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Transbilayer Distribution and Mobility of Phosphatidylcholine in Intact Erythrocyte Membranes

A Study with Phosphatidylcholine Exchange Protein

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1. The exchange of phosphatidylcholine between intact human or rat erythrocytes and rat liver microsomes was greatly stimulated by phosphatidylcholine-specific exchange proteins from rat liver and beef liver. It was found, however, that compared to the exchange reaction between phospholipid vesicles and rat liver microsomes, much higher concentrations of exchange protein were required in the case of intact red blood cells and microsomes.

2. In human erythrocytes, 75% of the phosphatidylcholine was available for exchange within 2 h at 37 °C. No additional exchange was observed during the next 2 h, indicating slow, if any, transbilayer movement of the residual phosphatidylcholine.

3. In rat erythrocytes 50–60% of the phosphatidylcholine was readily available for the exchange proteins. The residual phosphatidylcholine was exchanged at a much lower rate with a half time for equilibration of 7 h.

4. These results confirm in an independent way the asymmetric distribution of phosphatidylcholine over the membrane of human and rat erythrocytes as well as the occurrence of a slow transbilayer movement of this lipid in rat erythrocytes.

The human erythrocyte was the first cell in which an asymmetric distribution of phospholipids over both sides of the bilayer membrane was established. The outer monolayer mainly consists of the choline-containing phospholipids, phosphatidylcholine and sphingomyelin, whereas phosphatidylethanolamine and phosphatidylserine are preferentially located in the inner monolayer. Chemical labelling studies showed that the aminophospholipids were labelled much faster and, to a larger extent, in open ghosts than in intact cells [1–4]. Treatment of intact cells with phospholipases resulted in the hydrolysis of 50% of the total phospholipids among which 76% of the phosphatidylcholine 82% of the sphingomyelin and 20% of the phosphatidylethanolamine whereas all the phospholipid could be hydrolyzed in open ghosts [5]. The above results were confirmed, in part, by experiments on resealed right-side-out [6] and inside-out ghosts [7]. On the other hand it has been reported that the extent of phospholipid degradation in intact cells by phospholipases depends on the type of phospholipase used [8], the energized state of the erythrocyte [9] and the degree of crosslinking of intracellular membrane associated proteins like the spectrin-actin complex [10].

In order to establish the phospholipid distribution in an independent way, attempts were made to use phospholipid-exchange proteins as phospholipid-localizing tools. These proteins stimulate the transfer of single phospholipid molecules between different membrane surfaces [11], and are particularly usefull because they are thought to have minimal perturbing effects on membrane structure [12,13]. However, the exchange proteins specific for the transfer of phosphatidylcholine were found to be unable to stimulate the exchange of phosphatidylcholine present in intact erythrocytes [14–16]. On the other hand, non-specific exchange activity present in a high-speed supernatant of a liver homogenate was found to stimulate the exchange of all phospholipid species between rat erythrocytes and small lipid vesicles present in the supernatant [17]. Recently, a phosphatidylcholine-specific exchange protein was isolated and purified from rat liver in this laboratory (B. J. H. M. Poorthuis, unpublished results). Because this protein has a basic character in contrast to the acidic proteins purified previously [18], we investigated the exchange capacity of the protein for phosphatidylcholine present in the negatively charged erythrocyte surface. It turned out, however, that not the charge but the concentration of

protein is a critical factor in these experiments and that also phosphatidylcholine-exchange protein from beef liver, when present in high concentrations, can be used successfully to localize phosphatidylcholine in intact erythrocytes.

MATERIALS AND METHODS

Erythrocytes

Fresh human erythrocytes collected from venepuncture in standard acid/citrate/dextrose buffer were packed for 10 min at $2500 \times g$ and washed three times with a fourfold volume of buffer containing 150 mM NaCl, 1 mM EDTA, 25 mM glucose and 10 mM Tris HCl pH 7.4. The buffy coat was carefully removed after each wash. Fresh rat erythrocytes were obtained by cardiac puncture from male Wistar rats and treated like the human erythrocytes. The morphology of the erythrocytes was checked by light microscopy after suspension in formaldehyde buffer. The discocyte form of the cells was preserved throughout all incubations.

