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Quantitative comparison between aminophospholipid translocase activity in human erythrocytes and in K562 cells

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Spin-labeled phospholipids were used to determine the transbilayer movement of phospholipids in human erythrocytes, in K562 cells and in human neonatal red cells. The erythroleukemia cell line, K562, as well as human neonatal red cells, which are rich in reticulocytes, were considered as representative of human erythrocyte precursor cells. In the nucleated cells, the difference between outside-inside movement of aminophospholipids and that of phosphatidylcholine or sphingomyelin analogues allowed us to discriminate between lipid internalization due to aminophospholipid translocase activity and to endocytosis. From the initial rates of aminophospholipid inward movement, we inferred that the activity of the aminophospholipid translocase is higher in the precursor cells than in mature erythrocytes.

Introduction

Originally discovered in the plasma membrane of human red cells [1], the existence of a specific carrier protein, aminophospholipid translocase, that transports phosphatidylserine (PS) and phosphatidylethanolamine (PE) from the outer to the inner monolayer of the plasma membrane of eukaryotes is well documented; see the reviews by Devaux [2] and Schroit and Zwaal [3]. The aminophospholipid translocase requires cytosolic Mg-ATP (1 mM), is inhibited by NEM (0.3 mM), by vanadate (500 μM) and cytosolic Ca^{2+} (0.2 μM). Several laboratories are presently working on the purification of this protein [4–6]. A systematic comparison between erythrocytes from different animal was undertaken in Schroit's laboratory, where a fluorescent analogue of phosphatidylserine was used [7]. The same group has reported a translocase activity at 4°C in K562 cells which are human erythroleukemic cells.

The present study was undertaken to compare, at a quantitative level, the translocase activity in the erythroid lineage. Indeed, in order to understand the physiological role of the aminophospholipid translocase, it is important to determine whether, during red cell maturation, the activity of the protein increases or decreases. This question is of particular importance in view of the hypothesis put forward recently concerning a possible role of the protein during endocytosis [2]. If the main function of the aminophosholipid translocase is to permit endocytosis, its activity should *decrease* between the stage of erythroblast and that of erythrocyte, since only the former cells have an endocytic activity.

Unfortunately, erythroblasts are difficult to obtain in large enough quantities to allow EPR spectroscopy. Thus, K562 cells [8] have been chosen as representative of human erythroblasts. The erythroid nature of these cells was convincingly demonstrated [9–13], although K562 cells differ in several respects from normal adult erythroid stem cells [14] and do not undergo terminal differentiation or acquire morphological features of recognizable normal erythroblasts.

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Abbreviations: BSA, bovine serum albumin; DFP, diisopropylfluorophosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NEM, *N*-ethylmaleimide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; LPC, 2-lysophosphatidylcholine; C_n^m, general nomenclature of labeled acyl chains, *n* corresponds to the total number of carbons, *m* is the position of the carbon bearing the labeled group; βC_5^4 PC*, 1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphocholine; βC_5^4 PE*, 1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphoethanolamine; βC_5^4 PS*, 1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphoserine; C_5^4 SM*, *N*-(4-doxylpentanoyl)sphingosine-1-phospho-choline; αC_{16}^5 LPC*, 1-(5-doxylpalmitoyl)-*sn*-glycero-3-phospho choline; αC_{18}^{16} LPC*, 1-(16-doxylstearoyl)-*sn*-glycero-3-phosphocholine; RBC, red blod cell; *S*, order parameter; Tf, human transferrin.

The red blood cell population of the human newborns is more heterogeneous than that of adults [15]. Nevertheless, neonatal red cells are often considered as forming a unique cell type with a short life span and characteristics close to those of reticulocytes [16].

