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GLYCOPHORIN INCORPORATION INCREASES THE BILAYER PERMEABILITY OF LARGE UNILAMELLAR VESICLES IN A LIPID-DEPENDENT MANNER

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(1) The effect of glycophorin incorporation on the permeability properties of lipid bilayers is investigated by employing a new method, in which the trap of a small permeant and a high molecular weight, non-permeable molecule are simultaneously monitored. (2) Glycophorin induces a high permeability to potassium and glucose in large dioleoylphosphatidylcholine vesicles. (3) The glycophorin-induced enhanced permeability is found to be lipid dependent. By monitoring the % ratio of the glucose (permeant)/dextran (impermeant) trap in large unilamellar glycophorin-containing vesicles, it was determined immediately after removal of non-enclosed marker, that glycophorin-containing vesicles composed of dioleoylphosphatidylcholine were highly permeable to glucose (% glucose/dextran trap = 1.4), whereas glycophorin-containing vesicles prepared from total human erythrocyte lipids demonstrated a greatly reduced permeability in glucose (% glucose/dextran trap = 50). (4) The glycophorin-induced increased permeability appears to be related to transbilayer movement of lipid molecules. Glycophorin induces a fast lipid-transbilayer movement of lysophosphatidylcholine and a high permeability to potassium and glucose in dioleoylphosphatidylcholine vesicles, whereas glycophorin-containing vesicles comprised of total human erythrocyte lipids show no lipid-transbilayer movement and only a slight permeability enhancement to glucose, as compared to the protein-free vesicles.

Introduction

The biological membrane provides a selective barrier to a large variety of physiologically important solutes. This function is determined by the structure of the cell membrane, which is conferred by lipids and proteins, the main constituents of membranes. Lipid bilayers, spontaneously formed when membrane lipids are dispersed in water, are known to be freely permeable to water and small non-electrolytes but in general they form an efficient barrier against ions and larger polar solutes. When proteins are reconstituted into lipid model membranes this often results in an increase of the membrane permeability. This increase can be due to the formation of specific pore-type of structures by the protein molecules themselves [1] but it can also be the consequence of irregularities in the structural organization of the membrane at the lipid-protein interfaces. Such boundary effects are also found in pure lipid systems as a result of lipid phase separation at the temperature of the phase transition [2]. In the boundary region of the liquid and solid domains, defects in the packing of the lipid molecules allow the formation of transient pores through which small hydrated ions can permeate. This effect is dependent on the shape and nature of the lipids [3]. Little is known about the relationship between irregularities in the structural organization of the membrane at the lipid-protein

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interfaces and transport processes including lipid-transbilayer movement [4,5].

At present, information concerning the relationship between lipid-protein interactions and barrier properties of membranes has been mainly obtained from studies on glycophorin-containing model membranes. This membrane-spanning protein from the human erythrocyte membrane is well-suited for such studies as the protein itself has no known transport function.

It was reported that the conductance of black lipid membranes is significantly increased upon the incorporation of glycophorin [6]. Furthermore, large glycophorin-containing dioleoylphosphatidylcholine vesicles were found to be permeable to Dy^{3+} [7]. Also the hydrophobic tryptic peptide of glycophorin increases the permeability of Na^+ through the lipid bilayer [8]. In contrast, Mimms et al. [9] observed that glycophorin incorporated into vesicles prepared by the octylglucoside method had only a negligible effect on the permeability properties of the lipid bilayer. In addition to the permeability data it has been demonstrated that glycophorin incorporated into large unilamellar dioleoylphosphatidylcholine vesicles induces transbilayer movement of lysophosphatidylcholine and phosphatidylcholine [10]. In vesicles prepared from total erythrocyte lipids (including 45 mol% 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 [10]. The interpretation of all these data is complicated because the methods used in these studies do not necessarily give information on the permeability characteristics of the entire vesicle population. This is particularly relevant in view of the heterogeneity of these lipid-protein vesicles and the possibility that part of the vesicles are extremely permeable to the molecule under investigation. In order to gain further insight into barrier properties at the glycophorin-lipid interface we study the permeability properties of glycophorin-containing model membranes made of different lipids using a new assay which can give reliable information even in heterogeneous systems. These results are compared to the effect that glycophorin has on the transbilayer movement of lysophosphatidylcholine in the same vesicles. It is found that glycophorin incorporation in dioleoylphosphatidylcholine vesicles markedly increases the glucose and potassium permeability of these vesicles. Incorporation of the protein in vesicles made of the total lipid extracts of human erythrocytes did not significantly affect the permeability of these vesicles. In that the glycophorin-induced permeability paralleled the glycophorin-induced lysophosphatidylcholine transbilayer movement [10], it is suggested that packing defects at the protein-lipid interface are the sites of both transport processes.

Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine ($18:1_c/18:1_c$-phosphatidylcholine) and 16:0-lysophosphatidylcholine were synthesized as described before [11]. The total lipids of the human erythrocyte membrane were extracted from ghosts by *n*-butanol [12]. The solvent was evaporated under reduced pressure. The lipids were redissolved in chloroform and cholesterol was removed on a silicic acid column as described by van Dijck et al. [13]. L-[1-^{14}C]16 : 0-lysophosphatidylcholine (57 mCi/mmol), glycerol-tri-[9,10 (n)-^{3}H]oleate (500 mCi/mmol), D-[^{14}C]glucose (268 mCi/mmol) and [^{3}H]dextran (174 mCi/g) were obtained from Amersham International (Utrecht, The Netherlands). ^2H_2O was purchased from Merck, Sharpe and Dohme (Montreal, Canada). Cholesterol was obtained from Merck (Darmstadt, F.R.G.). 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. The enzyme lysophospholipase II was a kind gift from the group of Dr. H. van den Bosch from this laboratory. Glucose was obtained from Corn Products Company (Utrecht, The Netherlands). Dextran, M_r 60000–90000, was obtained from Serva Labor (Heidelberg, F.R.G.), and fatty acid-poor albumin (bovine) was purchased from Calbiochem (San Diego, U.S.A.). All other chemicals were of analytical grade.

Methods

Purification of glycophorin. Human erythrocyte ghosts were prepared according to the method of Parpart [14]. Glycophorin was isolated and puri-

fied from ghosts, according to a modification of the method of Verpoorte [15] as was described before [16]. The purified protein showed two bands (PAS I and PAS II) on sodium dodecylsulphate-polyacrylamide gels stained with Coomassie blue and with periodate schiff reagent [17]. The glycophorin contained 2.1 μmol sialic acid/mg protein and less than 0.4 mol phosphorus per mol glycophorin.

Preparation of vesicles. Glycophorin was incorporated into large unilamellar lipid vesicles by the method of MacDonald and MacDonald [18]. A mixture of glycophorin and lipids was dissolved in chloroform/methanol/water (150:75:1, v/v) and dried by evaporation. The lipid-protein film was hydrated in the appropriate buffer. This hydration led to the formation of large unilamellar glycophorin-containing vesicles (diameter 100–500 nm), which can be purified from other structures by stepwise centrifugation [19]. Multilamellar structures, containing little or no protein were removed by centrifugation at $10000 \times g$ for 10 min at 4°C. In order to pellet the glycophorin-containing vesicles, the supernatant was centrifuged at $37000 \times g$ for 30 min at 4°C.

Large unilamellar vesicles without glycophorin were prepared by the ether injection method according to Deamer and Bangham [20] as described in detail before [10], or by the reverse-phase evaporation method as described by Szoka and Papahadjopoulos [21]. These latter vesicles, which were used for entrapment experiments, were prepared by dissolving a dry film of 30 μmol lipid in 3 ml diethyl ether. 1.0 ml of the appropriate buffer was added and the resulting two-phase system was sonicated briefly (2–5 min) under nitrogen in a bath-type sonicator (Bransonic 12) at 0–5°C. The diethyl ether was removed under reduced pressure (water aspirator) by rotary evaporation at 20–25°C. After formation of the vesicles the suspension was kept under reduced pressure for an additional 15 min at 20°C in order to remove traces of diethyl ether. The vesicles were pelleted by centrifugation at $37000 \times g$ for 30 min at 4°C.

Fractionation of large unilamellar glycophorin-containing vesicles. Glycophorin-containing vesicles, prepared according to the method of MacDonald and MacDonald [18] and pelleted by stepwise centrifugation as described in the Method section above were resuspended (± 2 μmol lipid) in 350 μl buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.4) containing 37% (v/v) ^2H_2O in order to increase the density of the solution. The suspension was centrifuged in a Beckman Airfuge at $160000 \times g$ for 30 min at room temperature. The supernatant which contained vesicles with a low glycophorin content was discarded and the pellet, consisting of vesicles with a high glycophorin content was washed two times more with 300 μl buffer containing 37% ^2H_2O.

Determination of entrapment capacity of vesicles for different molecules. In order to correct for possible differences in vesicle sizes between different preparations, methods were used in which simultaneously the trap of a small permeant and a large non-permeable molecule could be determined.

(A) Hemoglobin and potassium entrapment measurements.

