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GLYCOPHORIN FACILITATES THE TRANSBILAYER MOVEMENT OF PHOSPHATIDYLCHOLINE IN VESICLES

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Summary

The rate of transbilayer movement of dioleoylphosphatidylcholine in sonicated lipid vesicles is enhanced by at least two orders of magnitude upon incorporation of glycophorin in the bilayer.

Phosphatidylcholine is predominantly localized in the outer layer of the human [1] and rat [2] erythrocyte membrane. It exchanges between the outer and inner layer in the rat erythrocyte membrane at 37°C with a halftime of 4—5 h [2, 3] and in the human erythrocyte membrane at 45°C with a comparable halftime [4]. Evidence for rapid transbilayer movements of phospholipids in bacterial [5] and microsomal [6] membranes have been reported. In lipid vesicles, however, the rate of transbilayer movement of phosphatidylcholine is much slower, with a halftime in the order of days or more [7]. Because membrane proteins may be responsible for this rate difference (see discussion in ref. 7), the transbilayer movement of phosphatidylcholine was studied in lipid vesicles containing glycophorin, a membrane-spanning protein from the human erythrocyte membrane.

N-($^{13}CH_3$)$_3$-labeled 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (dioleoylphosphatidyl[N-($^{13}CH_3$)$_3$]choline) was synthesized from dioleoylphosphatidic acid and N-($^{13}CH_3$)$_3$-labeled choline iodide [8]. The latter compound was formed from stoichiometric amounts of 90% enriched $^{13}CH_3I$ and ethanolamine during heating for 8 h at 90°C in an aqueous solution of 3 M Na_2CO_3. The fatty acids of the phosphatidylcholine were found to be chemically modified during this reaction, most likely due to the presence of iodine. The acyl chains were therefore removed and the $CdCl_2$-adduct of the N-($^{13}CH_3$)$_3$-labeled glycero-3-phosphocholine was subsequently reacylated with the acid chloride of oleic acid [9]. The purified dioleoylphosphatidyl-[N-($^{13}CH_3$)$_3$]choline was indistinguishable from dioleoylphosphatidylcho-

line prepared according to the method described in ref. 9, as judged by thin-layer chromatography and fatty acid analysis, and displayed an identical phase transition in an aqueous dispersion (measured by differential scanning calorimetry). [^{14}C]Dioleoylphosphatidylcholine (spec. act. 250 dpm/nmol) was prepared as described before [10]. [^{3}H]Cholesterol oleate [10], phosphatidylcholine exchange protein purified from beef liver [10] and purified and delipidated glycophorin [11] were obtained as described before. The transbilayer distribution of dioleoylphosphatidyl [N-($^{13}CH_3$)$_3$]choline in the vesicles was measured by ^{13}C NMR using $DyCl_3$ (B.D.H., Poole, U.K.) as shift reagent [10, 12, 13]. The transbilayer distribution of dioleoylphosphatidylcholine in the vesicles was determined by ^{31}P NMR using $Nd(NO_3)_3$ (Koch Light, Colnbrook, U.K.) as shift reagent [14]. Analytical procedures were used as described in refs. 10 and 12.

Sonicated unilamellar dioleoylphosphatidylcholine vesicles containing an average of 4—5 copies of glycophorin per vesicle were prepared as described in ref. 12. These vesicles have a similar size distribution as sonicated protein-free vesicles of dioleoylphosphatidylcholine (ref. 12 and Ververgaert, P.H.J.Th., unpublished spray-freezing results). The protein-free vesicles were therefore used as control. The orientation of glycophorin in the protein-containing vesicles is such that all sialic acid residues are facing outward, similar to the situation in the erythrocyte membrane [11].

