occur than the same test lipid in cholate should not affect the ATPase activity of the cholesterol/ATPase mixture; this is what we have observed for the cerebroside lipids. If, however, equilibration does occur then the test lipid will raise the activity of cholesterol/ATPase to the level of DOPC · ATPase. By a similar argument, if cholate

TABLE I

THE REVERSIBILITY OF THE BACK TITRATION PROCEDURE

DOPC · ATPase was incubated with an excess of cholesterol in the presence of cholate, and then more dioleoyl phosphatidylcholine in the presence of cholate was added, as described in Materials and Methods.

Incubation mixture	ATPase activity (μmol/min per mg enzyme at 37°C)
DOPC · ATPase	9.1
DOPC · ATPase + cholate + cholesterol	0.15
DOPC · ATPase + cholate + cholesterol + DOPC	9.3

catalyses only partial exchange of the test lipid then the forward and back titration procedures will not lead to the same ATPase activity. As shown in Fig. 1, all of the test lipids do lower the activity of DOPC · ATPase and raise the activity of cholesterol/ATPase to approximately the same level, so that lipid equilibration has occurred in all cases.

Although the presence of cholate in the incubation will override any natural tendency on the part of the protein to select particular lipids for its annulus, when aliquots are diluted into the assay medium more than 95% of the cholate dissociates from the lipid-protein complexes which then re-form vesicular structures [3]. (The residual cholate does not interfere with the effect of lipid on the ATPase since extensive dialysis of diluted samples to remove cholate did not affect the pattern of results in Fig. 1.) It is possible that under these conditions the protein may selectively interact with the dioleoyl phosphatidylcholine or cholesterol also present in the vesicles.

We have already shown that under the conditions used for these experiments cholesterol is excluded from the lipid annulus when there is sufficient phospholipid to complete an annulus of 30 lipid molecules [11]; in any case cholesterol is only present in the back titrations, and if it interacted selectively we would not have obtained similar results from the forward and back titrations. In contrast, dioleoyl phosphatidylcholine is present in all samples since it formed the original lipid annulus of the purified DOPC · ATPase. However, although we have previously shown selective interaction of the ATPase with dioleoyl phosphatidylcholine in the presence of lipid mixtures we have never observed this when dioleoyl phosphatidylcholine comprised less than 10% of the total phospholipid. In these experiments the test lipid comprised more than 96.5% of the total phospholipid in the titration incubations.

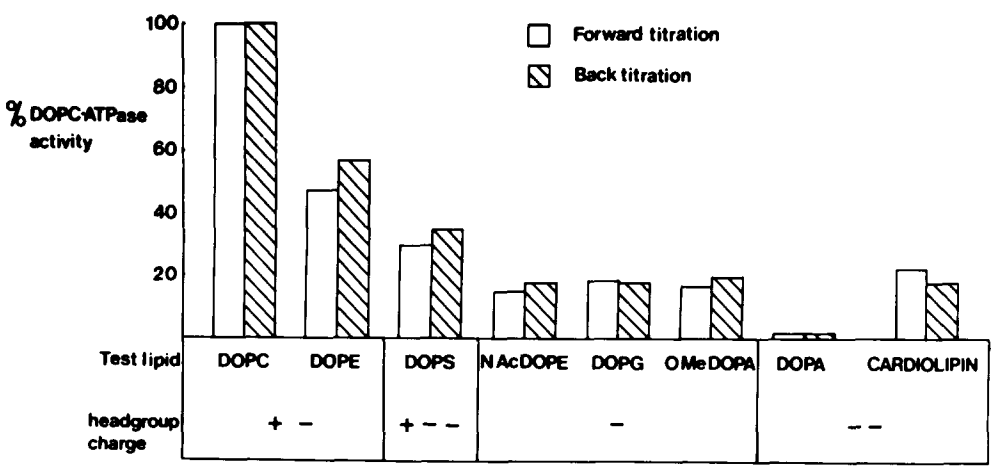

Fig. 1. The phospholipid headgroup specificity of the ATPase activity of the calcium pump protein. An excess of test lipid in the presence of cholate was added either directly to DOPC · ATPase (forward titration) or added to DOPC · ATPase which had been previously treated with cholesterol (back titration), as described in Materials and Methods. DOPE, dioleoyl phosphatidylethanolamine; DOPS, dioleoyl phosphatidylserine; NAcDOPE, *N*-acetyl dioleoyl phosphatidylethanolamine; DOPG, dioleoyl phosphatidylglycerol; OMeDOPA, *O*-methyl dioleoyl phosphatidic acid; DOPA, dioleoyl phosphatidic acid.