In this method the vesicles were prepared in a buffer containing as a large non-permeable molecule hemoglobin and as a small permeant potassium.

Hemoglobin-containing buffer solution was made by lysing 5 ml of washed packed erythrocytes in 100 ml distilled water at 4°C. After 1 h the solution was made 10 mM in Tris and titrated with 0.1 M HCl to pH 7.4. In order to remove erythrocyte membranes, the solution was centrifuged at $37000 \times g$ for 30 min at 4°C. The clear (red) supernatant was made 150 mM in KCl and was subsequently used to prepare vesicles as is described in the Methods section above.

After the appropriate centrifugations the pelleted vesicles were resuspended in 1.5 ml 150 mM choline chloride, 10 mM Tris-HCl (pH 7.4) at 4°C and centrifuged at $37000 \times g$ for 20 min at 4°C. This washing procedure was repeated three times in order to remove non-trapped hemoglobin and potassium. After the fourth washing procedure no hemoglobin could be detected in the supernatant. The potassium entrapment was measured by monitoring the amount of potassium released following the addition of 1% Triton X-100 using a potassium-selective electrode as was described previously [2]. The hemoglobin entrapment was measured by reading the absorbance at 416 nm in choline chloride buffer containing 1% Triton X-

100. The method was calibrated using serial dilutions of the original hemoglobin solution in the same buffer.

(B) Glucose and dextran entrapment measurements.

In this method ^{3}H-labeled dextran was used as a large non-permeable molecule and ^{14}C-labeled glucose as a small permeable molecule. Large unilamellar vesicles with and without glycophorin were made in a buffer containing 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 11 mM glucose, 0.20% dextran, 0.05% NaN_3, 10^6 dpm [^{3}H]dextran/0.5 ml and $5 \cdot 10^5$ dpm [^{14}C]glucose/0.5 ml. After the appropriate centrifugations (as described in above) and in some experiments after fractionation in the same buffer containing 37% ^2H_2O, a sample of the supernatant was withdrawn to determine the concentration of ^{3}H- and ^{14}C-radioactivity. The pellet, containing approx. 1 μmol of lipid vesicles, was resuspended in 1.5 ml of 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.05% NaN_3 and centrifuged at $37000 \times g$ for 10 min at 4°C. The washing procedure was repeated three times in order to remove non-trapped dextran and glucose. After the fourth washing procedure no dextran could be detected in the supernatant.

The ^{3}H- and ^{14}C-content of the vesicles was determined 1 h after the start of the washing procedure by radioactive counting. The efficiency of the washing procedure and possibly an aspecific adsorbtion of glucose or dextran to the vesicles was checked by the addition of dextran and glucose only to the outside of the vesicles after their formation. No dextran or glucose could be detected in the supernatant or pellet after the washing procedure, indicating a total removal of dextran and glucose from the outside of the vesicles.

Determination of the lysophosphatidylcholine transbilayer movement. Two methods were employed to study the transbilayer movement of lysophosphatidylcholine in large unilamellar vesicles.

(A) The available pool of lysophosphatidylcholine was determined by the use of lysophospholipase as was described in detail before [10].

(B) Alternatively, the available pool of lysophosphatidylcholine was determined by extraction of lysophosphatidylcholine from the outer monolayer of the vesicles with fatty acid-poor albumin (bovine) as was described for the erythrocyte membrane by Mohandas et al. [22] and Haest et al. [23]. 0.5 ml suspension of large unilamellar vesicles with or without glycophorin, consisting of 99 mol% $18:1_c/18:1_c$-phosphatidylcholine, 1 mol% 16:0-lysophosphatidylcholine, 10^5 dpm L-[1-^{14}C]16:0-lysophosphatidylcholine, 10^6 dpm glycerol-tri-[9,10 (n)-^{3}H]oleate in buffer (100 mM NaCl, 10 mM Tris-HCl (pH 7.4)) was mixed at room temperature with 0.5 ml of fresh 2% albumin solution in the same buffer. The final lipid concentration in the mixture was approx. 1 mM. Immediately after vortexing a 100 μl sample was withdrawn to determine the total amount of ^{3}H- and ^{14}C-radioactivity (100% value). At different times 175 μl samples were centrifuged at $160000 \times g$ for 10 min at room temperature in a Beckman Airfuge. After centrifugation 100 μl of the supernatant was pipetted into a scintillation vial in order to determine the amount of extracted lysophosphatidylcholine. The data were corrected for the presence of small amount of contaminating vesicles in the supernatant. In general, less than 10% of the total amount of ^{3}H-radioactivity from the non-extractable marker glycerol-trioleate was present in the supernatant.