Exchange Proteins

Phosphatidylcholine-specific exchange protein was purified from rat liver as will be published elsewhere (B. J. H. M. Poorthuis, unpublished results). Beef liver phosphatidylcholine-exchange protein was purified according to Wirtz et al. [19]. The proteins were stored in 50% (v/v) glycerol at $-20\,^{\circ}C$. Before use, glycerol was removed by elution of the protein over a Sephadex G-25 column equilibrated in the incubation buffer.

Microsomes

Rat liver microsomal membranes were prepared as described before [20] from Wistar rats that were injected intraperitoneally with 30 μCi [*methyl*-^{14}C]-choline. Before the incubations microsomes were sonicated for 1–2 min with a Branson sonifier (70 W output) under cooling in ice, 97% of the radioactivity present was found in the phosphatidylcholine. All the phosphatidylcholine in the microsomal membranes is available for exchange in one fast exchangeable pool [21, 22].

Incubation Conditions

0.8 ml packed cells, equivalent to 3 μmol phospholipid phosphate in human cells and 3.4 μmol in rat erythrocytes, were incubated with 0.32 ml microsomal membranes (2.4 μmol phospholipid phosphate) in glass tubes ar 37 °C under gentle rotation. To start the exchange 0.3–0.6 mg exchange protein was added which was dissolved in 5–8 ml of the buffer described above. In control incubations equal amounts of buffer without protein were used. After 1, 2, 3 and 4 h of incubation one quarter of the incubation mixture was withdrawn and diluted with 15 ml ice-cold buffer to stop the reaction. Cells were pelleted by centrifugation for 10 min at $2500 \times g$ and resuspended in another 15 ml of cold buffer. The cells were washed twice in the same buffer to remove the microsomes. During the incubation and subsequent washings no haemolysis occurred Packed cells were extracted according to Rose and Oklander [23]. Dried lipid extracts were suspended in 2 ml chloroform/methanol (2:1, v/v) and washed with 0.4 ml 50 mM KCl to remove water-soluble phosphate-containing material. Phospholipid-phosphate was determined according to Rouser [24] and radioactivity was measured in a Packard liquid scintillation counter in the external standard mode.

Calculations

The percentage of erythrocyte phosphatidylcholine which had equilibrated with microsomal lipid was calculated as follows. The original radioactivity (in dis./min) present in the microsomal phosphatidylcholine ($R_{m,o}$), the radioactivity (in dis./min) found in the erythrocytes after the experiment ($R_{e,t}$) and the absolute amount of phosphatidylcholine in the microsomes (c_m) were determined. The specific activity of phosphatidylcholine in the microsomes after the incubation is represented by: $(R_{m,o} - R_{e,t})/c_m$ and equals the specific activity of the pool of erythrocyte phosphatidylcholine which had equilibrated with the microsomal lipid $R_{e,t}/c_{e,t}$. The absolute amount of phosphatidylcholine equilibrated with microsomal lipid ($c_{e,t}$) can be calculated and expressed as a percentage of the total amount of erythrocyte phosphatidylcholine. The amounts of microsomal and erythrocyte phosphatidylcholine were determined in triplicate after separation of the total lipid extracts by two-dimensional thin-layer chromatography according to Broekhuyse [25]. Calculations were based on the radioactivity present in a constant amount of phospholipid extracted from the erythrocytes. At $t = 0$ microsomes were mixed with erythrocytes. In a control incubation the erythrocytes were spun down immediately and washed as described above. The amount of ^{14}C radioactivity in the erythrocyte pellet after washing was measured and represents the extent of contamination of erythrocytes with microsomes. The extent of contamination was supposed to be constant with time. The possibility of net transfer of phosphatidylcholine in either direction was excluded by analysis of phospholipid content of the erythrocytes before and after the incubations. The ratio phosphatidylcholine/phosphatidylethanolamine remained con-

