Biochimica et Biophysica Acta, 1105 (1992) 207–212

BBAMEM 75588

Transport and decarboxylation of liposomal phosphatidylserine: effect of cations

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(Received 29 November 1991)

Key words: Phosphatidylserine decarboxylation; Pyrene-labeled phospholipid; Liposome; Phospholipid transfer; Transfer protein, nonspecific lipid; Nonspecific lipid transfer protein; Mitochondrion; (Rat liver); (Ehrlich ascites tumor)

Decarboxylation of liposomal phosphatidylserine by rat liver and Ehrlich ascites tumor mitochondria was taken as a measure of phospholipid transfer. The process was found to be greatly enhanced by the cytoplasmic fraction of rat liver containing nonspecific lipid transfer protein, but not by the cytoplasmic fraction from tumor cells. Divalent cations, like rat liver cytoplasmic fraction, also stimulated phosphatidylserine decarboxylation by facilitating the lipid association with mitochondria. In contrast, these cations, at 0.5–3 mM concentration, inhibited the cytoplasmic fraction-mediated phosphatidylserine transport. Monovalent cations were also inhibitory but at 20–150 mM concentration. However, they had no effect on phosphatidylserine decarboxylation in the absence of the cytoplasmic fraction. Further experiments with purified rat liver nonspecific lipid transfer protein and pyrene-labeled phosphatidylcholine and phosphatidylserine have shown that cations by neutralizing net negative charge on phospholipid donor vesicles decrease the interaction of protein with them and, in consequence, lower the rate of release of molecules to the water phase.

Introduction

The intracellular mechanism of phosphatidylserine (PS) transport and assembly into mitochondrial membranes of mammalian tissues is still poorly understood. The phospholipid is solely synthesized in endoplasmic reticulum [1]. For decarboxylation it has to be transported to mitochondria [2,3]. The participation of cytoplasmic nonspecific lipid transfer protein(s) (nsL-TP) in this process seems to be documented [4–6]. The protein is basic (isoelectric point > 8.5) [7] due to very high contents of lysine [6,8] which makes an electrostatic interaction possible. However, the mode of action of nsL-TP in respect to PS transferring remained uncertain. To make it more clear we performed the present study with mono- and divalent cations knowing that the solubilized PS decarboxylase (EC 4.1.1.65) did not require any divalent cations for its activity [9]. In our experiments decarboxylation of liposomal [1-^{14}C]PS was taken as a measure of phospholipid transfer. The studies were performed with rat liver and Ehrlich ascites tumor (EAT) mitochondria. In addition, the effect of cations was also investigated with pyrene-labeled phospholipids and purified rat liver nsL-TP. The obtained data has shown that cations by neutralizing negative charge on donor liposomes decreased the interaction of nsL-TP with them. On the other hand, divalent cations by causing fusion or association of negatively charged liposomes with mitochondrial membranes stimulate PS decarboxylation.

Preliminary results of these studies were presented at the IUB Congress in Prague [10].

Abbreviations: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CL, cardiolipin; PA, phosphatidic acid; Pyr_xPC, 1-palmitoyl-2-(1-pyrenyl)$_x$-*sn*-glycero-3-phosphocholine, *x* indicates the total number of carbon units (including carbonyl one) in the aliphatic chain; Pyr_6PS, 1-palmitoyl-2-(1-pyrenyl)hexanoyl-*sn*-glycero-3-phosphoserine; TNP-PE, 2,4,6-trinitrophenylphosphatidylethanolamine; CF, the cytoplasmic fraction; nsL-TP, nonspecific lipid transfer protein (sterol carrier protein 2); EAT, Ehrlich ascites tumor.

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Materials and Methods

Isolation of mitochondria, microsomes and the cytoplasmic fraction

Liver mitochondria and microsomes from adult male Wistar rats were isolated in 225 mM mannitol, 75 mM

sucrose, 3 mM Tris-HCl, 0.5 mM EGTA (pH 7.4) by a conventional procedure [11]. The obtained fractions were suspended in the same medium as above but without EGTA. Mitochondria from EAT cells grown in the peritoneal cavity of adult female Swiss albino mice were isolated in a medium containing 210 mM mannitol, 70 mM sucrose, 5 mM K-Hepes (pH 7.2) and 0.5% defatted bovine serum albumin with the use of either Nagarse [12] or digitonin [13].

