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Absence of transbilayer diffusion of spin-labeled sphingomyelin on human erythrocytes. Comparison with the diffusion of several spin-labeled glycerophospholipids

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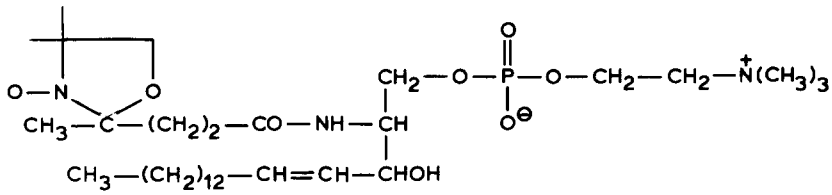
We have measured the transbilayer diffusion at 4°C of spin labeled analogs of sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidic acid in the human erythrocyte membrane. Measurements were also carried out in ghosts, released without ATP, and on large unilamellar vesicles made with total lipid extract. As reported previously (Seigneuret, M. and Devaux, P.F. (1984) Proc. Natl. Acad. Sci. USA 81, 3751–3755), the amino phospholipids are rapidely transported from the outer to the inner leaflet on fresh erythrocytes, whereas phosphatidylcholine diffuses slowly. We now show that phosphatidic acid behaves like phosphatidylcholine: approximately 10% is internalized in 5 h at 4°C. Under the same experimental conditions, no inward transport of sphingomyelin can be detected. In ghosts resealed without ATP, all glycerophospholipids tested diffuse slowly from the outer to the inner leaflet (approx. 10% in 5 h) while no transport of sphingomyelin is seen. Finally in lipid vesicles, the inward diffusion of all glycerophospholipids is less than 2% in 5 h and a very small transport of sphingomyelin can be measured. These results confirm the existence of a selective inward aminophospholipid transport on fresh erythrocytes and suggest a slow and passive diffusion of all phospholipids on ghosts, resealed without ATP, as well as on lipid vesicles.

We have recently shown that spin-labeled phospholipids with a short β-chain (C5) incorporate readily in the outer layer of cell membranes when added to a membrane suspension [1,2]. The orientation of the spin labels can be assessed by addition to the medium of ascorbate at 4°C, the nitroxide being reduced by ascorbate only if the phospholipid is on the outer layer. By this technique we have studied the translocation of spin-labeled analogs of phosphadidylcholine (PC), phosphatidyl serine (PS) and phosphatidylethanolamine (PE) in human erythrocyte membranes. It was established that on fresh erythrocytes the phosphatidylcholine label remains mainly in the outer leaflet while phosphatidylserine and phosphatidylethanolamine undergo rapid transverse diffusion that leads to their asymmetric distribution in favor of the inner leaflet. This latter effect is reversibly inhibited after ATP depletion of the erythrocytes and can be reproduced on resealed erythrocyte ghosts only if hydrolyzable Mg-ATP is included in the internal medium [1]. In the present study, we report further investigations using the same spin labels plus a spin-labeled analog of sphingomyelin and a spin-labeled analog of phosphatidic acid. Using the ascorbate assay, we have measured the kinetics of transbilayer diffusion at 4°C of the five different spin labels on:



Structure I.

(1) fresh erythrocytes, (ii) ghosts without ATP and (iii) large unilamellar vesicles prepared from total lipid extract.

A paramagnetic sphingomyelin (referred to as (0,2)SM) (Structure I) was synthesized by acylation of sphingosylphosphorylcholine with the *N*-hydroxysuccimide ester of 4-doxyl pentanoate, the procedure being adapted from Ref. 3.

Tetrahydrofuran and triethylamine were distilled from lithium aluminohydride; other products were dried under vacuum over phosphorus pentoxide before use.

