LIPID TRANSFER PROTEINS AS A TOOL IN THE STUDY OF MEMBRANE STRUCTURE. INSIDE-OUTSIDE DISTRIBUTION OF THE PHOSPHOLIPIDS IN THE PROTOPLASMIC MEMBRANE OF MICROCOCCUS LYSODEIKTICUS.

L.I.Barsukov, V.I.Kulikov and L.D.Bergelson
Shemyakin Institute of Bioorganic Chemistry
USSR Academy of Sciences, Moscow, USSR

Received June 1,1976

Summary: A method is presented which permits study of the sidedness of phospholipids in biological membranes by protein mediated phospholipid exchange between membranes and liposomes. For the protoplasmic membrane of M.lysodeikticus it was found that about one half of the total phosphatidylglycerol and diphosphatidylglycerol can be transferred by proteins, while phosphatidylinositol is not transferable. From a comparison of these results with those obtained with ghosts of M.lysodeikticus and pronase treated protoplasts it is concluded that diphosphatidylglycerol distributes almost evenly between the inner and outer surfaces of the protoplasmic membrane whereas phosphatidylglycerol is located predominantly on the outer surface and phosphatidylinositol on the inner one. Such asymmetrical distribution correlates with results obtained upon treatment of the protoplasts with phospholipases A_2 and C.

Cells of higher animals and plants contain a special group of proteins that are able to transfer lipids from one membrane to an other (1). According to recent data from this (2) and other laboratories (3,4) only the outer surfaces of the donor and acceptor membranes appear to be accessible to LTP. This feature of the LTP makes them a potentially

ABBREVIATIONS USED: PC, phosphatidylcholine (egg yolk lecithin); PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; LTP, lipid transfer proteins.

useful tool for evalution of the outside-inside distribution of lipids in biological membranes. The present communication describes the use of rat liver LTP for location of phospholipids in the membrane of M.lysodeikticus protoplasts.

MATERIALS AND METHODS. Purified egg lysozyme was obtained from Olaine Biochemical Factories (USSR) and pronase from Nagase Co. (Japan). Non-purified phospholipase A_2 from Naja naja oxiana snake venom and phospholipase C from Bacillus cereus were used. LTP (postmicrosomal supernatant fraction, pH 5.1) was isolated from rat liver as described by Wirtz and Zilversmit (5). M.lysodeikticus (Flemming strain 2665) was grown as described previously (6) and harvested after 18h growth by centrifugation. For incorporation of radioactive label into phospholipids bacteria were grown in the presence of [1-^{14}C] glycerol (100 μCi/l final concentration). Protoplasts were isolated by the method of Owen and Freer (7). Leakage of protoplasts was controlled by monitoring E_{260} of the protoplast supernatant fraction (8). Ghosts of M.lysodeikticus were prepared by lysozyme treatment of freshly harvested cells in hypotonic medium (9). PC vesicles were prepared by ultrasonication in 10 mM tris-HCl-0.8 M sucrose containing 2 mM $MgCl_2$, pH 7.4 (buffer A). Phospholipid extraction and analysis were performed as described by Thomas and Ellar (10). Lipid phosphorus was determined by the method of Gerlach and Deutike (11). Radioactivity of phospholipids was measured in a Mark II scintillation spectrometer (Nuclear Chicago).

RESULTS. The protoplasmic membrane of M.lysodeikticus contains only three phospholipids - DPG, PG and PI (10). The incubation of coarse DPG, PG or PI liposomes with sonicated PC vesicles and rat liver LTP showed that in the artificial system all three acidic phospholipids are transferred by LTP at commensurable rates. In order to investigate lipid transfer from protoplasts the latter were incubated with sonicated PC vesicles and LTP. Thereafter the protoplasts were sedimented at

Table 1. Phospholipid composition of protoplasts of M. lysodeikticus after incubation with PC vesicles without LTP and in presence of LTP.[a)]

	Protoplasts		Protoplasts + PC vesicles		Protoplasts + PC vesicles + LTP	
	radioactivity (cpm)	nmole	radioactivity (cpm)	nmole	radioactivity (cpm)	nmole
DPG	28600	9100	27250	8700	15100	4800
PG	3350	6790	3320	6710	1950	3940
PI	1230	2790	1200	2720	1250	2840

a) ^{14}C-protoplasts (0.2 mg P/ml) were incubated with PC vesicles (0.6 mg P/ml) and LTP (2.2 mg protein/ml) in buffer A at 37° for 30 min. The cooled (0°) incubation mixture was centrifuged at 12000g for 40 min. The supernatant was extracted by chloroform-methanol (2:1,v/v) and protoplasts by the method of Thomas and Ellar (10). All experiments were done at least twice and gave reproducible results (the relative standard deviations were about 10%).

