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Flip-flop rates of individual molecular species of phosphatidylcholine in the human red cell membrane

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Trace amounts of four different, well-defined species of phosphatidyl[*N-methyl*-^{14}C]choline ([^{14}C]PC), differing in their fatty acyl constituents, were introduced exclusively into the outer membrane leaflet of the intact erythrocyte by using a PC-specific phospholipid transfer protein. The rate of transbilayer equilibration of these probe molecules was calculated from the time-dependent decay in specific radioactivity of the PC pool in the outer monolayer, which was discriminated from that in the inner leaflet by treating the intact cells with phospholipase A_2 in the presence of sphingomyelinase C. At 37°C, 1,2-dipalmitoyl-, 1,2-dioleoyl-, 1-palmitoyl-2-linoleoyl- and 1-palmitoyl-2-arachidonoyl-PC revealed halftime values for the rate of their transbilayer equilibration of 26.3 ± 4.4, 14.4 ± 3.5, 2.9 ± 1.7 and 9.7 ± 1.6 h, respectively.

The two pools of phosphatidylcholine (PC) which are located in the outer and inner leaflets of the human red cell membrane, are identical in their molecular species composition [1]. The occurrence of transbilayer movements of PC molecules in the red cell membrane has been demonstrated, and a previous report [2] suggested that the rate at which this process proceeds, is positively related with the unsaturation of the constituting fatty acids. A recently developed technique [2] allows a direct determination of the actual 'flip-flop' rates of individual molecular species of PC in the red cell membrane. The results presented here, indeed show that the transbilayer movement of unsaturated species is faster than that of fully saturated PC, but there appears to be no direct proportionality between the rate of this process and the total degree of unsaturation of the molecule, indicating that additional factors play an important role.

The occurrence of transbilayer movements of PC molecules in the red cell membrane is thought to be responsible for maintaining identical molecular species compositions at both halves of the bilayer. This transbilayer movement is essential, because the topological asymmetry of the two PC-renewal pathways, viz. the exchange of intact PC molecules [3,4] between plasma lipoproteins and the outer monolayer, and the reacylation of lysoPC [4,5] taking place in the inner leaflet of the membrane, may generate differences in the fatty acid composition of both PC pools [6]. Obviously, such differences do not exist [1]. Although earlier experiments led us to conclude that, specifically in the human red cell membrane, PC flip-flop is virtually absent [3,7,8], more recent studies clearly demonstrated its existence. We have recently developed a procedure which enables a direct determination of the rates of PC flip-flop in the red cell membrane [2]. Briefly, a small fraction of the

endogenous PC in the outer half of the membrane is replaced by [*N-methyl*-^{14}C]PC, by incubating the intact cells in the presence of bovine liver PC-specific phospholipid transfer protein, and a suitable donor system containing the radiolabeled PC. The [^{14}C]PC introduced into the outer monolayer this way, is allowed to equilibrate with the inner membrane layer by subsequently incubating the cells at 37°C in the absence of the PC specific transfer protein and the [^{14}C]PC donor system. At various time intervals, samples are taken, and the intact cells treated with phospholipase A_2 and sphingomyelinase C, thereby converting all of the PC in the outer monolayer into lysoPC [9]. From the time-dependent decrease in specific radioactivity of this lysoPC, which is representative of the PC in the outer monolayer, the rate of transbilayer movement of the PC can be easily calculated [2].

Halftime values of 8–20 h have been determined this way for the flip-flop of PC in the normal human erythrocyte [2,10,11]. It should be realized, however, that these studies involved [^{14}C]PC donor systems composed of heterogeneous mixtures of various molecular species of PC, so that the recorded halftime values represent the means of the values for each individual species. Using such heterogeneous mixtures of PC from rat liver microsomes, hen eggs or soy beans, and comparing the results with those obtained with pure dipalmitoyl-PC, it was concluded that the average halftime of PC flip-flop in the human red cell membrane was related to the degree of saturation of the PC in those mixtures [2]. A similar conclusion has been drawn for the flip-flop of PC molecules in the rat erythrocyte membrane [7].

