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Transbilayer Movement of Various Phosphatidylcholine Species in Intact Human Erythrocytes

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Phosphatidylcholine specific phospholipid exchange protein was used to introduce (^{14}C)-labeled phosphatidylcholine of different fatty acyl compositions into the intact human erythrocyte. Hydrolysis by a combination of phospholipase A_2 and sphingomyelinase was applied to prove that originally all newly introduced phosphatidylcholine resided in the outer monolayer. Subsequently the erythrocytes were reincubated at 37°C, and redistribution of the introduced (^{14}C)phosphatidylcholine was monitored by applying the combination of phospholipases after different times of incubation. In the situation where 20% of the native erythrocyte phosphatidylcholine had been replaced by phosphatidylcholine from (^{14}C)choline-labeled rat liver microsomal membranes, a slow translocation of the (^{14}C)microsomal phosphatidylcholine was found, with a half-time of transbilayer equilibration of 10.8 hr. Furthermore, the transbilayer movement of probe amounts of (^{14}C)dipalmitoyl-phosphatidylcholine, (^{14}C)egg phosphatidylcholine and (^{14}C)soybean phosphatidylcholine was studied under conditions whereby the fatty acyl composition of the bulk erythrocyte phosphatidylcholine remained unchanged. In correlation to the increasing unsaturation of the probe, half-times for the transbilayer equilibration were calculated to be 26.9, 12.8, and 8.1 hr, respectively.

Key words: erythrocyte membranes, phosphatidylcholine transbilayer movement, phosphatidylcholine exchange protein, phospholipid localization

It is currently well established that the phospholipids in the human and rat erythrocyte are distributed over the two monolayers of the membrane in an asymmetric way. The outer monolayer is mainly composed of the choline-containing phospholipids phosphatidylcholine (PC) and sphingomyelin (SPH), whereas phosphatidylethanolamine (PE) and phosphatidylserine (PS) are confined to the inner layer [reviewed in 1]. This asymmetric arrangement is maintained during the lifetime of the cell [2] of 120

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days for the human and 50 days for the rat erythrocyte, which is remarkable if one realizes that the lipids are continuously modified. This is especially true for the PC. A spontaneous PC exchange exists between the erythrocyte and the plasma lipoproteins [3, 4]. Because plasma PC is far more unsaturated than that of the erythrocyte, this exchange results in the enrichment of unsaturated PC species in the outer monolayer [5]. In the inner monolayer, lyso-PC is acylated with fatty acid [4, 6], both of which are taken up from the plasma and translocated from the outer to the inner monolayer, and this acylation process mainly yields saturated PC species [5]. Since the PC from both monolayers has the same fatty acid composition [7, 8], transbilayer equilibration of PC must occur, which was indeed found in rat erythrocytes, with a half-time of 2–7 hr [8–13]. In contrast, a very slow, if any, transbilayer movement of PC was reported for human erythrocytes [8, 11, 14]. If present, the transbilayer movement has to be regulated by a defined mechanism in order to preserve the asymmetric distribution of the PC, and to maintain the identical fatty acid composition of the PC in outer and inner layer.

In order to determine the rate of transbilayer movement of the PC more precisely, a method has been developed that can be applied to intact erythrocytes under a variety of conditions. In previous studies [11, 15, 16] it was shown that defined PC species can be introduced into the outer monolayer of intact erythrocytes by means of the PC-specific phospholipid exchange protein [17]. In the present study this technique was combined with the use of phospholipases to hydrolyze the outer monolayer [18]. The newly inserted PC can thus be localized at various time intervals after the insertion, and the transbilayer mobility of individual molecular species of PC in intact erythrocytes can be measured.

MATERIALS AND METHODS

Erythrocytes

Fresh human erythrocytes, collected from venipuncture in standard acid-citrate-dextrose buffer, were packed for 5 min at 2,500g and washed three times with a four-fold volume of buffer containing 150 mM NaCl, 25 mM glucose, 3 mM NaN_3, and 10 mM Tris, PH 7.4, with HCl (referred to as buffer throughout). The buffy coat was carefully removed after each wash. Fresh rat erythrocytes were obtained by cardiac puncture from ether-anesthesized male Wistar rats weighing 125–150 g, and treated as the human cells. Fresh cells were used for the experiments. All incubations were carried out in 10-ml Teflon screw-cap tubes in a 37°C room on a clinical blood tube rotator at four revolutions per minute. Hemolysis was estimated by measurement of absorbance in a cell-free supernatant at 410 nm in a Shimadzu UV-110-02 spectrophotometer. The morphology of the erythrocytes was checked by light microscopy after suspension in isotonic formaldehyde buffer (100 mM NaCl, 35 mM Na_3 citrate, and 133 mM formaldehyde, pH 7.4).

