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THE TRANSPOSITION OF MOLECULAR CLASSES OF PHOSPHATIDYLCHOLINE ACROSS THE RAT ERYTHROCYTE MEMBRANE AND THEIR EXCHANGE BETWEEN THE RED CELL MEMBRANE AND PLASMA LIPOPROTEINS

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Summary

1. The molecular composition of phosphatidylcholine is similar in the inner and the outer layer of the rat erythrocyte membrane.

2. The rate of exchange of the various molecular classes of phosphatidylcholine between rat plasma and the red cell membrane does not depend on the degree of unsaturation of the different classes.

3. The transposition of the molecular classes of phosphatidylcholine between the inner and the outer layer of the rat erythrocyte membrane is more pronounced for the more unsaturated classes.

Introduction

The major pathways described for the renewal of erythrocyte phospholipids are: (1) the exchange of phospholipids between plasma lipoproteins and the red cell membrane [1—8], and (2) the acylation of lysophospholipids, which are either formed in the membrane or derived from the surrounding plasma [8—14]. Renooij et al. observed that the exchange of phospholipids occurs at the outside of the rat erythrocyte membrane [8], whereas the acylation of lysophospholipids takes place mainly at the inside of erythrocyte membranes [8,14].

Increasing evidence suggests an asymmetric distribution of the phospholipid components over the inner and outer layer of biological membranes [8,15—28]. The outer layer of the rat [8] and human [15—20,24] erythrocyte membrane consists mainly of choline-containing phospholipids and reflects the phospholipid composition of the surrounding plasma [29]. Phosphatidylcholine, which is the main phospholipid component in the plasma and the outer membrane layer of the human [18] and rat [8] erythrocyte, represents a mixture of

molecular classes, which differ with respect to the degree of unsaturation of their fatty-acyl constituents.

In this study we report the molecular composition of phosphatidylcholine from both layers of the rat erythrocyte membrane, as well as the degree to which the various classes of phosphatidylcholine participate in the exchange process between the red cell membrane and the plasma lipoproteins.

Various reports discuss the possibility of a transposition process, by which lipids can migrate between the inner and the outer layer of artificial [30—36] and natural membranes [8,23,37—41]. The results of the present study suggest that the extent to which the different molecular classes of phosphatidylcholine contribute to the transposition of this phospholipid across the rat erythrocyte membrane depends upon their degree of unsaturation.

Methods

Sampling of blood

Female Wistar rats weighing 125—150 g were obtained from the Central Institute for the Breeding of Laboratory Animals in Zeist, The Netherlands. Fresh samples of blood were obtained from ether-anaesthetized rats by cardiac puncture, using acid/citrate/dextrose as anticoagulant. The cells were spun down in a clinical centrifuge at maximum speed for 15 min. The plasma and the buffy coat were removed and the cells were washed three times with a tenfold volume of 0.9% NaCl. The plasma was heated at 56° C for 1 h with stirring to destroy the lecithin-cholesterol acyltransferase activity [42].

Incubation conditions for the exchange of phospholipids between plasma and erythrocytes

Rats were injected intraperitoneally with 1 mCi $^{32}P_i$. After 24 h blood was collected as described above. The incubations contained 6 ml of unlabelled erythrocytes and 7.5 ml ^{32}P-labelled heated plasma. After 2 h incubation at 37° C with gentle stirring the cells were separated from the plasma by centrifugation and washed successively with 70 ml 0.9% NaCl (two times), 70 ml of a saline solution of 1% defatted serum albumin (two times), 7.5 ml unlabelled plasma and finally three times with 70 ml 0.9% NaCl.

Incubation conditions for the incorporation of fatty acids into erythrocyte phospholipids

Packed cells (6 ml) were incubated in 7.5 ml heated plasma, to which 10 μCi of [1-^{14}C]palmitic acid had been added. After 1 h incubation at 37° C with gentle stirring, the cells were centrifuged and washed as described in the preceding paragraph.