General methods. The glycophorin-content of vesicles was measured by sialic acid determination according to Warren [24]. Phospholipid phosphorus was determined according to Rouser et al. [25] and cholesterol was measured according to Rudel and Morris [26].

The lipid composition of the extract from the human erythrocyte membrane was checked by two-dimensional thin-layer chromatography [27] and was found to be as was published before [28]. Also the lipid composition of the low-speed pellet (multilayered structures with little or no protein) and high-speed pellet (glycophorin-containing vesicles) in the MacDonald procedure were checked after lipid extraction [29] and found to be the same. The internal volumes of vesicles were determined from entrapment measurements of hemoglobin or dextran and used to calculate outer radii, assuming unilamellar vesicles, a bilayer thickness of 40 Å, an area of 67 Å^2/molecule phosphatidylcholine and an area of 39 Å^2/molecule cholesterol [30].

Results

Characterization of glycophorin-containing vesicles made of various lipid compositions

A. Unfractionated vesicles. Glycophorin-containing vesicles made of dioleoylphosphatidylcholine or total human erythrocyte lipids were prepared according to MacDonald and MacDonald [18] (see Methods) in a 1 : 450 glycophorin/lipid molar ratio. The size distribution histograms of both vesicle preparations derived from freeze-etch electron micrographs are shown in Fig. 1. Glycophorin-containing dioleoylphosphatidylcholine vesicles have a mean diameter of 350 ± 200 nm. A similar size (mean diameter of 340 ± 240 nm) is found for glycophorin-containing erythrocyte lipid vesicles, containing 45 mol% cholesterol.

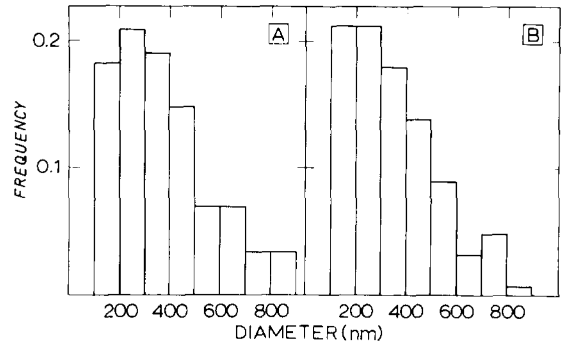

Fig. 1. Size distribution histograms of large unilamellar glycophorin-containing vesicles (protein/lipid molar ratio 1 : 450) made of dioleoylphosphatidylcholine (A) and total erythrocyte lipids including 45 mol% cholesterol (B), derived from freeze-etch electron microscopy. Diameters of 120 randomly chosen vesicles were measured in both vesicle populations, not including particles with a diameter of less than 100 nm.

B. Fractionated vesicles. In order to gain insight into the homogeneity of the protein-content of the vesicle population, glycophorin-containing vesicles prepared by the MacDonald procedure were resuspended in buffer containing 37% ^2H_2O. Vesicles with a high glycophorin-content were pelleted at 160000 × *g* and separated from vesicles containing little protein which remained in the supernatant (Table I). Protein-containing vesicles made of dioleoylphosphatidylcholine recovered in the supernatant after fractionation on 37% ^2H_2O (70%) still show a rather high glycophorin-content of 590 (lipid/glycophorin molar ratio). In the glycophorin-containing vesicle population made of erythrocyte phospholipids 30% of the vesicles have a very low protein-content (lipid/glycophorin molar ratio of > 900). When 45 mol% cholesterol is present, the vesicle fraction with a low glycophorin-content is even 60%, suggesting a more heterogeneous protein distribution in the vesicle population.

Glycophorin-induced changes in the permeabilty properties of the lipid bilayer

The permeability properties of the lipid bilayer

TABLE I

FRACTIONATION AND CHARACTERIZATION OF GLYCOPHORIN-CONTAINING LARGE, UNILAMELLAR VESICLES MADE OF DIFFERENT LIPID COMPOSITIONS IN BUFFER CONTAINING 37% ^2H_2O

Lipid			Lipid/glycophorin (molar ratio)	% of the total amount of phospholipid
DOPC	Unfractionated		500	100
	Fractionated	pellet	300	30
		supernatant	590	70
Erythrocyte phospholipids 0% cholesterol	Unfractionated		590	100
	Fractionated	pellet	430	70
		supernatant	>900	30
Erythrocyte phospholipids 45% cholesterol	Unfractionated		660	100
	Fractionated	pellet	320	40
		supernatant	>900	60

