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Fast translocation of phosphatidylcholine to the outer membrane leaflet after its synthesis at the inner membrane surface in human erythrocytes

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The translocation rate of [^{14}C]phosphatidylcholine to the outer membrane leaflet of human erythrocytes after its primary synthesis from lysophosphatidylcholine by acylation with ^{14}C-labeled oleic or palmitic acid in the inner leaflet has been measured by following the time-dependent increase of cleavability of ^{14}C-labeled phospholipids by external phospholipase A_2 (5 min, 37°C). Immediately after a short acylation time period of 10 min about 20% of the newly synthesized [^{14}C]phosphatidylcholine are already detectable in the outer leaflet. After an incubation of 1 h at 37°C following 10 min of acylation the fractions of labeled and native phosphatidylcholine accessible to the lipase are identical, which demonstrates that [^{14}C]phosphatidylcholine has attained the same asymmetric distribution as its endogenous analogue. The calculated halftime of the outward translocation is about 20 min and its activation energy is low, 30 kJ/mol. Translocation is inhibited by a 5 min treatment with phenylglyoxal following acylation. A fast translocation is not observed for newly synthesized phosphatidylethanolamine. Results suggest a selective, protein-mediated outward translocation of newly synthesized phosphatidylcholine.

Introduction

Phospholipid remodelling in the human erythrocyte [1–10] has been demonstrated to occur by two mechanisms. One, the exchange of phospholipids, essentially phosphatidylcholine, between plasma lipoproteins and the outer membrane leaflet of the erythrocyte [11–13]. Two, the incorporation of fatty acids into phospholipids [3–5]. Plasma albumin bound fatty acids rapidly distribute into the outer membrane leaflet followed by a very fast translocation to the inner leaflet with a halftime of < 15 s [14]. At the inner membrane surface these fatty acids are transformed into acyl-CoA by an ATP-dependent (AMP producing) enzymatic process [15–18]. These activated fatty acids are then coupled to lysophospholipids by esterification (acylation). The phosphatidylcholine synthesized in this way is translocated to the outer leaflet [18–21]. There is evidence that in rat erythrocytes the outward translocation of newly synthesized unsaturated phosphatidylcholine species is fast [20] as compared to the inward translocation of phosphatidylcholine species inserted into the membrane [22,23].

The present study aimed to investigate the process and mechanism of translocation of newly synthesized phosphatidylcholine to the outer membrane leaflet in human erythrocytes.

Materials and Methods

Fresh human blood anticoagulated with citrate was obtained from the local blood bank and was used within 5 days. Erythrocytes were isolated by centrifugation, the buffy coat carefully removed and the cells washed three times with 154 mmol l^{-1} NaCl.

1-[^{14}C]Oleic acid, 1-[^{14}C]palmitic acid, 1-[^{14}C]oleoyl-lysophosphatidylcholine and 1-[^{14}C]oleoyl-CoA (spec. act. 2 GBq/mmol) were purchased from Amersham-Buchler (Braunschweig), bovine serum albumin (essentially fatty free) was from Paesel (Frankfurt), Dextran 4 was from Serva (Mannheim) and phenylglyoxal was from Sigma (Deisenhofen).

Media (pH = 7.4) used for incubation

Medium A contained (mmol l^{-1}): KCl (90), NaCl (45), Na_2HPO_4/NaH_2PO_4 (12.5) and sucrose (44).

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Medium D contained (mmol l^{-1}): KCl (90), NaCl (45), Hepes (10) and Dextran 4 (40).

Incorporation of ^{14}C-labeled fatty acids into phospholipid

Variable amounts of a stock solution of ^{14}C-labeled oleic or palmitic acid in ethanol were evaporated in a vial under a stream of N_2 and 500 μl of an erythrocyte suspension (hematocrit 10%) was added. After an incubation at 22°C for 5 min under shaking the suspension was transferred to a new vial. Following centrifugation the cells were resuspended in 450 μl of Medium A containing 5 mmol l^{-1} glucose and incubated at 37°C. At increasing time intervals 50-μl aliquots of the suspension were mixed with 400 μl of medium A and 200-μl aliquots were then transferred to new vials. After centrifugation cells were treated (2 min, 0°C) three times with 400 μl of medium A containing 1.5% albumin to remove unesterified fatty acids from the cells and to determine esterified fatty acid. The cells were pelleted, washed three times with Medium A and hemolyzed with 220 μl H_2O. The radioactivity in the hemolysate was then measured by liquid scintillation counting. From the radioactivity in the sample and the specific radioactivity of the fatty acid the amount of esterified [^{14}C]fatty acid (nmol per ml of packed cells) was calculated.