Incubation conditions to measure the transposition of labelled phosphatidylcholine across the rat erythrocyte membrane

Discrimination between the inside and the outside of the erythrocyte membrane was made by treating aliquots of the packed cells with a combination of phospholipase A_2 (EC 3.1.1.4) from *Naja naja* venom and sphingomyelinase (sphingomyelin choline phosphohydrolase) from *Staphylococcus*

aureus [8,18] as described in detail below. In order to estimate the transposition of labelled phosphatidylcholine across the erythrocyte membrane, samples of 1.0 ml of radioactively labelled packed cells, obtained as described above, were incubated in 9 ml of a solution of 0.85% NaCl, 10 mM Tris · HCl (pH 7.4) at 37°C for 0, 1 and 2 h, respectively. After these incubations the cells were spun down and the supernatants replaced by 20 ml of 0.87% NaCl, 10 mM $CaCl_2$, 0.25 mM $MgCl_2$ (adjusted to pH 7.4 with Tris · HCl) containing 100 I.U. of phospholipase A_2 from *Naja naja* venom. After 1 h incubation at 37°C 10 I.U. of sphingomyelinase were added. Parallel incubations of 0.5 ml packed cells were carried out without the enzymes. The cells were spun down 1 h after the addition of sphingomyelinase and the reaction was terminated by adding EDTA in a final concentration of 10 mM. In parallel incubations 0.25 ml packed cells were treated with 25 I.U. of phospholipase A_2 plus 2.5 I.U. of sphingomyelinase in 5 ml buffer to measure the degradation of the red cell phospholipids. The hemolysis never exceeded 3—4%.

Analysis of lipids

The lipids were extracted from erythrocytes and plasma according to the procedure of Reed et al. [43] and separated via silica thin-layer chromatography, using chloroform/methanol/glacial acetic acid/water (70 : 35 : 8 : 4, v/v) as developing solvent. The phosphatidylcholine spots were visualized in ultraviolet light after spraying with a 0.005% (w/v) aqueous solution of Rhodamine-6-G and extracted from the silica according to the method of Bligh and Dyer [44]. Phosphatidylcholines were separated into their molecular classes by the following procedure: the phosphatidylcholines were hydrolysed with phospholipase D (EC 3.1.4.4) and subsequently treated with diazomethane [45]; the formed dimethylphosphatidates were purified chromatographically and then resolved into the different molecular classes by argentation thin layer chromatography [46].

The radioactivity of the individual constituents was assayed using a Packard liquid-scintillation counter, model 2425 B, and a scintillation system as described by Fricke [47].

The degradation of the red cell phospholipids by the action of the phospholipases was assayed by a phosphorus determination [48] on the individual phospholipids after two-dimensional silica thin layer chromatography according to the method described by Broekhuyse [49].

The fatty acids of red cell phosphatidylcholine were determined as described by Oldenborg et al. [50]. Discrimination between the fatty acid patterns of the 1- and the 2-position of the phosphatidylcholine molecule was accomplished as described previously [51].

Radioactive compounds and biochemicals

Sodium [^{32}P]phosphate (specific activity 150—600 Ci/mol) was obtained from Philips Duphar, Petten, The Netherlands, and [1-^{14}C]palmitic acid (specific activity 58 Ci/mol) from the Radiochemical Centre, Amersham, Great Britain. Most other biochemicals and enzymes were obtained from Boehringer, Mannheim, Germany. Phospholipase A_2, purified [52] from *Naja naja* venom and sphingomyelinase, purified [24] from *Staphylococcus aureus* were a

generous gift from Dr. R.F.A. Zwaal (Biochemisch Laboratorium, State University of Utrecht, Utrecht, The Netherlands).