Two approaches have been used in this study for investigating the transbilayer movement of dioleoylphosphatidylcholine in these vesicles. In the first approach dioleoylphosphatidyl[N-($^{13}CH_3$)$_3$]choline was introduced into the sonicated vesicles by means of a phospholipid exchange protein, using large (100—500 nm diameter), unilamellar glycophorin-containing vesicles [11] prepared from the labeled dioleoylphosphatidylcholine as donor. These vesicles are easily separated from the sonicated vesicles by means of centrifugation. A disadvantage of this method is that the exchange rate of dioleoylphosphatidylcholine from these large unsonicated vesicles is about 14 times less when compared to sonicated vesicles (assayed as described in ref. 10) which is in agreement with other observations [15]. Since the rate of exchange was also decreased by the presence of the sialic acid groups on the glycophorin molecule, both the sonicated acceptor vesicles and the unsonicated donor vesicles were treated with neuraminidase (*Vibrio cholerae;* Koch Light, Colnbrook Bucks, U.K.). Under the experimental conditions outlined in the legend of Fig. 1, 9—12% of the dioleoylphosphatidyl[N-($^{13}CH_3$)$_3$]choline molecules, initially present in the donor vesicles, were transported to the acceptor vesicles. Without the presence of exchange protein the transport was less than 1%. Fig. 1 shows that in the protein-free acceptor vesicles some 5% of the dioleoylphosphatidyl[N-($^{13}CH_3$)$_3$]choline-labeled molecules were present in the inner layer, 3.5 h after the incubation with the exchange protein. This fraction remained almost constant upon further incubation at 25°C. After resonication, 37% of the labeled molecules were found to be located in the inner layer. Although the origin of the small inside pool of dioleoylphosphatidyl[N-($^{13}CH_3$)$_3$]choline is not clear, the results confirm previous observations implying that the transbilayer movement of dioleoyphosphatidylcholine in sonicated vesicles is a very slow process

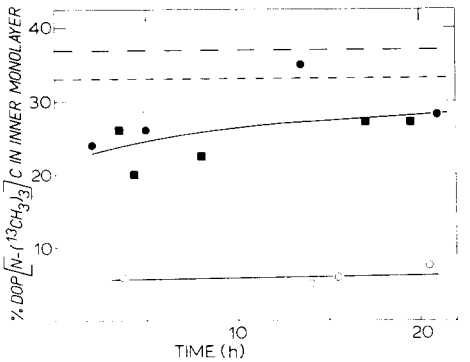

Fig. 1. Transbilayer distribution of dioleoylphosphatidyl[N-($^{13}CH_3$)$_3$]choline (DOP[N-($^{13}CH_3$)$_3$]C) introduced by the exchange protein in vesicles of dioleoylphosphatidylcholine with (●, ■) or without (○) glycophorin, as a function of time. The dotted line represents the total transbilayer distribution of dioleoylphosphatidylcholine in vesicles with (– – –) or without (— —) glycophorin. The data for the glycophorin-containing vesicles were obtained from two independent experiments (● and ■). Large unilamellar glycophorin-containing vesicles were prepared from a dried film consisting of 30 μmol dioleoylphosphatidyl[N-($^{13}CH_3$)$_3$]choline, 10^5 dpm [^{14}C]dioleoylphosphatidylcholine, trace (0.1% by weight) [^{3}H]cholesterol oleate (a non-exchangeable marker) and 3 mg glycophorin (dry weight), by suspending the film in 5 ml 25 mM NaCl/10 mM Tris/HAc, pH 7.0, buffer as described before [9, 12]. Sialic acid was removed from these structures by incubation with 50 units neuraminidase at 37°C for 1 h. Vesicles were pelleted by centrifugation at 125 000 × *g* for 45 min at 0°C [11], reincubated for 1 h at 37°C with fresh buffer containing 0.2 mM EDTA, and pelleted again. Vesicles were finally suspended in a 80—90% yield in 1.0 ml buffer (donor vesicles). Small unilamellar vesicles were prepared from 90 μmol dioleoylphosphatidylcholine with or without 9 mg glycophorin (dry weight) by ultrasonication of the large structures in 4.5 ml buffer as described elsewhere [12]. The sonicate was incubated with 150 units neuraminidase at 37°C for 1 h, and was subsequently applied to a Sepharose 4-B column [12]. The fractions containing the unilamellar vesicles (20—25 ml) were pooled and concentrated to 4.0 ml by vacuum dialysis as described before [19] and centrifuged at 125 000 × *g* for 45 min at 0°C in order to remove traces of aggregated material. The supernatant contained 40—50% of the original phospholipid phosphorus and had an identical protein:lipid ratio as the vesicles isolated immediately after the Sepharose 4-B column chromatography (acceptor vesicles). Donor and acceptor vesicles were incubated with 2.0 ml exchange protein (100 μg/ml; dialysed against the above buffer prior to the experiment) in a shaking water-bath for 1 h at 37°C. After the exchange, the donor and acceptor vesicles were separated by centrifugation at 200 000 × *g* for 60 min at 0°C. The supernatant, containing 90—100% of the acceptor vesicles, was passed through a small DEAE-cellulose column (prepared from 4 ml of a 20% (v/v) DEAE slurry), and eluted with 20% ^2H_2O-containing buffer in order to remove the exchange protein (yield of acceptor vesicles, 60—70%). The acceptor vesicles were subsequently stored at 25°C and the transbilayer distribution of the lipids was measured at 25°C at the indicated times, which represent the time periods from the end of the incubation with exchange protein to the midpoint of the 30 min data accumulation. The data were corrected for the presence of small amounts of donor vesicles (0—1% 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 from natural abundant ^{13}C atoms in the unlabeled dioleoylphosphatidylcholine molecules as described before [10]. The sum of both corrections was maximal 10% of the observed signal intensity from the molecules present in the inner monolayer.

