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PHOSPHATIDYLCHOLINE ACCESSIBILITY IN SINGLE BILAYER VESICLES PREPARED FROM RAT LIVER MICROSOMAL LIPIDS CONTAINING PROTEOLIPIDS

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SUMMARY

Proteolipids were detected in lipid extracts of rat liver mitochondria, microsomes and 100,000 xg soluble fraction. In view of the rapid transbilayer movement of phosphatidylcholine in liver microsomal membranes the possible action of microsomal proteolipids as mediators of phospholipid transmembrane movement was investigated. Single bilayer lipid vesicles were prepared by sonication or by a cholate dispersion technique from either total or proteolipid-free lipid extracts from microsomes. Accessibility of phosphatidylcholine in these membranes to a specific phosphatidylcholine exchange protein was restricted to the amount located in the outer monolayer of the vesicles.

INTRODUCTION

Proteolipids, initially isolated from bovine brain (1), occur in a wide variety of animal and plant tissues, where they appear to be membrane constituents. Except for mitochondria and chloroplasts where these chloroform/methanol-soluble proteins seem to play a role as proton translocators (2,3) not much is known about the function of proteolipids in other organelles (for reviews see refs. 4,5). In agreement with what would be expected from highly hydrophobic proteins, experimental evidence has been provided to suggest that proteolipids are deeply embedded in the apolar region of the bilayer (5-10).

It has been shown that intrinsic proteins from the erythrocyte membrane facilitate transbilayer movement of phospholipids in single bilayer vesicles (11-13). On the other hand rapid transbilayer movements of phospholipids have been shown to occur in a number of isolated membranes (14-20) while no such movements are found in vesicles derived from the phospholipids of these membranes. This has lead to the suggestion (21) that perhaps some membrane proteins ("flippases") could function in the translocation of phospholipids. Because proteolipids show a high hydrophobicity and may be ubiquitous membrane components, it became of interest to investigate their possible action as mediators of phospholipid transmembrane

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movements. Since the characteristic insolubility of proteolipids in ether (2) precluded the use of large unilamellar vesicles prepared by the method of Deamer and Bangham (22), it was decided to study the exchangeability of phosphatidylcholine in small vesicles as an index of phospholipid transbilayer movement.

EXPERIMENTAL

Materials

Dipalmitoylphosphatidyl[*Me*-^{14}C]choline (spec. act. 50 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, U.K.). Bovine liver phosphatidylcholine-exchange protein was purified by an established procedure (23) and proteolipid apoprotein from bovine myelin was prepared as reported (24). They were kindly donated, respectively, by Prof. K.W.A. Wirtz from this laboratory and by Dr. P.J. Brophy (University of Stirling, Scotland). Reagents for gel electrophoresis were obtained from UCB (Brussels, Belgium) and molecular weight markers from BDH (Poole, U.K.).

Methods

Rat liver subcellular fractions. Microsomes and post-microsomal supernatant were prepared as described (25); mitochondria as reported by Greenawalt (26). Lipids were extracted from these fractions by the method of Folch-Pi and Sakura (27).

Multilamellar liposomes and unilamellar vesicles. Liposomes were prepared in 50 mM Tris-HCl, 5 mM EDTA (pH 7.4), centrifuged and resuspended as described by Wirtz *et al.* (28). Single-bilayer vesicles were prepared either by sonication or by the cholate dispersion technique as described (25).

Phosphatidylcholine exchange. The exchangeable pool of phosphatidylcholine in unilamellar vesicles (containing traces of [^{14}C]phosphatidylcholine and of [^{3}H]cholesteryloleate) was determined as described (29). Recoveries of vesicles were 90% or better.

Gel electrophoresis. Electrophoresis in 15% acrylamide gels (acrylamide/bisacrylamide 1:20, w/w) containing 0.1% sodium dodecylsulfate (SDS) was performed according to Laemmli (30).

Analytical methods. Lipid phosphorus was determined by the method of Rouser *et al.* (31) and proteolipid protein according to Mokrasch (32).