Results

Analytical aspects of rat erythrocyte phosphatidylcholine

Table I shows the fatty acid composition of phosphatidylcholine in the rat erythrocyte membrane. There is no difference in the fatty acid patterns or in the positional distribution of the fatty-acyl chains of phosphatidylcholine from both sides of the membrane.

Also, Table II shows that the various molecular classes of phosphatidylcholine are distributed equally among the inner and outer layer of the rat erythrocyte membrane.

The exchange of the various molecular classes of phosphatidylcholine between rat plasma and erythrocytes

Table III shows that incubation of unlabelled erythrocytes in ^{32}P-labelled plasma results in a distribution of radioactivity among the molecular classes of red cell phosphatidylcholine, which is similar to the distribution of radioactivity between the molecular classes of plasma phosphatidylcholine. This suggests that there is no preference for any molecular class of phosphatidylcholine in the exchange process from rat plasma lipoproteins to the red cell membrane.

The transposition of molecular classes of phosphatidylcholine across the rat erythrocyte membrane

Fig. 1 shows that after the incubation of rat erythrocytes in radioactive plasma the majority of the ^{32}P-labelled phosphatidylcholine is found at the outside of the membrane, whereas [1-^{14}C]palmitic acid is incorporated mainly into phosphatidylcholine at the inside of the membrane, which is in agreement with earlier observations [8]. The total radioactivities remain constant upon reincubation of the labelled cells in the buffer. The asymmetric distribution of

TABLE I

FATTY ACID COMPOSITION OF RAT-ERYTHROCYTE PHOSPHATIDYLCHOLINE

The results are expressed as weight percentages ± S.E. Only the main fatty acids are mentioned in the overall compositions. The fatty acid composition of phosphatidylcholine in the outer layer has been calculated from the fatty acid composition of phosphatidylcholine in the total membrane and the inner layer.

Fatty acid	Outer layer			Inner layer		
	Overall	1-Position	2-Position	Overall	1-Position	2-Position
16:0	40.7 ± 1.3	48.5 ± 1.5	34.2 ± 1.5	42.3 ± 1.7	48.4 ± 1.7	39.2 ± 1.8
18:0	23.6 ± 1.3	40.6 ± 1.4	8.4 ± 1.3	23.8 ± 1.4	39.2 ± 0.5	8.7 ± 1.3
18:1	9.3 ± 0.5	6.0 ± 0.8	13.4 ± 0.6	10.5 ± 1.0	7.7 ± 1.2	13.7 ± 1.0
18:2	15.3 ± 0.9	4.9 ± 0.6	24.2 ± 1.4	12.1 ± 0.8	4.7 ± 0.5	19.4 ± 1.3
20:4	11.1 ± 1.0	+ *	19.9 ± 2.1	11.3 ± 0.9	+	19.0 ± 1.2

* +, Trace.

TABLE II

PROPORTION (% ± S.E.) OF MOLECULAR CLASSES OF PHOSPHATIDYLCHOLINE IN THE RAT ERYTHROCYTE MEMBRANE

Molecular class	Outer layer	Inner layer
Disaturated	34.7 ± 1.7	33.8 ± 1.8
Monoenoic	15.5 ± 0.9	15.0 ± 0.2
Dienoic	25.4 ± 1.5	24.3 ± 0.5
Tetraenoic	19.5 ± 1.1	20.5 ± 1.5
Hexaenoic	4.9 ± 0.6	6.3 ± 1.5

TABLE III

DISTRIBUTION OF RADIOACTIVITY (%) AMONG THE MOLECULAR CLASSES OF PHOSPHATIDYLCHOLINE AFTER THE EXCHANGE BETWEEN RAT PLASMA AND ERYTHROCYTES

Plasma phosphatidylcholine was labelled with $^{32}P_i$ in vivo. Unlabelled erythrocytes and ^{32}P-labelled plasma were incubated for 2 h as described in Methods. The rate of exchange of the various molecular classes appeared to be constant during the incubation time. In a representative experiment, the total phosphatidylcholine radioactivities were 188 000 dpm/ml plasma and 7600 dpm/ml packed cells, respectively.

