Biochimica et Biophysica Acta, 508 (1978) 97—108

BBA 77948

PROTEIN-MEDIATED TRANSBILAYER MOVEMENT OF LYSOPHOSPHATIDYLCHOLINE IN GLYCOPHORIN-CONTAINING VESICLES

E.J.J. van ZOELEN [a], B. de KRUIJFF [b] and L.L.M. van DEENEN [a]

[a] *Laboratory of Biochemistry, and* [b] *Institute of Molecular Biology, State University of Utrecht, Transitorium III, Padualaan 8, De Uithof, Utrecht (The Netherlands)*

(Received August 4th, 1977)

Summary

1. Sonicated glycophorin-containing vesicles of dioleoyl phosphatidylcholine have been made. The outside-inside distribution of the lipid molecules in these vesicles was measured with NMR and was found to be comparable with that of protein-free vesicles.

2. The transbilayer distribution of palmitoyl lysophosphatidylcholine in these vesicles is such that they have a significantly higher content of the lyso-compound in the inner monolayer when compared with vesicles without glycophorin.

3. Lysophosphatidylcholine, added to pre-existing glycophorin-containing vesicles, is incorporated in the outer monolayer of these vesicles. Subsequently it is able to move to the inner monolayer with an estimated half time of about 1.5 h at 4°C. This was measured with ^{13}C-NMR using [*N*-$^{13}CH_3$]lysophosphatidylcholine.

4. Treatment of co-sonicated vesicles of phosphatidylcholine and lysophosphatidylcholine containing glycophorin with the enzyme lysophospholipase results in a complete degradation of the lyso-compound. A half time of transbilayer movement of lysophosphatidylcholine during this experiment was estimated to be about 1 h at 37°C.

Introduction

The bilayer structure of biological membranes is firmly established. Both the lipids and the proteins can be asymmetrically arranged over the membrane [1]. In order to understand the principles underlying this asymmetry it is of interest to investigate whether transbilayer movement of lipids can take place. Evidence for the transbilayer movement of phosphatidylcholine in the rat erythrocyte membrane [2,3] and for that of cholesterol in the human erythrocyte mem-

brane [4] has been presented. Transbilayer movement of these lipids was not observed, however, in the membrane of Influenza virus [5,6]. In artificial membranes no indication was found for transbilayer movement of cholesterol [7], and also not for that of phosphatidylcholine [8—10], unless differences in lateral packing were induced over the membrane [11]. From NMR measurements [12] and from the action of the enzyme lysophosphatidylcholine [13] it was concluded that no transbilayer movement of lysophosphatidylcholine occurred in sonicated lipid vesicles.

One of the main differences between artificial and biological membranes in the absence of membrane-bound proteins in the former. Therefore, in this paper the transbilayer movement of lysophosphatidylcholine is investigated in vesicles containing glycophorin, a membrane-spanning protein from the human erythrocyte membrane [14]. This is done by a combined approach of the enzyme lysophospholipase and of ^{13}C-NMR, using (N-$^{13}CH_3$)-enriched lipids.

Materials and Methods

Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (18 : 1_c/18 : 1_c-phosphatidylcholine) and 1,2 dipalmitoyl-*sn*-glycero-3-phosphocholine (16 : 0/16 : 0-phosphatidylcholine) were synthesized as described before [15]. 1-Palmitoyl-*sn*-glycero-3-phosphocholine (16 : 0-lysophosphatidylcholine) was obtained by hydrolysis of 16 : 0/16 : 0-phosphatidylcholine by means of pancreatic phospholipase A_2 (a gift from Dr. A.J. Slotboom).

[N-$^{13}CH_3$]18 : 1_c/18 : 1_c-phosphatidylcholine and [N-$^{13}CH_3$]16 : 0-lysophosphatidylcholine were synthesized as described previously [12]. 1-[1-^{14}C]16 : 0-lysophosphatidylcholine (10^2 dpm/nmol), [9,10$^3H_2$]palmitic acid (10^6 dpm/nmol) and the enzyme lysophospholipase II were a generous gift of Dr. A.M.H.P. van den Besselaar from this laboratory (details about these compounds are given in ref. 13).