[10, 16]. In strong contrast with the above results it was observed with glycophorin-containing acceptor vesicles that 25% of the dioleoylphosphatidyl-[N-($^{13}CH_3$)$_3$]choline molecules were present in the inner layer 2 h after the incubation (Fig. 1). This fraction did not increase upon further incubation at 25°C. This clearly shows that in the presence of glycophorin, dioleoylphosphatidyl[N-($^{13}CH_3$)$_3$]choline can migrate from the outer to the inner layer. Since 33% of the dioleoylphosphatidylcholine molecules were found to be located in the inner layer of these vesicles [12], it can be concluded that a minor fraction of the acceptor vesicles exhibits a very slow transbilayer movement. This may be an indication that a fraction of the vesicles contain less or no protein molecules. In order to test the integrity of the vesicles, the

experiment described in Fig. 1 was repeated, using donor and acceptor vesicles of unlabeled dioleoylphosphatidylcholine. The transbilayer distribution of dioleoylphosphatidylcholine in the sonicated glycophorin-containing vesicles was measured by means of ^{31}P NMR. Before the addition of the donor vesicles and the exchange protein 36% of the lipid molecules were found to be located in the inner layer, and 37% after the exchange procedure. The linewidth and total intensity of the ^{31}P NMR signal of the vesicles were found to be constant; both parameters are very sensitive to the vesicle size [14]. These and other observations presented in the legend of Fig. 1 demonstrate that the vesicles remain intact during incubation, and that the outward-inward movement of dioleoylphosphatidylcholine is counterbalanced by an inward-outward movement. Since the acceptor vesicles were found to be impermeable to the shift reagent both before, during and after incubation with donor vesicles and exchange protein, it can be concluded that the barrier properties of the vesicle bilayer are maintained and that the much larger exchange protein is not able to enter the vesicles.

In the second approach, the size of the exchangeable pool of dioleoylphosphatidylcholine in the sonicated vesicles was determined by repetitive incubations of sonicated [^{14}C]dioleoylphosphatidylcholine vesicles with unsonicated glycophorin-containing vesicles prepared from unlabeled dioleoylphosphatidylcholine and exchange protein, as described in the legend of Fig. 2. The percentage of the label that could not be exchanged in the protein-free vesicles approaches the value of the percentage of dioleoylphosphatidylcholine molecules present in the inner layer (Fig. 2), in agreement with previous studies [16]. This is interpreted as only dioleoylphosphatidylcholine molecules in the outer layer of these vesicles being exchangeable. In the sonicated glycophorin-containing vesicles, however, at least 90% of the dioleoylphosphatidylcholine molecules were exchangeable (Fig. 2), and since 67% of the lipid molecules are present in the outer layer of these vesicles, this demonstrates that molecules originating from the inner layer are also exchangeable. This can only occur when dioleoylphosphatidylcholine is able to move from the inner to the outer layer in these glycophorin-containing vesicles. The non-exchangeable pool of 10% might again reflect a fraction of vesicles which contains less or no protein molecules. It should be realized that the rate of exchange of the protein-containing and protein-free vesicles used in Fig. 2 cannot be compared directly, since different amounts of sonicated vesicles were used.