Lipid extraction procedures

Following incubation of cells with ^{14}C-labeled fatty acid aliquots containing 50–100 μl of cells were treated three or four times with 1 ml of medium A containing 1.5% albumin, and washed three times with Medium A. After hemolysing the erythrocytes by mixing with H_2O (1 : 1) lipids were extracted with isopropanol/chloroform according to Ref. 24. Alternatively, lipids were extracted from the intact cells by mixing of 1 vol. packed cells with 11 vols. of isopropanol followed by 7 vols. of chloroform. In order to detect acyl-CoA the lipid extraction was carried out by mixing (30 min) packed intact cells with 5 vols. of 0.5 mol l^{-1} NH_2OH in methanol (prepared by mixing 1 mol l^{-1} solutions of $NH_2OH \cdot HCl$ and KOH in methanol). This treatment transforms acyl-CoA into acylhydroxamic acid [25–27]. Following mixing with 5 vols. of chloroform (30 min) the extract was centrifuged and a clear brown-red coloured supernatant was obtained. To quantify the yield of extractable radioactivity 10-μl samples of the extracts were counted for radioactivity and this radioactivity was then related to the total radioactivity in the erythrocytes (dpm/ml cells) determined directly without preceeding lipid extraction.

Thin-layer chromatographic procedures

Lipids were separated by thin-layer chromatography on silica gel 60 plates (Art. 5715 Merck, Darmstadt) using a solvent mixture of triethanolamine/ethanol/chloroform/water (35 : 34 : 30 : 6, v/v). Alternatively, to obtain a better separation of phosphatidylethanolamine and acylhydroxamic acids a solvent mixture of 35 : 34 : 32 : 4 was used. The distribution of radioactivity among the various spots was quantified with a thin-layer chromatographic scanner (TLC-linear analyzer, Berthold, Wildbad). From the fraction of radioactivity in a certain spot, the radioactivity in the lipid extract and the specific radioactivity of the fatty acid the amount of ^{14}C-labeled lipid/ml of packed cells was calculated.

Measurement of ^{14}C-labeled phospholipids translocated to the outer membrane leaflet

After incubation of cells with ^{14}C-labeled fatty acid the unesterified fatty acid was removed from the cells by three or four treatments with medium A containing 1.5% albumin. Cells were then washed three times with medium A (0°C), resuspended in medium D containing Dextran 4 and incubated at 37°C (hematocrit 10%). At different time intervals 1-ml aliquots of the suspension were mixed with Medium D containing Ca^{2+} and Mg^{2+} (0.25 mmol l^{-1} final concentration) and treated for 5 min with bee venom phospholipase A_2 (10 I.U./ml of cells). The enzymatic activity was terminated by the addition of EDTA (1 mmol l^{-1} final concentration) and the cells were isolated by centrifugation. The fraction of phosphatidylcholine translocated to the outer membrane leaflet was determined by two different procedures.

(a) Chromatographic procedure. Lipids were extracted from the intact cells by isopropanol/chloroform. After thin-layer chromatographic separation of lipids the decrease of radioactivity in the phosphatidylcholine fraction and the increase of radioactivity in the fatty acid fraction caused by the action of the phospholipase were quantified. From these data the amounts of cleaved phospholipids were calculated.

(b) albumin extraction procedure. Cells were resuspended in Medium D containing 1.5% albumin to extract [^{14}C]oleic acid released by the lipase from ^{14}C-labeled phosphatidylcholine in the outer membrane leaflet. From the albumin-extractable radioactivity and the specific radioactivity of ^{14}C-labeled fatty acid, the amount of ^{14}C-labeled phosphatidylcholine in the outer membrane leaflet was calculated.