Phospholipid headgroup specificity

The results presented in Fig. 1 indicate the dependence of ATPase activity upon the structure of the phospholipid headgroup. We used for this study the dioleoyl series of phospholipids in order to minimise the influence of the physical state of the fatty acid chains on enzyme activity. Assays were carried out at 37°C well above the liquid crystalline to crystalline phase transition for these lipids. Only in the case of cardiolipin was the fatty acid composition undefined; gas-liquid chromatographic analysis of the fatty acid methyl esters derived from a sample of cardiolipin by transesterification showed that 18 : 1 and 18 : 2 fatty acid chains comprised 77% of the total so that the fatty acid chains were sufficiently fluid at 37°C not to interfere with the analysis of headgroup specificity.

It is apparent from Fig. 1 that phospholipids which differ in their headgroup charge structure also differ in the extent to which they can support ATPase activity. The charge structure attributed to each phospholipid molecule in Fig. 1 assumes that each potential charge is fully expressed. This will be true for most phospholipids, but in the case of phosphatidyl serine and phosphatidic acid the published pK data suggest that these lipids will not fully express their potential charge under the assay conditions [12]. However, these pK values were obtained for pure lipid bilayers and they may not be the same for annular lipids in intimate contact with a membrane protein.

The zwitterionic dioleoyl phospholipids support the highest ATPase activity while dioleoyl phosphatidic acid with two negative charges supports an activity approximately 25-fold lower. Phospholipids with a single negative charge (dioleoyl phosphatidylglycerol and the unphysiological synthetic lipids *O*-methyl dioleoyl phosphatidic acid and *N*-acetyl dioleoyl phosphatidylethanolamine) support an activity about 5-fold lower than dioleoyl phosphatidylcholine, while dioleoyl phosphatidylserine, which is zwitterionic with an additional negative charge supports an ATPase activity intermediate between that supported by zwitterionic phospholipids (dioleoyl phosphatidylcholine and -ethanolamine) and that supported by those with a single negative charge.

The results obtained with cardiolipin are of particular interest. This lipid has two negative charges, like dioleoyl phosphatidic acid, but supports an ATPase activity which is about 5 times higher. The ATPase activity is, in fact, very similar to that supported by these phospholipids with a single negative charge. Cardiolipin is synthesized in vivo by condensing two molecules of phosphatidylglycerol (a lipid with a single negative charge) so that the resulting molecule resembles two diacyl phospholipids joined at the headgroup by a glycerol bridge [13]. These results clearly indicate that the bridge between the negatively charged headgroups on cardiolipin has little effect on the activity supported by this lipid.

The observation that cardiolipin, dioleoyl phosphatidylglycerol, *O*-methyl-dioleoyl phosphatidic acid and *N*-acetyl dioleoyl phosphatidylethanolamine which have structurally different headgroups all support a similar ATPase activity suggest that it is the charge structure of the headgroup that is the dominant factor in determining the phospholipid headgroup specificity of the Ca^{2+} pump. Each of these lipids carries a single negative charge on each diacyl glycerophosphate moiety.

Calcium uptake in reconstituted membrane vesicles

In order to determine whether the lipid specificity for calcium-accumulating activity is the same as that for Ca^{2+}-dependent ATPase activity we attempted to reconstitute the protein into vesicles comprising the different phospholipids. The method used was an adaptation of the cholate dialysis procedure of Racker [14]. This involves solubilising the calcium pump protein with cholate together with a large excess of the test lipid. The detergent is slowly dialysed away as a result of which the lipid-protein-detergent micelles grow larger and eventually become detergent-free lipid vesicles incorporating protein. The formation of vesicles depends critically on the conditions used (Warren, G.B. et al., in preparation), and we have used a protocol for these experiments which we have found to be reliable and reproducible. The ATP-dependent uptake of $^{45}Ca^{2+}$ into these vesicles is measured in the presence of potassium oxalate so that as the calcium concentration increases inside the vesicles, calcium precipitates and accumulated $^{45}Ca^{2+}$ can therefore be measured by a filtration assay.

The results presented in Table II clearly show that, while all the test lipids support significant ATPase activity, three of them were unable to support the accumulation of calcium. This inability could not be correlated with the chemical structure or charge on the phospholipid headgroup; dioleoyl phosphatidylethanolamine has the same headgroup charge as dioleoyl phosphatidylcholine but does not support calcium accumulation; dioleoyl phosphatidylglycerol supports calcium accumulation, unlike the two other lipids with a single negative charge (*N*-acetyl dioleoyl phosphatidylethanolamine and *O*-methyl dioleoyl phosphatidic acid). There are two possible explanations for these results because the assay measures calcium accumulation directly and calcium transport by the protein only indirectly. The test lipid could be uncoupling the hydrolysis of ATP from the transport of calcium so that the protein can no longer convey the ion across the membrane.