It has been shown in this paper that glycophorin greatly facilitates the transbilayer movement of dioleoylphosphatidylcholine in sonicated vesicles. It is important to note that during the experiments the vesicles remain in a state of equilibrium, with a uniform lipid composition and a constant ratio of molecules in inner and outer monolayer. Exact rates cannot be derived from our data, but it can be concluded from Fig. 1 that the transbilayer movement of dioleoylphosphatidylcholine in glycophorin-containing vesicles at 37° C must have a halftime of 1 h or less. In pure lipid vesicles a halftime of 11 days or more was reported for this process [16]. The mechanism by which a membrane-spanning protein like glycophorin facilitates the process of transbilayer movement is not yet understood. The observation that also

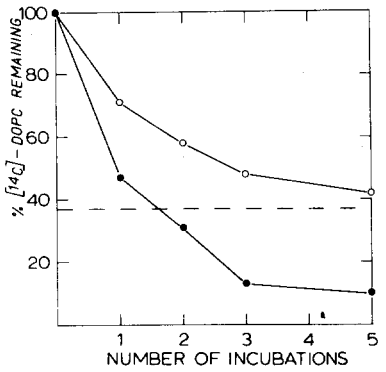

Fig. 2. Percentage of [^{14}C]dioleoylphosphatidylcholine (DOPC) exchangeable with phosphatidylcholine exchange protein in sonicated vesicles with (●) and without (○) glycophorin. The dotted line represents the percentage of molecules present in the inner monolayer of dioleoylphosphatidylcholine vesicles. Sonicated and unsonicated glycophorin-containing vesicles were prepared and treated with neuraminidase as described in the legend of Fig. 1. In this experiment the sonicated vesicles contained 10^5 dpm [^{14}C]dioleoylphosphatidylcholine and the large unsonicated vesicles were prepared from unlabeled dioleoylphosphatidylcholine and trace [^{3}H]cholesterol oleate. 1.5 ml sonicated vesicles (3.0 mM dioleoylphosphatidylcholine without and 1.5 mM dioleoylphosphatidylcholine with glycophorin) were incubated with 200 μl unsonicated vesicles (25.0 mM) and 1.5 ml exchange protein (100 μg/ml) for 1 h at 37°C. Unsonicated vesicles were removed by centrifugation at 200 000 × *g* for 1 h at 0°C and aliquots of the supernatant were assayed for radioactivity and phospholipid phosphorus. The supernatant was subsequently reincubated with a new, identical amount of unsonicated vesicles and an aliquot of new exchange protein, and this procedure was repeated 4 times. The percentage of [^{14}C]-dioleoylphosphatidylcholine remaining in the sonicated vesicles was calculated from [^{14}C]radioactivity and phospholipid phosphorus, after correction for small amounts (0—1%) of contaminating unsonicated vesicles as determined from [^{3}H]radioactivity.

the transbilayer movement of lysophosphatidylcholine in the vesicles is enhanced by two orders of magnitude upon the incorporation of glycophorin [12] suggests that there is no pronounced lipid specificity. It has been proposed that transbilayer movement occurs at the interface between boundary and bulk lipids, since irregularities in the bilayer may be expected to occur at these interfaces [12, 17]. This is supported by the observation that the rate of transbilayer movement of dimyristoylphosphatidylcholine shows a relative maximum under conditions where ordered and disordered lipids coexist [18]. It can, however, not be excluded that the protein itself is directly involved in the process of transbilayer movement of phospholipids.

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