Molecular class	Plasma phosphatidylcholine before exchange	Red cell phosphatidylcholine after exchange
Disaturated	1.7 ± 0.2	2.2 ± 0.3
Monoenoic	5.3 ± 0.4	4.0 ± 0.4
Dienoic	26.2 ± 2.3	25.7 ± 1.9
Polyenoic	66.8 ± 2.5	68.0 ± 2.2

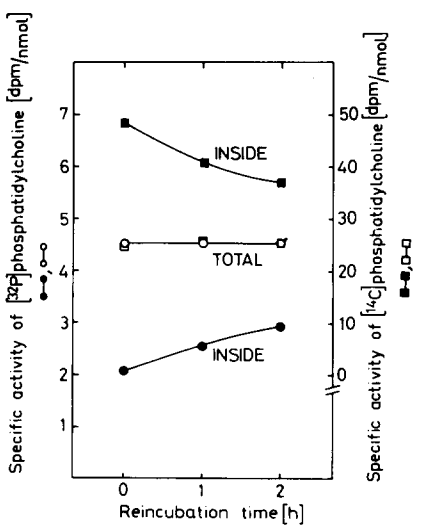

Fig. 1. Transposition of labelled phosphatidylcholine across the rat erythrocyte membrane. Rat erythrocyte phosphatidylcholine is labelled either by incubating the red cells in ^{32}P-labelled plasma (○——○ and ●——●) or by incorporation of [1-^{14}C]palmitic acid (□——□ and ■——■). The cells are, subsequently, reincubated in a buffer for the indicated times. The experimental details are given in Methods.

labelled phosphatidylcholine at the start of the reincubation time becomes less pronounced during the reincubation period. This suggests a transposition of phosphatidylcholine molecules from one side of the membrane to the other.

Erythrocyte phosphatidylcholine is not a single component, but represents a mixture of molecular species [51,53]. Therefore, it would be interesting to investigate whether the transposition process shows any preference for a particular class of phosphatidylcholine molecules.

When rat erythrocyte phosphatidylcholine is labelled by incubating the cells in ^{32}P-labelled plasma only the polyunsaturated molecular classes of phosphatidylcholine become sufficiently labelled (see, e.g., Table III) to measure accurately a transposition across the membrane.

When, however, rat erythrocyte phosphatidycholine is labelled by incubating the cells with [1-^{14}C]palmitic acid in plasma, the radioactivity is distributed more equally among the different molecular classes of phosphatidylcholine (Fig. 2). The specific activities of the molecular classes of phosphatidylcholine in the total cell membrane remain constant upon reincubation in the buffer. The specific activities of the molecular classes of phosphatidylcholine at the inside of the membrane decrease during the reincubation.

