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LIPID DEPENDENCE OF GLYCOPHORIN-INDUCED TRANSBILAYER MOVEMENT OF LYSOPHOSPHATIDYLCHOLINE IN LARGE UNILAMELLAR VESICLES

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(1) The effect of glycophorin incorporation on the transbilayer movement of lysophosphatidylcholine and phosphatidylcholine in large, unilamellar vesicles of varying lipid composition was investigated using lysophospholipase, exchange protein and ^{13}C-NMR. (2) Glycophorin induces a fast transbilayer movement of lysophosphatidylcholine ($t_{1/2}$, 3 min at 37°C) and phosphatidylcholine ($t_{1/2}$, 7 h at 37°C) in dioleoylphosphatidylcholine vesicles. This is in agreement with earlier observations in small unilamellar vesicles by Van Zoelen et al. (Van Zoelen, E.J.J., De Kruijff, B. and Van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 508, 97–108). (3) The glycophorin-induced transbilayer movement of lysophosphatidylcholine is lipid dependent. In vesicles made of the total erythrocyte lipids (phospholipids and cholesterol) glycophorin does not facilitate the lysophosphatidylcholine transbilayer movement, suggesting that a complex mixture of lipids is required to properly seal the lipid-protein interface.

Introduction

The transbilayer movement of lipids has been studied extensively in model and biological membranes (for review, see Ref. 2). In model lipid vesicles the transbilayer movement of phospholipids under equilibrium conditions is usually very slow (halftime values in the order of days) [3–5], except in the gel to liquid-crystalline phase transition [6], when non-bilayer structures are present [7,8], or when an equilibrium transbilayer distribution of lipid molecules is asymmetrically disturbed [9,10]. In biological membranes in general, the transbilayer movement of phospholipids is faster than in pure lipid vesicles. For instance, the transbilayer movement of phosphatidylethanolamine in the bacterial membrane [11] and of phosphatidylcholine in the microsomal membrane [12,13] show halftime values in the order of minutes. The transbilayer movement of phosphatidylcholine in the rat erythrocyte membrane shows halftime values in the order of hours [14–16]. Membrane proteins may play an important role in the transbilayer movement of phospholipids in biological membranes. It has been shown that glycophorin, a membrane-spanning protein of the human erythrocyte membrane, when incorporated into small unilamellar vesicles, greatly enhances the lysophosphatidylcholine and phosphatidylcholine transbilayer movement [1, 17]. Band 3, another integral protein of the human erythrocyte membrane also facilitates the phosphatidylcholine transbilayer movement in small vesicles [18].

In this study we concentrate on two aspects of the effect of glycophorin on the transbilayer movement of lipids. Firstly, as the highly curved bilayer in small vesicles has different physical properties than the more flat biological membrane, we study the effect of glycophorin incorporation on the transbilayer move-

ment of both lysophosphatidylcholine and phosphatidylcholine in large vesicles, which better approximate the biological membrane. In a recent study it was demonstrated that cytochrome *c* oxidase, an intrinsic protein of the mitochondrial membrane, does not induce transbilayer movement of phosphatidylcholine in vesicles [19]. This possibly could be due to either the different nature of the protein or to the more complex lipid composition of the vesicles [18]. In order to test this hypothesis we studied the effect of various lipids on the glycophorin-induced transbilayer movement of lysophosphatidylcholine.

Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine ($18{:}1_c$,-$18{:}1_c$-phosphatidylcholine) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine ($18{:}1_c$, $18{:}1_c$-phosphatidylethanolamine) were synthesized as described before [20,21]. Egg phosphatidylcholine was obtained as described previously [22]. $18{:}1_c$,$18{:}1_c$-Phosphatidyl[*N*-($^{13}CH_3)_3$]choline and 16:0-lysophosphatidyl[*N*-$^{13}CH_3(CH_3)_2$]choline were synthesized as described in Ref. 23. 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 gift from Dr. H. Van den Bosch from this laboratory (details about these compounds are given in Ref. 24. $18{:}1_c$,$18{:}1_c$-Phosphatidyl[*N-methyl*-^{14}C]choline (10^5 dpm/nmol) and [7α-^{3}H]cholesterol oleate ($1.3 \cdot 10^5$ dpm/nmol) were synthesized as in Refs. 21 and 9. Phosphatidylcholine exchange protein purified from bovine liver according to Kamp et al. [25] was a gift from Dr. K.W.A. Wirtz. It was stored at −20°C in 50% glycerol and dialyzed against incubation buffer before use. Fresh blood in acid-citrate dextrose was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service with no preference for special bloodgroups. Ghosts were prepared according to Dodge et al. [26]. The total phospholipids of the human erythrocyte membrane were purified according to Van Dijck et al. [27]. Glycophorin was extracted from the membrane according to Marchesi and Andrews [28], treated with chloroform/methanol and delipidated by ethanol precipitation as described previously [22]. Neuraminidase from *Vibrio cholerae* was obtained from Koch Light (Colnbrook Bucks., U.K.), Dy_2O_3 was purchased from British Drug Houses (Poole, U.K.) and was converted to its chloride by HCl. Cholesterol was obtained from Merck (Darmstadt, F.R.G.). All other chemicals were of analytical grade.

Methods

Preparation of vesicles

Glycophorin was incorporated into large, unilamellar lipid vesicles by the method of MacDonald and MacDonald [29]. A mixture of glycophorin and lipids (in a 1 : 1 400 molar ratio) was dissolved in chloroform/methanol/water (150 : 75 : 1, v/v) and dried by evaporation. The lipid-protein film was hydrated in 100 mM NaCl/10 mM Tris-HCl (pH 7.4). 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 [22].