The cytoplasmic fraction of rat liver (CF_{RL}) was obtained by centrifugation of post-mitochondrial supernatant at $105000 \times g$ for 1 h. The fraction was made free of lipoproteins by sedimenting them at pH 5.1 [14]. The same procedure was used for the cytoplasmic fraction (CF_E) from EAT cells after disruption them by osmotic shock [15].

Purification of nonspecific lipid transfer protein (nsL-TP) and assay of its transfer activity

NsL-TP from rat liver was purified according to Poorthuis et al. [16] with the following modifications. The hydroxyapatite step of original procedure was omitted and the active fractions eluted with 10 mM Tris-HCl/5 mM 2-mercaptoethanol/10% (v/v) glycerol (pH 7.4) from Sephadex G-50 column were collected, concentrated to small volume with Aquacide III and heated in a water bath at 90°C for 5 min. The denaturated proteins were discarded after centrifugation at $10000 \times g$ for 10 min and the supernatant containing active protein was stored at −20°C in a Tris-mercaptoethanol buffer enriched with 50% glycerol. In SDS electrophoresis on 15% polyacrylamide gel the purified protein moved as a double band behind α-lactalbumine (M_r 14400). NsL-TP activity was assayed by measuring the PS, PE or PC transfer from liposomes to rat liver mitochondria as described in Ref. 17. Liposomes used for these assays were prepared from total microsomal phospholipids containing ^{14}C-PS, ^{14}C-PE or ^{14}C-PC.

Assay of PS decarboxylase activity

Decarboxylation of [1-^{14}C]PS was followed by measuring the production of $^{14}CO_2$ [9]. The incubation medium contained in a total volume of 2.5 ml: 250 mM sucrose, 10 mM Tris-HCl (pH 7.4), PS liposomes (400 nmoles phospholipid phosphorus of a mixture of unlabeled and [1-^{14}C]PS, about 20000 dpm) and mitochondria (1.5 mg protein). Where indicated CF_{RL} or CF_E and/or different cations were added. Incubation was carried out under constant shaking at 37°C.

Labeling of microsomal phospholipids, lipid extraction, purification and analysis

Microsomal phospholipids were labeled in base-exchange reaction with [^{14}C]ethanolamine, choline or serine as described by Bjerve [18]. Lipids were extracted according to Bligh-Dyer [19]. Purification of labeled PS was achieved according to Comfurius and Zwaal [20]. Unlabeled PS was extracted [21] from beef brain and purified as labeled PS. Thin-layer chromatography analysis showed that both, labeled and unlabeled PS migrated as a single spot in a two-dimensional system of Rouser et al. [22]. Phospholipid phosphorus was determined as in Ref. 22.

Fluorescence measurements

Transfer of pyrene-labeled phospholipids (Pyr_xPC and Pyr_6PS) from unquenched (or quenched) donor vesicles to unlabeled, egg PC acceptor vesicles was measured at 37°C in the absence or presence of nsL-TP. Small unilamellar vesicles (both donor and acceptor) were prepared according to Batzri and Korn [23]. Usually, donor vesicles were made by rapid injection of a few microliters of ethanolic solution of lipid material into the cuvette filled with 2 ml of 20 mM Tris-HCl, 1 mM EDTA (pH 7.4), whereas acceptor vesicles were made separately in the same buffer and added thereafter. The experiments were performed with the use of a Hitachi F-4000 spectrofluorometer equipped with a termostated cuvette holder and a magnetic stirrer device. The excitation and emission wavelenghts were 344 and 378 nm and the slits 3 and 10 nm, respectively. The increase of pyrene monomer fluorescence intensity resulting from the transfer of labeled phospholipid molecules from donor vesicles was monitored as a function of time and initial slope of the progressing curve was taken as a measure for the transfer rate [24]. All fluorescence measurements were done in laboratory of Dr. P.J. Somerharju at the Department of Medical Chemistry of the University of Helsinki.

