Aminophospholipid Translocase of Human Erythrocytes: Phospholipid Substrate Specificity and Effect of Cholesterol†

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ABSTRACT: The outside–inside translocation rate and transmembrane equilibrium distribution, at 37 °C, of 16 different amphiphilic spin-labeled phospholipids have been determined in human erythrocytes. The transmembrane distribution was assessed by bovine serum albumin extraction of the spin-labels present in the outer monolayer. Within 15 min, more than 90% of the phosphatidylserine analogue was found in the inner monolayer; the equilibrium distribution of phosphatidylethanolamine spin-label was approximately 85–90% inside, with a half-time for translocation of ~50 min. In contrast, phosphatidylcholine reached a distribution corresponding to ~30% of the labels inside with a half-time of approximately 8 h, and only traces of sphingomyelin were found in the inner monolayer after 16 h. Thus, the spin-label analogues distributed themselves like endogenous phospholipids in red cells with a spontaneous segregation between the amino lipids and the choline-containing phospholipids. Progressive methylation of the amine group of phosphatidylethanolamine resulted in a stepwise decrease of the specific transport; modification of the β-carbon of the serine also decreased the efficiency of the rapid translocation without abolishing it. Phosphatidylpropanolamine was not transported. Substitution of the glyceride group by a ceramide abolished the rapid outside–inside translocation even with a molecule bearing a serine head group. Also it was found that esterification of the *sn*-2 position of the glycerol component was necessary for a rapid translocation since lysophosphatidylserine was only slowly transported from outside to inside. In contrast, the chain length did not seem to be important: a phosphatidylserine with an acetyl group on the *sn*-2 position was transported almost as well as a long-chain phosphatidylserine. Phospholipid outside–inside translocation in red cells was also measured after incubation of the membranes with *N*-ethylmaleimide. The decrease of the initial rate of PS translocation, which followed SH oxidation, was used as an indication of a protein involvement in the translocation process. Strong variations were observed only for the aminophospholipids. Finally, the aminophospholipid translocase activity could be modulated by modification of the cholesterol to phospholipid ratio. However, the transmembrane equilibrium distribution of spin-labeled aminophospholipids was not significantly affected by a 30% increase or decrease of the cholesterol content.

The selective translocation of aminophospholipids (phosphatidylserine and phosphatidylethanolamine) from the outer to the inner monolayer of human erythrocytes has been demonstrated by various techniques (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Tilley et al., 1986; Connor & Schroit, 1987). All investigators have found a Mg^{2+}-ATP requirement and an inhibition of the translocation by protein reagents such as *N*-ethylmaleimide, and by selective ions such as intracellular Ca^{2+} or vanadate. Using spin-labeled lipids with a short (C5) β chain bearing a nitroxide radical, we have shown previously by ESR[1] studies the differences between outside–inside translocation rates at low temperature of phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, phosphatidic acid, and sphingomyelin (Seigneuret & Devaux, 1984; Zachowski et al., 1985, 1986). We have also systematically investigated the role of intra- and extracellular ions on the translocation of aminophospholipid analogues (Bitbol et al., 1987). The phospholipid analogues used for these studies are slightly water soluble and therefore can be incorporated in a cell membrane very rapidly without requiring the help of a phospholipid exchange protein. Their transmembrane orientation is determined by the selective chemical reduction of the nitroxide radical. For example, the labels in the outer monolayer can be reduced by extracellular ascorbate added at 4 °C (Kornberg & Mc Connell, 1971; Seigneuret & Devaux, 1984). Alternatively, one can rely on the spontaneous reduction by red cell cytosol at 37 °C (Seigneuret et al., 1984). More recently, we have introduced a slightly different technique adapted from the back-exchange technique utilized by Sleight and Pagano (1984) with fluorescent lipids or by Bergmann et al. (1984) with radioactive lyso derivatives. After a period of incubation with the cells, the amphiphilic spin-labeled lipids present in the outer monolayer are extracted with defatted bovine serum albumin (BSA), and the fraction of extracted lipids is measured by ESR, after reoxidation by ferricyanide (Calvez et al., 1988; Bitbol & Devaux, 1988). The reoxidation step is important for some of the spin-labels, if the incubation is performed at high temperature, because of possible nitroxide reduction by the cytosol.

In the present study, we used a series of new spin-labeled analogues that readily transfer into membranes and whose transmembrane orientation can be assessed by the back-exchange technique. Our goal was to investigate the degree of modification tolerable on an aminophospholipid for it to be recognized and translocated by the specific carrier protein present in red blood cells. Finally, the influence of cholesterol

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[1] Abbreviations: LCAT, lecithin cholesterol acyltransferase; RBC, red blood cell; NEM, *N*-ethylmaleimide; BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate; ESR, electron spin resonance; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

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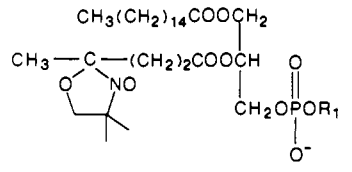
Chart I