Here, we consider the succession: K562 cells, neonatal red cells, adult red cells, as schematically representative of part of the human erythrocyte development. We have attempted to evaluate the aminophospholipid translocase activity in these different cells at a quantitative level by analysis of the initial rates of outside-inside passage of different spin-labeled lipids. The higher activity found in K562 cells compared to erythrocytes may be necessary for endocytosis and could be associated with the higher fluidity of the former cell membrane.

Materials and Methods

Materials

Culture media were purchased from Gibco BRL (Cergy-Pontoise, France), diisopropyl fluorophosphate (DFP), delipidated bovine serum albumin (BSA) and human transferrin (Tf) were purchased from Sigma. Radioactive iron (^{59}Fe) was purchased from Amersham.

Preparation of cells

Freshly drawn adult human blood was obtained from a blood bank (Centre National de la Transfusion Sanguine) and washed four times with 20 vols. of buffer A (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose and 20 mM Hepes (pH 7.4)).

Neonatal human blood

Blood was drawn from the placenta vessels of newborns at the time of delivery (Maternité Hotel Dieu, Paris). It was kept at 4°C, and used within 2 h. The preparation is identical to the one of the adult red blood cells.

K562 cells

K562 cell inocula were a generous gift of Dr. William Vainschenker (Service de Biochimie, Hôpital Henri Mondor, Créteil). Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated (0.5 h, 56°C) fetal calf serum, antibiotics (penicillin and streptomycin), and 2 mM glutamine. Cells were harvested by low-speed centrifugation and washed three times with the buffer A.

EPR spectroscopy

The EPR measurements were performed on a Varian E109 spectrometer equipped with a temperature control device and connected to a Tektronix 4051 computer.

Spin-labeling

All spin-labeled analogues were synthesized as described in Ref. 17. Prior to labeling the cells were incubated with 5 mM DFP. In previous experiments, we have found that DFP, which is reported to be an inhibitor of acyltransferase blocks the hydrolysis of the short chain spin-labeled lipids without affecting the aminophospholipid translocase activity. See the discussion in Ref. 18. In order to label the membranes, the desired amount of spin-label in a chloroform/methanol (1 : 1; v/v) solution was deposited in a tube and dried under reduced pressure. The dried film was resuspended in buffer by vigourously vortexing and then transferred to the tube containing the cell suspension. Unless otherwise specified, the number of cells was calculated so as to ensure a ratio of spin-labeled phospholipids to endogenous plasma membrane phospholipids of 1%. In the case of K562 cells, the fraction of lipids corresponding to the plasma membrane was estimated from the size of the cells and comparison with the lipid content of the erythrocyte plasma membrane.

Typically cells were resuspended in 1 ml at a final hematocrit of 50. Under such conditions the partition of the spin-labels was very much in favor of the membrane and total incorporation in the outer leaflet of the cell membrane was completed within a few minutes even at 4°C. This was checked by recording the EPR line shape in the presence of the membranes. The incorporation was assessed by the absence of narrow lines in the EPR spectra. Subsequent appearance of narrow lines took place in the absence of DFP and revealed hydrolysis of the short β chain followed by its solubilization in the aqueous phase.

Kinetics of spin-label translocation

The kinetics of outside-inside translocation were determined by the back exchange technique previously described [19,20]. The 1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphocholine (βC_5^4 PC*) and the corresponding analogues of PS, PE and SM were used. During the incubation, 50 μl aliquots of the suspension were drawn at regular intervals and mixed with 100 μl of a 1.5% (w/v) BSA solution in isotonic buffer. The mixture was incubated 3 min on melting ice, and then centrifuged 45 s at $7600 \times g$. To 90 μl of the supernatant obtained from each from each aliquot, 10 μl of 100 mM potassium ferricyanide was added in order to reoxidize any reduced spin-label. The intensity of the EPR spectrum of the BSA extract gave the fraction of spin-label on the outer leaflet for each aliquot. In spite of the DFP treatment, a fraction of the lipid analogues was hydrolyzed into lyso derivatives and free fatty acids, as revealed by the EPR spectrum; a correction was done before measuring the intensity of the spectrum as described in Ref. 17. The translocation rates were deduced from the initial slope of the curves

representing the fraction of non-accessible probe as a function of time. In most cases these curves could be simulated by a monoexponential plot as one might expect for a first-order kinetics. Such a simulation is only meaningful for the initial fraction of the curve in nucleated cells.