Glycophorin was purified and delipidated by a modification [23] of the method of Hamaguchi and Cleve. [^{3}H]Glycophorin was obtained using the method described for the labeling of C-terminal amino acids of proteins [16]. A high incorporation of ^{3}H was found in the sugar residues of glycophorin, also after prolonged dialysis of the protein against unlabeled water. Using ^3H_2O with a specific activity of 25 mCi/ml, glycophorin with a specific activity of 105 cpm/μg protein (as determined according to Lowry et al. [17]) was obtained.

^3H_2O was obtained from the Radiochemical Centre (Amersham, U.K.), $^{13}CH_3I$ from Prochem (London, U.K.) and ^2H_2O from Merck, Sharp and Dohme (Munich, G.F.R.). Dy_2O_3 was purchased from British Drug House (Poole, U.K.) and was converted to its chloride by HCl. Nd $(NO_3)_3$ was obtained from Koch Light (Colnbrook, U.K.) and Sepharose-4B from Pharmacia (Uppsala, Sweden). All other reagents were of Analytical Reagent Grade and used without purification.

Methods

Glycophorin-containing vesicles were prepared according to the method of McDonald and McDonald [18]. A mixture of glycophorin and lipids in chloro-

form/methanol/water (150/75/1, v/v) was dried by evaporation, and the lipid-protein film was hydrated in a 100 mM NaCl/10 mM Tris · HCl, pH 7.4, buffer. This leads to the formation of large unilamellar glycophorin-containing vesicles (diameter 100—500 nm), which can be purified from other structures by stepwise centrifugation [23]. Sonication of these vesicles was done with a Branson tip sonicator, power setting 4, under nitrogen for 3—5 min at 0°C. Metal particles from the probe were removed by centrifugation for 30 min at 30 000 × g at 4°C. Phosphorus was determined according to a modification [19] of the procedure of Fiske-Subba Row. Radioactivity was counted using a Packard Tricarb liquid scintillation spectrometer, according to well known procedures. ^{13}C Measurements were performed on a Bruker 360 WS spectrometer at a frequency of 90.5 MHz, as described in ref. 12, and ^{31}P-NMR on a Bruker WH-90 spectrometer at a frequency of 36.4 MHz (see ref. 20). All NMR experiments were done in a ^2H_2O-containing 100 mM (NaCl/25 mM Tris/0.2 mM EDTA-HAc, p^{2}H 7.0, buffer.

The assay for lysophospholipase treatment has been described in ref. 13.

Results

Sonication of glycophorin-containing vesicles

High resolution NMR spectra can be obtained from sonicated lipid vesicles. When large glycophorin-containing vesicles of phosphatidylcholine are

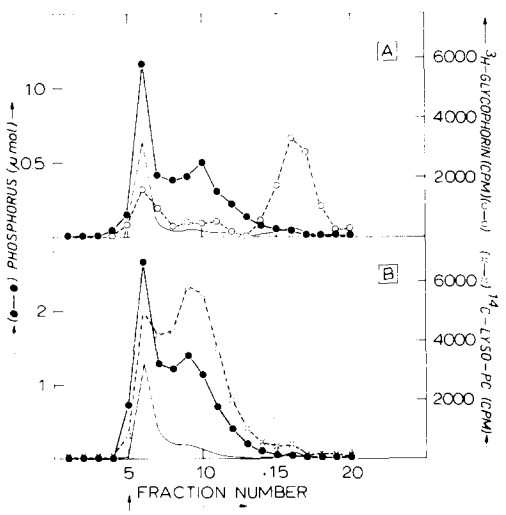


Fig. 1. Elution pattern of Sepharose-4B column (16 × 3 cm), eluted with 100 mM NaCl/10 mM Tris · HCl, pH 7.4. Fractions of 6.7 ml were sampled. 1A. 5 μmol 18 : 1_c/18 : 1_c-phosphatidylcholine vesicles containing [^{3}H]glycophorin (500 : 1 molar ratio) were sonicated and immediately put on the column. Besides the amount of phosphate (●———●) and [^{3}H]glycophorin (○———○), also the absorbance at 280 nm (———) is shown. Recovery of both phosphatidylcholine and glycophorin was 80%. 1B. To 10 μmol sonicated vesicles of 18 : 1_c/18 : 1_c-phosphatidylcholine containing glycophorin, 1 μmol [^{14}C]16 : 0-lysophosphatidylcholine (lyso-PC) was added. Phosphate (●———●), [^{14}C]lysophosphatidylcholine (X———X) and absorbance at 280 nm (———) are shown. Recovery of phosphorus was 92%, of radioactivity 87%. The arrow denotes the void volume of the column.