Other procedures

Protein concentration was determined by the method of Lowry et al. [25]. Radioactivity was measured by liquid-scintillation counting using Beckman LS 3801 spectrometer.

Results

Rat liver or EAT mitochondria decarboxylate liposomal PS with the rate that is linearly proportional up to 0.6 mg mitochondrial protein added per ml medium. The addition of CF_{RL} but not CF_E, markedly stimulated this process. With the former fraction the maximum stimulatory effect has been reached with 10 mg protein added in standard incubation conditions with both types of mitochondria. This amount was therefore used in all subsequent experiments. No stimulatory effect however, has been observed with CF_E up to 10 mg protein added with both rat liver and EAT mitochondria. The decarboxylation with 400 nmol PS in the presence of CF_{RL} appeared to be rectilinear for ap-

TABLE I

Stimulation of PS decarboxylation by rat liver cytoplasmic fraction (CF_{RL})

Mitochondria (1.5 mg protein) were incubated with [1-^{14}C]phosphatidylserine liposomes as described in Materials and Methods. The values in brackets represent the number of independent experiments.

Source of mitochondria		PS decarboxylase activity (nmol $^{14}CO_2$/mg per h ± S.D.)	Stimulation factor
Rat liver	$-CF_{RL}$	7.82 ± 1.72 (9)	
	$+CF_{RL}$	34.17 ± 6.11 (11)	4.4
Ehrlich ascites tumor cells	$-CF_{RL}$	17.37 ± 6.28 (8)	
	$+CF_{RL}$	65.59 ± 18.68 (10)	3.8

prox. 120 min. Insignificant slowing down of releasing $^{14}CO_2$ took place within 180 min of incubation. As shown in Table I, tumor mitochondria exhibited 2.5-fold higher activity of decarboxylation than liver mitochondria. This result has been obtained with tumor mitochondria independently of the method of isolation. The addition of CR_{RL} stimulated the reaction approximately 4-fold with both, liver and tumor mitochondria.

To elucidate whether charge of the substrate influence the protein-mediated transport, we examined the effect of divalent cations on liposomal PS decarboxylation. As shown in Fig. 1A, addition of 1–3 mM of Ca^{2+} inhibited decarboxylation of liposomal PS in the presence of CF_{RL} by both types of mitochondria, whereas at 5 mM concentration of this cation no inhibitory effect was further observed. On the other hand, a stimulation of PS decarboxylation was found in the absence of CF_{RL} upon addition of increasing concentration of Ca^{2+} up to 3 mM. Similar data has also been obtained for Mg^{2+} studied only with rat liver mitochondria (Fig. 1B). However, with Mn^{2+}, a maximum stimulating effect appeared at 0.5 mM concentration after which a sharp decrease in activity was observed (Fig. 1C). Further experiments undertaken with monovalent cations (concentration range 10–150 mM) showed a progressive inhibition of PS decarboxylation enhanced in the presence of either CF_{RL} (Fig. 2) or 5 mM Mg^{2+} (the latter studied only with K^+; data not shown). The inhibitory effect was independent on chloride or sulfate anion. In contrast to divalent cations, none of monovalent cations has affected the rate of PS decarboxylation in the absence of CF_{RL} (Fig. 2).

The data presented above has pointed out to the effect of the cations on PS transport process. Two possible explanations could employ either the affecting of binding of PS to the lipid transfer protein [6] or weakening an interaction of the protein with less negatively charged donor liposomes. To discriminate between these possibilities, the experiments were performed with purified rat liver nsL-TP and fluorescent phospholipids. As shown in Table II, a protein-dependent initial transfer rate of Pyr_6PC was low in case of