TABLE II

POTASSIUM PERMEABILITY OF DOPC BILAYER IN LARGE, UNILAMELLAR VESICLES

Large glycophorin-containing vesicles [a]			Large protein-free vesicles		
Calculated Hb trap (μl/μmol lipid)	Calculated K^+ trap (μl/μmol lipid)	K^+/Hb trap (%)	Calculated Hb trap (μl/μmol lipid)	Calculated K^+ trap (μl/μmol lipid)	K^+/Hb trap (%)
4.8	0.15	3.1	5.0	3.7	74

[a] The glycophorin/lipid molar ratio is 1 : 600.

of large unilamellar vesicles were investigated using a new method in which the trap of a high molecular weight non-permeable molecule and a small permeant were simultaneously monitored. This method allows one to correct for possible differences in vesicles sizes between different preparations and in particular this method can give information on the barrier properties of the vesicles when they are extremely permeable to small molecules. In an initial experiment, the influence of glycophorin incorporation on the permeability properties of the lipid bilayer was investigated using hemoglobin as a non-permeable molecule and potassium as a small permeable ion. Large unilamellar protein-free vesicles prepared by the reverse-phase evaporation method [21] and large unilamellar glycophorin-containing vesicles prepared by the method of MacDonald and MacDonald [18] were used to trap both hemoglobin and potassium simultaneously. Table II shows the calculated internal volumes derived from the amounts of trapped potassium and hemoglobin as is described in Methods.

The % ratio of K^+/hemoglobin calculated trap of large protein-free vesicles is 74% indicating a 26% release of potassium during the removal of non-trapped material from the dioleoylphosphatidylcholine vesicles (within 2 h at 4°C). The % ratio of K^+/hemoglobin calculated trap of protein-containing vesicles is 3.1%. This indicates that glycophorin incorporation greatly increases the potassium permeability of the vesicles such that now 96.9% of the potassium is lost in the same period of time. In the absence of hemoglobin the potassium trap of glycophorin-containing dioleoylphosphatidylcholine vesicles was found to be of the same order of magnitude, suggesting that the presence of hemoglobin has no influence on the potassium permeability measurements in these vesicles. However, as hemoglobin has been shown to influence the permeability properties of phosphatidylserine-containing vesicles [31] and a slight adsorbance of hemoglobin to the model membranes could influence the ratio of K^+/hemoglobin calculated trap, in further experiments, designed to study glycophorin-induced bilayer permeability, we choose alternative solutes to alleviate this possible complication.

Lipid-dependence of the glycophorin-induced permeability change in model membranes

In these experiments permeability determina-

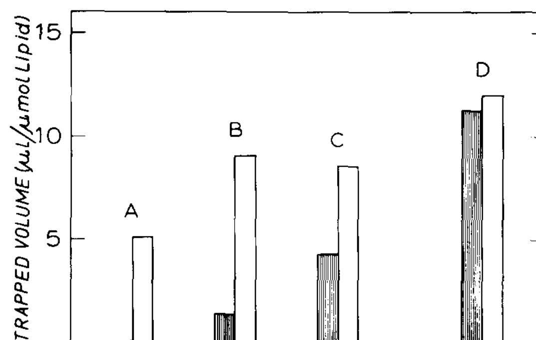

Fig. 2. Glucose trap (shaded) and dextran trap (unshaded) in large, unilamellar glycophorin-containing vesicles and protein-free vesicles made of various lipid mixtures. A, Glycophorin-containing vesicles made of dioleoylphosphatidylcholine (glycophorin/lipid molar ratio of 1 to 500). B. Glycophorin-containing vesicles made of cholesterol-free erythrocyte lipids after fractionation of 37% ^2H_2O-containing buffer (glycophorin/lipid molar ratio of 1 to 310). C, Glycophorin-containing vesicles made of erythrocyte lipids in the presence of 45 mol% cholesterol, after fractionation on 37% ^2H_2O-containing buffer (glycophorin/lipid molar ratio of 1 to 330). D, Protein-free large, unilamellar vesicles made of dioleoylphosphatidylcholine.