sonicated, small unilamellar vesicles are formed with only a limited amount of protein incorporated in them. This can be shown by passing the sonicated vesicles over a Sepharose-4B column (see Fig. 1A). Most of the [^{3}H]glycophorin appears with the column volume almost free of lipid. Using a molecular weight of 14 000 for the protein part of glycophorin [14], the molar ratio of glycophorin and phosphatidylcholine in the sonicated vesicles was found to be 1 : 1200. Since a sonicated vesicle of 18 : 1_c/18 : 1_c-phosphatidylcholine contains about 5600 lipid molecules (calculated from ref. 20), it can be concluded that each vesicle contains an average of 4—5 copies of glycophorin. A relatively high percentage of phosphorus (15%) is present in the void volume of the column. Sonication for a prolonged period of time is required to remove these larger unilamellar vesicles. They can be partly removed by centrifugation and this was carried out prior to all the NMR experiments.

Transbilayer distribution of phosphatidylcholine and lysophosphatidylcholine in sonicated glycophorin-containing vesicles

Due to the high curvature of sonicated vesicles, the outer monolayer of these structures contains more lipid molecules than the inner monolayer. The ratio of the number of molecules in outer and inner monolayer gives information about the vesicle size [20]. This ratio can be determined from NMR measurements using paramagnetic ions to which the vesicle is impermeable.

^{13}C-NMR measurements with Dy^{3+} as a shift reagent have shown that in sonicated vesicles of [*N*-$^{13}CH_3$]18 : 1_c/18 : 1_c-phosphatidylcholine, 37% of the lipid molecules are located in the inner monolayer [12]. A similar value was reported using ^{31}P-NMR [20]. In sonicated vesicles of [*N*-$^{13}CH_3$]18 : 1_c/18 : 1_c-phosphatidylcholine containing glycophorin, 33% of the signal could not be shifted by Dy^{3+}; using ^{31}P-NMR and Nd^{3+} as shift reagent a value of 30% ± 1% was obtained. The small difference between these two values is probably the result of the presence of a small fraction of larger unilamellar vesicles. These data demonstrate that the size of sonicated glycophorin-containing vesicles is in the same order of magnitude as that of protein-free vesicles. Support for this statement was also obtained from a comparison of the behaviour of both vesicles on a Sepharose-4B column. The intensity of the unshifted signal was constant for at least 3 h; this shows that the sonicated glycophorin-containing vesicles are impermeable to the shift reagent. In contrast with this observation it was found with ^{13}C-NMR that the large unsonicated glycophorin-containing vesicles are permeable to the shift reagent, since 85—90% of the total intensity was shifted immediately upon addition of the paramagnetic ions.

Lysophosphatidylcholine is known to be asymmetrically distributed over the two halves of the bilayer in sonicated vesicles with approximately 85% in the outer and 15% in the inner monolayer [12]. This was measured by using (*N*-$^{13}CH_3$)-labeled 16 : 0-lysophosphatidylcholine and 18 : 1_c/18 : 1_c-phosphatidylcholine (molar ratio, 15 : 85). Using the same approach for sonicated glycophorin-containing vesicles, the result was that 37% of the lyso-compound is located in the inner monolayer. Since 30% of all phospholipids was found to be located in the inner monolayer, this indicates that the ratio of lysophosphatidylcholine and phosphatidylcholine is almost the same in both monolayers. This demonstrates small amounts of glycophorin can give rise to signifi-

TABLE I

TRANSBILAYER DISTRIBUTION OF 18 : 1_c/18 : 1_c-PHOSPHATIDYLCHOLINE (DOPC) AND 16 : 0-LYSOPHOSPHATIDYLCHOLINE (LPC) IN SONICATED GLYCOPHORIN-CONTAINING VESICLES COMPARED WITH PROTEIN-FREE VESICLES AS DETERMINED BY ^{13}C NMR

For details see text.