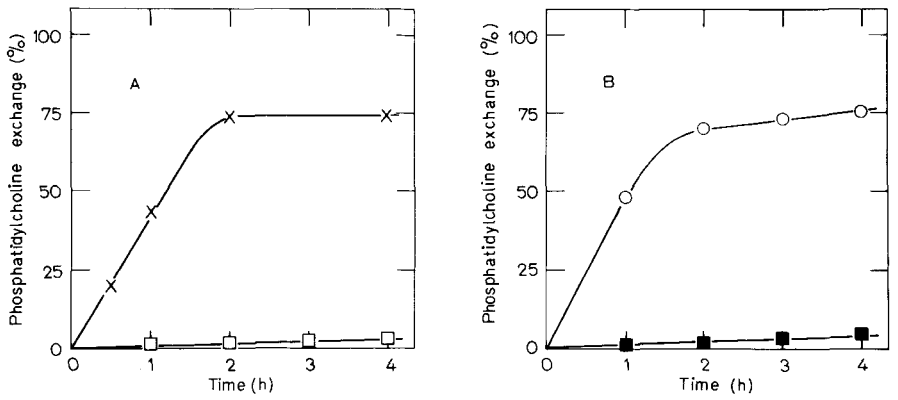

Fig. 1. *Exchange of phosphatidylcholine from human and rat erythrocytes.* Human (A) and rat (B) erythrocytes were incubated with [^{14}C]phosphatidylcholine containing rat liver microsomes as described under Materials and Methods. The amount of phosphatidylcholine which was exchanged in the absence (□, ■) and in the presence (×, ○) of phosphatidylcholine-specific exchange protein from beef liver is plotted as a percentage of total erythrocyte phosphatidylcholine

stant and since the exchange protein is unable to transfer phosphatidylethanolamine, it justifies this conclusion.

RESULTS

In order to test the exchange activity of the phosphatidylcholine exchange protein from rat liver on erythrocytes the following experiments were carried out. Human erythrocytes and ^{14}C-labelled microsomes were incubated for 2 h in the presence of partially purified rat liver protein which was available in large amounts, a small quantity of highly purified rat liver protein and a similar amount of the pure beef-liver exchange protein. Parallel incubations were carried out to estimate the activities of the various fractions in a standard microsome-phosphatidylcholine vesicle assay [19]. Table 1 presents the data and shows that both rat-liver and beef-liver exchange proteins are active in lecithin exchange between microsomes and erythrocytes, be it that very high concentrations of protein are necessary to obtain a good stimulation of transfer of phosphatidylcholine within a few hours. Furthermore, activity is roughly proportional to the amount of exchange protein added, as measured in the microsome-phosphatidylcholine vesicle assay. Since more of the purified beef-liver protein was available, it was used in all subsequent experiments.

The time dependence of phosphatidylcholine equilibration between microsomes and intact human and rat erythrocytes is presented in Fig. 1. After 2 h of incubation in the presence of beef liver phosphatidylcholine exchange protein about 75% of the lecithin in the human erythrocyte was exchanged (Fig. 1 A). No additional exchange was observed in the subsequent 2 h of incubation. This was not due to inactivation of the exchange protein because it was found in a control experiment that after 4 h of incubation at 37 °C the exchange protein in the incubation mixture is still fully active when tested in the standard microsome-vesicle assay. Control incubations without exchange proteins showed that spontaneous exchange of phosphatidylcholine also took place, but at a very low rate (Fig. 1). The data in Fig. 1 are corrected for contamination of erythrocytes, isolated after three wash procedures, with microsomal material which accounted for 3.3 ± 1.8% of the total phosphatidylcholine (see also Table 2). In contrast to human erythrocytes, with rat erythrocytes the exchange does not stop after 2 h of incubation, but a second pool of phosphatidylcholine becomes available

Table 1. *Protein-stimulated transfer of [^{14}C]phosphatidylcholine from rat liver microsomes to human erythrocytes and unilamellar vesicles*

Human erythrocytes (1 μmol phospholipid phosphate) were incubated with microsomes (0.85 μmol phospholipid phosphate) at 37 °C as described in Materials and Methods. Both beef-liver exchange protein as well as two different preparations of rat-liver exchange proteins (1 and 2) specific for the exchange of phosphatidylcholine were added. After 2 h of incubation the absolute amount of phosphatidylcholine which was exchanged was measured (in nmol) and expressed as a percentage of total erythrocyte phosphatidylcholine. Activities of the exchange proteins were measured under identical conditions using instead of erythrocytes, unilamellar vesicles of phosphatidylcholine/phosphatidic acid/cholesterol · ester (0.80:0.20:0.12 molar ratio). Incubations were carried out for 30 min at 37 °C and the data are expressed in nmol phosphatidylcholine exchanged from vesicles in 2 h

Protein	Exchange of phosphatidylcholine		
	from erythrocytes		from vesicles
	nmol	%	nmol
Beef liver protein	11.7	3.9	469
Rat liver protein (1)	6.9	2.3	224
Rat liver protein (2)	74.7	24.9	4814