To a solution of 38 μmol of 4-doxyl pentanoic acid in 100 μl of tetrahydrofuran were added 4.37 mg (1 equivalent) of *n*-hydroxysuccimide in 50 μl of tetrahydrofuran and 7.5 mg (1.1 equivalent) of dicyclohexylcarbodiimide in 50 μl of tetrahydrofuran. The mixture was stirred for four hours and, after addition of 0.1 equivalent of acetic acid, filtered over a sintered glass; the solvent was removed and the residue dried under vacuum over phosphorus pentoxide.

Crude product, dissolved in 1 ml of tetrahydrofuran, was mixed with 25 mg of sphingosylphosphorylcholine (supplied by Sigma) and 5.3 μl of triethylamine. The reaction, monitored by thin-layer chromatography (TLC) was quenched at 16 h by adding water. After evaporation to dryness, the residue was deposited on a column of CM-52 carbomethylcellulose [4]. Sphingomyelin was eluted with chloroform/methanol (95 : 5, v/v). It exhibited a single spot in TLC on silica gel. R_F = 0.21 with chloroform/methanol/water (65 : 25 : 4, v/v), giving a positive test with Dragendorf (for choline head) and molybdic (for phosphate group) reagents. The overall yield in pure (0.2)SM was 40% as determined by ESR spectroscopy.

Phosphatidylcholine analog (0,2)PC was synthesized as described in Ref. 5. Phosphatidylserine (0,2)PS, phosphatidylethanolamine (0,2)PE and phosphatidic acid (0,2)PA analogs were prepared enzymatically from (0,2)PC as in Ref. 4.

Freshly drawn human blood was obtained through the Centre National de Transfusion Sanguine (Paris). Before use, erythrocytes were washed carefully in 145 mM NaCl/5 mM KCl/1 mM $MgSO_4$/10 mM glucose/20 mM Hepes buffer (pH 7.4). Pink resealed ghosts were prepared according to Schwoch and Passow [6]. Erythrocyte lipids were extracted by the method of Rose and Oklander [7] and large unilamellar vesicles were formed by the reverse micelle evaporation technique of Szoka and Papahadjopoulos [8]. The average diameter of the vesicles was measured by freeze-fracture electron microscopy and found to approximate 0.2 to 0.3 μm. Spin labeling and electron spin resonance (ESR) experiments were performed as in Ref. 1. The amount of paramagnetic analog present on the inner half of the membrane was determined as the residual ESR signal after extracellular addition at 4°C of 10 mM sodium ascorbate. The kinetics of reduction of ascorbate were of the order of 5 min at 4°C, i.e. much faster than the kinetics of phospholipid transverse diffusion.

Experiments displayed in Figs. 1–3 were performed at least in triplicate on different blood samples (irrespective of the blood group). Each figure represents a typical set of experiments. Error bars due to the ESR signal-to-noise ratio (not shown) would not exceed the size of the symbol used.

The results obtained in whole cells are displayed in Fig. 1. The relatively rapid diffusion of (0,2)PS and (0,2)PE as well as the very slow diffusion of (0,2)PC have been reported previously [1]. For the aminophospholipids $t_{1/2}$ values are, respectively, 1.5 and 4 h at 4°C. This parameter cannot be determined in a reliable way for (0,2)PC

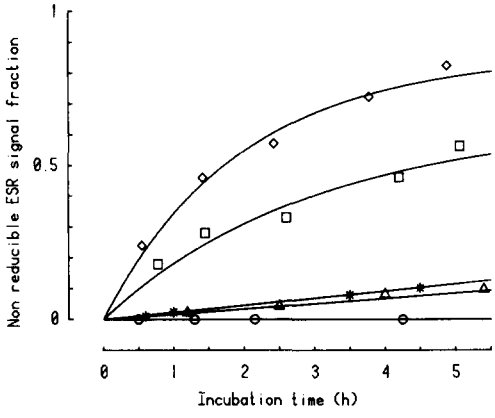


Fig. 1. Non reducible spin-label fraction as a function of incubation time at 4°C for (0,2)PS (◇), (0,2)PE (□), (0,2)PC (△), (0,2)PA (*) and (0,2)SM (○) after addition to intact erythrocytes. The values for data points were obtained after ascorbate reduction at different incubation time as described in Ref. 1. Curves associated with (0,2)PS and (0,2)PE were obtained by fitting the data points with a single exponential law.