tions were performed using ^{14}C-labeled glucose as small permeant and ^{3}H-labeled dextran as a high-molecular weight, non-permeable molecule. Large unilamellar protein-free and glycophorin-containing vesicles were prepared in buffer containing [^{14}C]glucose and [^{3}H]dextran. After removal of non-trapped material, samples were drawn to determine the internal volumes of vesicles derived from the amounts of trapped dextran and glucose. The internal volumes of various vesicles, derived from the amount of trapped dextran and glucose are shown in Fig. 2. The glucose trap (shaded) is presented together with the dextran trap (unshaded) for glycophorin-containing vesicles made of various lipid compositions (A, B and C) and protein-free vesicles (D). The results depicted in Fig. 2 clearly show large differences in glucose trap, which has been determined for the various vesicles compared to small differences in dextran trap. The calculated dextran trap of glycophorin-containing vesicles varies from 5.1 μl/μmol lipid for dioleoylphosphatidylcholine vesicles to 9.1 μl/μmol lipid for vesicles made of erythrocyte lipids without cholesterol. Protein-free dioleoylphosphatidylcholine vesicles have a calculated dextran trap of 12 μl/mol lipid. The % ratio of the glucose/dextran calculated traps of the same vesicles are summarized in Table III. Large protein-free dioleoylphosphatidylcholine vesicles show a % ratio of glucose/dextran calculated trap of 94%, indicating a very low glucose leakage during the removal of non-trapped material (1 h at 4°C) which is in agreement with previous results observed by Demel et al. [32]. Variation of the lipid composition of the protein-free vesicles by using total erythrocyte phospholipids with and without cholesterol has little or no influence on the % ratio of the gucose/dextran calculated trap. In large, unilamellar, glycophorin-containing dioleoylphosphatidylcholine vesicles the % ratio of glucose/dextran calculated trap is 1.4%. This indicates that glycophorin incorporation in these vesicles greatly increases the glucose permeability of the vesicles. The % ratio of glucose/dextran calculated trap values in glycophorin-containing and protein-free dioleoylphosphatidylcholine vesicles are in reasonable agreement with the above described % ratios of potassium/hemoglobin calculated trap values in the same vesicles suggest-

TABLE III

GLUCOSE PERMEABILITY OF BILAYERS MADE OF DIFFERENT LIPID COMPOSITIONS IN LARGE, GLYCOPHORIN-CONTAINING VESICLES AND LARGE PROTEIN-FREE VESICLES

Protein content of the glycophorin-containing vesicles (glycophorin/lipid molar ratio) (A) 1 to 500 (not fractionated); (B) 1 to 310 (after fractionation on 37% $^{2}H_2O$-containing buffer); (C) 1 to 330 (after fractionation on 37% $^{2}H_2O$-containing buffer).

	Lipid	$\frac{\text{Glucose}}{\text{Dextran}}$ Trap (%)	
		Large glycophorin-containing vesicles	Large protein-free vesicles
A	DOPC	1.4	94
B	Erythrocyte lipids 0% cholesterol	15	100
C	Erythrocyte lipids 45% cholesterol	50	94

ing a good similarity between the two methods used for these vesicles. The lipid-dependence of the glycophorin-induced high permeability of the bilayer is evident from the % ratio of glucose/dextran calculated trap values found for glycophorin-containing vesicles comprised of erythrocyte lipids without cholesterol (50%) after fractionation of the vesicles on 37% $^{2}H_2O$ in order to remove structures with little or no glycophorin content (Table III). It can be concluded, that glycophorin-containing vesicles prepared from the total human erythrocyte lipids including cholesterol demonstrate a greatly reduced permeability to glucose compared to glycophorin-containing dioleoylphosphatidylcholine vesicles.

The dextran trap of the glycophorin-containing dioleoylphosphatidylcholine vesicles (5 μl/μmol lipid, see Fig. 2) is too low with respect to the calculated trapped volume of the same vesicles derived from the mean diameter of the protein-containing vesicle population which is presented in Fig. 1 (11 μl/μmol lipid). However, the dextran trap is in good agreement with the hemoglobin trap of glycophorin -containing dioleoylphosphatidylcholine vesicles (4.8 μl/μmol lipid). The rea-

son for this discrepancy is not understood, but it should be realized that small errors in the determination of the vesicle size by freeze-etch electron microscopy will result in large differences in calculated enclosed volumes. For glycophorin-containing vesicles made of the total erythrocyte lipids the dextran trap (8.6 μl/μmol lipid, see Fig. 2) is in good agreement with the calculated trapped volume, derived from the mean size of the same vesicles presented in Fig. 1 ($\pm$9 μl/μmol lipid).

Transbilayer movement of lysophosphatidylcholine in large unilamellar glycophorin-containing vesicles

Glycophorin incorporation increases the transbilayer movement of phosphatidylcholine and lysophosphatidylcholine in small and large, unilamellar vesicles [10,33,34]. To relate the increase in permeability to this increase in lipid transbilayer movement, transbilayer movement of lysophosphatidylcholine is investigated in the same vesicles as have been used for the permeability measurements. The main difference with respect to previous studies [10] is that in this study glycophorin, which has been purified without the use of lithium diiodosalicilate, is used. At the same time a new method for determining the transbilayer movement of lysophosphatidylcholine is introduced. Transbilayer movement of lysophosphatidylcholine in large, unilamellar dioleoylphosphatidylcholine vesicles, with and without glycophorin, is measured by determining the extractable fraction of lysophosphatidylcholine by externally added fatty acid-free albumin.