RESULTS AND DISCUSSION

Table I summarizes the quantitative distribution of chloroform/methanol-soluble protein obtained from rat liver mitochondria, microsomes and 100,000 x *g* supernatant. The SDS-polyacrylamide gels after electrophoresis of lipid extracts from these subcellular fractions are shown in Fig. 1 together with one which had been loaded with myelin proteolipid apoprotein. In agreement with the data from Table I which suggest the presence of proteolipid in the post-microsomal supernatant, a major band, corresponding to a molecular weight of about 12,000, can be seen in the gel (Fig. 1). This is in contrast to the general idea that these proteins are exclusively membrane-associated components (5). Svensson *et al.* (33) have described the presence of a lipoprotein complex, believed to be a microsomal membrane precursor, in the post-microsomal supernatant of rat liver. It was noticed by these authors that 67% of

TABLE I

COMPOSITION OF CHLOROFORM/METHANOL EXTRACTS OBTAINED FROM RAT LIVER SUBCELLULAR FRACTIONS

Fraction[a]	Phospholipid	Proteolipid	Proteolipid/Phospholipid
	(nmol/mg protein)	(µg/mg protein)	(µg/µmol)
Mitochondria	207	6	30
Microsomes	517	9	18
Post-microsomal supernatant	12	1.6	139

[a]The subcellular fractions and their extracts were obtained as described under *Methods*. The values represent means of two to four determinations.

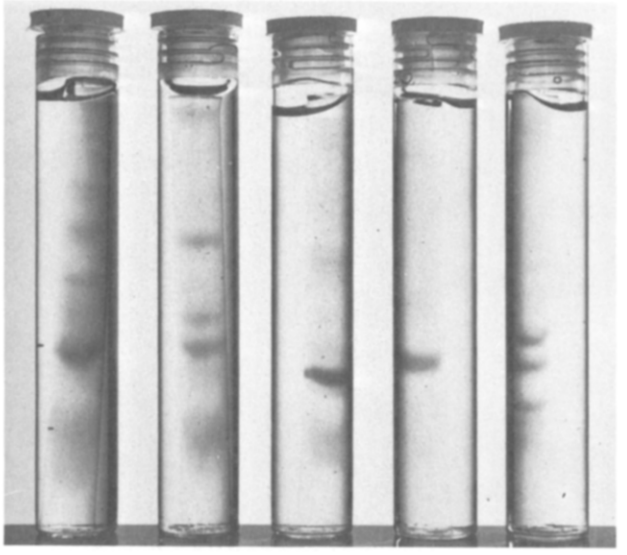

Fig. 1. SDS-polyacrylamide gels after electrophoresis of the following preparations (from left to right): microsomal extract (0.8 µmol lipid P), mitochondrial extract (0.9 µmol lipid P), post-microsomal supernatant extract (0.23 µmol lipid P), bovine myelin proteolipid apoprotein (24 µg) and molecular weight markers (40 µg). These markers correspond to the following molecular weights (from top to bottom): 16 949, 14 404, 8 159, 6 214 and 2 512. Electrophoresis, carried out as described under *Methods*, was stopped when the bromophenol blue front had travelled till 1 cm from the bottom of each gel. Diffuse bands seen in the lower part of gels loaded with lipid extracts arise from phospholipid which moves together with the tracking dye (these bands are not Coomassie Brilliant Blue positive).

TABLE II

COMPOSITION OF MICROSOMAL EXTRACTS BEFORE AND AFTER ETHER TREATMENT

	Proteolipids	Phospholipids	Phospholipid classes (%)[b]			
	(μg)	(μmol)	PC	PE	PI+PS	SPH
Before	3 036	230	62.5	20.5	13.9	3.2
After	n.d.[a]	213	64.2	20.9	12.0	3.4

[a] Not detectable neither in the proteolipid assay nor in SDS-polyacrylamide gels.
[b] Values are expressed as percentage of total lipid phosphorus recovered. P, phosphatidyl; C, choline; E, ethanolamine; I, inositol; S, serine; SPH, sphingomyelin.

this complex could be recovered in the organic phase of a $CHCl_3/CH_3OH/H_2O$ mixture. After SDS-polyacrylamide gel electrophoresis two bands corresponding to molecular weights of 12,000 and 68,000 were found. Based on similar amino acid composition and the possibility of interconversion between the two forms it was suggested that only one peptide was present (33). It is obvious from the gel depicted in Fig. 1 that almost exclusively the proteolipid with an apparent molecular weight of 12,000 is observed under the conditions used. Microsomal and mitochondrial extracts showed several bands in the same type of gel (Fig. 1) with molecular weights ranging from about 15,000 to over 50,000. While the isolation of proteolipids from rat liver mitochondria has been described (5), research on proteolipids from rat liver microsomes is limited to documenting their presence (34).