This aspect can be studied in greater detail as the PC specific transfer protein offers the unique possibility of replacing endogenous PC molecules in the outer membrane layer of the intact cell, by any particular well defined (radiolabeled) species. When performing such experiments, two very essential prerequisites should be fulfilled. First, any appreciable disturbance in the native fatty acid composition of the red cell PC should be avoided, since such changes may drastically affect a variety of cell parameters [12–14]. A non-modifying condition can be satisfactorily created when a small amount of the highly radiolabeled, well defined PC species is incorporated in donor vesicles of which the bulk is composed of egg PC [2]. Secondly, to follow the redistribution of the labeled PC over both halves of the bilayer, it is essential to completely convert the PC in the outer layer into lyso PC, which can only be achieved by using the phospholipase A_2 in combination with sphingomyelinase C [9]. When using phospholipase A_2 alone, the PC in the outer monolayer is not degraded to completion. More importantly, however, the residual PC that remains in the outer leaflet when only *Naja naja* phospholipase A_2 is used and which may account for some 10% of the outer PC pool [9,15,16], is considerably enriched in disaturated species [17]. This makes the lyso PC thus produced not truely representative of the PC present in the outer leaflet and will particularly introduce tremendous errors in experiments involving dipalmitoyl-PC. It has been mentioned before [15] that, once the maximum level of PC hydrolysis by treatment of intact cells with phospholipase A_2 (either alone or in combination with sphingomyelinase C) has been reached, no further degradation will take place during prolonged incubations. This obviously indicates that, at least in the normal erythrocyte, the lyso PC produced in the outer monolayer this way does not equilibrate with the residual PC in the inner leaflet. Furthermore, it is essential to note that under the conditions of the present experiments the greater part, if not all, of the unsaturated PC species will have been degraded in considerably less than 1 h after the addition of the phospholipase A_2 [16,17]. This is important in view of the relatively short halftimes of transbilayer equilibration of the unsaturated species (Table I). A considerable part of the dipalmitoyl-PC, on the other hand, will not be degraded before the subsequent addition of sphingomyelinase C [17]. This delay in its hydrolysis, however, will not seriously affect the experimental data obtained for this species which experiences a very slow transbilayer movement (Table I). Indeed, it has been observed that immediately after the exchange procedure and subsequent washings, the combined action of phospholipase A_2 and sphingomyelinase C degraded 95–97% of the newly introduced PC (either unsaturated or saturated) demonstrating that essentially all of this PC was still present in the outer membrane leaflet [2,17].

In agreement with earlier studies [2], un-

TABLE I

FLIP-FLOP OF INDIVIDUAL SPECIES OF PHOSPHATIDYLCHOLINE IN THE HUMAN ERYTHROCYTE MEMBRANE

Fresh human erythrocytes were obtained from healthy volunteers, washed twice with 150 mM NaCl, and once with a buffer containing 90 mM KCl, 45 mM NaCl, 44 mM sucrose, 10 mM glucose, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 mM Tris-HCl (pH 7.4) referred to as 'buffer' throughout. 1-Palmitoyl-2-linoleoyl-PC and 1-palmitoyl-2-arachidonoyl-PC (both from Avanti Polar Lipids Inc., Birmingham, AL, U.S.A.), were converted into their (*N-methyl*-^{14}C)-labeled derivatives by the method of Stoffel [18], using [^{14}C]methyliodide (The Radiochemical Centre, Amersham, U.K.). 1,2-[1-^{14}C]Dioleoyl-PC and 1,2-dipalmitoylphosphatidyl[*N-methyl*-^{14}C]-choline, were from The Radiochemical Centre (Amersham, U.K.). Donor vesicles, containing trace amounts of one of the ^{14}C-labeled PC species, were prepared in buffer by sonication of equimolar amounts of egg PC (Sigma, St. Louis, MO, U.S.A.), and cholesterol (Merck, Darmstadt, F.R.G.) plus 6% egg phosphatidate (Sigma, St. Louis, MO, U.S.A.), and trace amounts of glyceroltri[^{3}H]oleate (The Radiochemical Centre, Amersham, U.K.; 0.5 mCi/mmol vesicle PC) as non-exchangeable marker, and the [^{14}C]PC species to be studied (0.1 mCi/mmol vesicle PC), essentially following the previously published procedure [13]. The [^{14}C]PC species were introduced into intact erythrocytes by incubating (1 h, 37°C, gentle shaking) a 33% suspension of the cells in the presence of PC specific transfer protein (1 nmol/100 μl packed cells), purified and prepared for use according to standard procedures [10,19], and the [^{14}C]PC donor vesicles (the ratio vesicle PC to erythrocyte PC varied from 1 to 5). Termination of this exchange reaction, subsequent incubation of the cells at 37°C (upto 8 h) to allow the [^{14}C]PC to equilibrate over both halves of the bilayer, treatment of samples at various time intervals (0, 1, 2, 4, 6 and 8 h) with *Naja naja* phospholipase A_2 (Sigma, St. Louis, MO, U.S.A., 30 IU/250 μl packed cells) and *Staphylococcus aureus* sphingomyelinase C (purified according to Ref. 9, 10 IU/250 μl packed cells), extraction and analysis of lipids, determination of specific radioactivity of the lyso PC in the samples, correction of the analytical data for contamination of red cells with [^{14}C]PC containing donor vesicles (never exceeding 2% of total erythrocyte phospholipid), and calculation of halftime values of PC flip-flop, were performed exactly following the procedures already published in detail elsewhere [2].