Phosphatidylcholine-Specific Exchange Protein

This protein, purified from beef liver according to Kamp and Wirtz [17], was a gift from Dr. K. W. A. Wirtz. It was homogeneous on dodecyl sulfate disk-gel electrophoresis, and stored in 50% (v/v) glycerol at −20°C. The day before use, the protein solution was dialyzed against a 1,000-fold volume of buffer at 4°C for 1 hr and against another 1,000-fold volume of fresh buffer overnight. The dialysis bag had been equili-

brated before use, in 0.1% bovine serum albumin (BSA) solution for 1 hr. After dialysis the protein solution was concentrated against flake polyethyleneglycol (Calbiochem, San Diego) to give a concentration of 150 μg/ml (5.4 μM). Fatty-acid-free BSA (Calbiochem) was added to the solution at a final concentration of 0.1% (w/v).

Phospholipases

Phospholipase A_2 (EC 3.1.1.4) from Naja naja venom or from bee venom was obtained from Sigma (St. Louis) at specific activities of 970 IU and 1,500 IU/mg protein, respectively. They were stored in buffer at 0.5 IU/μl and 4°C, and used without further purification. Sphingomyelinase C (EC 3.1.4.12) from Staphylococcus aureus was purified according to Colley et al [19] and stored in 50% (v/v) glycerol at −20°C and 0.11 IU/μl. The procedure of Zwaal et al [20] was used to purify phospholipase C (EC3.1.4.3) from Bacillus cereus, which was stored in 50% (v/v) glycerol at −20°C and 0.67 IU/μl. The latter two phospholipases were a gift from Dr. B. Roelofsen.

Microsomes

Liver microsomal membranes were prepared from Wistar rats injected intraperitoneally with 30 μCi of (methyl-^{14}C)choline [17] and were a gift from Dr. B. J. H. M. Poorthuis. Before incubation, microsomes were sonicated for 2 min with a Branson sonifier (65 W) under N_2 in ice. The suspension was then centrifuged for 10 min at 3,000 g, and the supernatant was used. It contained 150 nmol phosphatidylcholine/40 μl, with a specific activity of 0.06 mCi/mol.

Preparation of Unilamellar Donor Vesicles

Dipalmitoyl-phosphatidylcholine (DPPC) was synthesized according to Van Deenen and De Haas [21] and was a gift from Dr. R. A. Demel. Egg phosphatidylcholine (egg PC) was obtained from Sigma (St. Louis); soybean phosphatidylcholine (Soybean PC) was kindly donated by Natterman (Köln). Cholesterol was purchased from Merck (Darmstadt), and egg phosphatidic acid was prepared from egg PC with phospholipase D. (Methyl-^{14}C)DPPC, cholesteryl (1-^{14}C)oleate, and (^{14}C)methyliodide, having specific activities of 58, 26, and 58 mCi/mmol, respectively, were obtained from the Radiochemical Centre (Amersham). (Methyl-^{14}C)egg PC and (methl^{14}C) soybean PC were synthesized according to Stoffel [22]. Better than 98% of the total radioactivity in the final preparation was found in the PC with a specific activity of 58 mCi/mmol. The fatty acid composition of the labeled PCs is given in Table I.

Vesicles were prepared from egg PC mixed with an equimolar amount of cholesterol, phosphatidic acid (5 mol %), and trace amounts of one (methyl-^{14}C)phosphatidylcholine species and of (^{14}C)cholesteryloleate, as a nonexchangeable marker. The resulting specific activity of PC in this mixture was 0.2–0.4 mCi/mmol, and the ratio (^{14}C)PC to (^{14}C)cholesteryloleate was kept between 4 and 6; 10 μmol lipids were dried from a chloroform/methanol 2:1 (v/v) solution and dispersed by vortexing in 2 ml of buffer. Vesicles were obtained by sonication of the lipid dispersion with a Branson sonifier (65 W) under N_2 in 10 min at 20°C and centrifugation at 100,000g for 45 min. Vesicle recovery was determined by radioactivity measurements using toluene, containing 2,5-diphenyloxazol (0.5%, w/v) dimethyl POPOP (0.025%, w/v) and BioSolve (2%, v/v, Beckman, Fullerton, California) in a Packard scintillation spectrometer. The resulting vesicles after centrifugation were compared to the original mixture and showed the same ratio of (^{14}C)PC to (^{14}C)cholesteryloleate after separation by