Membrane lipid composition	Glycophorin	% DOPC (outer monolayer)	% DOPC (inner monolayer)	% LPC (outer monolayer)	% LPC (inner monolayer)	% Total lipid (outer monolayer)	% Total lipid (inner monolayer)
18 : 1_c/18 : 1_c-phosphatidylcholine	—	63	37	—	—	63	37 *
18 : 1_c/18 : 1_c-phosphatidylcholine	+	67	33	—	—	67	33
18 : 1_c/18 : 1_c-phosphatidylcholine : 16 : 0-lysophosphatidylcholine (85 : 15)	—	52	48	85	15	57	43 *
18 : 1_c/18 : 1_c-phosphatidylcholine : 16 : 0-lysophosphatidylcholine (85 : 15)	+	71	29	63	37	70	30

* Taken from ref. 12.

cant alterations in the transbilayer distribution of phospholipids. All data about the transbilayer distribution of phosphatidylcholine and lysophosphatidylcholine in sonicated vesicles with and without glycophorin are summarized in Table I.

Incorporation of externally added lysophosphatidylcholine in pre-existing vesicles

When lysophosphatidylcholine (in a micellar solution) is added to pre-existing lipid vesicles, it rapidly penetrates the outer monolayer of the vesicles. By using [*N*-$^{13}CH_3$]-lysophosphatidylcholine, a possible transbilayer movement of the lyso-compound from the outer to the inner monolayer can be detected with ^{13}C-NMR from the appearance of unshifted resonances [12]. It should be checked, however, that the externally added lyso-compound is quantitatively incorporated in the vesicles, and that the bilayer remains intact during this incorporation.

When sonicated glycophorin-containing vesicles are passed over a Sepharose-4B column immediately after the addition of 15 mole% of [^{14}C]-lysophosphatidylcholine, almost all label chromatographs with the vesicle peak (see Fig. 1B) which is in agreement with previous data using pure lipid vesicles [13]. When unsonicated glycophorin-containing vesicles of 18 : 1_c/18 : 1_c-phosphatidylcholine were incubated with 15 mole% of [^{14}C]16 : 0-lysophosphatidylcholine

for 10 min at 27° C, 85—90% of the introduced label was recovered in the pellet after centrifugation of the reaction mixture at 125 000 × *g* for 45 min at 4° C [23]. A similar result was obtained when the whole procedure was carried out at 4° C.

Using ^{31}P-NMR it could be shown that the external addition of 15 mole% of lysophosphatidylcholine to sonicated glycophorin-containing vesicles in the presence of a shift reagent resulted in a small increase in the intensity of the resonances of the inside lipids. However, no significant alteration in the transbilayer distribution of the phospholipids in the vesicles was observed, which demonstrates that fusion of the vesicles upon addition of the lyso-compound does not occur. In addition, these results show that the barrier properties of the vesicles are not affected by the incorporation of the lyso-compound in the outer monolayer. Since after sonication of the large glycophorin-containing vesicles free glycophorin will be in solution (see Fig. 1) it was checked by ^{31}P-NMR that free glycophorin is not able to prevent externally added lysophosphatidylcholine from being shifted by paramagnetic cations.

Transbilayer movement of lysophosphatidylcholine in sonicated glycophorin-containing vesicles

For the ^{13}C-NMR measurements of the transbilayer movement of lysophosphatidylcholine, the following conditions were used. To 1.5 ml sonicated glycophorin-containing vesicles, prepared from unlabeled 18 : 1_c/18 : 1_c-phosphatidylcholine (26 mM), 3.5 mM [*N*-$^{13}CH_3$]16 : 0-lysophosphatidylcholine (molar ratio, 85 : 15) was added at the desired temperature (4 or 27° C). After certain times 8 mM $DyCl_3$ was added and the ^{13}C-spectrum was recorded immediately (half hour data accumulation at the desired temperature). The appearance of unshifted inside-resonances was taken as an indication for the transbilayer movement of lysophosphatidylcholine.