PC (I), $R_1 = (CH_2)_2N^+(CH_3)_3$

PS (II), $R_1 = CH_2CHN^+H_3$ (L isomer), COO^-

PE (III), $R_1 = (CH_2)_2N^+H_3$

PA (IV), $R_1 = H$

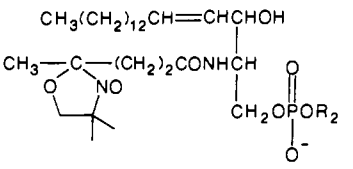
PME (V), $R_1 = (CH_2)_2N^+H_2CH_3$

PDE (VI), $R_1 = (CH_2)_2N^+H(CH_3)_2$

PS-MeE (VII), $R_1 = CH_2CHN^+H_3$ (L isomer), $COOCH_3$

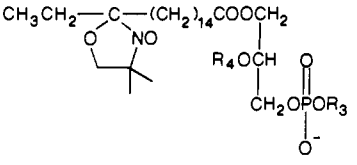
PE-βMe(L) (VIII-L), $R_1 = CH_2CHN^+H_3$ and VIII-D, CH_3

PP (IX), $R_1 = (CH_2)_3N^+H_3$



SpC (X), $R_2 = (CH_2)_2N^+(CH_3)_3$

SpS (XI), $R_2 = CH_2CHN^+H_3$ (L isomer), COO^-



lyso-PC (XII), $R_3 = (CH_2)_2N^+(CH_3)_3$; $R_4 = H$

lyso-PS (XIII), $R_3 = CH_2CHN^+H_3$; $R_4 = H$, COO^-

Ac-PC (XIV), $R_3 = (CH_2)_2N^+(CH_3)_3$; $R_4 = COCH_3$

Ac-PS (XV), $R_3 = CH_2CHN^+H_3$; $R_4 = COCH_3$, COO^-

on the activity of the aminophospholipid translocase was also examined.

A requisite to these investigations is that spin-labels can provide significant information on the behavior of endogenous lipids. This important point has been discussed in our previous studies and is further debated in the present article. The strongest argument in favor of the use of these spin-labeled analogues is that their spontaneous transmembrane distribution at equilibrium is identical with that of endogenous lipids.

Materials and Methods

Spin-Labels. The spin-labeled lipids used are shown in Chart I.

The detailed procedures used in synthesizing these spin-labeled lipids will be presented elsewhere (Fellmann and Hervé, to be published). Here, we give the general protocols. The spin-labeled phosphatidylcholine analogue (molecule I) was synthesized by acylation of egg yolk lysophosphatidylcholine, with 4-doxylpentanoic acid following the one-pot protocol of Samuel et al. (1985), based on catalysis with (dimethylamino)pyridine. Molecules II–IX were synthesized from I according to Comfurius and Zwaal (1977) by phospholipase D catalyzed head-group exchange. The alcohols were L-serine (II), ethanolamine (III), none (IV), methylethanolamine (V), dimethylethanolamine (VI), L-serine methyl ester (VII), L-alaninol (VIII-L) or D-alaninol (VIII-D), and 3-amino-1-propanol (IX). The synthesis of molecule X was described in Zachowski et al. (1985). Molecule XI was derived from X by phospholipase D catalyzed head-group exchange. Molecules XII–XV were synthesized from the biradical bis-(16-doxylstearoyl)phosphatidylcholine, which was synthesized from glycerophosphorylcholine cadmium salt and 16-doxylstearic acid, following Samuel's procedure (Samuel et al., 1985). The biradical was hydrolyzed into lysophosphatidylcholine by phospholipase A_2 reaction to yield molecule XII. To obtain molecule XIII, the biradical PC was first converted into the corresponding biradical PS by phospholipase D catalyzed head-group exchange, and then the chain 2 was cleaved by hydrolysis with phospholipase A_2. Molecule XII was acetylated to yield molecule XIV, according to Samuel et al. (1985). Molecule XV was obtained by head-group exchange from molecule XIV.

Membrane Labeling. Fresh human blood was obtained from healthy volunteers or from a local blood bank (Centre National de Transfusion Sanguine). Blood collected on EDTA was stored at 4 °C and used within 5 days. Erythrocytes were obtained by four washes of blood (1000*g* for 5 min) in buffer A (20 mM Hepes, pH 7.5, 5 mM Na_2HPO_4, 145 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM inosine, 1 mM $MgSO_4$, 0.1 mM EGTA). Prior to labeling, erythrocytes were incubated with 5 mM diisopropyl fluorophosphate (DFP) to minimize the hydrolysis of spin-labeled phospholipids (Wykle et al., 1980). To label the membranes, suitable amounts of spin-labels in chloroform/methanol (1:1 v/v), corresponding to approximately 1% of total phospholipids, were dried under vacuum and resuspended by vigorous vortexing in buffer A. Two volumes of the aqueous dispersions of labels preincubated at 37 °C was then added to one volume of preheated red cells. The incorporation in red cell membranes was completed within 2 min.