Results and Interpretation

In preliminary experiments [28] it was established that already after a 60 min incubation of intact cells with ^{14}C-labeled oleic acid 25% of the newly synthetized [^{14}C]phosphatidylcholine could be cleaved by phospholipase A_2, which is evidence for translocation of the phospholipid to the outer membrane leaflet [29,30] following its formation at the inner membrane surface. Since the phospholipase treatment of erythrocytes was

restricted to 5 min and such a treatment is known to inhibit translocation of newly synthesized phosphatidylcholine [28] a possible translocation of phosphatidylcholine during the incubation with the phospholipase was limited. When this lipase treatment was done in Dextran 4 (see Methods), which has a stimulating effect on bee venom phospholipase A_2 [31], the lipase cleaved up to 53% of the native phosphatidylcholine under our experimental conditions. This is comparable to the fraction cleavable by a 1 h exposure to the lipase in salt media in the absence of Dextran 4 [30] and represents 70% of the fraction of this phospholipid thought to be present in the outer membrane leaflet [29]. In order to limit the outward translocation of newly synthesized phosphatidylcholine during the acylation period, it seemed desirable to keep this period as short as possible, in order to minimize the fraction of phosphatidylcholine already translocated at the start of the translocation measurement. Prior to the translocation studies we therefore investigated the consequences of such a restriction of the acylation phase for the yield of labeled phosphatidylcholine.

Yields of labeled phosphatidylcholine obtained by short incubations of cells with ^{14}C-labeled fatty acids (acylat

Insertion of ^{14}C-labeled fatty acids into the membrane of human erythrocytes (2–100 nmol/ml cells) results in a time-dependent increase of radioactivity not removable by albumin [14,27] due to the incorporation of ^{14}C-labeled fatty acids into phospholipids. Albumin has been shown to remove essentially all of the unesterified fatty acids from both inner and outer membrane leaflet, since flip-flop of fatty acids between the leaflets is a very fast process [14,32]. About 90% of the radioactivity not removable by albumin can be extracted from the hemolyzed cells by isopropanol/chloroform (data not shown) using the Rose and Oklander procedure [24,33,34]. As expected, chromatograms of these extracts demonstrated that labeled oleic acid had become incorporated into phosphatidylcholine and to a lesser extent into phosphatidylethanolamine (see also Refs. 14, 18, 27, 28 and 35). Unexpectedly, the radiochromatograms also demonstrated the presence of considerable amounts of unesterified fatty acids in these extracts (Fig. 1) after short acylation periods. This fraction of unesterified fatty acid decreased with increasing acylation times in parallel with an increase of the fraction of labeled phospholipid (Fig. 1).

Data obtained by a comparison of different lipid extraction procedures support the view that these unesterified fatty acids arise from a hydrolysis of acyl-CoA thioesters during the lipid extraction procedure. The procedures applied comprised:

(A) The original Rose and Oklander extraction of lysed cells [24].

(B) The Rose and Oklander extraction of intact cells.

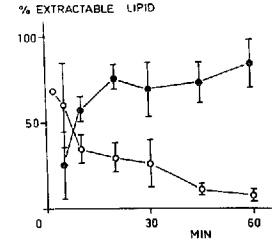

Fig. 1. Extractability of labeled lipids (in % of total cellular radioactivity) from erythrocytes following incubation with [^{14}C]oleic acid. Erythrocytes were incubated with [^{14}C]oleic acid at 37 °C for the time periods given on the abscissa and then treated with albumin to remove unesterified fatty acids. Subsequently, cells were hemolyzed and lipids extracted by isopropanol/chloroform using the procedure of Rose and Oklander [24]. The percentages of extractable radioactivity present as unesterified fatty acid (○) and phospholipid (●) were determined. Mean values (±S.D.) of 2–9 experiments. The 100% values were derived from the total radioactivity in the cells prior to lipid extraction.

(C) The extraction of intact cells by hydroxylamine/methanol/chloroform.