TABLE 1. Fatty-Acyl Composition in Various Phosphatidylcholine Preparations

Phosphatidylcholine[a]	16:0	18:0	18:1	18:2	18:3	20:4
Rat erythrocyte PC	41	24	10	14		11
Human erythrocyte PC	37	13	22	22		6
Egg PC	39	13	31	17		
Soybean PC	15	3	9	67	6	
(^{14}C-methyl) egg PC	35	16	36	11		2
(^{14}C-methyl)soybean PC	18	6	16	57	4	
Total microsomal lipid	24	29	5	15		28

[a]The different PCs and their fatty-acyl methylesters were obtained and analyzed as described in Materials and Methods. Only the main fatty acids were quantitated. Each number, giving the weight percentage of the respective fatty acid, is the mean of at least two determinations, the SD being ± 2.

TLC (see Sample Analysis). Furthermore, the (^{14}C)cholesteryloleate proved to be non-exchangeable from vesicles that were prepared this way.

Incubation Conditions

For each sample 150 μl packed erythrocytes containing 140 nmol phosphatidylcholine were incubated at 37°C with 40 μl microsome or vesicle suspension containing 150 or 50–90 nmol phosphatidylcholine, respectively, and 150 μl exchange protein solution (final concentration 2.4 μM). In the control incubation the exchange protein solution was replaced by buffer, and in a second control incubation the donor microsomes and vesicles were also omitted. Experiments included 10–20 samples. After 1 hr, for each sample, 0.5 ml 37°C buffer was added and the erythrocytes pelleted. The pellet was washed with 1 ml of 37°C buffer, resuspended in 375 μl of 37°C buffer per sample, and incubated for a second time. The temperature of the wash buffer was essential to minimize contamination, and a second wash had no lowering effect. At timed intervals, four samples of 0.5 ml were taken from both the incubation and control incubations. To two samples, 2 ml of buffer containing 10 mM EDTA was added and after centrifugation the cells were lysed with 350 μl of 10 mM EDTA in H_2O and stored frozen. The two other samples (thus resulting in a duplicate measurement) were incubated at 37°C in 1.5 ml buffer containing 13 mM Ca^{2+}. Then 20 IU of phospholipase A_2 was added, followed after 1 hr by the addition of 8 IU of sphingomyelinase. To make sure that all phospholipids in the outer monolayer were hydrolyzed, the phospholipase concentrations used were at least two times higher than necessary [18]. Two hours after the start of the phospholipase incubation, the cells were pelleted, washed with 2 ml of buffer containing 10 mM EDTA, lyzed in 350 μl of 10 mM EDTA in H_2O, and stored frozen at −20°C overnight.

Sample Analysis

After thawing, the samples were extracted following the procedure of Rose and Oklander [23]. The extract was dissolved into 1 ml $CHCl_3/CH_3OH$ 2:1 (v/v), and 2 × 100 μl was used for a total phosphate determination [24] to normalize all results to a defined sample size. Eight hundred microliters were analyzed by TLC (Merck Kieselgel 60 DC-Fertig platten) using the solvent systems of Broekhuyse [25]. To determine the radioactivity in the different lipids, a one-dimensional separation was performed in $CHCl_3$-CH_3OH-25% NH_4 OH-H_2O (90:54:5.7:5.3, v/v). The lipid spots were visualized by I_2 staining and scraped off after I_2 sublimation. Radioactivity was determined

as described above. When the phosphate content of the different phospholipid spots and the cholesterol content had to be determined, a two-dimensional separation was applied with a second solvent $CHCl_3$-CH_3OH-CH_3COOH-H_2O (90:30:8:2.85, v/v). Gas-liquid chromatographic analysis of the fatty acids was performed on a Packard B24 gas chromatograph after conversion of the fatty acyl chains to their methylesters [26]. The extent of lipid peroxidation was estimated by measuring the A_{233}/A_{215} ratio in a Unicam SP 1700 double-beam spectrophotometer according to Klein [27].