Cholesterol Enrichment. The technique was based on the exchange of cholesterol between liposome and RBC (Cooper et al., 1975). Fifty milligrams of egg lecithin was mixed in chloroform/methanol (1:1 v/v) with either 20 mg (control) or 100 mg of cholesterol (cholesterol enrichment). Solvent was removed under vacuum, and lipids were resuspended in 20 mL of 20 mM Hepes buffer, pH 7.5. The suspension was then submitted to sonication (Ultra Son, Annemasse) under nitrogen flow, twice for 20 min at 0 °C. Titanium particles and large-size vesicles were removed by centrifugation at 100000*g* for 1 h. Vesicles in the supernatant exhibited a molar ratio cholesterol/phospholipid of 0.8 (control) or 2 (cholesterol enrichment). Five milliliters of pelleted erythrocytes was added to 20 mL of the vesicle suspension, plus 20 mL of buffer B (290 mM NaCl, 10 mM KCl, 10 mM Na_2HPO_4, 20 mM glucose, 20 mM inosine, 2 mM $MgSO_4$, 0.2 mM EGTA, 20 mM Hepes, pH 7.5) and 10 mL of decomplemented plasma. The cells were incubated overnight at 37 °C with gentle agitation. After incubation, cells were centrifuged at 1000*g* for 5 min and washed four times with buffer A.

Cholesterol Depletion. The method for cholesterol depletion was based on cholesterol extraction by plasma lipoproteins as described by Gottlieb (1976). Blood plasma containing 100 μg/mL penicillin and streptomycin was first incubated for 72

h at 37 °C and afterward for 30 min at 56 °C to denaturate LCAT. Five milliliters of packed erythrocytes was then added to 50 mL of plasma and 10 mL of 145 mM NaCl, 5 mM KCl, 5 mM Na_2HPO_4, 50 mM inosine, 50 mM glucose, 1 mM $MgSO_4$, 0.1 mM EGTA, and 50 mM Hepes buffer, pH 7.5. The cells were incubated overnight at 37 °C with gentle agitation and afterward centrifuged at 1000*g* for 5 min and washed four times with buffer A. Control cells (5 mL) were incubated with 10 mL of decomplemented plasma and 40 mL of buffer A.

ESR Determination of the Transmembrane Distribution of Spin-Labeled Lipids. During the incubation at 37 °C, 150-μL aliquots of the suspension were drawn at regular intervals and mixed with 60 μL of a 10% (w/v) fatty acid free bovine serum albumin solution in buffer A. The mixture was incubated 1 min on ice and then centrifuged 1 min at 7600*g* (Calvez et al., 1988). To 90 μL of the supernatant obtained from each aliquot was added 10 μL of 100 mM potassium ferricyanide in order to reoxidize any reduced spin-label, and the ESR spectrum was recorded on a Varian E109 spectrometer. The intensity of the ESR spectrum obtained after BSA extraction of each aliquot gave the fraction of spin-labels on the outer monolayer. The total amount of spin-label per aliquot was obtained from the ESR intensity of the sample drawn immediately after membrane labeling (i.e., without allowing incubation). For long incubation periods (>10 h) in spite of the DFP treatment a fraction of the spin-labeled lipids was hydrolyzed into lyso derivatives and free fatty acids, as revealed by the appearance of three narrow peaks superimposed on the spectrum of the BSA-bound spin-labels for molecules I–XI. Computer subtraction of the narrow component was necessary to obtain a correct estimation of the amplitude of the broad ESR component. All kinetics have been repeated several times. Each curve, however, corresponds to a single representative experiment.

Miscellaneous. Phospholipid concentration was determined by the method of Rouser et al. (1967). Cholesterol concentration was determined by the Merck test, based on the use of cholesterol oxidase and cholesterol esterase. ATP determination was performed by the luciferin–luciferase test (Brown, 1982). For the treatment with *N*-ethylmaleimide, erythrocytes at 4% hematocrit in buffer A were supplemented with various amounts of NEM, incubated at 37 °C for 15 min, and afterward washed twice in buffer A before spin-labeling.

Results

Transmembrane Distribution of Spin-Labeled Analogues of the Major Phospholipids. All the spin-labeled phospholipids used in this paper have a partial water solubility which enables their rapid incorporation in biological membranes without requiring a phospholipid exchange protein. Molecules I–XI give rise in water, at a concentration of ~40 μM, to a typical single-line broad spectrum corresponding to spin-labels in micelles, superimposed to a narrow triplet associated with monomeric spin-labels (Seigneuret et al., 1984). Molecules XII–XV are more soluble than the former ones and at the same concentration give rise only to a narrow triplet (Figure 1a). Upon incubation with red cells, the spectrum changes within a few minutes: the spectrum shown in Figure 1b (full-line curve) is typical of a 16-doxylstearic acid (or phospholipid) in a fluid bilayer. Small modifications of the latter spectrum can take place when the molecule flips from the outer monolayer to the inner monolayer (see below). Finally, after BSA extraction and separation by centrifugation, the spectrum of the supernatant (Figure 1c) is typical of a strongly immobilized probe.