Multilamellar structures, containing little or no protein were removed by centrifugation at $10\,000 \times g$ for 10 min at 4°C. In order to pellet the glycophorin-containing vesicles the supernatant was centrifuged at $125\,000 \times g$ for 60 min at 4°C.

Large unilamellar vesicles without glycophorin were prepared by the either injection method according to Deamer and Bangham [30]. A lipid solution in ether (2 μmol/ml) was injected at a rate of 0.25 ml/min into 4 ml of a 100 mM NaCl/10 mM Tris-HCl (pH 7.4) buffer at 55°C. This leads to the formation of large unilamellar vesicles. The vesicles were pelleted at $35\,000 \times g$ for 30 min at 4°C and dialysed extensively against buffer.

Small unilamellar vesicles were prepared by sonicating a dispersion of lipids in buffer under nitrogen for 3–5 min at 0°C, using a Branson tip sonicator at power setting 4. Metal particles from the probe were removed by centrifugation at $30\,000 \times g$ for 30 min at 4°C.

Determination of the lysophosphatidylcholine transbilayer movement

The assay for lysophospholipase treatment has been described previously [31]. Large unilamellar vesicles, with or without glycophorin, consisting of 5 mol% 1-[1-^{14}C]16:0-lysophosphatidylcholine, a trace amount of [9,10-$^3H_2$]palmitic acid as an internal standard and 95% mol% test lipids were incubated at 37°C with 0.4 mg lysophospholipase II dis-

solved in 10 mM potassium phosphate buffer (pH 7.4). The incubation mixture consisted of 0.1–0.6 μmol lipid in 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.4 mM 2-mercaptoethanol in a total volume of 0.5 ml. At different times the reaction was stopped and fatty acid was extracted by a modified Dole extraction procedure [32]. A 500-μl sample of the heptane layer was pipetted into a scintillation vial in order to determine ^{3}H- and ^{14}C-radioactivity. The $^{14}C/^{3}H$ ratio of the released fatty acids, divided by the $^{14}C/^{3}H$ ratio of the original vesicles gave the degree of lysophosphatidylcholine hydrolysis.

Determination of the transbilayer movement of phosphatidylcholine

Two methods were employed to study the transbilayer movement of phosphatidylcholine:

A. The phosphatidylcholine exchange protein was used to determine the exchangeable pool of phosphatidylcholine in large glycophorin-containing vesicles by a similar procedure as described previously [8]. Large glycophorin-containing vesicles (1 μmol $18:1_c$,$18:1_c$-phosphatidylcholine, 10^5 dpm ^{14}C-labeled phosphatidylcholine, 150 mM NaCl, 10 mM Tris-acetic acid (pH 7.4) were incubated with small sonicated vesicles (1.5 μmol $18:1_c$,$18:1_c$-phosphatidylcholine, 10^6 dpm [^{3}H]cholesterol oleate in 150 mM NaCl, 10 mM Tris-acetic acid (pH 7.4) and 50 μg of dialyzed exchange protein in a total volume of 2 ml. After 1 h of incubation at 37°C, a 400-μl sample was withdrawn to determine the percentage of ^{14}C-labeled phosphatidylcholine in the glycophorin-containing vesicles. The large glycophorin-containing vesicles in the remainder of the incubation mixture were sedimented at 35 000 × *g* for 30 min at 4°C, the supernatant was discarded and the large vesicles were dispersed in buffer and subsequently reincubated with a new amount of sonicated vesicles and exchange protein as done in the original incubation. After 1 h again a sample was withdrawn, containing 1/5 of the original amount of large vesicles. This procedure was repeated three times. To determine the percentage of ^{14}C-labeled phosphatidylcholine remaining in the large glycophorin-containing vesicles, the vesicles in the drawn samples were sedimented at 35 000 × *g* for 30 min at 4°C, washed three times with 1 ml of buffer and finally assayed for ^{14}C, ^{3}H and phospholipid phosphorus [33]. The percentage of ^{14}C-labeled phosphatidylcholine remaining in the vesicles was calculated from ^{14}C-radioactivity and phospholipid phosphorus after correction for amounts (3% after 1 h upto 23% after 5 h incubation) of contaminating sonicated vesicles as determined from ^{3}H-radioactivity.

B. Alternatively the appearance of phosphatidyl-[*N*-($^{13}CH_3)_3$]choline in the inside monolayer of large glycophorin-containing vesicles was measured by ^{13}C-NMR, using Dy^{3+} as shift reagent, after introducing the ^{13}C-labeled lipid with phosphatidylcholine exchange protein in the outside monolayer of the vesicles, as described before [17].