Fig. 2. shows some spectra recorded during these experiments. Fig. 2A shows the resonances arising from the natural abundance ^{13}C-nuclei in the choline groups of the unlabeled 18 : 1_c/18 : 1_c-phosphatidylcholine in the glycophorin-containing vesicles. In all experiments corrections were made for these natural abundance signals, assuming an outside-inside distribution of 67 : 33 (see Table I). Fig. 2B shows the spectrum after the external addition of [^{13}C]lysophosphatidylcholine, resulting in a large increase in intensity of the signals from the choline groups relative to the external standard. Upon the addition of the shift reagent, the resonances of the outside phospholipids are shifted downfield. These shifted resonances may loose their nuclear Overhauser enhancement, which results in a decrease in intensity [12]. Therefore, only the intensity of the unshifted resonances (relative to the external standard) before and after the addition of the shift reagent were used for calculations. Hardly any unshifted resonance is seen 10 min after addition of the lyso-compound at 4° C (Fig. 2C), but it is clearly visible after 1.5 h at 27° C (Fig. 2D). Fig. 2E shows the spectrum when the shift reagent is added after cosonication of the phosphatidylcholine and the labeled lysophosphatidylcholine with glycophorin.

Fig. 3 shows the percentage of unshifted resonances from the lysophosphatidylcholine as a function of time for measurements done at 4 and 27° C. The transbilayer movement of lysophosphatidylcholine is clearly shown as a tem-

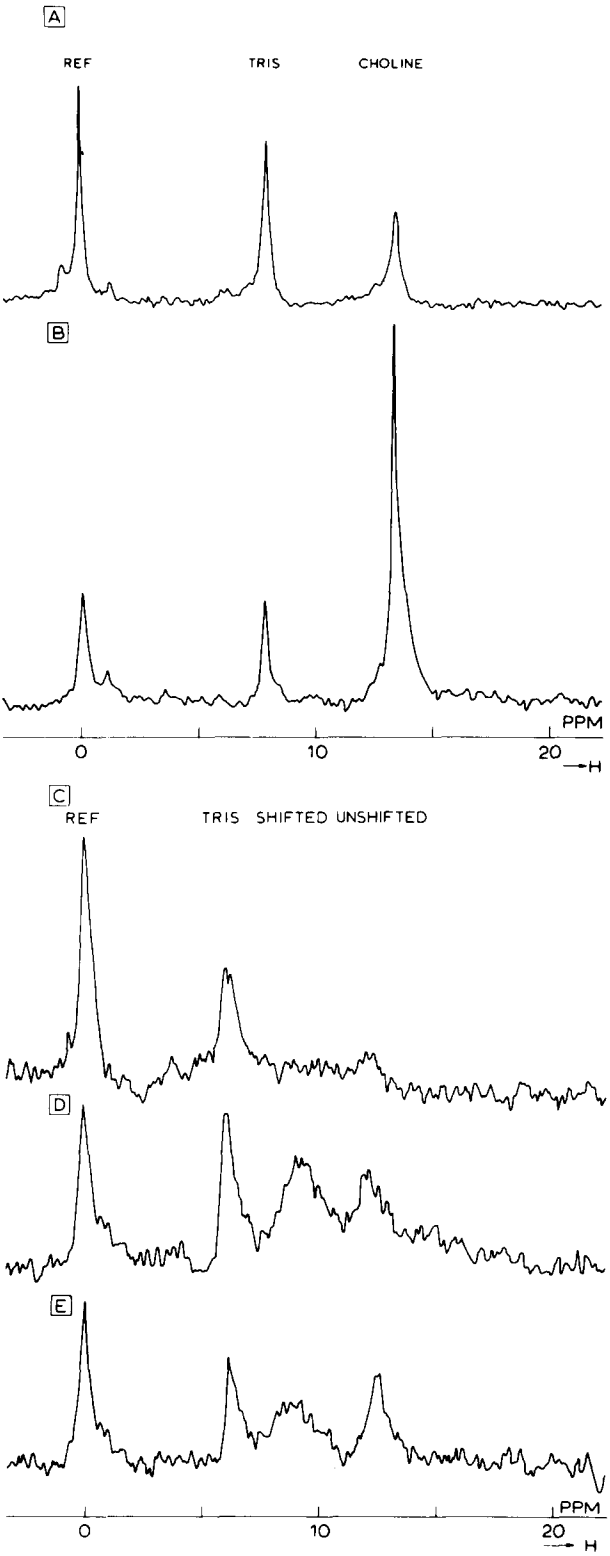


Fig. 2. 90.5 MHz ^{13}C-spectra of A, sonicated vesicles of 18 : 1_c/18 : 1_c-phosphatidylcholine (26 mM lipid) containing glycophorin; B, idem after the addition of 3.5 mM [*N*-$^{13}CH_3$]16 : 0-lysophosphatidylcholine; C, spectrum recorded 10 min after addition of the lyso-compound (at 4°C) and after addition of 8 mM Dy Cl_3; D, spectrum recorded 1.5 h after addition of the lyso-compound (at 27°C) and after addition of 8 mM Dy Cl_3; E, spectrum recorded after cosonication of 18 : 1_c/18 : 1_c-phosphatidylcholine and [*N*-$^{13}CH_3$]16 : 0-lysophosphatidylcholine with glycophorin, after addition of 8 mM Dy Cl_3. Chemical shifts are upfield from 1.4 dioxane (REF). For details see text.