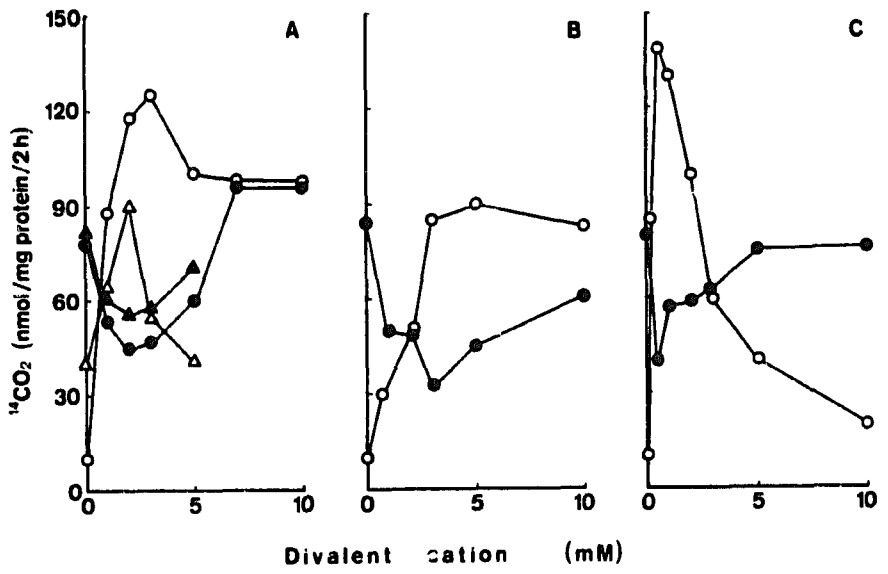

Fig. 1. Effect of divalent cations on the decarboxylation of liposomal PS by rat liver (circles) and EAT (triangles) mitochondria in the absence (open symbols) and the presence (full symbols) of CF_{RL}. (A) Ca^{2+}; (B) Mg^{2+}; (C) Mn^{2+}.

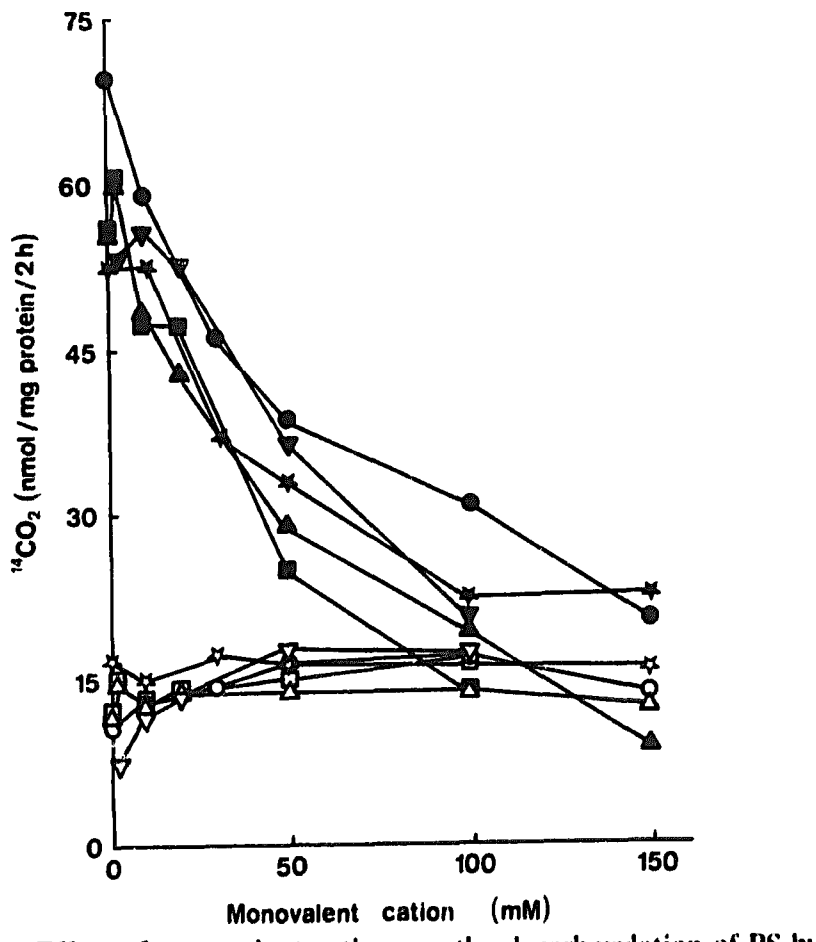

Fig. 2. Effect of monovalent cations on the decarboxylation of PS by rat liver mitochondria in the absence (open symbols) and presence (full symbols) of CF_{RL}. LiCl (□, ■); NaCl (△, ▲); Tris-Cl (▽ ▼); KCl (○, ●); NH_4Cl (☆, ★).