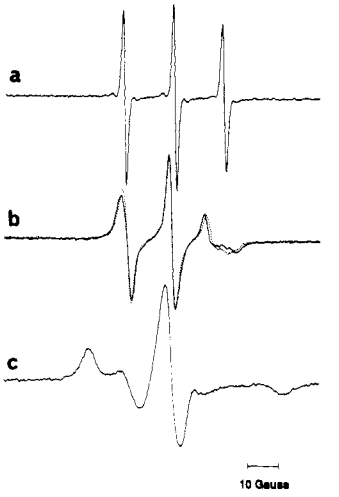


FIGURE 1: Membrane incorporation and reextraction of spin-labeled phospholipids. The top spectrum (a) is the spectrum of lyso-PC (XII) in buffer. The narrow peaks and the absence of broad component show the high solubility of this molecule in water. Spectra b were obtained in the presence of human erythrocytes. The full-line spectrum was recorded at 37 °C immediately after addition of XII to an erythrocyte suspension; the spectrum remained unchanged after 10 h of incubation. The dotted-line spectrum was obtained with lyso-PS (XIII), following 10 h of incubation at 37 °C and BSA extraction of the remaining probes in the outer monolayer. Spectrum c is the spectrum of lyso-PC bound to BSA. Molecules XII–XV give the same spectrum with BSA.

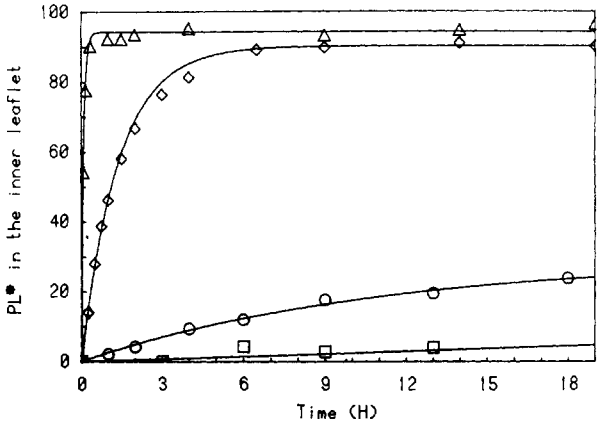


FIGURE 2: Spontaneous passage from the outer monolayer to the inner monolayer of spin-labeled analogues of the major phospholipids in red blood cells. Temperature: 37 °C. PC (○), PS (△), PE (◇), and SpC (□). The curves shown here, as well as in following figures, are fitted to an exponential function by least-squares, nonlinear regression.

Figure 2 shows the percentage of spin-labeled lipids in the inner monolayer as a function of incubation time at 37 °C for the analogues of the four major phospholipids of human erythrocytes. As previously shown at low temperature (Seigneuret & Devaux, 1984; Zachowski et al., 1985), the aminophospholipids, PS and PE, behave very differently from the choline derivatives, PC and SpC. The half-times required to reach equilibrium transmembrane distribution at 37 °C are respectively 5 min, 1 h, 8 h, and ≫10 h. The plateaus correspond respectively to 93% (II) and 84% (III) for the aminophospholipids and ~25% (I) and ≃5% (X) for choline derivatives.

The results of Figure 2 differ slightly at equilibrium from the values obtained at 4 °C as reported in Seigneuret and Devaux (1984). In particular, the PE analogue indicated an apparent equilibrium distribution at 4 °C corresponding to approximately 65% PE inside, instead of 84% in the present experiment, the difference being less important for the PS

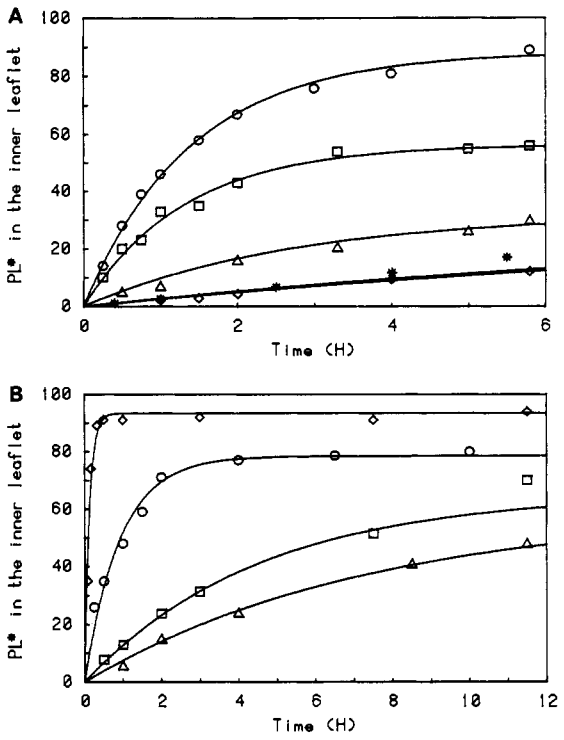


FIGURE 3: Effect of chemical modifications of the head group on the kinetics of transmembrane reorientation at 37 °C. (A) Successive methylations of PE: PE (○), PME (□), PDE (△), PC (◇), and PP (*). (B) Modifications of the carboxyl group of PS: PS (◇), PS-MeE (○), PE-βMe(D) (□), and PE-βMe(L) (△).

derivative. Consequently, we have tested systematically the plateau level obtained with PE as a function of temperature. In reality, the plateau was almost independent of temperature. However, at 5 °C the equilibrium level was clearly visible only after 15 or 20 h (data not shown). The initial velocity of PE reorientation vs temperature followed a single exponential law corresponding to an activation energy of 34 kJ·mol^{-1}, similar to the value reported for PS translocation (Zachowski et al., 1986).