Large, unilamellar glycophorin-containing acceptor vesicles (prepared from a dried film consisting of 108 μmol $18:1_c$,$18:1_c$-phosphatidylcholine, 12 μmol $18:1_c$,$18:1_c$-phosphatidylethanolamine, required to make the vesicles impermeable to Dy^{3+} [34], and 0.24 μmol glycophorin) were treated with neuraminidase as described before [17] and incubated with small sonicated donor vesicles (90 μmol $18:1_c$, $18:1_c$-phosphatidyl[*N*-($^{13}CH_3)_3$]choline, [^{14}C]$18:1_c$,-$18:1_c$-phosphatidylcholine, [^{3}H]cholesterol oleate) and 200 μg of dialyzed exchange protein in a total volume of 10.0 ml buffer (150 mM NaCl, 10 mM Tris-acetic acid (pH 7.0) and 0.2 mM EDTA) for 1 h at 37°C. After the exchange, the donor and acceptor vesicles were separated by centrifugation at 37 500 × *g* for 1 h at 4°C. The large glycophorin-containing vesicles (16 μmol lipid, containing 47% dioleoylphosphatidyl[^{13}C]choline) were washed once and finally suspended in 20% $^{2}H_2O$-containing buffer in a total volume of 2.8 ml. The transbilayer distribution of the [^{13}C]$18:1_c$,-$18:1_c$-phosphatidylcholine was measured at 37°C at the indicated times, which represent the time periods from the end of the incubation with exchange protein to the midpoint of the 16 min data accumulation. The data were corrected for the presence of small amounts of donor vesicles (4.6% of the total ^{3}H-radioactivity in the donor vesicles was recovered as a contamination with the acceptor vesicles) and for the intensity of the methyl choline signals arising for natural abundant ^{13}C atoms in the unlabeled dioleoylphosphatidylcholine molecules as described before [9,17].

^{13}C-NMR

^{13}C-NMR measurements to determine the inside/

outside distribution of $18{:}1_c$,$18{:}1_c$-phosphatidyl-[*N*-($^{13}CH_3$)$_3$]choline in large glycophorin-containing vesicles, were performed on a Bruker 360 WS spectrometer at a frequency of 90.5 MHz, as described in detail previously [9,23]. Prior to the NMR experiments 10–20% of ^2H_2O-containing 150 mM NaCl, 10 mM Tris-acetic acid, 0.2 mM EDTA (p^{2}H 7.0) was added to the samples.

^{13}C-NMR measurements, to determine the inside/outside distribution of 16:0-lysophosphatidyl[*N*-$^{13}CH_3(CH_3)_2$]choline in large vesicles, with and without glycophorin, were performed on a Bruker WP-200 spectrometer at a frequency of 50.3 MHz. Spectra were obtained from 500 transients using a 15 μs 90° pulse, a 2 500 Hz sweep width and 16 K data points. Prior to the NMR experiments 10–20% of ^2H_2O-containing 100 mM NaCl, 10 mM Tris-acetic acid, 0.2 mM EDTA (p^{2}H 7.4) was added to the sample. Intensities were determined with respect to external 1,4-dioxane and Dy^{3+} was used as shift reagent [1,34].

General methods

The glycophorin content of the vesicles was checked by sialic acid determination according to Warren [35], and phospholipid phosphorus assay [33]. Cholesterol was measured according to Rudel and Morris [36]. The lipid composition of the vesicles made from the major phospholipids of the human erythrocyte membrane and cholesterol was checked by two-dimensional thin-layer chromatography [37].

Results

Transbilayer movement of lysophosphatidylcholine and phosphatidylcholine in large unilamellar glycophorin-containing vesicles

To test the effect of glycophorin on the transbilayer movement of lipids in large, unilamellar vesicles, which are employed as model membrane systems, protein-free ether evaporation method vesicles [30] and the glycophorin-containing MacDonald vesicles [29] are used. Vesicles prepared of $18{:}1_c$,-$18{:}1_c$-phosphatidylcholine and 16:0-lysophosphatidylcholine by the ether evaporation method are large (3 400 ± 2 300 Å diameter, mean ± S.D. obtained from 100 vesicles) and unilamellar (Fig. 1). Hydration of a dry glycophorin-lipid film (1 : 400 molar ratio) followed by differential centrifugation [22] also results in the formation of large, unilamellar vesicles in which the glycophorin is incorporated in a transmembrane position [22] and in which the glycophorin/lipid molar ratio varies from 1 : 500 to 1 : 200. The size of the vesicles is comparable to the pure lipid vesicles (Fig. 1). On the fracture face small particles, representing glycophorin, are visible in agreement with previous studies [22]. Preparation of the vesicles, containing the different lipids used in this study, always results in the formation of unilamellar vesicles of comparable size. The transbilayer movement of lysophosphatidylcholine for these vesicles was measured by determining the fraction of lysophosphatidylcholine which can be degraded by externally added lysophospholipase. In protein-free vesicles, 50% of the lysophosphatidylcholine was degraded (Fig. 2). Since 50% of the (N-$^{13}CH_3(CH_3)_2$)-signal intensity of similar vesicles made from 16:0-lysophosphatidyl[N-$^{13}CH_3(CH_3)_2$]choline and $18{:}1_c$,-$18{:}1_c$-phosphatidylcholine could be shifted with Dy^{3+} (Fig. 3) it can be concluded that 50% of the lysophosphatidylcholine is located in the outer monolayer and that lysophosphatidylcholine does not move across the $18{:}1_c$,$18{:}1_c$-phosphatidylcholine bilayer, nor that the lysophospholipase treatment induces a transbilayer movement of lysophosphatidylcholine.