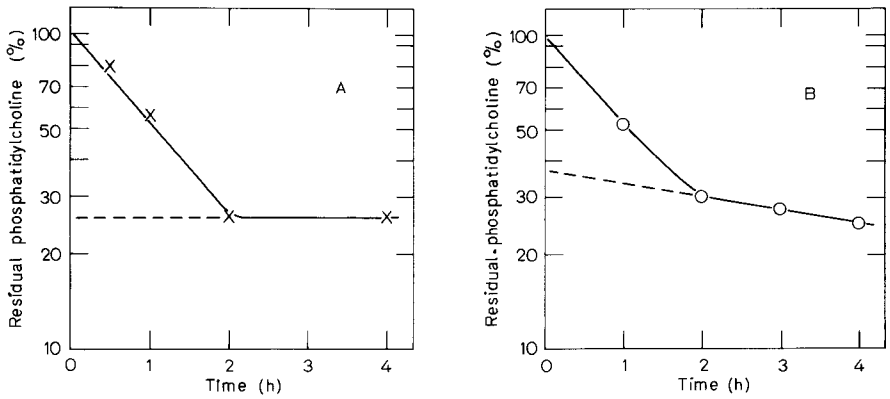

Fig. 2. *Phosphatidylcholine in human and rat erythrocytes not equilibrated with donor phosphatidylcholine.* The data obtained in the experiments shown in Fig. 1 are expressed as the percentage of the total phosphatidylcholine in the human (A) and rat (B) erythrocytes which had not equilibrated with microsomal phosphatidylcholine. Solid lines connect the experimental points. The dashed lines show the extrapolation of the slow phase to zero time

Table 2. *Exchange of phosphatidylcholine from intact human and rat erythrocytes*
Incubations were carried out as described in Materials and Methods. The exchange data are corrected for contamination of erythrocytes with microsomes 3.3 ± 1.8% for human and 5.8 ± 1.0% for rat erythrocytes. The data include the spontaneous exchange which amounted up to 4.5% within 4 h of incubation. The size of the fast exchangeable pools was calculated from semilogarithmic plots as given in Fig. 2

Cells	Lecithin content	Fast exchangeable lecithin	
	% total phospholipid	% total lecithin	% total phospholipid
Human (3)	30.0 ± 1.7	74.2 ± 3.1	22.3 ± 1.6
Rat 1	39.8	63.1	25.3
Rat 2	47.2	51.1	24.3
Rat 3	45.0	47.1	22.1

for exchange at a much lower rate. Both the size of the readily available pool of phosphatidylcholine as well as the rates of exchange can be accurately assessed by plotting the data in a semilogarithmic way as presented in Fig. 2. It is clear that the intact human erythrocyte contains only one exchangeable pool of phosphatidylcholine, amounting up to 75% whereas 25% of this lipid, presumably located in the inner layer, cannot be exchanged. The data, obtained with rat erythrocytes, show a biphasic exchange pattern. This means that part of the phosphatidylcholine in the rat erythrocyte membrane is located in a pool which is readily available for exchange. The residual phosphatidylcholine, in contrast to the phosphatidylcholine in human erythrocytes, is also available for exchange but at a much lower rate. This rate can be calculated (using the least squares method) from the slope of the second part of the biphasic curve (Fig. 2B). With three different preparations of rat erythrocytes identical half-times were measured of 7.4 ± 0.7 h. As is shown in Table 2, the size of the readily exchangeable phosphatidylcholine pool in rat erythrocytes, could be less accurately determined than in human erythrocytes. In the three preparations variable data were obtained on the total phosphatidylcholine content and consequently on the fraction of phosphatidylcholine that represents the fast exchangeable pool. However, when expressed as a percentage of total phospholipid, the data are in good agreement and show that in rat erythrocytes an amount of phosphatidylcholine representing 22–25% of the total phospholipid is present as a fast exchangeable pool.