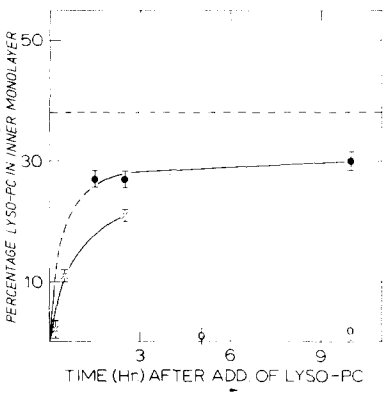


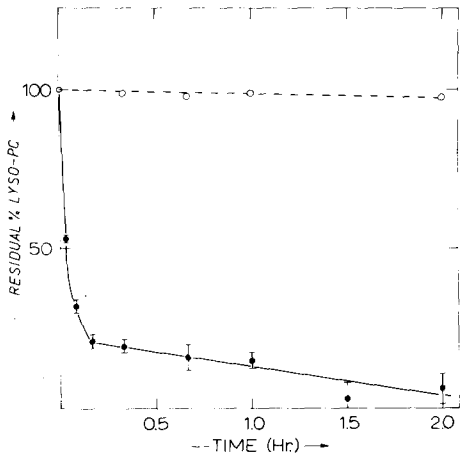
Fig. 3. Percentage of total 16 : 0-lysophosphatidylcholine (LYSO-PC) present in the inner monolayer of 18 : 1_c/18 : 1_c-phosphatidylcholine vesicles (26 mM) containing glycophorin after incubation for various periods of time with 3.5 mM [*N*-$^{13}CH_3$]16 : 0-lysophosphatidylcholine at 4°C (X———X) or 27°C (●———●). The dotted line represents the percentage of 16 : 0-lysophosphatidylcholine present in the inner monolayer after cosonication of both lipids. As a comparison (see ref. 12) the results are shown when vesicles without protein are used (○———○).

perature-dependent process. At 27°C the process is so fast that the system has come to equilibration within 1 h. The distribution of the lyso-compound under these conditions turns out to be different from the distribution in the cosonicated vesicles (dotted line). At 4°C the process is much slower; since it was shown that also at this temperature the incorporation of lysophosphatidylcholine is accomplished within 1 h the observed process is certainly the result of the transbilayer movement of the lyso-compound. A half time of about 1.5 h was estimated for the process under these conditions. The experiment at 4°C also shows that at zero time all resonances can be shifted, indicating that the lysophosphatidylcholine is indeed incorporated initially in the outer monolayer of the vesicle. When during the experiments the temperature was increased from 4 to 27°C, the distribution which is characteristic for the system after equilibration was immediately observed.

Approach with lysophospholipase

In cosonicated vesicles of phosphatidylcholine and lysophosphatidylcholine (molar ratio, 95 : 5) between 80 and 90% of the lyso-compound could be degraded by the enzyme lysophospholipase. Lysophosphatidylcholine introduced in the outer monolayer of sonicated vesicles could be fully degraded by the action of the enzyme [13]. This was taken as an indication that the transbilayer distribution of lysophosphatidylcholine in cosonicated vesicles is such that 80—90% is located in the outer monolayer, and that no transbilayer movement of lysophosphatidycholine occurs in these vesicles. These data were in agreement with the NMR data [12,13].