This latter procedure has been used to estimate [16,25–27] acyl thioesters [15–17,27] which are not detectable by procedures A and B. Transformation of the thioesters into apolar *N*-acylhydroxamic acids by hy-

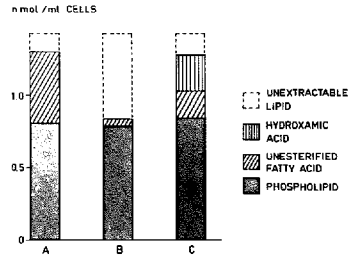

Fig. 2. Balance sheet of ^{14}C-labeled lipids extracted from erythrocytes according to three different methods. In all three cases erythrocytes were pulse-labeled for 10 min with [^{14}C]oleic acid (37 °C) and unesterified fatty acid removed by albumin. Lipid extracts were then prepared from hemolyzed (A) or intact (B) cells by isopropanol/chloroform, or from intact cells by hydroxylamine/methanol/chloroform (C). The distribution of radioactivity amoung different lipid fractions was determined by thin-layer chromatography and subsequent radiochromatogram scanning. Amounts of extractable radioactivity contained in the various lipid fractions and amounts of unextractable radioactivity were related to the total amount of radioactivity in the cells (Mean values of four experiments).

droxylamine (NH_2OH) in methanol makes them extractable [25–27,32,35].

The differences and similarities of the three extraction methods are exemplified in the balance sheets in Fig. 2. After 10 min of acylation the recovery of labeled phospholipid is the same in all three extraction procedures, although the total recovery of radioactivity is much smaller in B than in A and C (Fig. 2). Moreover, the total recovery of labeled unesterified fatty acid and *N*-acylhydroxamic acid is about the same in A and C, while in B it is much smaller, due to the inextractability of acyl-CoA. This constellation of data suggests hydrolysis of acyl-CoA during extraction according to procedures A and C. In line with this notion, hydrolysis of exogenously added [^{14}C]oleoyl-CoA is observed when intact cells are directly extracted with hydroxylamine/methanol followed by chloroform (data not shown).

From all these studies it follows that a shortening of the acylation period in the translocation studies is limited by the low yield of labeled phosphatidylcholine and by the accumulation of labeled precursors in the early phase of acylation. These labeled precursors could progressively label phosphatidylcholine during the translocation measurement.

Translocation of newly synthetized phosphatidylcholine to the outer membrane leaflet

As a compromise between minimal outward translocation of labeled phosphatidylcholine during the acylation period and maximal phosphatidylcholine labeling an acylation period of 10 min was choosen for translocation studies. Surprisingly, even after these 10 min of acylation with [^{14}C]oleic acid, i.e. a pulse-labeling, a considerable fraction of at least 15% of ^{14}C-labeled phosphatidylcholine was cleavable by a 5 min treatment with phospholipase A_2, as could be shown by thin-layer chromatography of the lipids extracted by isopropanol/chloroform from intact cells. This cleavable fraction must have been translocated to the outer leaflet during the 10 min acylation period.

Incubation of the cells following removal of the labeled unesterified oleic acid by albumin after the 10 min acylation period results in a fast increase of the amount of phosphatidylcholine (nmol/ml cells) cleavable by the lipase, up to a saturating value reached after 60 min of incubation (Fig. 3). The cleavable amounts determined by the chromatographic procedure in three experiments correspond to 50–55% of the total amount of phosphatidylcholine labeled with oleic acid during the acylation period. Interestingly, the same percentage of the total native phosphatidylcholine is also cleaved by the lipase under these conditions (up to 53%, data not shown). Thus, newly formed [^{14}C]phosphatidylcholine seems to have reached the same asymmetric distribution as its native unlabeled analogue. From Fig. 3 a halftime for translocation at 37°C of 20 min can be

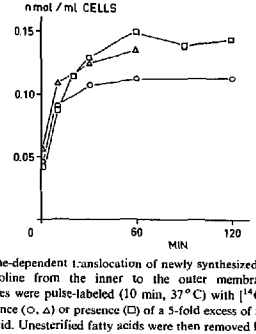

Fig. 3. Time-dependent translocation of newly synthesized [^{14}C]phosphatidylcholine from the inner to the outer membrane leaflet. Erythrocytes were pulse-labeled (10 min, 37°C) with [^{14}C]oleic acid in the absence (○, △) or presence (□) of a 5-fold excess of non-labeled palmitic acid. Unesterified fatty acids were then removed by albumin. The time-dependent increase of the amount of [^{14}C]phosphatidylcholine (nmol/ml cells) transferred to the outside was then measured by following the increase of the accessibility of the labeled phospholipid towards phospholipase A_2 as analyzed by the chromatographic (○, □) or the albumin extraction procedure (△).

derived. Since the fraction of labeled phosphatidylcholine already translocated during the acylation (10 min, 37°C) and washing (10 min, 0°C) procedures was not included in this estimate, the real half-time will be even smaller.