12000g and the radioactivity of the lipids of the pellet and supernatant were measured. Control experiments showed that upon incubation of protoplasts and PC vesicles without LTP or of the protoplasts in the absence of PC only small amounts (<5%) of the total lipid radioactivity were transferred to the supernatant. However, when the protoplasts were coincubated with PC vesicles in the presence of LTP about 1/3 of the lipid radioactivity of the initial protoplasts was found in the lipid extract of the supernatant. TLC of the extract revealed the presence of DPG, PG and PC and the absence of PI. Phospholipid

analysis of the precipitated protoplasts demonstrated that a part of the acidic phospholipids had been replaced by PC during the incubation process. As can be seen from table 1 in the presence of LTP and PC vesicles more than 40% of the initial PG and about 50% of DPG were released by protoplasts while PI was completly retained.

When pronase treated protoplasts were incubated with PC vesicles and LTP still more lipids were transferred, the increase occuring mainly at the expense of PG and PI (79% and 22% transfer respectively); at the same time no appreciable change in the transfer of DPG was observed.

Experiments wherein cosonicated mixtures of PC and M.lysodeikticus total phospholipids were treated with phospholipases A_2 and C revealed no significant differences in the susceptibility of the three acidic phospholipids to these enzymes. However, in intact protoplasts phospholipase C destroyed much more PG (70%) than was degraded by phospholipase A_2 (46%) while DPG was affected to the same extent by both enzymes (54 and 48%, respectively) and PI was not hydrolyzed at all.

DISCUSSION. Judging by the lack of significant leakage, neither LTP nor phospholipases A_2 and C produced lysis of intact protoplasts on incubation for 40-50 min (more prolonged phospholipase digestion resulted in leakage of nucleotidic material). Therefrom it may be inferred that under the experimental conditions used these three agents interacted only with the exterior surface of the protoplasts. Hence we presume that the DPG and PG fractions transferred to the PC vesicles by LTP must have been located on the outer surface of the membrane. With respect to lipids unaffected by LTP, two possibilities have to be considered: either (i) they are located at the inner surface

Table 2. Transfer of M.lysodeikticus membrane phospholipids by LTP and their hydrolysis by phospholipase A_2 and C.

Phospho-lipid	Transfer onto PC vesicles (%) in the presence of LTP			Extent of hydrolysis by phospho-lipases (%)	
	Protoplasts [a]	Pronase treated protoplasts [b]	Ghosts [c]	A_2 [d]	C [f]
DPG	47	41	33	48	54
PG	42	79	54	46	70
PI	1	22	52	- [e]	0

a) For experimental conditions see footnotes to table 1;
b) Protoplasts (25 mg protein/ml) were incubated with pronase (0.5 mg/ml) in buffer A at 37° for 20 min. The cooled (0°) mixture was centrifuged and washed with cold (0°) buffer. Incubation of the pronase treated protoplasts with PC vesicles and LTP was performed as described for intact protoplasts;
c) To avoid membrane fusion the experiments were carried out by incubation of M.lysodeikticus ghosts and LTP saturated with PC by pre-incubation with coarse PC liposomes;
d) Treatment of M.lysodeikticus protoplasts (0.2 mg P/ml) with phospholipase A_2 (0.4 mg/ml) was carried out in buffer A in the presence of 10 mM $CaCl_2$ at 37° for 40 min;
e) Digestion of PI by phospholipase A_2 could not be measured because the products were not easily separable by TLC;
f) Treatment of the M.lysodeikticus protoplasts (15 mg protein/ml) with phospholipase C (0.5 mg protein/ml) was carried out in buffer A in the presence of 0.4 mM $CaCl_2$ at 37° for 40 min. Digestion by phospholipases A_2 and C was stopped by adding 0.2 ml of saturated EDTA solution.

or (ii) being located at the outside, they are unavailable because of tight packing or of shielding by the membrane proteins.