Fluidity measurements

Two lysoderivatives where used to assess the plasma membrane viscosity: αC_{16}^{5} and αC_{18}^{16} spin-labeled lysophosphatidylcholine (respectively αC_{16}^{5}- and αC_{18}^{16}-LPC*). The comparison of the spectra in the different cell types allows us to compare the average viscosity. The order parameters (S) were calculated for the C_{16}^{5}-probe by the following formula [21]:

$$S = \frac{T'_{\parallel} - T'_{\perp}}{T_{zz} - 1/2(T_{xx} + T_{yy})} \frac{a_o}{a'_o}$$

with

$$a'_o = \frac{T'_{\parallel} + 2T'_{\perp}}{3}; \qquad a_o = 15.2 \text{ G}; \qquad T_{xx} = 6.3 \text{ G}$$

$T_{yy} = 5.8$ G and $T_{zz} = 33.6$ G.

Uptake of iron by K562 cells

Human transferrin labelling by ^{59}Fe was performed as described in Dautry-Varsat et al. [22]: specific activity varied from 20 to 40 cpm/ng of Tf.

Uptake of ^{59}Fe-Tf was performed as previously described [23] with minor modifications. The K562 cells were washed twice with buffer A at room temperature, and then incubated for 45 min at 37°C in RPMI 1640 supplemented with BSA (5 mg/ml) to deplete the cells of the bovine transferrin accumulated during cell culture [24]. Cells were then pelleted, washed at 4°C and resuspended at $2 \cdot 10^6$ cells/ml at 4°C in RPMI 1640 containing 200 μg/ml of ^{59}Fe-Tf and BSA (5 mg/ml). Samples were incubated for 3 h at the indicated temperatures. Nonspecific binding was determined in the presence of a 500-fold excess of unlabeled ligand and ranged from 2 to 8% of specific binding. At the end of the incubation cells were cooled back to 4°C, pelleted, washed four times with 1 ml of ice-cold buffer A. Final cell pellets were solubilized in NaOH (0.1 M). Protein content was assayed by the method Lowry et al. [25] and radioactivity was determined using a Beckman LS 5000 TD model liquid scintillation counter. All data points in the figures represent averaged triplicate determination (±S.D.).

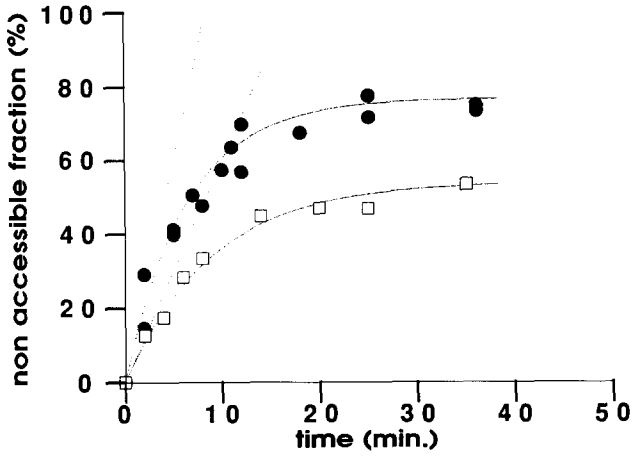


Fig. 1. Kinetics of translocation of spin-labeled analogues of PS (●) and PE (□) versus time, in K562 at 26°C. Plots (solid lines) correspond to the function: $\lambda\,(1-e^{-t/\tau})$. The translocation rates (λ/τ) are deduced from the initial slope (dotted line): βC_5^4 PS* 12±2%/min, βC_5^4 PE* 6±1%/min. All data points represent the average of at least three independent determinations.