Specificity of the Outside–Inside Aminophospholipid Translocation. Two series of modified aminophospholipids have been tested. In a first series, we have modified only the head group of the spin-labeled PE or PS, maintaining a long α chain and a short β chain bearing the nitroxide. The curves displayed in Figure 3A reveal the influence of successive methylations of the amino group of PE (III, V, VI, and I), as well as the drastic effect of adding one CH_2 between the phosphate and the amino group (IX). Figure 3B shows the influence of the modification of the carboxylic group of PS: while the esterification of the carboxylic group produced only a small decrease in the efficiency of lipid translocation (VII), substitution of the carboxyl by a methyl had a larger effect (VII-D and VIII-L). Note that the D isomer of this latter molecule was transported at a faster rate than the L isomer.

As an extreme case, it was interesting to study the outside–inside passage of spin-labeled PA. Although previous investigations, from our laboratory, have shown that PA flips from the outside monolayer to the inside monolayer very slowly, at 4 °C, with a rate comparable to that of PC (Zachowski et al., 1985), we have found that at 37 °C the initial rate of PA translocation was comparable to that of PE (data not shown). On the other hand, in spite of the DFP present, a large fraction of the probes was very rapidly hydrolyzed, as revealed by the narrow component appearing on the ESR spectrum. This precluded the establishment of any reliable kinetics curve for PA translocation at 37 °C.

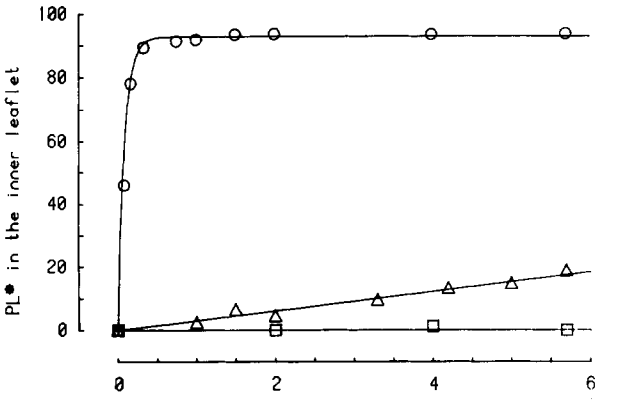


FIGURE 4: Effect of the substitution of the glyceride moiety by a ceramide. SpC (□), SpS (△), and PS (○). Temperature: 37 °C.

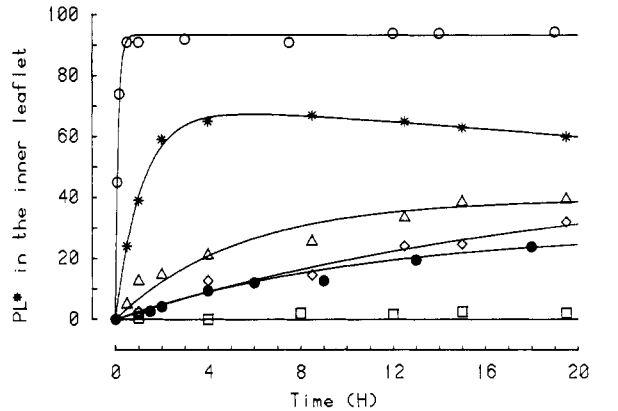


FIGURE 5: Deletion or modification of the β chain. The nitroxide is now at the 16th position of the α chain. Lyso-PC (□), Ac-PC (◇), PC (●), lyso-PS (△), Ac-PS (*), and PS (○). Temperature: 37 °C.

The second series of molecules was designed to test the influence of the glycerol backbone. Figure 4 shows that the serine moiety is not sufficient for the lipid to be recognized by the aminophospholipid translocase. Indeed, SpS, which is derived from sphingomyelin by substitution of the choline moiety by a serine, flips as a phosphatidylcholine molecule, i.e., with a very low rate, yet with a faster rate than sphingomyelin (SpC).

The lyso derivatives XII and XIII have a nitroxide on the fatty acid chain in the *sn*-1 position of the glycerol. As previously reported by Bergmann et al. (1984), the transmembrane diffusion of the lyso derivatives is slow, much slower for lyso-PS than for PS (Figure 5). However, there is a significant difference between XII and XIII, i.e., between lyso-PC and lyso-PS, revealing some catalysis of lyso-PS translocation which does not exist for the lyso-PC. We have also studied the reorientation of the short-chain spin-labeled PE (molecule III) in the presence of various concentrations of unlabeled lyso-PS (up to 4 unlabeled molecules for 1 labeled one). The results (not shown) indicated that the lyso lipid has little or no effect on the reorientation of molecule III and, thus, does not compete effectively with PE at the binding site of the aminophospholipid translocase. Interestingly, the short chain on the *sn*-2 position of the 1-stearoyl-2-acetyl-PS analogue (Ac-PS) is sufficient to restore a rapid translocation (Figure 5). Similarly, 1-stearoyl-2-acetyl-PC (Ac-PC) flips like the phosphatidylcholine with a C5 *sn*-2 chain (PC). Note the decrease of the plateau after ~8 h of incubation of molecule XV (Ac-PS). This is probably due to the hydrolysis of the β chain, in spite of the partial inhibition of the endogenous phospholipase A_2 by DFP.