In large, glycophorin-containing dioleoylphosphatidylcholine vesicles 80–90% of the lysophosphatidylcholine was degraded (Fig. 4). Since large glycophorin-containing vesicles made of 95 mol% dioleoylphosphatidylcholine and 5 mol% 16:0 lysophosphatidylcholine are leaky for the shift reagent Dy^{3+} [34], it is impossible to determine the transbilayer distribution of the lyso-compound. As the large glycophorin-containing vesicles are impermeable to enzymes (trypsin [22]) of a size comparable to lysophospholipase and based on the size of the vesicles, a symmetrical lysophosphatidylcholine distribution seems most logical, these data strongly suggest a fast transbilayer movement of lysophosphatidylcholine. To test this further, vesicles were made of 95 mol% dioleoylphosphatidylcholine and dioleoylphosphatidylethanolamine in a 9 : 1 molar ratio and 5 mol% 16:0-lysophosphatidylcholine. As these vesicles are not permeable to Dy^{3+} [34], the transbilayer distribution of

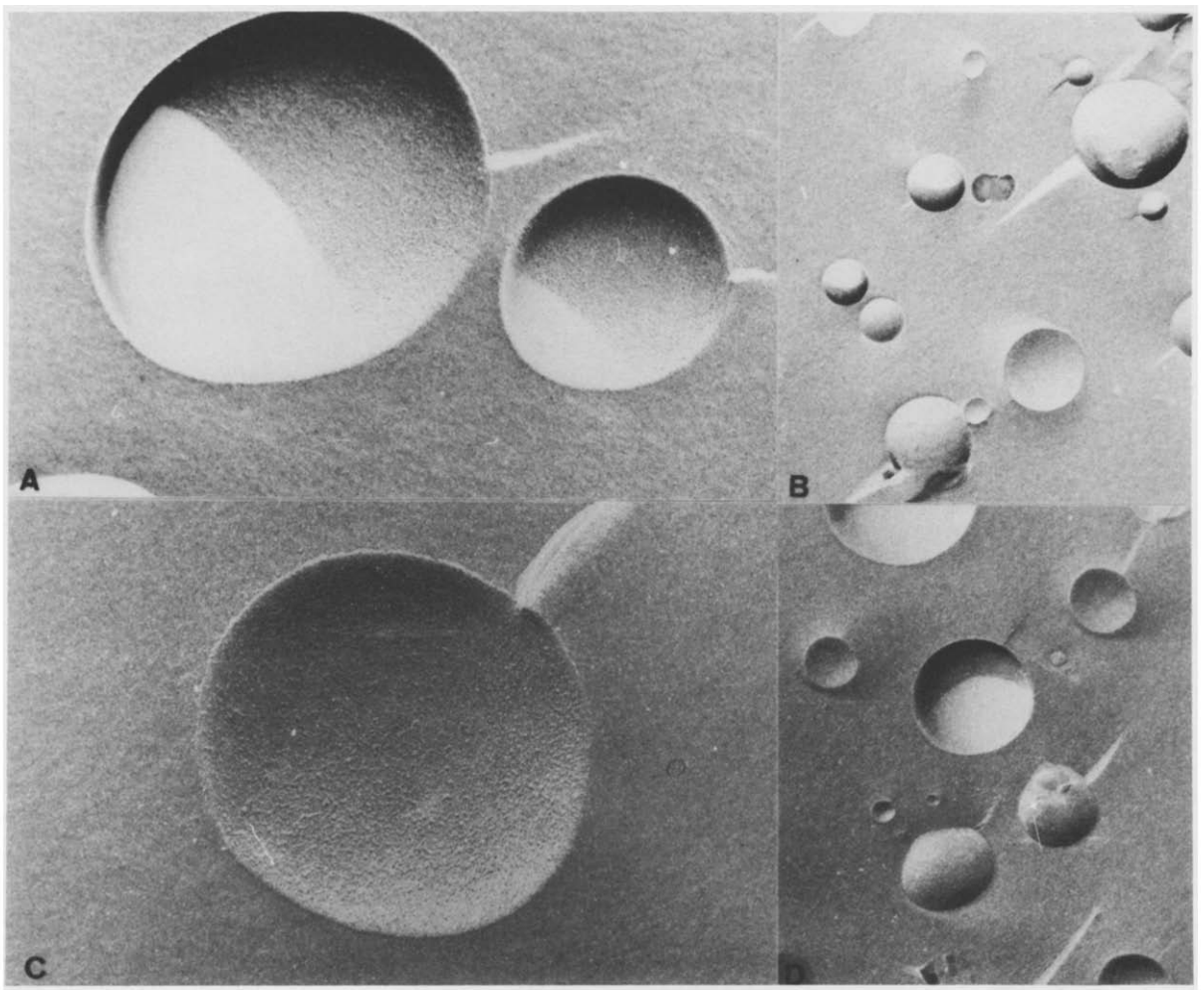


Fig. 1. Freeze-fracture electron micrographs of vesicles containing 95 mol% $18{:}1_c$,$18{:}1_c$-phosphatidylcholine and 5 mol% 16:0-lysophosphatidylcholine. Large, unilamellar vesciles in the absence of glycophorin (A, B). Large, unilamellar glycophorin-containing vesicles (C, D). Magnification: (A, C) × 100 000; (B, D) × 31 250.

lysophosphatidylcholine could be determined. It was found that 47% of the (N-$^{13}CH_3(CH_3)_2$)-signal intensity of the ^{13}C-labeled lysophosphatidylcholine could be shifted by Dy^{3+}, suggesting that 47% of the lysophosphatidylcholine is located in the outer monolayer of the glycophorin-containing vesicles.

With lysophospholipase, 80% of the lysophosphatidylcholine could be degraded within a few minutes (Fig. 4). From these results it is concluded, that glycophorin induces a fast transbilayer movement of lysophosphatidylcholine in large, unilamellar vesicles. Although the time resolution of our assay is rather low and accurate determination of the rate of transbilayer movement is impossible, it can be estimated from Fig. 4 that the halftime of the transbilayer movement is 3 min or less.