In large, unilamellar protein-free vesicles which do now show transbilayer movement of lysophosphatidylcholine [10] and which have a symmetrical transbilayer distribution of lysophosphatidylcholine , 50% of the lysophosphatidylcholine is extracted (Fig. 3), demonstrating that only the lysophosphatidylcholine from the outer monolayer is extracted and that no transbilayer movement lysophosphatidylcholine is introduced by this method.

In large glycophorin-containing vesicles about 80% of the lysophosphatidylcholine is extracted, indicating that a considerable fraction of the lysophosphatidylcholine shows a transbilayer movement under the influence of the lithium diiodosalicilate-free glycophorin.

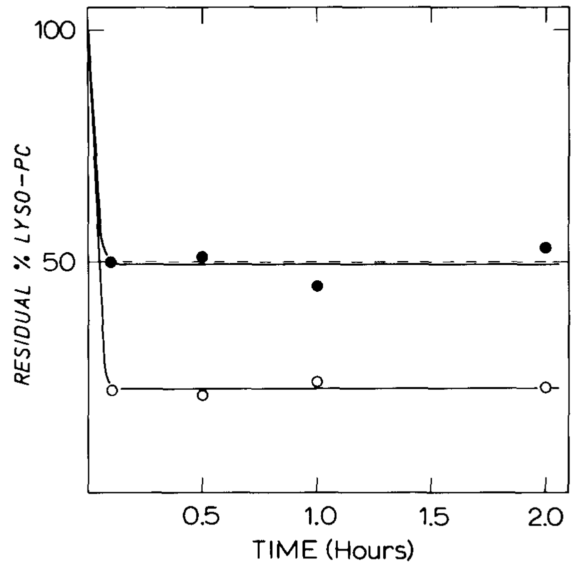

Fig. 3. Extraction of lysophosphatidylcholine by albumin from large, unilamellar vesicles made of 99 mol% dioleoylphosphatidylcholine and 1 mol% [^{14}C]lysophosphatidylcholine at room temperature. ●——●, Large, unilamellar protein-free vesicles. ○——○, Large, unilamellar glycophorin-containing vesicles (1:450 glycophorin/lipid molar ratio).

Transbilayer movement of lysophosphatidylcholine in fractionated glycophorin-containing unilamellar vesicles made of different lipid compositions

The albumin extraction procedure on lysophosphatidylcholine in vesicles made of erythrocyte lipids was found to be unreliable since it resulted in irreproduceable pelleting of the vesicles during the assay. Therefore, in this study the lysophospholipase assay [10] was used to investigate the effect of different lipid compositions on the glycophorin-induced transbilayer movement of lysophosphatidylcholine in the same vesicles as were used for permeability measurements.

Degradation of lysophosphatidylcholine in large glycophorin-containing vesicles made of the total lipids from the human erythrocyte membrane is shown in Fig. 4. In the absence of cholesterol, 35% of the lysophosphatidylcholine could not be degraded by the enzyme. Following fractionation of the vesicles in order to remove those vesicles which contain little or no protein, 15% of the lysophosphatidylcholine could still not be degraded by the enzyme, indicating the presence of a fraction of