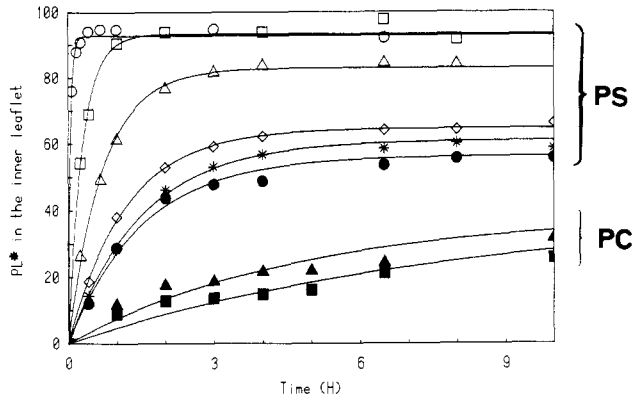


FIGURE 6: Effect of preincubation at 37 °C with NEM. Top set of curves: kinetics of PS passage after incubation with NEM: 0 mM (○); 0.25 mM (□); 0.5 mM (△); 1 mM (◇); 1.5 mM (*); 2 mM (●). Lower set of curves: Kinetics of PC passage without incubation with NEM (■) and after incubation with 1 mM NEM (▲). Temperature of kinetics measurements: 37 °C.

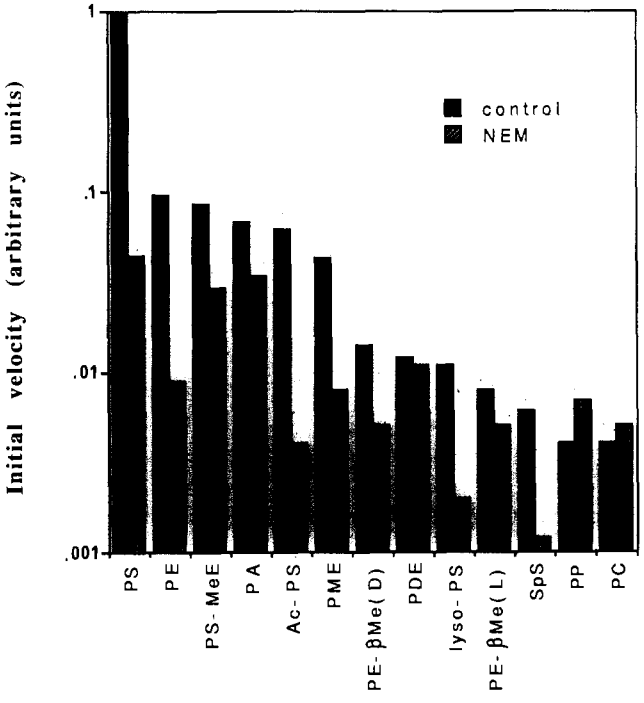


FIGURE 7: Effect of preincubation with NEM (1 mM) on the initial velocity of transmembrane outside–inside passage. The initial velocity of PS at 37 °C in the absence of NEM is 84 ± 15 nmol h^{-1} (mg of membrane protein)$^{-1}$. This value is used as a reference for other molecules.

Influence of N-Ethylmaleimide Treatment. Pretreatment of erythrocytes with increasing NEM concentrations leads to a progressive inhibition of the aminophospholipid translocation as revealed by the decrease of the initial rate of PS reorientation (Daleke & Huestis, 1985; Zachowski et al., 1986). Figure 6 (top) shows that not only is the rate of PS translocation modified but also the level of the plateau. On the other hand (Figure 6, bottom), the rate of PC translocation is not decreased by NEM preincubation, and it even appears to be slightly *increased*. Thus, we have used the influence of NEM on the spin-label outside–inside passage in erythrocytes as a criterion for the aminophospholipid translocase involvement. Figure 7 summarizes the results of a series of investigations: for each spin-labeled molecule, the initial rates of outside–inside transport was measured with or without 1 mM NEM preincubation. The uncertainties are not shown but are discussed later. This figure shows that relatively high initial rates

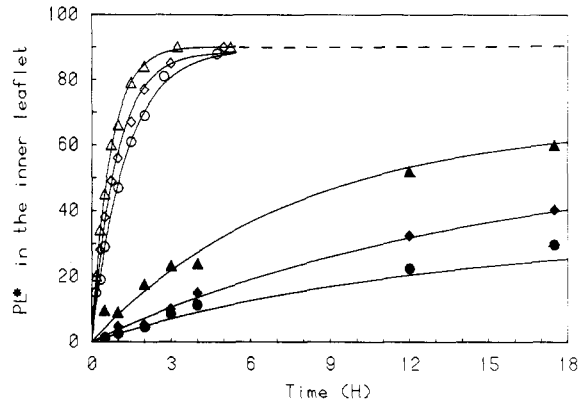


FIGURE 8: Effect of cholesterol enrichment or depletion on the transmembrane reorientation kinetics, at 37 °C, for PE (open symbols) and PC (closed symbols). The molar ratios of cholesterol to phospholipid were 0.92 (○, ●), 0.75 (◇, ♦), and 0.55 (△, ▲).

may not always correspond to a specific translocation via the aminophospholipid translocase.