RESULTS

Introduction of (^{14}C)PC into the Bilayer of the Erythrocyte

Phosphatidylcholine-specific exchange protein has been applied to introduce (^{14}C)PC molecules into the outer layer of erythrocyte membranes. To verify that the (^{14}C)PC molecules were indeed present in the bilayer and to demonstrate that the ^{14}C label originated from the exchange process only, the following control experiments were carried out.

Vesicles containing egg PC:cholesterol:egg PA (1:1:0.1, mole/mole) and trace amounts of (^{14}C)PC and (^{14}C)cholesteryloleate were incubated with erythrocytes and exchange protein under the standard conditions described in Materials and Methods. After the incubation the erythrocytes were isolated and washed to remove residual vesicles and exchange protein. To test if the residual (^{14}C)PC was adhering to the erythrocyte or actually present in the bilayer, the erythrocytes (150 μl) were incubated with phospholipase C from B cereus (10 IU) as described for phospholipase A_2, except that the reaction was stopped with 2 mM final concentration of 1,10-phenanthroliniumchloride in addition to EDTA. This enzyme is unable to hydrolyze PC molecules within intact erythrocyte membranes [28]. It was found that PC was completely degraded when donor vesicles were incubated with phospholipase C. Contrariwise, less than 5% of the radioactive PC, which was introduced in the erythrocyte by the exchange protein, could be hydrolyzed, demonstrating that at least 95% of the radioactive PC was present in the erythrocyte membrane. This conclusion was confirmed by measuring the amount of the nonexchangeable (^{14}C)cholesteryloleate in the erythrocyte extract. The ratio of (^{14}C)PC/(^{14}C)cholesteryloleate measured in the erythrocyte pellet after exchange was at least 20-fold higher than the ratio in the original donor vesicles. In subsequent experiments, the amount of (^{14}C)cholesteryloleate present in the erythrocyte extract was measured and used to determine the extent of contamination.

A second set of control experiments was carried out to estimate the amount of (^{14}C)PC that did not originate from the exchange process but was formed in the erythrocyte membrane by acylation of (^{14}C)lyso-PC. The latter process occurs in the inner membrane layer [4, 6], and substantial formation of (^{14}C)PC might interfere with the measurement of transbilayer movement of the exchanged (^{14}C)PC from the outer to the inner layer. About 1% of the radioactivity of the various (^{14}C)PC preparations appeared to be in the form of lyso-PC, but although a rapid uptake of the lyso-derivative by the erythrocytes was observed, the overall contribution of this process to the final amount of (^{14}C)PC present in the membrane after exchange was limited to values less than 3% under the experimental conditions used. Final control experiments were carried out to estimate the extent of membrane modification due to the exchange process. The cholesterol content of the erythrocyte appeared to be unaltered using donor ves-

icles that contained 50 mole percent cholesterol, and at the most 5% of the erythrocyte cholesterol was lost during a 1-hr incubation with microsomes. The presence of exchange protein had no influence on the phospholipid composition, which was identical before and after the incubation, ensuring that no net PC transfer occurred. During the exchange reaction 10–20% of the native erythrocyte PC was replaced by donor PC. In order to prevent modification of the fatty acyl composition of the erythrocyte PC [15] in the transbilayer movement studies, the (^{14}C)PC trace was embedded in most experiments in a matrix of egg PC, which has a fatty acyl composition that resembles that of the human erythrocyte. Because peroxidation of PC was reported to induce transbilayer movement of phospholipids in model membranes [29], we also checked the degree of peroxidation of the egg PC. A_{233}/A_{215} was 0.145 in our preparation, indicating that the egg PC did not contain peroxides [27].