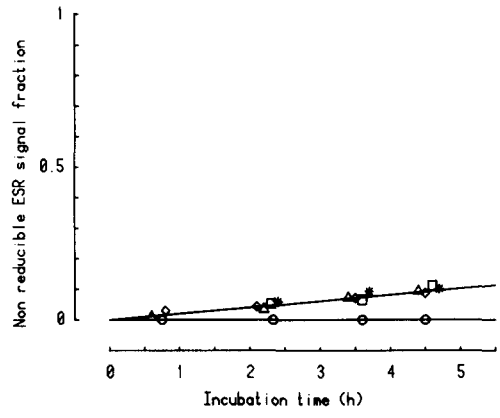


Fig. 2. Non reducible spin-label fraction as a function of incubation time for (0,2)PS (◇), (0,2)PE (□), (0,2)PC (△), (0,2)PA (*) and (0,2)SM (○) after addition to red cell ghosts resealed in the absence of ATP.

with only 5 h incubation at this temperature, indicating a much slower diffusion rate. However a significant uptake of (0,2)PC is measurable (approx. 10% in 5 h). A very similar result is obtained for (0,2)PA. This latter finding proves that the transverse segregation between (0,2)PS and (0,2)PC is not based upon the difference in charge of the two phospholipids, since (0,2)PA is negatively charged and yet behaves just like the zwitterionic phospholipid (0,2)PC. Interestingly enough, (0,2)SM behaves differently from (0,2)PC: absolutely no diffusion of the former lipid can be detected in the course of 5 h incubation at 4°C. The absence of transverse diffusion of the sphingomyelin derivative is consistent with the observation of Boegheim et al. [9]. These authors showed that the fatty acid composition of the three endogeneous glycerophospholipids in erythrocytes is randomized over the two membrane halves, while sphingomyelin located on the outer leaflet is chemically different from that found in the inner leaflet. This was interpreted as a strong indication of the high stability of the transverse distribution of sphingomyelin.

The differences in behaviour of the five phospholipids are partly abolished when the diffusion is studied on ghosts resealed in the absence of ATP (Fig. 2) or on large unilamellar vesicles (Fig 3). Large unilamellar vesicles are used rather than sonicated vesicles to avoid lipid asymmetry induced by curvature effects [10]. In the two latter systems, the four glycerophospholipids have comparable diffusion rates while (0.2)SM shows essentially no inward diffusion. On resealed ghosts the level of non reducible spin label reached after 5 h incubation at 4°C is approx. 10%, irrespective of the head group of the glycerophospholipid. The similarity of the transverse diffusion rates of the four glycerophospholipids on ghosts resealed without ATP suggests a common mechanism of passive diffusion. This passive diffusion is probably also responsible for the transport of phos-

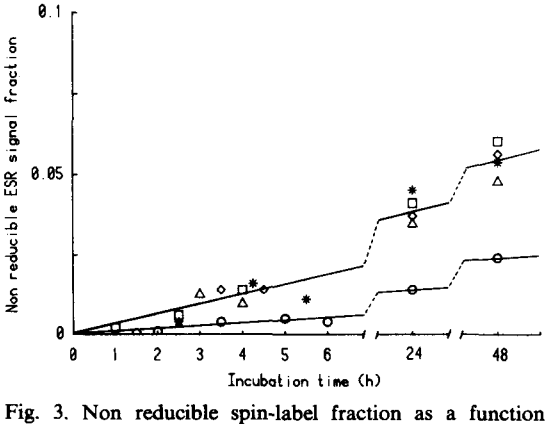


Fig. 3. Non reducible spin-label fraction as a function of incubation time at 4°C for (0,2)PC (△), (0,2)PS (◇), (0,2)PE (□), (0,2)PA (*) and (0,2)SM (○) after addition to large unilamellar liposomes prepared from erythrocyte extracted lipids.

phatidylcholine and phosphatidic acid on fresh erythrocytes. On the other hand a very specific mechanism of transport must be postulated for the aminophospholipids on erythrocytes.