In order to choose between these two possibilities the

action of LTP toward pronase treated protoplasts was studied. In M.lysodeikticus protoplasts surface proteins are known to comprise less than 30% of total membrane proteins (12). Since pronase treatment destroyed about 20% of the membrane proteins we can assume that proteins shielding the outside lipid molecules were almost completely removed, leading to exposure of additional lipids to the LTP. If so, the results of the present work indicate that no less than 80% of the total PG and at least 20% of PI are located on the outside of the membrane. DPG appears to be distributed evenly between the two surfaces. From the fact that the amount of transferable DPG is unaltered by pronase treatment it can be concluded that all DPG molecules on the outside of intact protoplast are accessible to LTP. The predominant localization of PG at the outside and of PI at the inside of the membrane was confirmed by transfer experiments carried out with ghosts of M.lysodeikticus. In such preparations consisting of mixtures of inside-out and right-side-out vesicles as well as unsealed membranes more PI and less PG were transferable than in pronase treated protoplasts.

Since phospholipase A_2 and lipid transfer proteins are of commensurate molecular size (13,14) the accessibility of surface membrane lipids to these agents should be of similar degree. This was indeed found to be the case for PG and DPG. When native and pronase treated protoplasts were digested by phospholipase C PI was also affected exactly to the same degree as in the lipid transfer procedure (0 and 20% respectively). A more complex picture was observed for PG. Whereas in native protoplasts this phospholipid was equally affected by LTP and phospholipase A_2 (47-48%), much higher amounts (70%) were hydrolyzed by phospholipase C. This result may be inter-

preted as indicating that 70% of the PG is on the outer surface, but that the outward facing PG is only partly accessible to LTP and phospholipase A_2 in the intact protoplasts.

We thus conclude that the lipid transfer proteins can be used to study the outside-inside distribution of phospholipids in biological membranes. The method has a number of merits: LTP acts at physiological pH and temperature, it is non-lytic and apparently does not damage the membranes of cells or organelles. Hence protein mediated lipid transfer could be a valuable addition to the existing methods of measuring phospholipid asymmetry, i.e. chemical labeling (15), phospholipase treatment (16) and NMR with hydrophylic paramagnetic probes (17).

An important feature of the protein mediated exchange is that the newly introduced phospholipids are incorporated only into the outer monolayer. In the present work it has been shown that the use of LTP enables one to introduce PC into bacterial membranes. This approach opens the possibility of replacement of some lipids by others in membrane and lipoprotein complexes without destroying their integrity.

ACKNOLEDGMENT. We are indebted to Dr.I.D.Ryabova and Dr.D.M.Ostrovskii for their help and valuable discussion.

REFERENCES

1. Dawson,R.M.C. (1973) Sub-Cell.Biochem. 2, 69-89.
2. Barsukov,L.I., Shapiro,Y.E., Viktorov,A.V., Volkova,V.I., Bystrov,V.F. and Bergelson,L.D. (1975) Chem.Phys.Lipids 14,211-226.
3. Hellings,J.A., Kamp,H.H., Wirtz,K.W.A. and Van Deenen,L.L.M. (1974) Eur.J.Biochem. 47, 601-605.
4. Rothman,J.E. and Dawidowicz,E.A. (1975) Biochemistry 14, 2809-2816.
5. Wirtz,K.W.A. and Zilversmit,D.B. (1969)Biochim.Biophys.Acta 193, 105-116.

6. Ryabova,I.D., Reshetov,P.D., Zhdanov,G.L. and Khokhlov,A.S. (1965) Antibiotiki 10, 1066-1069.
7. Owen,P. and Freer,J.H. (1972) Biochem.J. 129, 907-917.
8. Reaveley,D.A. and Rogers,H.J. (1969) Biochem.J. 113, 67-79.
9. Ostrovskii,D.N., Bystrov,V.F., Zhukova,I.G., Chervin,I.I. and Yakovlev,G.I. (1973) Biokhimija 38, 161-168.
10. Thomas,T.D. and Ellar,D.J. (1973) Biochim.Biophys.Acta 316, 180-195.
11. Gerlach,E. and Deutike,B. (1963) Biochem.Z. 337, 477-481.
12. Lukojanova,M.A., Biryuzova,V.I., Simakova,I.M. and Gel'man,N.S. (1967) Biokhimija 32, 816-822.
13. Wells,M.A. and Hanahan,D.J. (1969) Biochemistry 8, 414-424.
14. Kamp,H.H., Wirtz,K.W.A. and Van Deenen,L.L.M. (1973) Biochim.Biophys.Acta 318, 313-325.
15. Carraway,K.L. (1975) Biochim.Biophys.Acta 415, 379-410.
16. Zwaal,R.F.A., Roelofsen,B., Comfurius,P. and Van Deenen, L.L.M (1975) Biochim.Biophys.Acta 406, 83-96.
17. Bergelson,L.D. and Bystrov,V.F. (1974) Proc.IXth FEBS Meeting 35, 35-46.