TABLE II

Transfer of pyrene-labeled phospholipids from donor vesicles of different composition to egg PC acceptor vesicles in the presence and absence of nsL-TP

In all measurements donor vesicles contained 1 nmol pyrene-labeled phospholipid. Where indicated TNP-PE and PA were mixed with Pyr_6PC in a molar ratio of 0.1 and 0.2, respectively. NsL-TP (9 μg) was added after acceptor vesicles (100 nmol egg PC). The values given are the average of three determinations; S.E. omitted for clarity's sake, did not exceed 15%.

Donor vesicles	Initial rate of fluorescence monomer increase (arbitrary units)		Stimulation factor
	− nsL-TP	+ nsL-TP	
Pyr_6PC	18.5	24.4	1.32
Pyr_6PC/TNP-PE	19.0	27.5	1.45
Pyr_6PC/PA/TNP-PE	21.2	61.8	2.91
+ 10 mM $CaCl_2$	17.6	17.2	–
+ 10 mM $MgCl_2$	13.6	15.0	1.10
Pyr_6PS	128.8	32.4 [a]	–
+ 10 mM $CaCl_2$	28.8	18.4	–
+ 10 mM $MgCl_2$	27.6	18.7	–
+ 150 mM NaCl	16.0	20.0	1.25

[a] Note that rate of spontaneous transfer of Pyr_6PS before addition of protein was 31 units. In other cases it was slightly lower than initial or unchanged

donor vesicles not containing charged phospholipid or upon addition of cations to negatively charged donor vesicles. In contrast, a spontaneous transfer of Pyr_6PS being very fast for first 30 s gradually decreased and addition of nsL-TP 2 min later did not accelerate the transfer rate efficiently. Both divalent and monovalent cations decreased the rate of spontaneous transfer of PS in a concentration-dependent manner. Although, the lack of effect of nsL-TP in the case of PS-donor vesicles in the absence of cations could be partly explained by the fact that significant amount of PS was already transferred to acceptor vesicles, no such assumption can be made for the protein-mediated transfer in the presence of the cations. In this case, a neutralization of the charge made an interaction of the protein less favourable. Mg^{2+} and Ca^{2+} ions acted in a very similar way, exhibited an exponential inhibition curve (not shown).

The high initial rate of spontaneous transfer was not the attribute of Pyr_6PS only. Also Pyr_6PC or Pyr_8PC species can leave rapidly donor vesicles when they contained substantial amounts of PA or CL. The initial rate of spontaneous transfer of PC species rose with increasing amount of PA or CL up to 40 mol% and 20 mol%, respectively. For example, the initial rate of spontaneous transfer of Pyr_8PC rose from 18.8 estimated for vesicles composed of 1 nmol Pyr_8PC, 1 nmol egg PC and 0.2 nmol TNP-PE to 27.6, 53.6 and 131.0 units for vesicles in which part of egg PC was substituted equivalently by 0.1, 0.2 and 0.4 nmol CL, respectively. As in case of Pyr_6PS no acceleration with nsL-TP was found for vesicles made with addition of two highest CL concentration (data not shown).