TABLE II

PROPERTIES OF MEMBRANES RECONSTITUTED FROM SARCOPLASMIC RETICULUM USING DIFFERENT LIPIDS

Reconstitution experiments were carried out as described in Materials and Methods. In each case the amount of lipid used was the same; in the last three experiments reported equal amounts (by weight) of the two lipids were used. The Ca^{2+} accumulating and ATPase activities are in initial rates expressed in μmol/min per mg enzyme at 25°C.

Lipid	Ca^{2+} accumulating activity	ATPase activity
Dioleoyl phosphatidylcholine	0.84	1.55
Dioleoyl phosphatidylethanolamine	0	2.03
Dioleoyl phosphatidylserene	0.43	0.37
N-Acetyl dioleoyl phosphatidic acid	0	0.32
Dioleoyl phosphatidylglycerol	0.17	0.32
O-Methyl dioleoylphosphatidic acid	0	0.38
Dioleoyl phosphatidic acid	0.24	0.15
Dioleoyl phosphatidylethanolamine + dioleoyl phosphatidylcholine	0.68	1.06
N-Acetyl dioleoyl phosphatidic acid + dioleoyl phosphatidylcholine	0.05	0.45
O-Methyl dioleoyl phosphatidic acid + dioleoyl phosphatidylcholine	1.20	1.01

Alternatively, the test lipid may not affect the coupling of ATP hydrolysis to calcium transport but may be unable to form a sealed vesicle in which the Ca^{2+} can be accumulated. It is this second possibility that interests us here. Using electron microscopy we examined the reconstituted complexes to determine which lipids formed morphologically-intact vesicles. In all cases where calcium accumulation was observed, the electron micrographs clearly showed the presence of smooth intact vesicles; an example using dioleoyl phospahtidylcholine is shown in Fig. 2A. Intact vesicles were not observed using lipids which did not support calcium accumulation. An example using dioleoyl phosphatidylethanolamine is shown in Fig. 2B; the membranes from irregularly stacked sheets showing no evidence of a membrane-enclosed volume into which calcium could be transported.

Of course, it is possible that those lipids which do not support calcium accumulation because they do not form sealed vesicles, may, at the same time, uncouple ATP hydrolysis from calcium transport. To test this possibility, we prepared binary lipid mixtures comprising a lipid which did support calcium accumulation in reconstituted systems together with a lipid which did not. Dioleoyl phosphatidylcholine was dispersed in $CHCl_3$ together with an equal weight of either dioleoyl phosphatidylethanolamine, *N*-acetyl dioleoyl phosphatidylethanolamine or *O*-methyl dioleoyl phosphatidic acid. After removal of $CHCl_3$ and dispersal in cholate the lipid mixture was used in the reconstitution procedure described in Materials and Methods. In each case the lipid mixture supported significant ATPase activity (Table II) which was, however, very different from that supported by dioleoyl phosphatidylcholine alone. This suggests that the protein is interacting with both of the lipids in the binary mixture; in other words both of the lipids in the mixture appear to be present in the lipid annulus which determines the functioning of the protein. In all cases (Table I) the binary lipid mixture supported significant calcium accumulation despite the interaction of the protein with lipid molecules which did not support calcium accumulation when present on their own. This suggests that those lipids which do not support calcium accumulation do not uncouple ATPase activity from calcium transport; they are inactive because they do not form sealed vesicles. When mixed with dioleoyl phosphatidylcholine these lipids are able to form sealed vesicles as shown in Fig. 2C using dioleoyl phosphatidylcholine and dioleoyl phosphatidylethanolamine as an example.

We must emphasize that these results do not eliminate any particular lipid from a role in calcium transport in vivo simply because it cannot support calcium accumulation on its own. Biological membranes contain many lipid species and the precise composition is optimal for membrane function; many of these essential lipid species are unable to form sealed vesicles on their own. As an example, phosphatidylcholines and phosphatidylethanolamines comprise more than 85% of the headgroups in native sarcoplasmic reticulum membranes. Phosphatidylcholine supports calcium accumulation in reconstituted complexes; phosphatidylethanolamine does not because it cannot form sealed vesicles. Nevertheless, a mixture of phosphatidylcholine and -ethanolamine is essential for efficient calcium accumulation because phosphatidylethanolamine appears to be involved in the microscopic sealing of the calcium pump protein into the membrane bilayer.