Transbilayer Movement of (^{14}C)PC in the Erythrocyte Membrane

The localization of newly introduced (^{14}C)PC molecules was investigated using the phospholipase A_2 from Naja naja. It has been well documented [cf 1, 18] that complete detection of all PC molecules in the outer membrane by this enzyme is possible only if the sphingomyelin, which is present in the outer layer, is also hydrolyzed by a sphingomyelinase. The combination of the two enzymes as described in Materials and Methods was therefore used in subsequent experiments. Under the conditions described here 65 ± 2% (n = 18) of the total PC could be hydrolyzed by phospholipase A_2 alone, and 77 ± 1% (n = 5) was hydrolyzed during the combined action of phospholipase A_2 and sphingomyelinase. In contrast, directly after the exchange procedure and subsequent washings, 97 ± 2% (n = 2) of the newly introduced (^{14}C)PC molecules could be hydrolyzed, demonstrating that this PC is in the outer layer of the membrane. This result was obtained with (^{14}C)PC originating from the microsomal membranes and also when (^{14}C)egg PC, (^{14}C)dipalmitoyl PC, or (^{14}C)soybean PC had been introduced into the erythrocytes, 95 ± 2 (n = 3), 96 ± 2% (n = 4), and 97 ± 2% (n = 2) of the (^{14}C)PC was hydrolyzed, respectively, by the phospholipases. To follow the fate of the various PCs, cells were incubated at 37°C in fresh buffer, samples were taken at different time intervals up to 24 hr, and treated with phospholipase A_2 and sphingomyelinase as described. The results are summarized in Figure 1 and Table II. When microsomal (^{14}C)PC was introduced in the erythrocyte, a gradual decrease in the amount of (^{14}C)PC accessible for the enzymatic hydrolysis could be observed. In combination with the observation that the total amount of (^{14}C)PC did not change during the 24-hr incubation, the conclusion can be drawn that, at 37°C, a slow transbilayer movement of the microsomal PC within the erythrocyte membrane proceeds. From the semilogarithmic plot of the exponential decrease of outer monolayer (^{14}C)PC, the half-time of this transbilayer movement can be calculated as described in the appendix. Under the conditions applied, the half-time of translocation of this PC was 10.8 hr.

In addition, the values measured using various PC species show that transbilayer movement depends on the fatty-acid composition. Dipalmitoyl PC is translocated to the other layer very slowly with a half-time of equilibration of 26.9 hr. An increase in the degree of unsaturation (cf Table I) results in a progressive increase in the ability of these PC molecules to move to the inner region of the membrane (Table II).

During any of these incubations, the erythrocyte remained unaltered as far as tested. As a first parameter we used the morphology of the erythrocyte. When azide was present to prevent microbial growth and glucose to keep the erythrocyte membrane

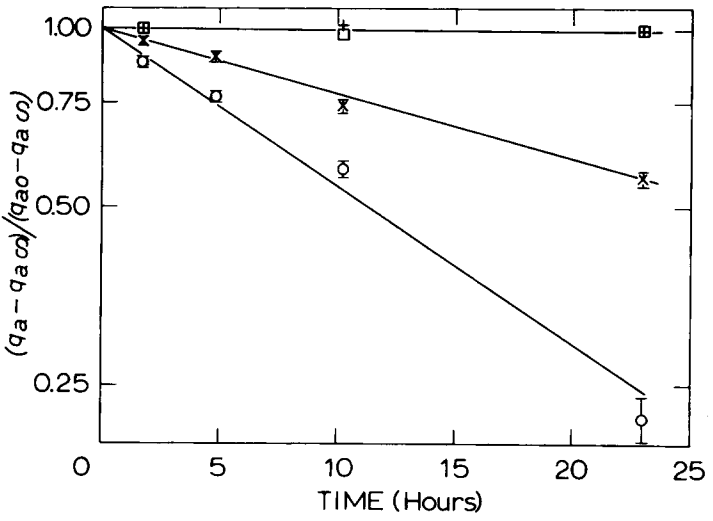

Fig. 1. Transbilayer movement of two PC species in intact human erythrocytes. Human erythrocytes (150 μl) were incubated for 1 hr at 37°C with (+, ×) vesicles (40 μl, 79 nmol PC) consisting of egg PC/-cholesterol/egg PA (1:1:0.1, mole/mole) with a trace amount of (^{14}C)DPPC and (^{14}C)cholesteryloleate or (□, ○) (^{14}C)choline-labeled microsomes (40 μl, 153 nmol PC), and PC exchange protein to a final concentration of 2.5 and 2.9 μM, respectively. The vesicles or microsomes were washed away and the erythrocytes, containing 4.10^3 dis.min^{-1} 150 μl packed cells^{-1}, were reincubated at 37°C. At different time intervals after the start of the exchange incubation, samples were taken to be extracted (+, □) or treated with phospholipase A_2 + sphingomyelinase, and extracted (×, ○). The radioactivity in the remaining PC was determined and the redistribution in time plotted, all as described under Materials and Methods. In the case that the samples were not treated with the phospholipases (+, □) each point represents the mean of two extractions. The SD of the (^{14}C)PC dis.min^{-1} was ± 2%. When the samples have been subjected to the phospholipase action (×, ○), every point represents two independent incubations with the phospholipases, as described in Materials and Methods. The SD for the mean of the resulting values is indicated in the figure.