Results

Fig. 1 shows the kinetics of aminolipid internalization in K562 cells, at 26°C. At this intermediate temperature, the initial slope of the kinetics curve which corresponds to the velocity of inward passage of the spin-labels, is mesurable with accuracy. At 37°C, i.e., at the physiological temperature, the transmembrane mo-

TABLE I

Translocation rates of aminophospholipid analogues in adults and neonatal erythrocytes, and in K562 cells at different temperatures

		Translocation rates (%/min)					
		0°C	15°C	22°C	26°C	32°C	37°C
Adult RBC	βC_5^4 PS*	1.0±0.1	2.1±0.2	5.0±0.3	6.9±0.5	11 ±2	18 ±2
	βC_5^4 PE*	0.3±0.1	0.8±0.1	1.1±0.2	1.5±0.2	1.7±0.3	1.9±0.3
Neonatal RBC	βC_5^4 PS*	[a]	[a]	[a]	10 ±2	[a]	[a]
	βC_5^4 PE*	[a]	[a]	[a]	3.0±0.4	[a]	[a]
K562	βC_5^4 PS*	<0.1	4.0±0.3	7.0±0.3	12 ±2	16 ±5	21 ±5
	βC_5^4 PE*	<0.1	1.5±0.3	2.6±0.3	6 ±1	9 ±2	13 ±3

[a] not determined.

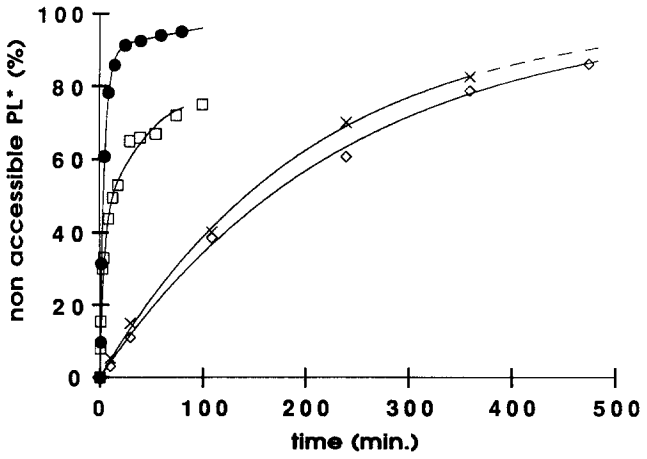


Fig. 2. Kinetics of translocation of the four spin-labeled analogues: C_5^4 SM (◇), βC_5^4 PC (×), βC_5^4 PE (□) and βC_5^4 PS (●), in K562 cells at 37°C. The dashed part of the PC curve corresponds to the fact that for prolonged incubation, the non-accessible fraction for PC is impossible to measure because of the hydrolysis of the short chain (see Materials and Methods).