The selectivity of phosphatidylcholine labeling by [^{14}C]oleic acid is improved when acylation occurs in the presence of an excess of non-labeled palmitic acid, which reduces incorporation of oleic acid into phosphatidylethanolamine [28]. In this case the rate of phosphatidylcholine translocation is quite similar to that obtained after labeling with [^{14}C]oleic acid alone. Moreover, translocation rates determined by the chromatographic procedure and the albumin extraction procedure (see Methods) coincide (Fig. 3).

Translocation of labeled phosphatidylethanolamine was not detectable in our experiments (data not shown). This might be related to the low extent of labeling of phosphatidylethanolamine by [^{14}C]oleic acid. Therefore, experiments were designed to improve the extent of phosphatidylethanolamine labeling. When [^{14}C]palmitic acid is used to acylate phospholipids, phosphatidylethanolamine is labeled to a considerably higher extent [28]. Even at this higher extent of labeling, however, no evidence for a time-dependent increase of the cleavable fraction of phosphatidylethanolamine could be obtained (data not shown), although a fast increase of the cleavable fraction of phosphatidylcholine was again observed.

The fast outward translocation of newly synthetized phosphatidylcholine is distinctly different from the slow

inward translocation ($t_{1/2}$ = 3–26 h) of exogenously added membrane-inserted phosphatidylcholine [22,23]. This indicates a mediated process of outward translocation of newly synthesized phosphatidylcholine, a view supported by the surprisingly low temperature dependence of this translocation (Fig. 4), from which an activation energy of 30 kJ/mol can be derived. Inward translocation of phosphatidylcholine has an apparent activation energy of 55 kJ/mol [36].

To provide more evidence for a mediated translocation, effects of chemical modification of membrane protein SH-groups by *N*-ethylmaleimide and of guanidino groups of arginine by phenylglyoxal [37] as well as the influence of the ATPase inhibitor vanadate [38] on the translocation were investigated. The presence of *N*-ethylmaleimide (2 mmol l^{-1}) during the translocation measurement did not significantly affect the translocation rate (data not shown). A treatment with the SH-reagent during the acylation period was not feasible in view of the inhibition of acylation by the reagent. Pretreatment of cells with vanadate (0.5 mmol l^{-1}) during acylation or its presence during translocation did not affect the translocation rate (data not shown). However, a 5 min treatment of cells with 20 mmol l^{-1} phenylglyoxal (37°C) after the acylation period, followed by removal of the reagent by washing produced a considerable inhibition of phosphatidylcholine translocation (Fig. 5). Exposure to phenylglyoxal during the acylation period inhibited the formation of labeled phospholipid (data not shown). Under our experimental conditions phenylglyoxal treatment does neither affect phospholipase activity nor the inward translocation of lysophosphatidylcholine and phosphatidylcholine (Schimmack and Haest, unpublished data).

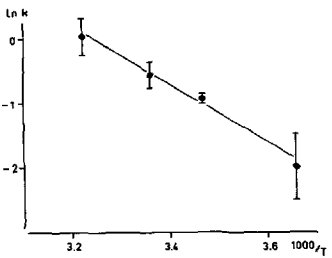

Fig. 4. Temperature dependence of the outward translocation of [^{14}C]phosphatidylcholine. After pulse-labeling with [^{14}C]oleic acid for 10 min erythrocytes were treated with albumin, incubated at different temperatures and the rate of translocation (k, min^{-1}) of phosphatidylcholine to the outer membrane leaflet measured as the percentage of labeled phosphatidylcholine cleavable (labeled fatty acid released by phospholipase cleavage) related to total labeled lipid. Mean values (±S.D) from 3–5 experiments.