TABLE II. Transbilayer Movement of Various PC Species in Intact Human Erythrocytes[a]

Probe	Replaced (% of erythrocyte PC)	$t_{1/2}$ (hr)
(^{14}C)microsomal PC[b]	19	10.8
(^{14}C)DPPC[c]	10	26.9
(^{14}C)egg PC[d]	12	12.8
(^{14}C)soybean PC[e]	14	8.1

[a]Human erythrocytes (150 μl) were incubated for 1 hr at 37°C with: [b](^{14}C)choline-labeled microsomes (40 μl, 143 nmol PC); or
[c,d,e]vesicles (40 μl, 79, 70, and 77 nmol PC, respectively) consisting of egg PC/cholesterol/egg PA (1:1:0.1, mole, mole), a trace of (^{14}C)cholesteryloleate and (^{14}C)DPPC, (^{14}C)egg PC, or (^{14}C)soybean PC, respectively. Also, PC exchange protein was added to a final concentration of
[b]2.9, and
[c,d,e]2.5 μM.
The microsomes or vesicles were washed away and redistribution of (^{14}C)PC (4.10^3dis.min^{-1}, 150 μl packed cells^{-1}) was monitored with the combination of phospholipase A_2 + sphingomyelinase which selectively hydrolyzes the outer monolayer. The percentage of the erythrocyte PC that was replaced by donor PC was calculated as described in Materials and Methods. The half-time of transbilayer equilibration ($t_{1/2}$) was calculated from a semilogarithmic plot (Fig. 1) of the data as described in Materials and Methods.

in the energized state, the discocyte form of the erythrocyte was maintained for 24 hr at 37°C. Secondly, based on phospholipid analysis, it was found that the phospholipid composition did not change during 24 hr and that hydrolysis by the phospholipases was also unchanged. Only slightly less PC was degraded after 24 hr, 74% compared to 77% (at t = 1.6 hr), and the sensitivity for hemolysis during the EDTA wash after the phospholipases was slightly increased.

DISCUSSION

For a better understanding of the origin, maintenance, and functional role of the phospholipid asymmetry that exists in erythrocyte membranes [1, 9–12, 18], it is important to gain insight in the principles that govern the mobility of the individual phospholipids at the molecular level. Several experimental approaches have been applied to estimate the transbilayer movement of PC in mammalian erythrocyte membranes [3,5, 7–11,14,30]. In this paper a method is described by which the rate of transbilayer equilibration of PC in intact erythrocyte can be quantitated. The method is based on the partial replacement of native PC molecules by radioactive analogues, followed by the localization of the latter compounds with phospholipases. The observation that this newly introduced PC can be hydrolyzed completely—directly after its insertion— by the phospholipase-sphingomyelinase treatment shows that all of the newly introduced PC is located in the outer layer and that neither the phospholipases nor the exchange proteins do induce a reorientation of PC over the bilayer. Thus, the use of these proteins as tools for the study of transbilayer distribution of phospholipids in the erythrocyte membrane is justified.

When (^{14}C)PC molecules are introduced that have a fatty-acid composition similar to the native PC molecules, the method does not disturb the original erythrocyte membrane composition. This nonmodifying condition can be approached by introducing very small amounts of highly labeled defined PC molecules, embedded in a matrix of egg PC. This way, it is possible to measure rates of transbilayer movements of individual PC species.

Indications of a complete absence of transbilayer movement of PC in human erythrocytes came from the observation that in patients who had received divided doses of $^{32}P_i$ in the blood the fraction of the total erythrocyte PC that became equilibrated with the plasma PC was limited to 63% in 12 days [3]. In addition, no transposition was measured in a 3-hr incubation at 37°C when the erythrocyte PC had been labeled in vitro with (^{14}C)palmitic acid [8]. When the cells were incubated at 45°C, however, a transbilayer equilibration was detected with a half-time of 4–5 hr [8, 31]. A half-time of 7 hr at room temperature was reported for a PC with a spin-labeled fatty acyl chain [14]. When the spin-label was present in the choline moiety of hydrogenated PC, the observed half-time was much longer and can be calculated to be ~17 hr at 37°C. The transbilayer movement of the PC was therefore attributed to the bulky spin-label [14]. A very slow, if any, transbilayer movement of the PC was observed when the PC of intact human erythrocytes was allowed to equilibrate with PC from (^{14}C)choline-labeled microsomes [11]. In the present paper the latter incubation was repeated, and under identical conditions, using the method described above, transbilayer movement of the (^{14}C)microsomal PC was evident, with a half-time of 10.8 hr. Also, for three different (^{14}C)PC species, translocation was observed with half-times of 8–27 hr. The conclusion from these observations has to be that PC displays transbilayer movement in intact