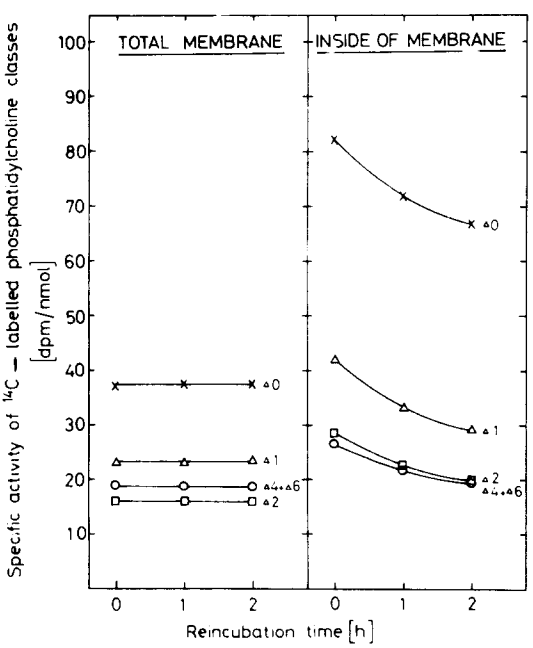

Fig. 2. Specific activities of the various molecular classes of rat erythrocyte phosphatidylcholine and their transposition across the membrane during reincubation of [1-^{14}C]palmitate-labelled erythrocytes in buffer. The incubation conditions and analytical procedures are described in Methods. Notation: Δ0 stands for disaturated, Δ1 for monoenoic, Δ2 for dienoic and Δ4 + Δ6 for polyunsaturated molecular classes. At the start of the reincubation period the total radioactivities in phosphatidylcholine of the total membrane and the inside of the membrane are 39 870 and 32 630 dpm/ml packed cells, respectively. 1.0 ml packed cells comprises 1650 nmol phosphatidylcholine. The degradation of red cell phosphatidylcholine by the phospholipase action was 60%.

TABLE IV

DISTRIBUTION OF RADIOACTIVITY (%) AMONG THE MOLECULAR CLASSES OF PLASMA PHOSPHATIDYLCHOLINE AFTER EXCHANGE WITH [1-^{14}C]PALMITATE-LABELLED ERYTHROCYTES

3.0 ml unlabelled erythrocytes were incubated with 20 μCi [1-^{14}C]palmitic acid in 3.7 ml plasma for 1 h at 37°C. After the incubation the erythrocytes were washed. Subsequently, 0.4 ml packed cells were extracted, 0.8 ml treated with phospholipase A_2 from *Naja naja* venom plus sphingomyelinase, and 1.0 ml incubated in 1.25 ml unlabelled plasma for 2 h at 37°C. In a representative experiment the total radioactivity in the erythrocyte phosphatidylcholine was 84 500 dpm/ml packed cells; the total radioactivity in plasma phosphatidylcholine after the exchange with [1-^{14}C]palmitate-labelled erythrocytes was 1370 dpm.

Molecular class	Erythrocyte phosphatidylcholine			Plasma phosphatidylcholine after exchange with [1-^{14}C]-palmitate-labelled erythrocytes
	Total	Inside	Outside	
Disaturated	42	53	34	36
Monoenoic	17	16	18	14
Dienoic	15	13	16	16
Polyenoic	26	18	32	34

The decrease of the specific activity of the disaturated class is 40 ± 3% smaller than the decrease in specific activities of the unsaturated classes. At the start of the reincubation period the disaturated class exhibits the most extreme asymmetrical distribution of radioactivity between the inside and the outside of the membrane. This might suggest that the transposition of the disaturated class, which will probably take place during the initial incubation in plasma as well, is slower than the transposition of the other classes, which seem to have already approached an equilibrium in the transposition of the labelled components by the start of the reincubation period.

After incubation of the erythrocytes with [1-^{14}C]palmitic acid the percentage of radioactivity recovered in the polyunsaturated phosphatidylcholines is smaller on the inside of the membrane than on the outside (see Table IV). This could be explained by faster transfer of the polyunsaturated classes from the inner to the outer layer if we assume that they were originally formed at the inside of the membrane via acylation of lyso compounds [8]. Since the exchange of phosphatidylcholine between the outside of the erythrocyte membrane and the plasma lipoproteins is random for all molecular classes (Table III), we would expect that incubation of [1-^{14}C]palmitate-labelled cells with unlabelled plasma would result in an enrichment of radioactivity in the polyunsaturated plasma phosphatidylcholines in comparison to the radioactivity content of this molecular class in the total erythrocyte. Table IV shows that, indeed, the enrichment of labelled polyunsaturated classes in the outer layer of the membrane is reflected in the labelling pattern of the plasma phosphatidylcholine classes. The data which can be derived from Fig. 2 and Table IV suggest that the transposition of the molecular classes of phosphatidylcholine across the rat erythrocyte membrane shows a preference for the polyunsaturated classes.