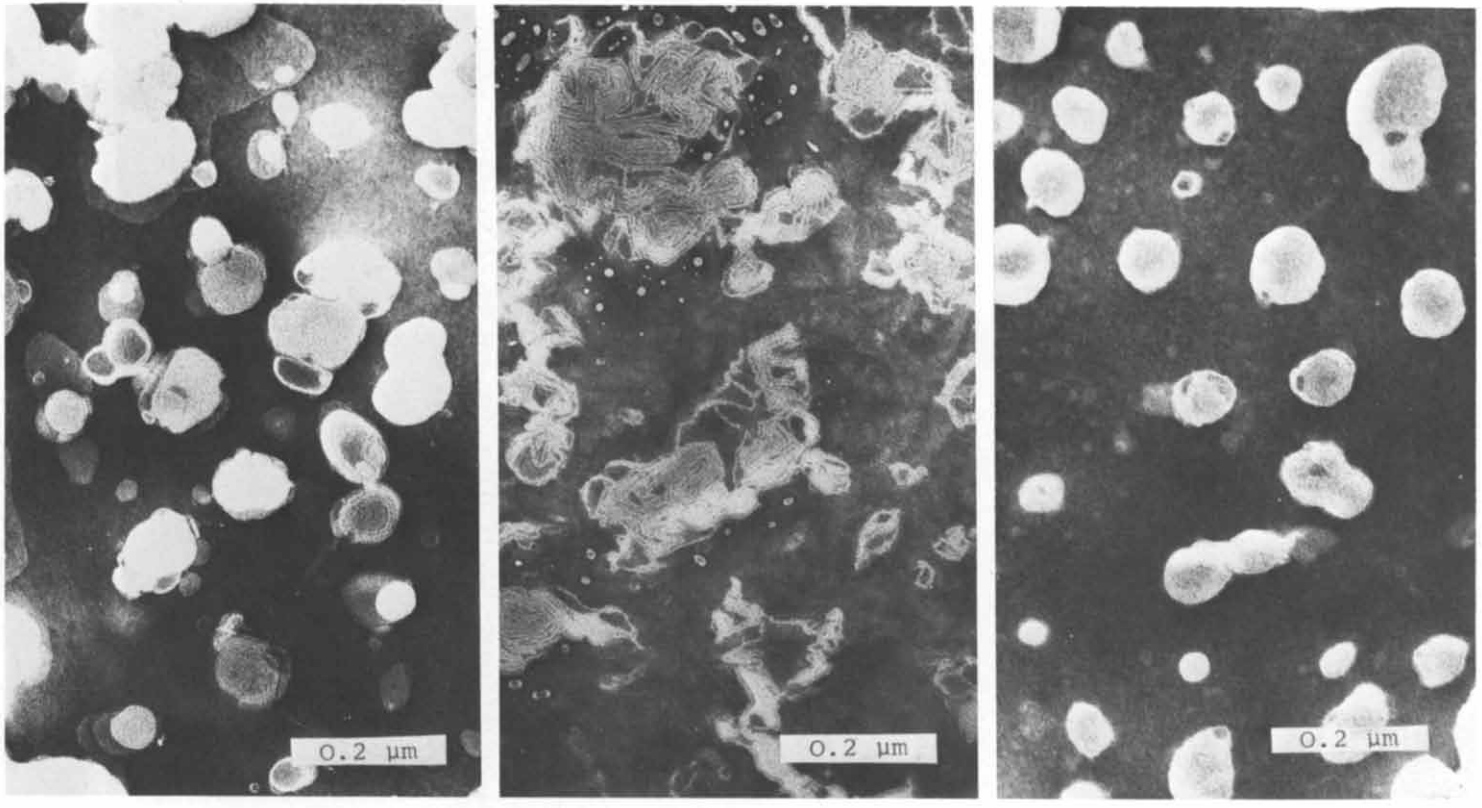

Fig. 2. Electron micrographs of membranes reconstituted from sarcoplasmic reticulum negatively stained with 2% phosphotungstate. The lipids used were (A) dioleoyl phosphatidylcholine; (B) dioleoyl phosphatidylethanolamine; (C) equal amounts (by weight) of dioleoyl phosphatidylcholine and dioleoyl phosphatidylethanolamine.

Our results are in partial disagreement with those of Racker and co-workers [4—6,15] who observed calcium uptake in reconstituted vesicles comprising phosphatidylethanolamine from soya lipids, whereas phosphatidylcholine was ineffective. These workers found, as we do, that mixtures of phosphatidylethanolamine and phosphatidylcholine were more effective than phosphatidylethanolamine alone, and that calcium uptake was not observed for vesicles reconstituted using *N*-acetyl-phosphatidylethanolamine [15]. These discrepancies will be examined and discussed in a subsequent paper (Warren, G.B. et al., in preparation).

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