human erythrocytes, but that the rate is low, which probably explains why the translocation was not detected in some of the previous investigations [8, 11, 31] or considered neglectable in another [14].

The various PC species appeared to redistribute with half-times that were directly proportional to the saturation of the PC. For DPPC, egg PC, and soybean PC the ratio of the half-times observed for the transbilayer equilibration, was 3.3:1.6:1.0, yielding relative rate constants for the translocation ($1/t^{1/2}$, see Eq. 7 in the Appendix) of 1.0, 2.1, and 3.3, respectively. This is in good agreement with the relative rates of transposition that were reported for disaturated, monoenoic, dienoic, and polyenoic PCs in rat erythrocytes—namely 1.0, 2.0, 2.7, and 3.2, respectively [8].

The actual half-times for transbilayer equilibration of PC in human erythrocytes of 9–27 hr are much longer than the values (2–7 hr) reported for PC in rat erythrocytes [8–13, 31]. Still, even in human erythrocytes the translocation of PC is fast compared to the life-time of the erythrocyte of 120 days, and also fast compared to the renewal of the erythrocyte PC by exchange with plasma PC or acylation of lyso-PC, which in both cases has a rate of approximately 1% h^{-1} [31], or a half-time of about 100 hr [4]. PC translocation may therefore be fast enough to abolish any differences in PC species composition of the inner and outer monolayer generated by the renewal processes [5, 31] and to bring about a more or less random distribution of the various molecular species of PC over the two monolayers [7,8].

Some observations, however, seem to be incompatible with the picture of PC translocation with half-times of 2–7 and 8–27 hr for rat and human erythrocytes, respectively. First, in human erythrocytes no transbilayer equilibration of (^{32}P)PC at all was observed on a time scale of 12 days in an in vivo study [3]. Second, the translocation of (^{14}C)PC and (^{32}P)PC incorporated by the incubation of human erythrocytes in plasma with (^{14}C)fatty acids or in $^{32}P_i$-labeled plasma that was reported to occur at 45°C with a half-time of 4–5 hr [8, 31] disappeared when the cells had been preincubated in rat plasma [31]. Third, only part of the PC in rat erythrocytes equilibrated with (^{32}P)PC when the cells were incubated in a $^{32}P_i$-labeled rat liver extract for 18 hr [30], and finally, when rats were injected with $^{32}P_i$ the PC in the outer monolayer of the erythrocytes kept a significantly higher specific activity than the inner monolayer PC, even after 96 hr [5, 31]. Also after 96 hr the distribution of ^{32}P over the molecular species of PC in the outer monolayer was significantly different from the distribution in the inner monolayer [5, 31], which is inconsistent with a half-time for PC transbilayer equilibration of 2–7 h. These observations suggest that, especially in the rat erythrocyte, translocation rates of PC in vivo may be much lower than the rates reported in vitro. Furthermore, as far as the PC translocation in erythrocytes is concerned, the physiologically relevant question may not be if and how fast transbilayer movement occurs, but by which factors the rate of the transbilayer movement of PC is regulated.

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APPENDIX: Calculation of Phosphatidylcholine Radioactivity and Rate of Transbilayer Movement in the Erythrocytes

Radioactivity found in the lipid spots of the different samples was first normalized to a defined sample size, by means of the total phosphate in each sample (see Sample Analysis). Subsequently, the phosphatidylcholine radioactivity was corrected for contamination of the erythrocytes by intact vesicles. The amount of contaminating phosphatidylcholine radioactivity was calculated from the (^{14}C)cholesteryloleate in each sample and the ratio of (^{14}C)phosphatidylcholine/(^{14}C)cholesteryloleate in the original vesicles. When erythrocytes had been incubated with microsomes as a donor

system, the radioactivity values of the samples that had been incubated with exchange protein were simply corrected for the values of the control samples without exchange protein.