Transbilayer movement of lysophosphatidylcholine can only be demonstrated by this enzymatic approach if the transbilayer distribution of the lyso-compound is known. Since it was found that in cosonicated vesicles of phosphatidylcholine and lysophosphatidylcholine containing glycophorin, 37% of the lyso-compound is located in the inner monolayer (see Table I), it was of



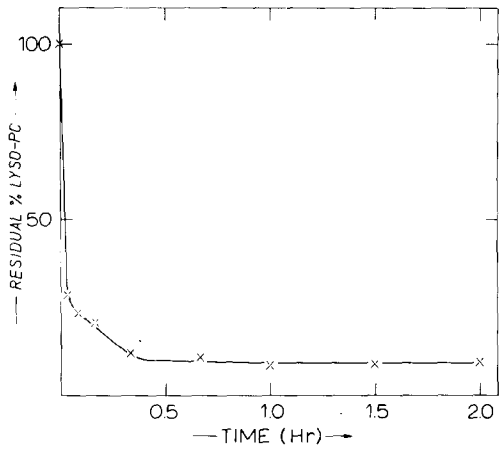


Fig. 4. Time course of lysophosphatidylcholine (LYSO-PC) hydrolysis by lysophospholipase. Sonicated glycophorin-containing vesicles of 0.125 μmol 18 : 1_c/18 : 1_c-phosphatidylcholine containing 5 mole% of 16 : 0-lysophosphatidylcholine were treated with 0.2 mg of enzyme (●——●). In the control experiments no enzyme was used (○——○).

Fig. 5. Time course of lysophosphatidylcholine (LYSO-PC) hydrolysis by lysophospholipase. Unsonicated glycophorin containing vesicles of 0.10 μmol 18 : 1_c/18 : 1_c-phosphatidylcholine (1 : 800 molar ratio) containing 5 mole% of 16 : 0-lysophosphatidylcholine were treated with 0.2 mg enzyme.

interest to study the enzymatic degradation of lysophosphatidylcholine by the enzyme in these vesicles. Glycophorin-containing vesicles of 18 : 1_c/18 : 1_c-phosphatidylcholine and 1-[1-^{14}C]16 : 0-lysophosphatidylcholine (molar ratio, 95 : 5) were sonicated and passed over a Sepharose-4B column. The vesicle-containing fractions (8—12; see Fig. 1B) were pooled and concentrated over a collodion membrane filter (Sartorius) up to a concentration of 0.3 μmol phospholipid/ml.

Fig. 4. shows the time course for the hydrolysis of lysophosphatidylcholine in the cosonicated vesicles at 37° C. After a very rapid hydrolysis of about 70% of the lyso-compound, a slower rate of hydrolysis is observed resulting in a 95% degradation after about 90 min. This biphasic behaviour demonstrates that in this system movement of lysophosphatidylcholine from the inner to the outer monolayer takes place with an estimated half-time of about 1 h.

Treatment of unsonicated glycophorin-containing vesicles with lysophospholipase results in a 90% degradation of the lyso-compound with 30 min (see Fig. 5). Since these vesicles are permeable to shift reagents, no information on the transbilayer distribution of lysophosphatidylcholine in these structures can be obtained from NMR measurements. It has been shown, however, that they are impermeable to enzymes [23]. Since 10—15% of the resonances can not be shifted in these structures and assuming that only protein-containing bilayers are permeable to the shift reagent, this would imply that a fraction of the lipid molecules is located in protein-free bilayers, probably trapped in the protein-containing bilayers (see ref. 23). The experiment with lysophospholipase suggests, therefore, that all lysophosphatidylcholine in the protein-containing bilayers can be degraded.

Discussion

Glycophorin, the major sialoglycoprotein of the human erythrocyte membrane is one of the best characterized membrane-bound proteins. It spans the lipid bilayer by means of a hydrophobic region, thus making contact with both monolayers of the membrane [14]. It can easily be purified [14,23] and can be reconstituted in large unilamellar vesicles as a membrane-spanning protein by the method of McDonald and McDonald [18,23]. Sonication of glycophorin-containing vesicles prepared from phosphatidylcholine leads to the formation of small vesicles with only a limited amount of protein incorporated in them. However, much more glycophorin remains incorporated upon sonication when negatively-charged phospholipids are present in the bilayer [23]. Since glycophorin tends to form aggregates of at least two [14], and perhaps even four, copies [23] it can not be excluded that a minor part of the vesicles obtained after sonication do not contain protein molecules. No indication for such a heterogeneity was observed, however, when the vesicles were chromatographed over a DEAE-column (not shown).