The transbilayer movement of dioleoylphosphatidylcholine in large glycophorin-containing MacDonald vesicles was studied by two methods:

A. The size of the exchangeable pool of ^{14}C-labeled dioleoylphosphatidylcholine in large glycophorin-containing vesicles made of dioleoylphosphatidylcholine was determined by repetitive incubations with sonicated 'acceptor' vesicles made of dioleoyl-

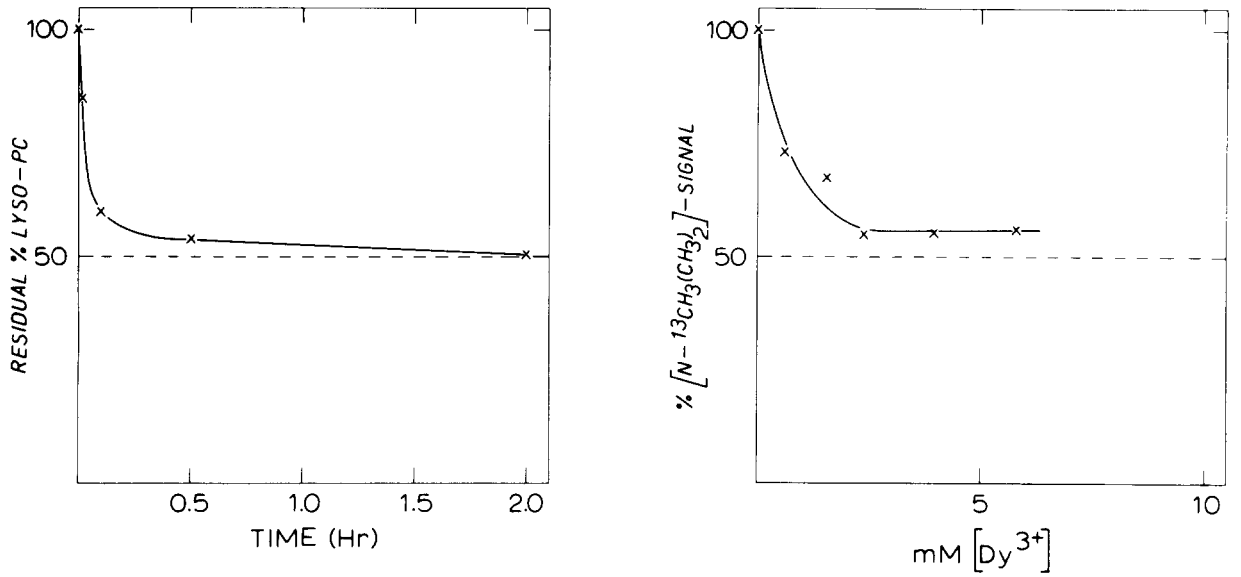


Fig. 2. Time course of lysophosphatidylcholine (lyso-PC) hydrolysis by lysophospholipase II in large, protein-free unilamellar vesicles at 37°C. Vesicles containing 95 mol% 18:1_c,18:1_c-phosphatidylcholine and 5 mol% [^{14}C]16:0-lysophosphatidylcholine were incubated with 0.4 mg enzyme (for details see Methods).

Fig. 3. Effect of Dy^{3+} on the (N-$^{13}CH_3(CH_3)_2$-signal intensity of large, protein-free unilamellar vesicles, containing 95 mol% 18:1_c,18:1_c-phosphatidylcholine and 5 mol% 16:0-lysophosphatidyl[N-$^{13}CH_3(CH_3)_2$]choline. To the 13 mM phospholipid vesicle suspension increasing amounts of $DyCl_3$ were added.

phosphatidylcholine and a trace amount of [^{3}H]cholesterol oleate and phosphatidylcholine exchange protein. Fig. 5 shows that 80% of the ^{14}C-labeled dioleoylphosphatidylcholine could be exchanged after five successive 1-h incubations, each followed by centrifugation for 0.5 h.

B. Alternatively the appearance of phosphatidyl-[N-($^{13}CH_3$)$_3$]choline in the inner monolayer of large, MacDonald vesicles was measured by ^{13}C-NMR after introducing the lipid to the outside monolayer of the vesicles using the phosphatidylcholine exchange protein. Under our experimental conditions, the ^{13}C-labeled dioleoylphosphatidylcholine is transported by the exchange protein from the sonicated donor vesicles to the large glycophorin-containing acceptor vesicles. After the exchange, ^{13}C-labeled dioleoylphosphatidylcholine comprises approx. 47% of the lipid content of the glycophorin-containing MacDonald vesicles. In the experiment depicted in Fig. 6, it is shown that the ^{13}C-labeled dioleoylphosphatidylcholine is initially present in the outer monolayer and is slowly translocated to the inside, such that after 4 h 17% of the ^{13}C-labeled dioleoylphosphatidylcholine can be found in the inner monolayer. It can be estimated that the halftime of the transbilayer movement is about 7 h. Dioleoylphosphatidylcholine [4] and rat liver phosphatidylcholine [5] have been shown to undergo transbilayer movement in small unilamellar sonicated vesicles in the order of days. Additionally, transbilayer movement of rat liver phosphatidylcholine in large protein-free unilamellar vesicles has been demonstrated to be of the same time scale as sonicated vesicles [19]. From these and our data it can be suggested, that glycophorin facilitates the transbilayer movement of dioleoylphosphatidylcholine in large unilamellar MacDonald vesicles. Similar results have previously been observed in small sonicated glycophorin-containing vesicles [17].