In the samples that had been treated with phospholipases, correction for contaminating phosphatidylcholine was carried out in the following way: The contamination was calculated from the (^{14}C)cholesteryloleate in the sample, and also the hydrolysis of the contaminating (^{14}C)PC by the phospholipases was studied. It was found that in vesicles containing (^{14}C)DPPC, (^{14}C)egg PC, or (^{14}C)soybean PC and in microsomes containing (^{14}C)microsomal PC 32, 28, 25, and 3%, respectively, of the (^{14}C)PC remained after the incubation with phospholipase A_2. This was independent of the presence of sphingomyelinase or erythrocytes, and these percentages of contaminating (^{14}C)PC were therefore used for the correction after the incubation with phospholipases.

Replacement of native erythrocyte PC was determined as follows: % introduced = (specific activity of the erythrocyte PC)/(specific activity of the donor PC at zero time).

After the (^{14}C)PC has been introduced into the erythrocyte membrane, we are dealing with a two-pool closed system (Fig. 2), with, as an approximation, all (^{14}C)PC in the outer monolayer at zero time [32].

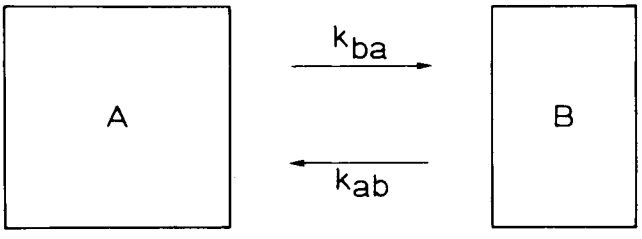

Fig. 2. Two-pool model for the exchange of (^{14}C)PC between the outer monolayer PC pool and the inner monolayer PC pool of the erythrocyte membrane.

The relation between the (^{14}C)PC in the outer monolayer at time t_x (q_a) and the time (t) is given by the equation:

$$q_a/q_{ao} = H_1 \exp(-g_1 t) + H_2 \tag{1}$$

in which

$$H_1 = (g_1 - k_{ab})/g_1 \quad H_2 = k_{ab}/g_1 \tag{2}$$

where q_{ao} is total (^{14}C)PC in the erythrocyte, which is all present in the outer monolayer at zero time; k_{ab},k_{ba} are the rate constants of transfer from the two pools; g_1 is the rate constant for the transbilayer equilibration, and the inverse of the relaxation time of the process,

$$g_1 = 1/\tau = k_{ab} + k_{ba} \tag{3}$$

in a steady state-condition: $k_{ba} \times A = k_{ab} \times B$ (Eq. 4) in which A and B are the sizes of the outer and inner PC pool, respectively. Substituting equation (3) and (4) in equation (2) gives $H_1 = B/(A + B)$ and $H_2 = A/(A + B)$ which substituted in equation (2) yields:

$$\ln \left[\frac{q_a - q_{a\sim}}{q_{ao} - q_{a\sim}} \right] = -g_1 t \tag{5}$$

in which $q_{a\sim}$ is the amount of (^{14}C)PC in the outer monolayer at $t = \sim$ ($q_{a\sim} = A/(A + B) \times q_{ao}$) (Eq. 6). The half-time of PC equilibration can be calculated as $t_{1/2} = 0.693/g_1$ (Eq. 7) in which g_1 is the slope of the semilogarithmic plot of equation 5.

Equation 5 is used to describe our experimental system:

$t = 0$ For zero time is taken the start of the incubation with exchange protein + .

$t = t_x$ For t_x is taken the start of the incubation with phospholipase A_2.

q_{ao} This is found as the (^{14}C)PC of the samples that had not been treated with phospholipases.

$q_{a\sim}$ $= 0.77/(0.77 + 0.23) \times q_{ao} = 0.77q_{ao}$ (Eq. 6) in human erythrocytes, where 77% of the PC can be hydrolyzed by the combined phospholipases.

q_a This is the (^{14}C)PC in the outer monolayer at time t_x, and is found by subtraction of the (^{14}C)PC in the inner monolayer (represented by the (^{14}C)PC remaining after phospholipase treatment) from total (^{14}C)PC, q_{ao}.

At different time points q_a was determined and the half-time of transbilayer equilibration for the PC species under investigation could be calculated from the semilogarithmic plot of equation 5 and equation 7.