Discussion

The results shown in Table I and Table II suggest strongly that the molecular composition of rat erythrocyte phosphatidylcholine is identical in the inner and outer layers of the red cell membrane.

The exchange of molecular classes of phosphatidylcholine between serum lipoproteins and the red cell membrane shows no preference for any particular class (Table III). In this respect it is worthwhile to notice the remarkably high content of disaturated phosphatidylcholine in the rat erythrocyte membrane (Table II), especially considering the virtual absence of this class in plasma phosphatidylcholine [53].

Fig. 1 shows that the transposition of labelled phosphatidylcholine across the rat erythrocyte membrane can be observed in either transmembrane direction. We must be aware that we study just a minor part of the phosphatidylcholine pool, as the actual renewal of red cell phosphatidylcholine in vitro is in the order of 1% per h via exchange or even less via acylation (refs. 7 and 54, and our own observations). Therefore the similarity of the curves in Fig. 1 does not necessarily imply that in fact one curve is complementary to the other. It might be possible that the labelled phosphatidylcholines, derived from exchange or acylation, are concentrated in separate pools which do not intermingle by the same translocation process.

Fig. 2 suggests that the transposition of molecular classes of phosphatidylcholine across the rat erythrocyte membrane depends upon the degree of unsaturation of the different classes. The transposition rate of the unsaturated classes during the reincubation period may be an underestimation of the actual transposition rate, because at the start of the reincubation period the unsaturated classes may already approach an equilibrium in the distribution of radioactivity over both sides of the membrane. Therefore, a correction on the apparent transposition rates from Fig. 2 might give an indication of the actual relationship between the transposition rates of the different molecular classes. The most simple correction might be to divide the percentage decrease in the specific activity of the inner layer by the ratio of the specific activity at the inside to that at the outside of the membrane before reincubation. This calculation shows that the transposition rates of the monoenoic, dienoic and polyenoic molecular classes are, respectively, 2.0, 2.7 and 3.2 times higher than that of the disaturated class. This suggests a gradual increase of the transposition rate, which parallels the increase in the degree of unsaturation of the various molecular classes.

Table IV shows that, after incubation of [1-^{14}C]palmitate-labelled rat erythrocytes in plasma, relatively more radioactivity is found in the polyunsaturated classes of plasma phosphatidylcholine. This apparent preferential exchange of the more unsaturated classes of phosphatidylcholine has also been reported for rabbit erythrocytes [54]. We suggest, as explanation, that the fatty acid is incorporated into the molecular classes of phosphatidylcholine predominantly at the inside of the membrane. The higher transposition rate of the polyunsaturated classes leads to a relative enrichment of labelled polyunsaturated classes in the outer layer of the membrane, which becomes reflected in the labelling of the molecular classes of plasma phosphatidylcholine as a

result of the non-specific exchange of the molecular classes of phosphatidylcholine between the red cell membrane and plasma lipoproteins.

One must be aware that the phospholipase technique used in this report discriminates in principle between a phospholipase-degradable phospholipid pool and a pool which is inaccessible to the enzyme. The most simple interpretation is to assume that these pools represent the phospholipid part of the outer and the inner membrane layer, respectively.

It must be stressed that the results mentioned above were obtained with rat erythrocytes. This does not necessarily imply that these results are also valid for erythrocytes from other animal species. Whereas a measurable translocation of rat erythrocyte phosphatidylcholine becomes relevant between 20 and 30° C [55], no translocation of human erythrocyte phosphatidylcholine can be observed at 37° C. However, at 45° C a translocation rate can be measured which is comparable to that in rat erythrocytes at 37° C (unpublished observations).

As a general conclusion it might be stated that phosphatidylcholine in the rat erythrocyte membrane exists in a dynamic state in which transposition across the membrane and exchange with plasma phosphatidylcholine occur.

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