DISCUSSION

The present results show that phosphatidylcholine in the erythrocyte membrane is less readily available for exchange by phosphatidylcholine-specific exchange proteins than phosphatidylcholine in microsomes or unilamellar vesicles. Consequently a much higher protein concentration is necessary to be able to detect protein catalyzed exchange than under standard conditions (Table 1). This explains why previous attempts to stimulate the exchange of phosphatidylcholine by specific exchange protein between intact erythrocytes and other membranes failed: the concentrations of exchange protein used in those experiments were far too low [14–16]. A comparison of earlier data on the exchange of phosphatidylcholine from ghosts with our data presented here shows that the accessibility of phosphatidylcholine in the intact erythrocytes for exchange proteins is very low, much lower than in erythrocyte ghosts in which the phospholipids are readily available for exchange [14]. This difference in phospholipid accessibility, noted here for exchange proteins, is also

found with various phospholipases. Those phospholipases which have a limited penetration capacity – up to surface pressure of 0.3 mN/cm (30 dynes/cm) – do not have access to phospholipids in the intact red cells but hydrolyse rapidly phospholipids in erythrocyte ghosts [26–28]. It is obvious that haemolysis results in extensive alterations in the properties of the erythrocyte membrane. Which parameter of membrane structure is altered is not clear but the observations discussed here lead to the speculation that the condensed and tight packing of lipids in the intact membrane is lost resulting in an erythrocyte ghost in which the lipids are organized in a loosely packed bilayer structure with a low surface pressure. That the exchange activity of the beef-liver phosphatidylcholine-exchange protein can be affected by the way of lipid packing or organization within a membrane was demonstrated recently [19]. Kinetic studies of the exchange between lipid vesicles and multilamellar liposomes showed that the interaction of the exchange protein with the membrane interface proceeded 50–100 times slower with liposomes than with highly curved vesicles of identical lipid composition. Again the accessibility of phospholipids for the exchange protein parallels the accessibility for phospholipases [29, 30].

The observation that phosphatidylcholine exchange could be stimulated by various exchange proteins prompted us to reinvestigate the localization and transbilayer movement of this phospholipid in intact erythrocytes. Experiments with the human red cells clearly show that phosphatidylcholine is present in two pools: 74% is readily exchangeable and presumably present in the outer layer and 26% is located in the inner layer. These data are in excellent agreement with the data obtained with phospholipases [5]. In those experiments 76% was readily hydrolyzed and was assigned to the outer monolayer while 24% was completely resistant to phospholipase attack. Although in the case of the rat erythrocytes the data on the size of the fast exchangeable pool of phosphatidylcholine are less clear cut, the amounts of phosphatidylcholine which can be exchanged are roughly identical to the amounts detected at the outside by phospholipase action (62%) [31]. Bloj and Zilversmit reported higher values of 64–75% exchangeable phosphatidylcholine obtained with exchange studies on released ghosts [14]. Indeed, these experiments could be performing using low concentrations of exchange protein, which in view of the discussion presented above indicates that resealed ghosts have a lipid packing which differs from the natural membrane and for that reason cannot be compared directly with intact red cells. Our data generally agree with earlier exchange experiments on intact red cells. Reed reported the spontaneous exchange of lipids between serum and human erythrocytes [32]. The amount of exchangeable phosphatidylcholine (63%) is low but no complete equilibrium was reached under the experimental conditions. Steck et al. used crude supernatant fraction of rat liver homogenate to stimulate the exchange of phospholipid between intact erythrocytes and lipid vesicles [17]. They concluded that all the phospholipid classes are available for exchange which, with regard to phosphatidylcholine, is in agreement with our observations and the data obtained with phospholipases. No bilayer movement of phosphatidylcholine in human erythrocytes could be detected. In rat erythrocytes, on the other hand, a slow transbilayer movement with a half-time value of 7–8 h was observed. These data confirm in an independent way earlier data obtained by Rousselet et al. [33] who showed the absence of transbilayer movement in human erythrocytes with a spin-label technique and data of Renooy et al. [31, 34, 35] obtained on rat erythrocytes with a phospholipase degradation technique. Also in the latter experiments a slow transbilayer movement of phosphatidylcholine was observed.

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Note Added in Proof. The halftimes mentioned above are empirical values, which are immediately derived from the semilogarithmic plot of the equilibrated phosphatidylcholine pool against time. In a more accurate way half lifes for equilibration of [^{14}C]phosphatidylcholine between the two monolayers of the bilayer can be calculated, using a three-pool closed system model according to Shipley and Clark [Shipley, R. A. and Clark, R. E. (1972) *Tracer Methods for* in vivo *Kinetics – Theory and Applications*, New York, N.Y., Academic Press. p. 129]. Based on this model a half life of equilibration was calculated of 4.0 ± 0.6 h.