Proteolipids can be removed from chloroform/methanol solutions by diethylether precipitation (2). Table II shows that this treatment selectively removes microsomal proteolipids from the solution without affecting its phospholipid composition. Proteolipid-free extracts obtained in this way do not give rise to Coomassie Blue positive bands after electrophoresis in SDS-polyacrylamide gels (not shown). Therefore it is justified to use ether-treated extracts for the preparation of proteolipid-free model membranes. Chloroform/methanol-soluble proteins from various sources can be readily incorporated in model membranes using a number of different techniques (3,5,7,9,10,24). The presence of proteolipid in lipid extracts makes it possible to prepare vesicles from these extracts in the same way as usual for proteolipid-free preparations (9).

TABLE III

MAXIMAL PHOSPHATIDYLCHOLINE EXCHANGEABILITY IN VESICLES WITH DIFFERENT COMPOSITION AND PREPARED BY DIFFERENT METHODS

Vesicle composition[a]	Method of preparation	Exchangeability (%)[b]
Total extract	Sonication	66.1
Total extract	Cholate	75.0
Proteolipid-free extract	Sonication	64.8
Proteolipid-free extract	Cholate	71.0

[a] In each case 5 mol % lysophosphatidylcholine was added.
[b] Sonicated vesicles (100 nmol total phospholipid, 2 nmol phosphatidyl-[*Me*-^{14}C]choline and a trace of [^{3}H]cholesteryloleate) were incubated with multilamellar liposomes (6 µmol phospholipid) prepared from proteolipid-free extract, and 16.5 µg phosphatidylcholine-exchange protein (final volume 500 µl). "Cholate-vesicles" (100 nmol total phospholipid, 0.5 nmol phosphatidyl[*Me*-^{14}C]choline and a trace of [^{3}H]cholesteryloleate) were sized by Sepharose 4B chromatography as described (37) and incubated with proteolipid-free multilayered liposomes(7.2 µmol phospholipid) in the presence of 16.5 µg exchange protein (final volume 350 µl). Incubations were performed at 25° C for 4 h and exchangeabilities were calculated as indicated in the Experimental section. In blank experiments without exchange protein less than 5% exchange took place.

The exchangeability of vesicular phosphatidylcholine was determined after incubation with proteolipid-free multilayered liposomes and exchange protein at 25°C for 4 h (Table III). From control experiments it could be concluded that, under the conditions employed, a period of 4 h would be largely in excess of that required to achieve complete transfer of [^{14}C]phosphatidylcholine from the outer monolayer of the vesicles to the liposomes. Table III shows that the fraction of phosphatidylcholine maximally exchangeable in sonicated vesicles prepared from any of the two extracts agrees with published data for sonicated microsomal lipids (18). The limited exchangeability of phosphatidylcholine in vesicles prepared by a cholate dispersion technique indicates that the residual traces of cholate do not facilitate transbilayer movements. A similar conclusion was drawn regarding phosphatidylcholine mobilities in isolated microsomal vesicles (35) and in unilamellar vesicles prepared by cholate dialysis (36). At the same time the results in Table III demonstrate that the presence of proteolipids in the vesicular membranes does not significantly increase the phosphatidylcholine pool which is available for exchange from the outer monolayer. The slightly larger fraction of phosphatidylcholine available in vesicles prepared by cholate dispersion as

compared to that in sonicated vesicles is in line with the somewhat smaller size of the former (37). The combined results indicate that proteolipids, the *in vivo* functioning of which is still largely unknown, do not facilitate transbilayer movements of phosphatidylcholine in single bilayer vesicles. This is in contrast to the enhanced transbilayer movements observed upon incorporation of the intrinsic erythrocyte membrane proteins glycophorin (11, 12) and band 3 protein (13) in such vesicles. On the other hand, another intrinsic membrane protein, cytochrome c oxidase, did not enhance transbilayer movements in small unilamellar vesicles (36).

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