Difference in Viscosity of the Two Monolayers. Figure 1b shows the difference of viscosity, at 37 °C, of the two monolayers of human erythrocytes as revealed with probes deeply buried within the hydrophobic phase (molecules XII–XV). The two spectra displayed in this figure were obtained with the lyso derivatives; the same spectra can be obtained with the short-chain phospholipids XIV and XV. The full-line spectrum was recorded with the PC derivative (XII). The PS derivative (XIII) gave initially the same spectrum. However, after overnight incubation the spectrum of lyso-PS was significantly modified and indicated more motion. This must be due to the latter molecule flipping slowly toward the inner monolayer. Such an interpretation was confirmed by experiments involving overnight incubation followed by washing with BSA, which selectively removes the probes from the outer monolayer. When such an experiment was carried out with the lyso-PS (XIII), the pelleted membranes gave a spectrum corresponding to the dotted-line spectrum (Figure 1b); when the same experiment was carried out with the lyso-PC (XII), practically all the signal disappeared, even after 10 h of incubation. The spectra, at 37 °C, of the phospholipids labeled in the C5 position of the β chain (I and II) remained very similar before and after incubation (data not shown).

Cholesterol Influence on Spin-Labeled Phospholipid Translocation. The influence of cholesterol to phospholipid ratio (C/P) on the outside–inside passage of spin-labeled phospholipids was investigated in the case of PS (molecule II). PE (molecule III), and PC (molecule I). Cholesterol enrichment or depletion implied long incubation at 37 °C of the red cells; thus in all instances a control sample was used to check the possible influence of this incubation period on the exogenous lipid translocation. Figure 8 shows typical results for PE and PC translocation obtained with cells where the C/P ratio was varied between 0.55 and 0.92 (mol/mol); experiments were carried out with intermediate values which led to intermediate curves (not shown). The data obtained for PS translocation are not shown in this figure. They were qualitatively identical with those obtained for PE; namely, they revealed a small influence of the level of cholesterol on the initial slope but no measurable effect on the plateau. Thus, cholesterol depletion accelerated the phospholipid outside–inside passage while cholesterol enrichment had the opposite effect. However, the plateaus reached by the aminophospholipids were not significantly modified, while the extrapolated plateaus of phosphatidylcholine were possibly in-

fluenced by the cholesterol level.

Discussion

In this article, we utilize amphiphilic spin-labeled lipids to assess the substrate selectivity of the outside–inside transport of aminophospholipids in human red blood cell membranes. Because of their partial water solubility, the spin-labels are readily incorporated in the external leaflet of erythrocytes within a minute of incubation. The transmembrane localization of the probes is later determined by extraction with BSA of the spin-labels remaining in the outer monolayer. This technique is more convenient than the use of ascorbate to selectively reduce the labels in the outer monolayer (Seigneuret & Devaux, 1984), which can only be performed at temperatures close to 0 °C. In contrast, the present technique can be applied to cells incubated at any temperature; spin reduction by the cytosol is overcome by ferricyanide reoxidation after membrane extraction. The necessity of carrying out such reoxidation of the spin-labels present in the outer monolayers when reduction takes place only in the inner monolayer shows that the distribution of spin-labeled molecules at a given time is the result of continuous inward and outward transmembrane motions (Bitbol & Devaux, 1988).

In the series of molecules tested throughout this paper, we have paid attention to the influence of head-group composition and/or modifications at the level of the glycerol backbone. However, to what extent can these spin-labeled lipids be considered as representative of endogenous lipids? It is only a posteriori that the answer can be given. Figure 2 shows how the equilibrium distribution of the spin-labeled lipids is remarkably similar to that of endogenous lipids (Verkleij et al., 1973). In addition, the data of figure 2 can be superimposed to the kinetics data of Roelofsen et al. (1987) obtained with long-chain radioactive phospholipids incorporated into human red blood cells by a phospholipid exchange protein and whose transmembrane orientation was assessed by the phospholipase A_2 technique. However, the data of Roelofsen et al. lack the first hour of incubation because of the techniques used for incorporation and transmembrane determination. Thus, the spin-label technique appears to provide the same information as the use of more physiological long-chain lipids, while having the advantage of allowing the determination of the initial rates of outside–inside translocation, which is a direct measure of the aminophospholipid translocase activity. In contrast, the equilibrium distribution is the result of the inward and outward opposite motions.

Daleke and Huestis have monitored phospholipid translocation from erythrocyte shape changes (Daleke & Huestis, 1985). They showed also the selective translocation of the aminophospholipids. The half-time for the translocation of a low concentration of DMPS was of the order of a few minutes, while DMPE flipped in approximately 2 h at 37 °C. These values are consistent with our observations made with spin-labels.

A general comment about the phospholipid specificity of the aminophospholipid translocase is that chemical modifications of the acyl chains have little consequence on the rate of outside–inside translocation in accordance with the fact that spin-labeled phospholipids with a nitroxide on one of the acyl chains are good substrates. This conclusion is in agreement with the results of Daleke and Huestis, who found the same rate of transport for PS of various acyl chain length (Daleke & Huestis, 1985). Middelkoop et al. (1986), on the other hand, reported that PC's flip-flop in erythrocytes is strongly dependent on the nature of the acyl chains. This confirms that the translocation of the aminophospholipids proceeds by a different mechanism than that used by PC.