The influence of varying lipid compositions on the glycophorin-induced transbilayer movement of lysophosphatidylcholine

The lysophospholipase assay was used to investigate the effect of different lipids on the glycophorin-

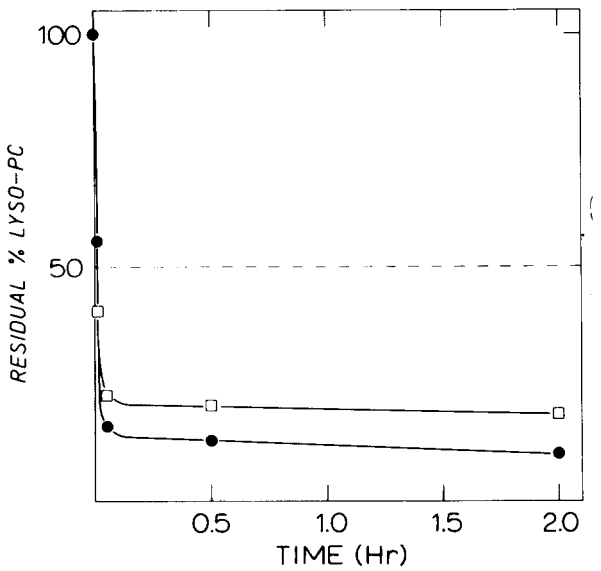


Fig. 4. Time course of lysophosphatidylcholine (lyso-PC) hydrolysis by lysophospholipase in large, glycophorin-containing unilamellar vesicles, with different lipid compositions, at 37°C. Vesicle lipid composition: ●——●, 95 mol% 18:1_c-18:1_c-phosphatidylcholine and 5 mol% [^{14}C]16:0-lysophosphatidylcholine. □——□, 95 mol% 18:1_c,18:1_c-phosphatidylcholine and 18:1_c,18:1_c-phosphatidylethanolamine in a 9 : 1 molar ratio and 5 mol% [^{14}C]16:0-lysophosphatidylcholine. Vesicles were incubated with 0.4 mg enzyme (for details see Methods).

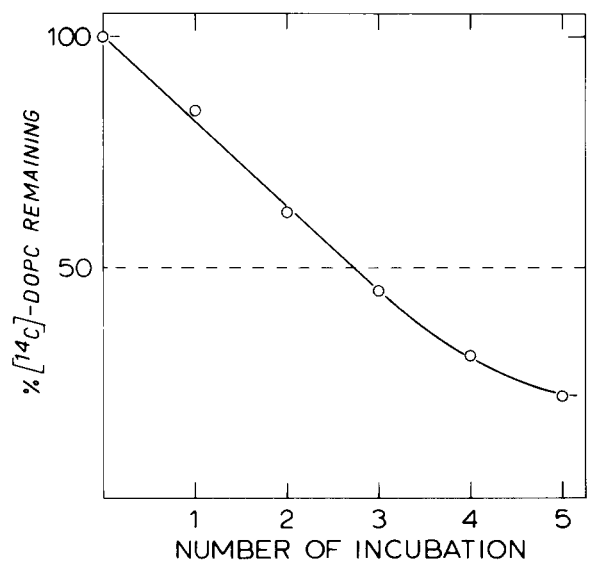


Fig. 5. Percentage of [^{14}C]18:1_c,18:1_c-phosphatidylcholine ([^{14}C]DOPC) exchangeable with phosphatidylcholine exchange protein in large, glycophorin-containing unilamellar 18:1_c,18:1_c-phosphatidylcholine vesicles. For experimental details see Methods.

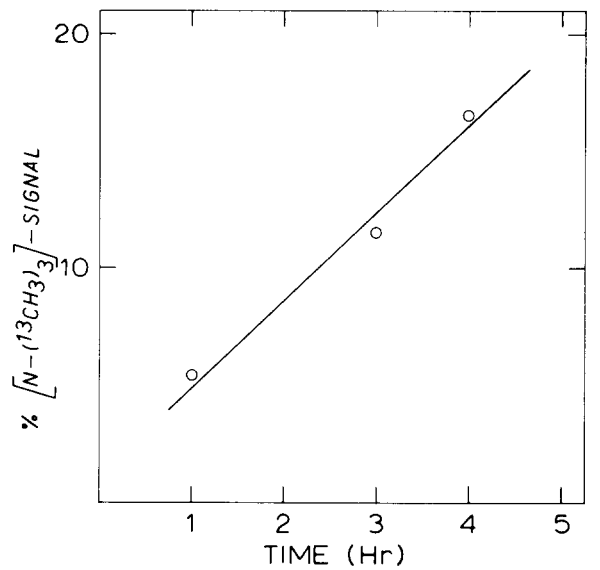


Fig. 6. Time dependence of the (N-($^{13}CH_3$)$_3$)-signal intensity of 18:1_c,18:1_c-phosphatidyl[N-($^{13}CH_3$)$_3$]choline in the inner monolayer of large, glycophorin-containing unilamellar vesicles after introduction of the labeled lipid by the exchange protein to the outer monolayer of the vesicles. For experimental details see Methods.

induced transbilayer movement of lysophosphatidylcholine. Large glycophorin-containing MacDonald vesicles containing 95 mol% test lipids and 5 mol% 1-[1-^{14}C]16:0-lysophosphatidylcholine were incubated with the enzyme at 37°C. Cholesterol was utilyzed as the first test lipid since it occurs in large quantities in the erythrocyte membrane. Increasing the cholesterol/phosphatidylcholine molar ratio from 0.5 to 0.8 in the vesicle leads to a decrease in the total fraction of lysophosphatidylcholine which can be degraded by the enzyme (Fig. 7). The rate at which the lysophosphatidylcholine is degraded in the accessible pool is comparable to the cholesterol-free vesicles (Fig. 7). As these vesicles are unilamellar as judged by freeze-fracturing, these data would indicate the existence of different types of vesicles in the preparation which differ by their lysophosphatidylcholine transbilayer movement properties. Cholesterol apparently increases the fraction of the vesicles in which transbilayer movement of lysophosphatidylcholine is inhibited.