Comparison of Figs. 2 and 3 reveals that the transverse diffusion on ghosts depleated of ATP is still much faster than that observed on pure lipid vesicles (approximately five times faster). On the later system (note the difference in scale between Figs. 2 and 3) less than 2% of the glycerophospholipids have traversed the bilayer in 5 h. The acceleration of phospholipid diffusion by the presence of proteins from the erythrocyte was already noted by De Kruijff et al. [11]. This mechanism seems to be non specific and should not be confused with the ATP-dependent specific transport of aminophospholipids on erythrocytes or on ghosts resealed in the presence of ATP [1].

Finally, we would like to mention that the remarkable stability of sphingomyelin in erythrocytes as well as on ghosts is slightly reduced in pure lipid vesicles (Fig. 3). Indeed, we have found in a reproducible manner that a measurable amount of (0,2)SM becomes unaccessible to ascorbate after a few hours at 4°C. This amount is quite sizeable after 24 or 48 h (see Fig. 3). It is quite difficult to speculate about the origin of the stability of sphingomyelin on erythrocytes or on ghosts. However, the involvement of a specific protein is unlikely for the following reasons.

(i) ESR spectra of the (0,2)SM and of (0,2)PC on erythrocyte membranes have been carefully compared and found quasi identical (not shown). Thus, no evidence of protein-lipid interactions can be inferred from the ESR line shapes.

(ii) Large size unilamellar vesicles made with the total lipid extract from erythrocytes have a priori an isotropic distribution of lipids between both leaflets. Thus sphingomyelin-sphingomyelin interactions which may be important on erythrocytes (12) could be lost or diminished on lipid vesicles. This may explain the difference in stability of sphingomyelin on the latter system.

A basic assumption of this work is that spin-labeled lipids having a short β chain reflect to some extent the behaviour of natural lipids. This is sustained by the strong tendency of aminophospholipid analogues to choose the inside lipid layer of erythrocyte as endogeneous aminophospholipids do. The marked difference between the stability of the sphingomyelin analogue and the phosphatidylcholine analogue is also in agreement with the known stability of natural sphingomyelin on the outer layer of erythocytes. On the other hand the rates of spontaneous diffusion as well as ATP dependent uptake measured with these spin-labeled lipids are likely to be slightly different from the values one would measure (if possible) with natural lipids. This does not affect our conclusions as long as we focus our attention on the equilibrium situations and not on the exact time scales of the implied phenomenon.

In a previous publication [1] we have suggested that aminophospholipids were selectively transported on the human erythrocyte from the outer to the inner leaflet by an ATP-driven protein carrier. We have shown already that in ghosts this selective transport takes place only if ghosts are resealed with ATP inside. The fact that the phosphatidic acid derivative and the sphingomyelin derivative behave largely like phosphatidylcholine confirms the selectivity of the putative carrier for aminophospholipids. In the present report we show that the inward diffusion of the sphingomyelin analog is even slower than that of the phosphatidylcholine analog. Thus if one assumes that the spin-labeled lipids reproduce at least the relative rates of diffusion of the endogeneous phospholipids, our results allow us to explain completely the known asymmetry of phospholipids in erythrocytes [10,13]. Two processes would be involved.

(i) Selective inward transport of aminolipids by an ATP-driven protein.

(ii) Passive diffusion of phosphatidylcholine and sphingomyelin counterbalancing the uptake of aminolipids, yet favoring the maintenance of sphingomyelin over that of phosphatidylcholine in the outer leaflet.

Note that our model differs from that of Haest and collaborators [14,15], involving an hypothetical 'reorientation' site, essentially by the requirement of ATP which we have demonstrated necessary for the selective transport of aminolipids [1].

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