The transfer activity of the purified protein was also controlled by another vesicle-vesicle assay [26]. In this assay, a separation of negatively charged donor vesicles containing originally 1 nmol pyrene-labeled phospholipid from uncharged acceptor egg PC vesicles was achieved with the use of DEAE cellulose (DE 52, preswollen) columns (0.4 × 1 cm) previously saturated with egg PC. The liposomes were prepared in 20 mM Tris-HCl, 1 mM EDTA (pH 7.4) and the same buffer was used for elution. After 10 min incubation in 37°C of donor and acceptor vesicles together with nsL-TP, the whole mixture (0.5 ml) was applied to DEAE-cellulose column. Negatively charged donor vesicles plus protein retained on the column, whereas uncharged acceptor vesicles were recovered in the elluent. With this method a linearity of Pyr_8PC transfer from donor vesicles consisted of Pyr_8PC, CL and egg PC in molar ratio 1 : 1 : 8 (10 nmol of total lipid) to acceptor vesicles (200 nmol egg PC) was observed with increasing amount of nsL-TP up to 9 μg added per incubation. Furthermore, with this amount of protein, a lipid transfer proceeded linearly for 10 min, reaching a plateau for transferred lipid contents at 30 min. Within this time, about 50% of Pyr_8PC was transferred to acceptor vesicles from which 36.8% was estimated as a protein-dependent transport.

Discussion

The present investigation provides a further support that translocation-dependent decarboxylation of liposomal PS is enhanced by CF_{RL} [4–6,27]. No such effect, however, was visible with CF_E which is probably lacking of nsL-TP. The reason for its absence may be either secretion of the protein as in case of hepatoma cells [28] or decreased number of peroxisomes which in various cells are considered to serve as a deposit of its precursor [29–31]. Although EAT mitochondria exhibited much higher activity of PS decarboxylase than liver mitochondria, the stimulating effect of CF_{RL} was further observed. This may therefore indicate that transport of liposomal PS is, at least, one of the factors limiting the decarboxylation of this lipid. Further evidence for this assumption comes from the experiments with divalent cations which are known to stimulate fusion or association of negatively charged phospholipids with biological membranes [32,33]. As shown in Fig. 1, divalent cations stimulated PS decarboxylation in a concentration-dependent manner and mimic the effect of CF_{RL}. Basing on concentrations required to give a maximum effect, they may be arranged in a series: $Mn^{2+} > Ca^{2+} > Mg^{2+}$. In such aligment they are

also effective in causing fusion of PS small unilamellar vesicles and in lowering in the dielectric constant of the membrane surface, which apart of increase in surface tension lead to increase of hydrophobicity of the membrane surface [34]. In addition, a sharp decreasing in PS decarboxylation at higher concentrations of Mn^{2+} can be ascribed to direct action on enzyme activity itself [9].

Divalent cations in the concentration range 1–3 mM inhibited PS decarboxylation in the presence of the cytoplasmic fraction (Fig. 1). Analogically, although, at much higher concentration acted monovalent cations. Both by neutralizing the charge of membranes made unable an interaction of nsL-TP with them. A more direct evidence supporting this explanation is given from the experiments with purified nsL-TP and fluorescent phospholipids. As shown in Table II, lack of charge on donor vesicles decreased the rate of Pyr_6PC transfer observed in the presence of nsL-TP. Simultanously, cations strongly affected a spontanous transfer rate that is particularly evidenced for Pyr_6PS. A non-stimulatory (or even inhibitory) effect of nsL-TP on Pyr_6PS transport between membranes is most probably caused by its strong electrostatic binding to the vesicles made of this phospholipid. Such interaction affected the rate of release of Pyr_6PS molecules to the aqueous medium and in consequence decreased their transport to the acceptor membrane. In accordance with the above statement of strong electrostatic interaction between cationic protein and negatively charge liposomes was an observation of binding of nsL-TP to DEAE-cellulose column previously pretreated with PC vesicles containing 10 mol% of CL. No such binding of nsL-TP was found for DEAE-cellulose saturated with PC alone. In a similar way, transport of PS may depend on the ratio of negatively charged donor to acceptor vesicles and/or protein. This may explain the data of Voelker [35], who did not find any effect of partially purified nsL-TP on PS transfer between microsomes and mitochondria, but observed it when more purified protein was added in excess.

Recent studies performed with rat liver nsL-TP on sterol transport have shown the positive effect of the presence of negatively charge phospholipids in donor liposome [36,37]. On the contrary, divalent metal ions, high salt and polycation neomycin were inhibitory [36].