To test the effect of a complex fatty acid composition of the lipid on the glycophorin-induced transbi-

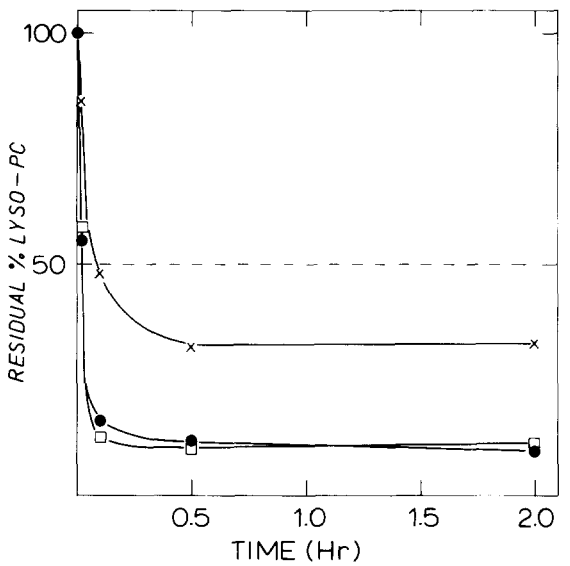


Fig. 7. The influence of cholesterol on the lysophosphatidylcholine (lyso-PC) hydrolysis by lysophospholipase II in large, glycophorin-containing unilamellar vesicles at 37°C. Vesicle lipid composition: •———•, 95 mol% 18:1_c,18:1_c-phosphatidylcholine and 5 mol% [^{14}C]16:0-lysophosphatidylcholine. □———□, 95 mol% 18:1_c,18:1_c-phosphatidylcholine and cholesterol in a cholesterol/phosphatidylcholine = 0.5 molar ratio and 5 mol% [^{14}C]16:0-lysophosphatidylcholine. X———X, 95 mol% 18:1_c,18:1_c-phosphatidylcholine and cholesterol in a cholesterol/phosphatidylcholine = 0.8 molar ratio and 5 mol% [^{14}C]16:0-lysophosphatidylcholine. Vesicles were incubated with 0.4 mg enzyme (for details see Methods).

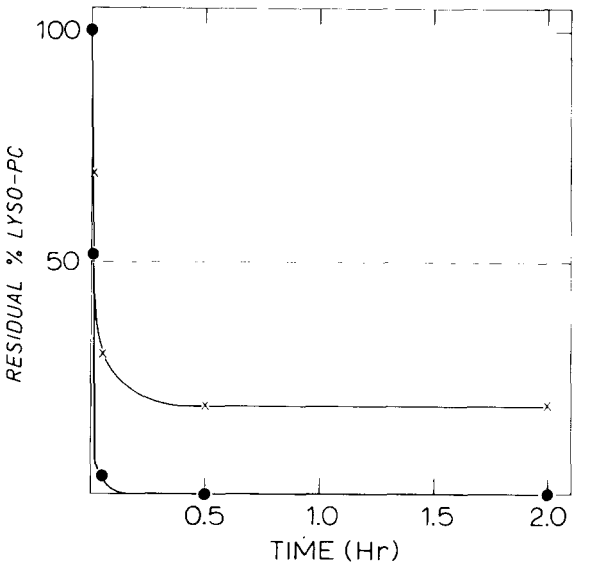
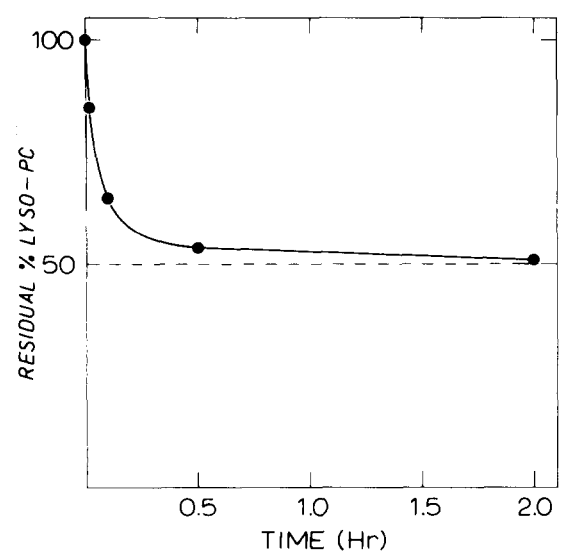


Fig. 8. Time course of lysophasphatidylcholine (lyso-PC) hydrolysis by lysophospholipase II in large, glycophorin-containing unilamellar vesicles, made of egg phosphatidylcholine in the presence and absence of cholesterol at 37°C. Vesicle lipid composition: ○———○, 95 mol% egg phosphatidylcholine and 5 mol% [^{14}C]16:0-lysophosphatidylcholine. X———X, 95 mol% egg phosphatidylcholine and cholesterol in a cholesterol/phosphatidylcholine = 0.8 molar ratio and 5 mol% [^{14}C]16:0-lysophosphatidylcholine. Vesicles were incubated with 0.4 mg enzyme (for details see Methods).