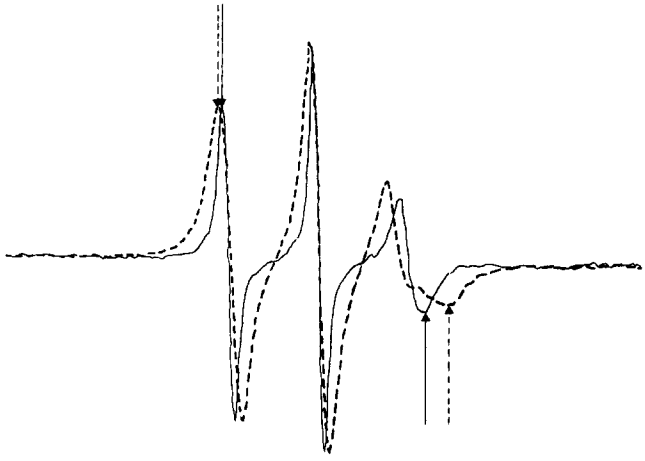
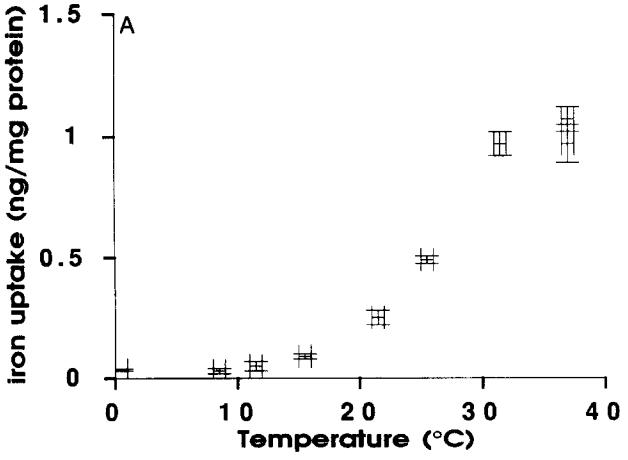


Fig. 4. EPR spectra of the αC_{18}^{16} LPC* probing: solid line, K562 cells; dashed line, RBCs. Temperature 37°C. The outer extreme splitting is indicated by arrows.

tion of βC_5^4 PS* in K562 cells is so fast that accurate determination of the initial slope is difficult with the sampling technique used here. Nevertheless, we have carried out experiments at several temperatures between 0°C and 37°C. Table I shows the rates (±S.D.) of aminophospholipid translocation deduced from the initial slope of the kinetics curves (at least three curves for each temperature-aminophospholipid pair). The table includes some data obtained at 26°C with neonatal red blood cells. Because of the rarity of the latter cells, only one temperature was used in this case. We again selected 26°C in order to obtain a good accuracy allowing us a comparison with mature erythrocytes and with erythroblasts. The regular decrease of activity associated with the cell maturation is illustrated in Table I which shows that K562 cells have always higher translocation rates than erythrocytes. When the internalization of the four classes of phospholipids in K562 cells is represented in the same figure, as in Fig. 2, a striking difference appears between the aminolipids (βC_5^4 PS* and βC_5^4 PE*) on the one hand and the choline containing lipids (βC_5^4 PC* and βC_5^4 SM*) on the other hand. At 37°C, the latter lipids are slowly internalized in K562 cells with exactly the same kinetics for PC and SM analogues characterized by a half-time of approx. 4 h. Very likely, the kinetics seen for the disappearance of the choline containing lipids in K562 cells is in fact due to endocytosis.

We have directly measured endocytosis at different temperatures in K562 cells by measuring the uptake of iron (Fig. 3A). For 3 h of incubation at 37°C, the ^{59}Fe uptake was 1.0 (±0.1) ng for 1 mg cell protein. These data were consistent with those of Klausner et al. [26] on the same cells. A correlation between the uptake of iron and the uptake of spin-labeled PC and SM can be



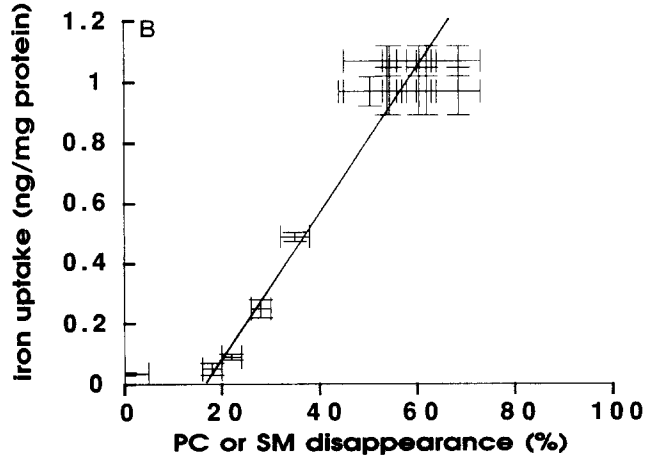


Fig. 3. K562 cells. (A) Endocytosis versus temperature, as assessed by the accumulation of iron in the cell within 3 h. Error bars: vertical ±S.D. (see text), horizontal ±0.5°C. (B) Correlation plot between the accumulation of iron in the cell, and disappearance of PC or SM analogues from the outer monolayer, after 3 h. Error bars: vertical, see above; horizontal, ±S.D. from a minimum total of seven determinations.