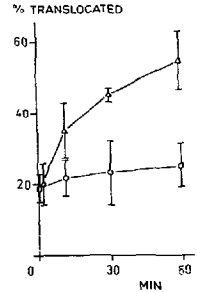

Fig. 5. Inhibition of the outward translocation of newly synthesized phosphatidylcholine by phenylglyoxal. After pulse-labeling with [^{14}C]oleic acid for 10 min and treatment with albumin erythrocytes were exposed to phenylglyoxal (5 min, 20 mmol l^{-1}, 37°C) followed by washing (0°C) with medium A containing albumin (2×) and medium A (2×). The translocation of phosphatidylcholine to the outer membrane leaflet was then measured at 37°C. Results represent mean values (±S.D.) of five experiments with phenylglyoxal (○) and four experiments in the absence of phenylglyoxal (△).

Discussion

In the present study the translocation of phosphatidylcholine to the outer membrane surface of human erythrocytes after its synthesis from lysophosphatidylcholine and palmitic or oleic acid at the inner membrane surface is shown to be a fast process with a halftime of 20 min or less. Translocation is inhibitable by the arginine reagent phenylglyoxal and has a surprisingly low temperature dependence. The present results on human erythrocytes generalize the original evidence for a fast translocation of newly synthesized unsaturated phosphatidylcholine species in rat erythrocytes [20]. In this original study a quantification of the halftime of translocation was not possible due to the long incubation periods used for acylation and phospholipase treatment. The outward translocation rate reported here is at least 10-fold higher than the rates of inward and outward translocation of spin-labeled phosphatidylcholine analogs [23] or of the inward translocation of ^{14}C-labeled phosphatidylcholines [22]. In view of the slow inward translocation of these phosphatidylcholines and the fast outward translocation of newly synthesized phosphatidylcholine it might be expected that the newly synthesized phospholipid would transiently distribute more asymmetric than its native analogue. This was not observed.

High rates have previously been reported for the selective, mediated inward translocation of phosphatidylethanolamine and phosphatidylserine in blood cells [39,40] and fibroblasts [41], for the non-selective inward translocation of phospholipids in the endoplasmic reticulum [42–44] and for the outward translocation of newly synthesized phosphatidylethanolamine in bacteria [45]. In contrast to the translocation of phospholipids in the endoplasmic reticulum and the inward translocation of phosphatidylethanolamine and phosphatidylserine in erythrocytes [44,36] outward translocation of newly synthetized phosphatidylcholine in erythrocytes is not inhibitable by *N*-ethylmaleimide and vanadate.

The fast outward translocation of newly synthesized phosphatidylcholine in erythrocytes is also selective. Outward translocation of newly synthesized phosphatidylethanolamine is not demonstrable during 3 h of incubation. Since phosphatidylethanolamine is a good substrate for bee venom phospholipase [30,31] the lack of its cleavage indicates a lack of translocation. Notwithstanding the probable preferential stationary orientation of ^{14}C-labeled phosphatidylethanolamine to the inner membrane leaflet similar to that of its native analogue (inside/outside 4:1, Ref. 29] a cleavage of [^{14}C]phosphatidylethanolamine should have been detectable, in particular if its outward translocation were as rapid as that of newly synthesized phophatidylcholine and thus faster than the inward translocation of phosphatidylethanolamine (halftime 1 h, Ref. 46). Alternatively, following its outward translocation, newly synthesized phosphatidylethanolamine could reside in a pool that is not accessible for the phospholipase.

The low activation energy of outward translocation of newly synthesized phosphatidylcholine is similar to that for the mediated translocation of aminophospholipids in erythrocytes, but lower than that of simple translocation of phosphatidylcholine [36]. This low activation energy may be taken as evidence for a protein-mediated translocation of newly synthetized phosphatidylcholine.

In view of the fast translocation of newly synthesized phosphatidylcholine to the outer membrane leaflet it might be expected that the extent of degradation of this phospholipid should increase upon prolongation of the treatment with the phospholipase from 5 to 60 min. However, this has not been observed [18–21]. The discrepancy is explained by an inhibition of translocation by the phospholipase treatment [28]. Since non-mediated translocation of phosphatidylcholine inserted into the erythrocyte membrane is slow in both directions [23], whereas outward translocation of newly synthesized phosphatidylcholine is fast it may be concluded that at least the major fraction of newly synthesized phosphatidylcholine resides in a pool of lipid from which it is translocated to the outer membrane leaflet prior to delivery to the bulk lipid domain.

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