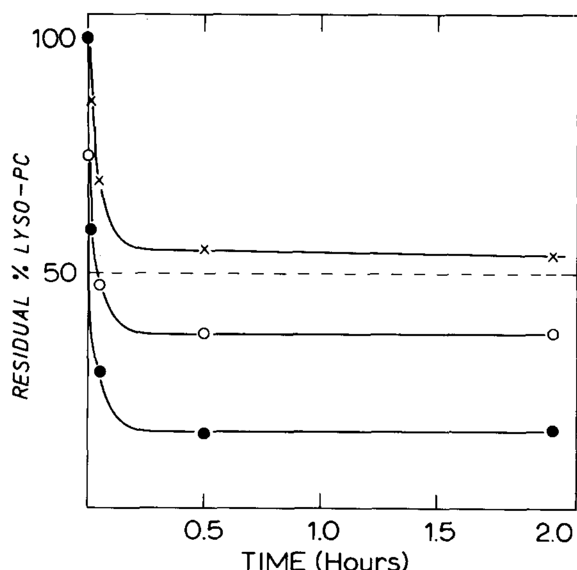

Fig. 4. Time-course of lysophosphatidylcholine hydrolysis by lysophospholipase II in large, unilamellar glycophorin-containing vesicles at 37°C. The protein-lipid molar ratio is 1 : 600. O——O, Non-fractionated vesicles made of 95 mol% erythrocyte lipids without cholesterol, 5 mol% [^{14}C]16:0-lysophosphatidylcholine. The protein-lipid molar ratio is 1 : 400. ●——●, Fractionated vesicles made of 95 mol% cholesterol-free erythrocyte lipids and 5 mol% [^{14}C]16:0-lysophosphatidylcholine. ×——×, Fractionated vesicles made of 95 mol% erythrocyte lipids in the presence of 45 mol% cholesterol, 5 mol% [^{14}C]16:0-lysophosphatidylcholine.

the vesicle population in which not all the lysophosphatidylcholine is available to the enzyme. In fractionated vesicles prepared from total erythrocyte lipids including 45 mol% cholesterol, only 50% of the lysophosphatidylcholine could be degraded, indicating no transbilayer movement of lysophosphatidylcholine which is in agreement with previous observations in unfractionated vesicles [10].

The results show a related lipid-dependence between glycophorin-induced transbilayer movement of lysophosphatidylcholine and glycophorin-induced permeability to glucose in large, unilamellar protein-containing vesicles.

Discussion

The main result of the present study is that glycophorin incorporated in large, unilamellar dioleoylphosphatidylcholine vesicles greatly increases the potassium and glucose permeability through the bilayer. In strong contrast, incorporation of the protein into vesicles of the total lipid extract of the human erythrocyte membrane only slightly affected the barrier function of the bilayer towards these permeants. Furthermore, there is a striking similarity between the influence of the protein on the permeability characteristics of the vesicles and the effect on the transbilayer movement of lipids. Literature data [10] and the present experiments (Fig. 3) demonstrate that the protein facilitates lysophosphatidylcholine transbilayer movement in a similar lipid-dependent way. In considering the molecular mechanism of the glycophorin-induced changes in barrier function two aspects should be discussed. In the first instance the geometry of the lipid-protein interface might be an important parameter. Although in models of biological membranes intrinsic proteins are often schematically represented as smooth cylinders it is clear that the surface of the protein must be highly irregular. To properly seal such an interface a mixture of lipids, all with varying molecular shape, might be required as was originally suggested by Israelachvili [35]. In the total erythrocyte lipids a broad spectrum of differently shaped molecules are present and thus it is possible that the protein is surrounded by lipid molecules of a particular shape in such a way that no significant irregularities exist in the interface. Inherent to such a model is a preferential association of particularly shaped lipids with specific parts of the hydrophobic part of the protein.

Unsaturated phosphatidylethanolamine which due to the relatively small headgroup and large hydrocarbon chain area has a pronounced cone shape [36], might be important in this respect as incorporation of dioleoylphosphatidylethanolamine in glycophorin-containing dioleoylphosphatidylcholine bilayers reduced the Dy^{3+}-permeability [7]. Furthermore, it was recently shown that large quantities of glycophorin can be incorporated in dioleoylphosphatidylethanolamine model membranes so that the dioleoylphosphatidylethanolamine which in the absence of the protein forms the hexagonal H_{II} phase is now stabilized into a bilayer organization [16]. This also suggests that the cone-shaped di-

oleoylphosphatidylethanolamine is counterbalanced by a part of the polypeptide chain with a reversed shape. In the case of the dioleoylphosphatidylcholine-glycophorin bilayer statistical defects occur as a result of a mismatch between the dynamic shape of the lipid and the protein molecule.

A second important aspects is the state of aggregation of the protein in the bilayer. Although these data are lacking, it can be inferred from published data that glycophorin is present as aggregates in the lipid bilayer [19]. The presence of such aggregates might be of importance for the barrier properties of the system as for instance in these aggregates more hydrophilic pore-type protein structure may be formed. As the aggregation state of the protein might be lipid-dependent this could be an alternative explanation for the observed lipd-dependence of the permeability increase. However, it should be realized that the increase in lipid transbilayer movement induced by the protein is more difficult to imagine by this mechanism.

An attraction of this model is that it might explain why, in the study of Mimms et al. [9] in which very low amounts of glycophorin were incorporated in vesicles using detergent methods, no large changes in permeability were observed. Under these conditions the protein might be less aggregated. In the study of Romans et al. [8] it was also demonstrated that the aggregates of the tryptic hydrophobic segment of glycophorin increase the bilayer permeability relative to the situation in which monomers are present. These findings suggest that protein concentration and aggregation state are important in permeability and lipid transbilayer movement processes and this will be a subject for future investigations.

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