In conclusion, the data of the present investigation are in favour of earlier proposal [38,39] that nsL-TP, by interacting with the membrane, lowers the energy barrier to lipid-monomer dissociation. The inability of the protein to binding cholesterol [37,39] makes unlikely its functioning as a carrier. Although other authors were able to show direct binding of fluorescent sterol analogue [40] there is no doubts that nsL-TP is distinct in mode of action from other phospholipid transfer proteins. However, by which way this protein virtually facilitating transfer of lipids between membranes remains to be established (for possible models of action see recent review of Wirtz and Gadella [41]). In any case, the protein became a part of transient collisional complex with donor membrane that seems to be very likely influenced by electrostatic interaction.

A separate question represents an intramitochondrial transport of PS. Our previous data [42] and those of Voelker [35] have shown that decarboxylating enzyme is located in the inner mitochondrial membrane with the active centre exposed to the intermembrane space. PS introduced into outer membrane either by means of nsL-TP or fusion must be then translocated inside of the mitochondrion. An involvement of specific contact sites in the present process has recently been proposed for yeast [43] and rat liver mitochondria [44]. The evidence that Ca^{2+} increased the number of contact sites has also been presented [45]. Thus, the intramitochondrial transport of PS would differ from PA transport which occurs most probably as a free diffusion process [46].

Finally, it should be stressed that our experimental system employed liposomes as a donor of PS. Under conditions used, the addition of nsL-TP highly stimulated phospholipid transport. No such stimulation, however, was found in the study of Vance, who used rat liver microsomal membranes prelabeled in PS by base-exchange reaction [47]. According to this author a newly-synthesized phospholipids are transferred more rapidly than pre-existing. Our data does not seem to be in contradiction with the above statement. In addition to the distinct pools of phospholipids, that are proposed by Vance [47], an effect of protein as a constituent of the donor membrane may also has an influence on the rate and extent of PS transport. Indeed, Voelker [35] has observed a great difference in transport of radioactive PS from microsomes and liposomes to mitochondria.

Acknowledgement

The authors would like to express their gratitude to Dr. Pentti J. Somerharju for offering the fluorescent phospholipids, interesting discussions and inviting one of us (J.Z.) to his laboratory at the Department of Medical Chemistry, University of Helsinki, where fluorescent measurements were performed.

References

1 Bjerve, K.S. (1973) Biochim. Biophys. Acta 306, 396–402.
2 Borkenhagen, L.F., Kennedy, E.P. and Fielding, L. (1961) J. Biol. Chem. 236, PC28–PC30.
3 Dennis, E.A. and Kennedy, E.P. (1972) J. Lipid Res. 13, 263–267.
4 Butler, M.M. and Thompson, W. (1975) Biochim. Biophys. Acta 388, 52–57.
5 Zilversmit, D.B. and Hughes, M.E. (1977) Biochim. Biophys. Acta 469, 99–110.