In contrast with the limited effect of acyl chain modifications, all modifications of the head group effectively decrease the rate of aminophospholipid translocation. The removal of the carboxyl group, or its esterification, decreases the uptake of the corresponding lipids (III, VII) by 1 order of magnitude when compared to PS (II). It is obvious that this group is involved in the interaction between the phospholipid and the carrier protein. Progressive methylation of the amino group leading from PE (II) to PC (I) through molecules V and VI results in a progressive decrease of the uptake. As pointed out by Sleight and Pagano (1985), monomethylphosphatidylethanolamine is close to phosphatidylethanolamine, while dimethylphosphatidylethanolamine resembles phosphatidylcholine. Note that the presence of a dissociable proton on molecules V, VI, and IX, as on III, is not sufficient to ensure a high translocation rate.

More surprising is the importance of the glycerol backbone: substitution of a glyceride by a ceramide, even linked to serine, drastically reduces the lipid transfer. Similarly, and as already reported by Bergmann et al. (1984), the absence of the β chain has a definite effect on the lipid uptake. Nevertheless, lyso-PS is transported more rapidly than lyso-PC. In conclusion, the aminophospholipid translocase recognizes not only the amino group on the lipid polar moiety but also the glycerol and the ester bonds. A simple acetyl chain is sufficient to restore a high transfer rate from the outer to the inner monolayer.

Figure 7 presents the various spin-labels classified as a function of their initial outside–inside translocation rate. The molecules that do not flip, like sphingomyelin, are not shown. In principle, it is possible to reckon a relative affinity for the aminophospholipid translocase. However, as shown in this figure, NEM treatment has rather different effects on the translocation rates, suggesting that different mechanisms may be involved.

Phosphatidic acid (IV) raises a particular problem. First, it is surprising that this molecule which, on the one hand, flips like PC at 4 °C (Zachowski et al., 1985) has on the other hand an initial rate of translocation, at 37 °C, comparable to that of PE. However, unlike PE, pretreatment with NEM has only a limited effect. These two observations lead us to conclude that, in erythrocytes as in fibroblasts (Pagano & Longmuir, 1985), PA traverses the plasma membrane by a different mechanism than PS. Possibly, PA is hydrolyzed into diacylglycerol (Hokin et al., 1963), which can then flip rapidly through the membrane (Ganong & Bell, 1984). The chemical instability of PA is manifested in our experiments by the high yield of fatty acid hydrolysis obtained with molecule IV. Preliminary investigations, which are not described in the present paper, have been carried out in order to analyze the chemical nature of the spin-labels after incubation in erythrocytes. Total lipid extract was run on HPLC and the spin concentration of the various fractions determined by ESR spectroscopy. However, the short-chain spin-labeled lipids do not elute exactly like long-chain lipids; in addition, calibration experiments are complicated by the large excess of long-chain endogenous lipids. Consequently, accurate chemical assignment of the paramagnetic lipids after incubation is difficult, apart from the quantitative determination of free fatty acid formation, which can be measured directly on the ESR spectra.

The above remark is valid for the other spin-labeled lipids used throughout this paper. Evidence for the chemical integrity of the spin-labels in the red cells comes partially from the crude lipid analysis by TLC or HPLC after incubation (Zachowski et al., 1986) and partially from indirect arguments.

In fact, it is known that most enzymes of phospholipid metabolism are not present in the plasma membrane of red cells with the exception of phospholipases and transacylases (Renooij et al., 1974; Marinetti & Cattieu, 1982). However, when the short-chain phospholipids are hydrolyzed, the free fatty acids are released in the aqueous environment, and this is immediately detected by ESR (see above). As for the methylation of the head group of PE, it is a process of very low efficiency in red cells (Hirata & Axelrod, 1978).

The ESR line shapes obtained at 37 °C with the phospholipids labeled at the 16th position of a C18 α chain (molecules XII and XIII) confirm the difference in viscosity between the inner and outer monolayers of erythrocytes, which we found previously by ESR, at 4 °C, with the nitroxide on the 4th position of the α chain (Seigneuret et al., 1984) and, at various temperatures, by the photobleaching technique (Morrot et al., 1986). However, it should be noted that molecules I and II did not show the difference in viscosity which was observed at 4 °C with these probes. Probably, the short chain at high temperature is tilted toward the aqueous environment. This would be consistent with the fast chemical reduction, at high temperature, of such probes when located in the inner monolayer, i.e., facing the cytosol.

Finally, the cholesterol content of the bilayer has a limited but significant effect on the protein activity, which is enhanced by a low level of cholesterol and slowed down in cholesterol-rich membranes. Such an effect of cholesterol on a membrane-bound enzyme has been already described (Yeagle, 1983). It is possible that the lipid composition affects the membrane fluidity and hence the turnover rate of the aminophospholipid translocase. Note that fluctuations in the level of cholesterol do not seem to change the equilibrium transmembrane distributions of PS and PE, probably because the inside–outside motion of amino lipids is also dependent on the aminophospholipid translocase (Bitbol & Devaux, 1988) and, thus, similarly affected by membrane composition. On the other hand, the level of cholesterol appears to influence the transmembrane distribution of PC; this is another indication of the different pathways used by PS and PC to traverse the membrane.

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