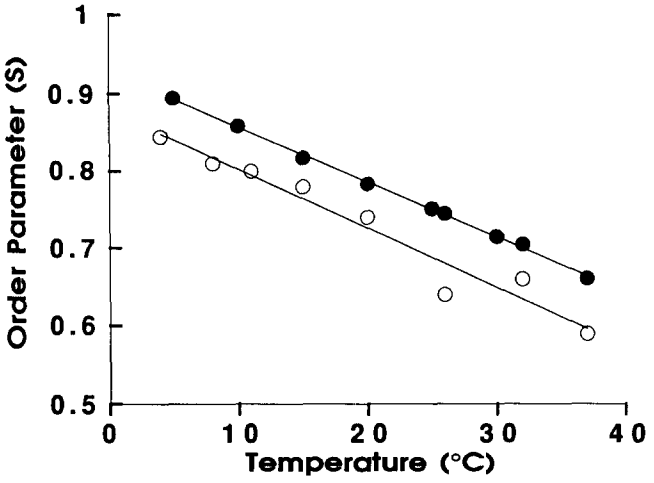


Fig. 5. Order parameter as a function of temperature, deduced from the EPR spectra of αC_{16}^{5} LPC* in K562 cells (○) and in RBCs (●). The size of the symbol takes in account the precision of the determination. The plain lines correspond to the best fit as determined by linear regression.

established and is demonstrated by the linear plot shown in Fig. 3B.

Finally, to see whether the difference in aminolipid translocation in K562 cells and in RBCs could be correlated to a difference in membrane viscosity, we have compared the viscosity of the plasma membrane of K562 cells and of erythrocytes using two spin-labeled probes. The lyso derivatives utilized are slightly amphiphilic and label rapidly the *outer* monolayer. They are not transported by the aminophospholipid translocase and thus, remain in the outer monolayer. The comparison of the spectra obtained with the two probes in the two cell types reveals a striking difference of average viscosity which appears, for example, in the measurement of the order parameters and line-widths. Fig. 4 shows a comparison of the spectra of the αC_{18}^{16} LPC* in the two cell types at 37°C. Fig. 5 shows the effective order parameter of the αC_{16}^{5} LPC* calculated from the EPR spectra for the two cell types and for different temperatures. The conclusion is that in the whole range of temperature explored erythroblast plasma membrane is more fluid than erythrocyte plasma membrane.