In this paper it has been demonstrated that in spite of the low incorporation of protein molecules in the sonicated vesicles, both the transbilayer distribution and the rate of transbilayer movement of lysophosphatidylcholine are strongly affected. In spite of all geometric factors [12] the ratio of lysophosphatidylcholine and phosphatidylcholine in the inner monolayer of sonicated glycophorin-containing vesicles is even higher than in the outer monolayer. This suggests that interactions between lysophosphatidylcholine and glycophorin may exist. Since the protein is oriented with its sugar-containing part to the outside of the vesicle (see ref. 23) interaction with the cytoplasmic C-terminal region of the protein seems most likely. In this respect it is worth mentioning that the acylation of lysophosphatidylcholine in the erythrocyte membrane occurs at the cytoplasmic side of the membrane [2].

Incorporation of glycophorin in lipid bilayers leads to a large increase in the rate of transbilayer movement of lysophosphatidylcholine. It should be realized that during this study three different systems have been investigated which can not be compared directly. In the NMR experiments, external lysophosphatidylcholine is added to vesicles of phosphatidylcholine which are in a state of equilibrium. The penetration of the lyso-compound in the outer monolayer gives rise to a gradient of lysophosphatidylcholine over the two halves of the bilayer, and in addition to a surplus of lipid molecules in the outer monolayer (mass gradient). By means of transbilayer movement, a new equilibrium situation can be reached, but since these vesicles will always contain more lipid molecules than the vesicles obtained after sonication of both lipids, the transbilayer distribution of the lipids in both vesicles can not be compared. During the experiments with the lysophospholipase, the lysophosphatidylcholine in the outer monolayer of the cosonicated vesicles is degraded first, thus inducing a gradient of the lysocompound over the membrane. Since hardly any mass gradient will be induced during this process, the rate of transbilayer movement observed with this technique can not be compared directly with the one found during the NMR measurements. It should also be realized that different concentrations of lysophosphatidylcholine were used, and that the rate-constant for

transbilayer movement from outer to inner and from inner to outer monolayer does not have to be the same in a sonicated vesicle.

Although the transbilayer distribution of lysophosphatidylcholine in the unsonicated glycophorin-containing vesicles is not known, the observation that 90% of the lyso-compound can be degraded in these structures strongly suggests that also in these large vesicles transbilayer movement of lysophosphatidylcholine takes place. This would imply that stress on the membrane due to high curvature is not a requirement for this process.

In order to explain the stimulating effect of proteins on the transbilayer movement of lipids, two mechanisms can be considered. Since membrane spanning proteins make contact with both monolayers of the membrane, transbilayer movement of lipids can take place at the immediate boundary of protein and lipids. However, since lipids in the direct environment of proteins are thought to be less mobile than lipids in the bulk of the bilayer, this possibility seems unlikely. A more reasonable explanation is the assumption that, due to the incorporation of proteins, discontinuities in the lipid bilayer are formed. Going from the lipids in the bulk of the bilayer to the lipids in the direct environment of the proteins, a gradient of lipid mobility will exist [21]. Depending on the steepness of this gradient statistical discontinuities in the bilayer can be formed when lipid molecules exchange between the bulk of the bilayer and the environment of the protein. The activation energy required for the transbilayer movement of lipid molecules at these discontinuities will be much lower than in a continuous bilayer. It is known that discontinuities in the lipid bilayer due to the coexisting of lipids in the gel and liquid crystalline state (at the phase transition temperature) can give rise to an enhanced permeability [22]. It is possible that the discontinuities in the bilayer due to the incorporation of proteins can cause the observed enhanced transbilayer movement in a comparable way.

The results presented in this paper demonstrate that transbilayer movement of phospholipids can take place when membrane-spanning proteins are present in the bilayer. It is realized, however, that possibly not all phenomena of transbilayer movement can be explained by the above mechanism, since it was claimed that in the rat erythrocyte membrane phosphatidylcholine from one monolayer can specifically exchange with phosphatidylcholine from the other monolayer [2]. Such a process would require a much more specific mechanism for transbilayer movement than the one presented here.

Acknowledgements

The present investigations were carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO). The ^{13}C-NMR measurements were carried out at the SON NMR facility in Groningen. We would like to thank Dr. A.M.H.P. van den Besselaar for his contribution during the lysophospholipase experiments and Mrs. A.W.M. Lancée-Hermkens for synthesizing some of lipids.

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