6 Barańska, J. and Grabarek, Z. (1979) FEBS Lett. 104, 253–257.
7 Bloj, B. and Zilversmit, D.B. (1977) J. Biol. Chem. 252, 1613–1619.
8 Błoj, B., Hughes, M.E., Wilson, D.B. and Zilversmit, D.B. (1978) FEBS Lett. 96, 87–89.
9 Dygas, A. and Zborowski, J. (1989) Acta Biochim. Polon. 36, 131–141.
10 Zborowski, J. and Jasińska, R. (1988) 14th Int. Congress Biochemistry, Prague, Czechoslovakia, Abstracts, Vol. 2, p.97.
11 Hogebcom, G.H. (1955) Methods Enzymol. 1, 16–19.
12 Villalobo, A. and Lehninger, A.L. (1980) Arch. Biochem. Biophys. 203, 473–482.
13 Moreadith, R.W. and Fiskum, G. (1984) Anal. Biochem. 137, 360–367.
14 Wirtz, K.W.A. and Zilversmit, D.B. (1969) Biochim. Biophys. Acta 193, 105–116.
15 Borst, P. (1960) J. Biophys. Biochem. Cytol. 7, 381–383.
16 Poorthuis, B.J.H.M., Glatz, J.F.C., Akeroyd, R. and Wirtz, K.W.A. (1981) Biochim. Biophys. Acta 665, 256–261.
17 Crain, R.C. and Zilversmit, D.B. (1980) Biochemistry 19, 1433–1439.
18 Bjerve, K.S. (1973) Biochim. Biophys. Acta 296, 549–562.
19 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
20 Comfurius, P. and Zwaal, R.F.A. (1977) Biochim. Biophys. Acta 488, 36–42.
21 Folch, J. (1942) J. Biol. Chem. 146, 35–44.
22 Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496.
23 Batzri, S. and Korn, E.D. (1973) Biochim. Biophys. Acta 298, 1015–1019.
24 Somerharju, P., Brockerhoff, H. and Wirtz, K.W.A. (1981) Biochim. Biophys. Acta 649, 521–528.
25 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
26 Hellings, J.A., Kamp, H.H., Wirtz, K.W.A. and Van Deenen, L.L.M. (1974) Eur. J. Biochem. 47, 601–605.
27 Dygas, A., Zborowski, J. and Wojtczak, L. (1980) Acta Biochim. Polon. 27, 153–161.
28 Crain, R.C. and Clark, R.W. (1985) Arch. Biochem. Biophys. 241, 290–297.
29 Poorthuis, B.J.H.M., Van der Krift, Th.P., Teelink, T., Akeroyd, R., Hostetler, K.Y. and Wirtz, K.W.A. (1980) Biochim. Biophys. Acta 600, 376–386.
30 Van Amerongen, A., Helms, J.B., Van der Krift, Th.P., Schutgens, R.B.H. and Wirtz, K.W.A. (1987) Biochim. Biophys. Acta 919, 149–155.
31 Van Heusden, G.P.H., Bos, K., Raetz, C.R. and Wirtz, K.W.A. (1990) J. Biol. Chem. 265, 4105–4110.
32 Hoekstra, D. and Wilschut, J. (1989) in Water Transport in Biological Membranes (Benga, G., ed.), Vol. I, pp. 143–176, CRC Press, Boca Raton, FL.
33 Corazzi, L., Pistolesi, R. and Arienti, G. (1991) J. Neurochem. 56, 207–212.
34 Ohki, S. and Arnold, K. (1990) J. Membr. Biol. 114, 195–203.
35 Voelker, D.R. (1989) J. Biol. Chem. 264, 8019–8025.
36 Schroeder, F., Butko, P., Hapala, I. and Scallen, T.J. (1990) Lipids 25, 669–674.
37 Billheimer, J.T. and Gaylor, J.L. (1990) Biochim. Biophys. Acta 1046, 136–143.
38 Nichols, J.W. and Pagano, R.E. (1983) J. Biol. Chem. 258, 5368–5371.
39 Van Amerongen, A., Demel, R.A., Westerman, J. and Wirtz, K.W.A. (1989) Biochim. Biophys. Acta 1004, 36–43.
40 Schroeder, F., Butko, P., Nemecz, G. and Scallen, T.J. (1990) J. Biol. Chem. 265, 151–157.
41 Wirtz, K.W.A. and Gadella, T.W.J., Jr. (1990) Experientia 46, 592–599.
42 Zborowski, J., Dygas, A. and Wojtczak, L. (1983) FEBS. Lett. 157, 179–182.
43 Simbeni, R., Paltauf, F. and Daum, A. (1990) J. Biol. Chem. 265, 281–285.
44 Faber, B., Hovius, R. and Nicolay, K. (1990) 6th European Bioenergetics Conference, Noordwijkerhout, The Netherlands, EBEC Reports, Vol. 6, p. 86.
45 Verkleij, A.J. (1984) Biochim. Biophys. Acta 779, 43–63.
46 Wojtczak, L., Barańska, J. and Zborowski, J. (1990) Biochim. Biophys. Acta 1044, 284–287.
47 Vance, J.E. (1991) J. Biol. Chem. 266, 89–97.