Discussion

While the techniques used to measure lipid flip-flop give unambiguous results with erythrocytes, there are several difficulties when dealing with nucleated cells. Firstly, lipid translocation is generally investigated with slightly water soluble phospholipids, which permit a rapid membrane labeling. However, once in the cytosolic leaflet of the plasma membrane, these lipids redistribute spontaneously between all the cell organelles because of their water solubility. This feature precludes a determination of the true transmembrane equilibrium distribution in the plasma membrane. Secondly, in most nucleated cells, the plasma membrane is continuously recycled by endocytosis-exocytosis, at least at physiological temperatures. This membrane traffic can sequester phospholipids from the plasma membrane external leaflet without involving transmembrane reorientation. Thus, measurements of flip-flop rates are obscured by this alternative pathway. Using a low temperature to differentiate flip-flop from endocytosis is not completely satisfactory since endocytosis and flip-flop appear progressively and simultaneously as the temperature is raised. Also, inhibition of endocytosis by addition of vanadate or by ATP depletion blocks simultaneously endocytosis and aminophospholipid translocation. Cytosolic ATP measurements are not significant as its subcellular distribution is not determined. On the other hand, the rapid internalization of a subclass of lipids is the indication of a selective mechanism which is not explainable by endocytosis. Such observations were made with platelets [27], lymphocytes [28] and guinea pig reticulocytes [29]. The former results provided *qualitative* evidence of the existence of an aminophospholipid translocase activity. With K562 cells, which are human erythroblastoid transformed cells, we have obtained at 37°C a rapid internalization of PS and PE analogues and a very slow internalization of PC and SM analogues. An important characteristic of the internalization of the latter lipids (not seen with the above mentionned systems) is the quasi parallel kinetics curves observed for the two choline containing phospholipids (see Fig. 2). Since it has always been found in cells which do not undergo endocytosis that the spontaneous diffusion of PC is faster than that of SM [20], we infer that in K562 cells, the homogeneous disapearance from the outer monolayer of PC and SM at 37°C and, a fortiori at lower temperature, is solely due to endocytosis. This argument is strengthened by the remarkable correlation between iron uptake and the disapearance of choline containing lipids from the outer leaflet (see Fig. 3). In principle, PC and SM should be recycled back to the outer monolayer of the plasma membrane, thus one might anticipate a plateau for PC and SM below 100% for the non-accessible fraction (Fig. 2). However the plateau would only be reached after a prolonged incubation at 37°C (more than 10 h). During such a long incubation, a fraction of the nitroxides is non-reversibly reduced and no reliable quantitation of the steady state can be deduced from an apparent plateau reached by PC or SM.

The rates indicated in Table I correspond to the rates of spin-labeled aminolipid disappearance from the plasma membrane outer monolayer, as calculated from the initial slopes of the kinetics curves. In principle to obtain the rate of lipid inward movement (or

'flipping') it is necessary to subtract the contribution of the endocytosis which is given by the time-scale of PC or SM disappearance. However, if one compares the horizontal scales of Figs. 1 and 2, it appears that the contribution of endocytosis is negligible in the first 5 min. Thus, the rates calculated in Table I correspond to the true rates of aminophospholipid translocation, even at 37°C, temperature at which endocytosis is an active phenomenon.

The conclusion of the present study is therefore that the translocation of the aminophospholipids is more efficient in the premature forms of erythroid cells, the percentage of lipid translocated per min being two to 3-fold higher in K562 cells than in erythrocytes. At this stage it is impossible to discriminate between the different mechanisms which could be responsible for this phenomenon. However, we have established that the membrane viscosity is considerably reduced in K562 cells compared to erythrocytes. For example, the order parameter, S, determined with the αC_{16}^{5} LPC* in RBCs is higher than the values obtained in K562 cells. Thus, although we cannot rule out the possibility that the specific activity or the number of enzymes per lipids varies during the red cell maturation, it appears possible to account for the difference in activity solely by the difference in average lipid viscosity. It would be necessary to isolate the plasma membrane of K562 cells in order to determine whether the phospholipids are more unsaturated in the latter cells or whether the cholesterol content is responsible for the difference in viscosity.

The present data are interesting to compare with the aminophospholipid translocase measurements made by Middelkoop et al. [30] in the plasma membrane of Friend erythroleukemic cells and in intact murine erythrocytes. In the case of the murine membrane, although the ATP-dependent translocase is present and active in the precursor cell, an asymmetric distribution of PS but not of PE was observed; this could suggest a lower translocase activity. Middelkoop et al. attributed their observation to the difference in skeletal network. Another parameter which can explain the difference in lipid asymmetry is the average lipid viscosity: if the spontaneous flip-flop is faster, i.e., if the 'leak' through the lipid bilayer is rapid, a higher translocase activity is not automatically associated with a higher phospholipid asymmetry.

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