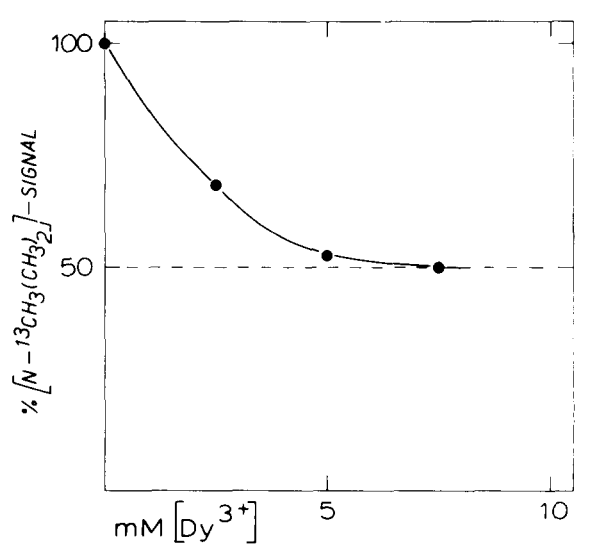


Fig. 9. Time course of lysophosphatidylcholine (lyso-PC) hydrolysis by lysophospholipase II in large, glycophorin-containing vesicles, with a complex lipid composition at 37°C. Vesicles containing 95 mol% total lipids of the human erythrocyte membrane and 5 mol% [14]16:0-lysophosphatidylcholine were incubated with 0.4 mg enzyme (for experimental details see Methods).

Fig. 10. Effect of Dy^{3+} on the (N-$^{13}CH_3(CH_3)_2$)-signal intensity of large, glycophorin-containing unilamellar vesicles, containing 95 mol% total lipids of the human erythrocyte membrane and 5 mol% 16:0-lysophosphatidyl[N-$^{13}CH_3(CH_3)_2$]choline. To the 23 mM phospholipid vesicle suspension increasing amounts of $DyCl_3$ were added.

layer movement of lysophosphatidylcholine, experiments were performed with glycophorin-containing MacDonald vesicles made of egg phosphatidylcholine in the presence and absence of cholesterol. Fig. 8 shows a 100% degradation of lysolecithin in vesicles made of egg phosphatidylcholine. Again, cholesterol introduces an increased amount of lysophosphatidylcholine which cannot be degraded. It is not possible to determine the transbilayer distribution of lysophosphatidylcholine by NMR in cholesterol-containing structures as these MacDonald vesicles are permeable to shift reagents.

Degradation of lysophosphatidylcholine in large glycophorin-containing vesicles made of the total lipids from the human erythrocyte membrane containing cholesterol is shown in Fig. 9. In these vesicles, made of lipids with not only a complex fatty acid composition, but also a complex phospholipid composition with different headgroups, 50% of the lysophosphatidylcholine is degraded by the enzyme. These structures are not permeable to Dy^{3+}. It is shown in Fig. 10 by ^{13}C-NMR, that in these structures 50% of the 16:0-lysophosphatidyl[*N*-$^{13}CH_3$ $(CH_3)_2$]choline is located on the outside of the vesicles, suggesting an equimolar distribution of the lyso compound. From these results it can be concluded that glycophorin incorporation cannot introduce transbilayer movement of lysophosphatidylcholine in large vesicles, made of the total lipids from the human erythrocyte.

Discussion

In this study it is shown that glycophorin, a membrane-spanning protein from the human erythrocyte membrane, induces a transbilayer movement of phosphatidylcholine and lysophosphatidylcholine when it is incorporated in large, unilamellar vesicles of 18:1$_c$,-18:1$_c$-phosphatidylcholine. As a similar effect has been observed in small sonicated vesicles [1,17] it can be concluded that the bilayer curvature plays no major role in this process.

In discussing the mechanism of the glycophorin-induced transbilayer movement, several possibilities should be considered. Firstly, the protein itself could be directly involved in the translocation step in a way similar to that of membrane transport proteins. This possibility is unlikely in view of the following observations: transport proteins in general show a great specificity towards the transported component, whereas glycophorin catalyzes the transport of structurally different lipids such as phosphatidylcholine and lysophosphatidylcholine. Furthermore, band 3, the other major integral membrane protein of the erythrocyte membrane, also catalyzes the transbilayer movement of phosphatidylcholine in vesicles [18]. In addition, it is shown that the glycophorin-induced transbilayer movement is lipid dependent and does not occur in vesicles prepared from the total lipids of the erythrocyte.

A more likely explanation for the protein-induced transbilayer movement of lipids is that it occurs via discontinuities of the lipid bilayer induced by the protein. This could be due either to the irregular surface of the membrane-spanning part of the protein or a mismatch between the length of this hydrophobic domain and the bilayer thickness as has been hypothesized by Israelachvili [38]. Such defects could be responsible for the observed transbilayer movement of the lipids and the enhanced permeability and accessibility for phospholipases [39]. The lipid dependence of these processes then can be understood by assuming that special shaped lipids could accommodate the protein more properly in the lipid bilayer thereby decreasing the amount of packing deficiencies. Such a lipid could be unsaturated phosphatidylethanolamine which has a conical shape (with the polar head group at the smaller end of the cone) and which has been shown to diminish the glycophorin-induced Dy^{3+} permeability in 18:1$_c$,18:1$_c$-phosphatidylcholine bilayers [34]. Further investigations are necessary to determine whether or not phosphatidylethanolamine is the component inhibiting transbilayer movement in the erythrocyte lipid mixture. Finally, it should be realized that glycophorin has a strong tendency for aggregation and even in SDS it can form dimers [40] and in vesicles dimers to hexamers [22]. It is therefore possible that this protein aggregation plays a role in these transport processes.

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