PC species	Halftime (h)
1,2-Dipalmitoyl	26.3 ± 4.4 [a]
1,2-Dioleoyl	14.4 ± 3.5 [b]
1-Palmitoyl-2-linoleoyl	2.9 ± 1.7 [b]
1-Palmitoyl-2-arachidonoyl	9.7 ± 1.6 [c]

[a] Mean ± S.D. value derived from the best linear fit calculated for a semi-logarithmic plot (compare Fig. 1) of duplicate determinations of the relative specific radioactivities of the PC in the outer monolayer at the time points indicated above.

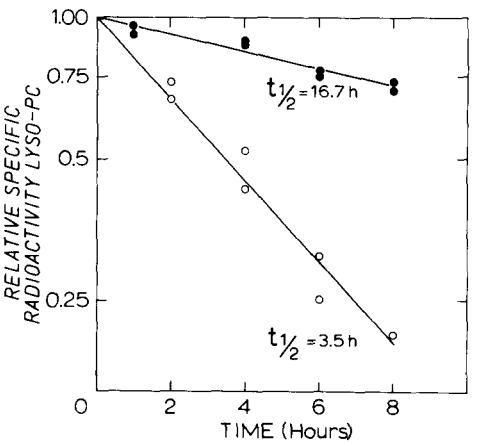

Fig. 1. Transbilayer movement of 1,2-dioleoyl-PC and 1-palmitoyl-2-linoleoyl-PC in the human erythrocyte. Transbilayer movements were determined by following the transbilayer equilibration of the ^{14}C-labeled PC species previously introduced into the outer membrane leaflet (see legend to Table I). The redistribution of radiolabeled PC was followed by measuring the specific radioactivity of the PC in the outer layer, modified to lyso PC by phospholipase treatment (see legend to Table I). The relative specific radioactivities of the (lyso)PC's [10], derived from a typical experiment, are plotted semilogarithmically versus the time of incubation for 1,2-dioleoyl-PC (●) and 1-palmitoyl-2-linoleoyl-PC (○), respectively. Halftime values for transbilayer movement ($t_{\frac{1}{2}}$) can be calculated from the slopes of these lines.

saturated PC species experience transbilayer movements in the human red cell membrane which are considerably faster than that of a fully saturated one, such as 1,2-dipalmitoyl-PC (Table I). On the other hand, and in contrast to earlier suggestions [2], there appears to exist no direct proportionality between the rate of flip-flop of a particular PC species and its total degree of unsaturation. 1-Palmitoyl-2-linoleoyl-PC experiences a transbilayer movement which is about four to five times as fast as that of 1,2-dioleoyl-PC (Table I; Fig. 1), although these two species have the same degree of unsaturation. This indicates that not only the total number of double bonds in a PC molecule, but more specifically their distribution over the two acyl chains, is an important determinant of its transbilayer mobility. Possibly even more surpris-

[b,c] Mean ± S.D. values calculated from the mean halftime values derived from three (b) and two (c) independent experiments, determined as described under [a].

ing, is the finding that the flip-flop rate of the higher unsaturated 1-palmitoyl-2-arachidonoyl-PC is appreciably slower than that of the 1-palmitoyl-2-linoleoyl species (Table I).

At present, one may only speculate whether these differences are a consequence of differences in the molecular geometry of the various PC species which, in turn, may influence their packing in the bilayer as well as their interactions with adjacent membrane constituents. Alternatively, they may represent a reflection of a possible heterogeneity in their lateral distribution in the membrane, i.e., in different domains in which the 'facilities' for transbilayer movements may differ from one another. Further studies will be necessary to gain insight into the factors which govern the transbilayer mobility of the PC molecules in the erythrocyte membrane, and, more specifically, that of the individual molecular species.

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