Abnormality of Phospholipid Transverse Diffusion in Sickle Erythrocytes

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Abstract

We have used spin-labeled analogues of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine to compare the transverse diffusion rates of lipids in normal and sickle erythrocytes. The β-chain of the spin-labeled lipids was a short chain (five carbons) providing the spin labels with a relative water solubility, and hence permitting their rapid incorporation into cell membranes. The orientation of the labeled lipids in the membranes was assayed by selective chemical reduction of the nitroxide labels embedded in the outer leaflet. We have found that all three spin-labeled phospholipids are initially incorporated in the outer leaflet. Upon incubation at 4°C the aminophospholipids, not the phosphatidylcholine, diffuse toward the inner leaflet within 3 h. The transverse diffusion rate of aminophospholipids is reduced by 41% (phosphatidylserine) and 14% (phosphatidylethanolamine) in homozygote sickle cells (SS) when compared with normal cells (AA) or heterozygote cells (AS or SC). At equilibrium the asymmetric distribution of spin-labeled phospholipids resulting from this selective diffusion is also reduced in SS cells when compared with AA, SC, or AS cells. This reduced asymmetry was not found in a reticulocyte-rich blood sample (hemoglobin A), indicating that the age of the cell cannot be responsible for this phenomenon. Moreover, because at low temperatures the sickling process does not occur, the observed perturbations in phospholipid organization reflect preexisting membrane abnormalities in sickle cells. Ghosts loaded with ATP give the same results. Varying the concentration of intracellular calcium had no effect on lipid diffusion, except at very high free calcium concentrations (3 μM) when diffusion was practically abolished. We suggest that membrane protein alterations may be part of the explanation of the observed abnormalities.

Introduction

Many lines of evidence demonstrate that the structure and functions of red cell membranes in various hemoglobinopathies (sickle cell disease, thalassemia, hemoglobinose C, etc.) are significantly perturbed (1, 2). However, the relationship between hemoglobin mutation and membrane alterations, as well as the events leading to hemolysis, are not well understood.

The electrophoretic pattern of the membrane proteins in sickle cells differs only slightly from that of normal cells (3, 4). For example variations in intrachain cross-linking (4) or protein phosphorylation (5) have been reported. The lipid composition and lipid-to-protein ratio in sickle cells and normal cells are very close (6–8). However, abnormalities have been observed at the lipid organization level. Measuring the degree of fluorescence anisotropy of probes incorporated in the membrane, Rice-Evans et al. (6) showed that the hydrophobic region of the lipid bilayer is less fluid in sickle cells than in normal cells. Another striking difference concerns the phospholipid asymmetry between the two halves of the bilayer: different techniques have concurrently revealed that a greater percentage of aminophospholipids is exposed on the outer surface of sickle cell membrane than of normal cell membrane (9–12). However a recent report by Raval and Allan (13) does not support these assumptions.

The ratio of inner versus outer lipids is one of the major parameters determining the normal erythrocyte discocyte shape (14, 15). Thus, an active transport of phospholipids is required to compensate for spontaneous equilibration of the lipids between both layers as well as to allow the cells to recover the discocyte shape after cell deformation. Indeed most shape changes are accompanied by a change in the ratio of the area of the outer and inner layers (16). Thus, the reversibility of cell deformation after mechanical constraint may require the rapid flux of lipids from one side to another, implying that dynamic deformability should be influenced by the rate of transverse diffusion of lipids. Recently, Seigneuret and Devaux designed an experiment that allowed them to measure the rate of transverse diffusion of spin-labeled phospholipids selectively incorporated into the outer layer of red blood cells (15). They showed that aminophospholipids (phosphatidylserine [PS*][1] and phosphatidylethanolamine [PE*]) diffuse from the outer layer to the inner layer at a much faster rate than phosphatidylcholine (PC*), providing the cells contain ATP. A rigorous parallel between shape change and lipid transverse diffusion could be established.

Bergmann et al. (17) have also found that lysophosphatidylserine reorients more rapidly than lysophosphatidylcholine in normal erythrocyte membranes. To date, no such comparative studies have been performed in sickle cells; only the transverse diffusion (or flip-flop) of PC* has been investigated. Depending upon the authors' results, either a moderate (18) or a very strong acceleration (11) of transbilayer movement of PC* in sickle cells compared with normal red blood cells has been reported.

In this paper, we report abnormalities in the efficiency of the selective transport of aminophospholipids from the outer leaflet to the inner leaflet of sickle cell membranes. We used the spin-label technique developed by Seigneuret and Devaux (15) and Seigneuret et al. (19). It takes advantage of the partial water solubility of spin-labeled phospholipids possessing one relatively short chain (C_5). This feature allows an early and

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1. *Abbreviations used in this paper:* PC*, phosphatidylcholine; PE*, phosphatidylethanolamine; PS*, phosphatidylserine.

rapid incorporation of the spin labels in the outer membrane layer. The sidedness of the spin label is detected by chemical reduction of the labels exposed on the outer layer, Na^+ ascorbate being added at 4°C as a reducing agent (15).

Experiments were carried out with different hemoglobinopathic red cells, either with whole cells or resealed ghosts. The effects of oxygenation, intracellular calcium, cell density, and cell age were tested.

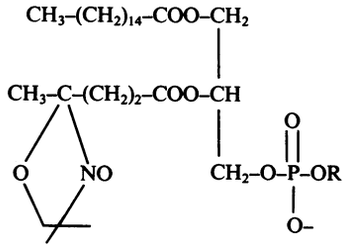
Methods

Erythrocyte and ghost preparation. Blood samples of patients with sickle cell syndromes diagnosed by standard laboratory procedure (20) were obtained after the patients gave informed consent according to the code of ethics of the World Medical Association. The patients under study were at distance of crisis (1 mo) and transfusion (4 mo). Volunteers from our laboratories served as control blood donors.

Fresh venous blood was collected in a medium containing EDTA, NaCl, and glucose to conserve the energy reserve of the cells; it was processed in the following 24 h. The samples were centrifuged at 2,500 *g* for 5 min to remove plasma and the buffy coat. Erythrocytes were subsequently washed three times in 5 vol of phosphate-buffered saline (10 mM Na phosphate, 145 mM NaCl, pH 7.35, 300 mosM).

Cell separation according to their density was performed according to the centrifugation technique of Murphy (21). Pink ghosts were prepared according to the method of Schwoch and Passow (22).

Spin labeling and ESR experiment. The following spin-labeled phospholipids were synthesized as described previously (15).



PC*: R = $(CH_2)_2-\overset{+}{N}(CH_3)_3$

PS*: R = $CH_2-\underset{\underset{COO^-}{|}}{\overset{+}{C}H}-NH_3$

PE*: R = $(CH_2)_2-\overset{+}{N}H_3$

Membrane labeling and ESR determination of the phospholipid fraction present on the inner half of the membrane were run as described previously (15). Briefly, the spin labels were added from a concentrated ethanol solution to an erythrocyte suspension, the spin-label concentration corresponding to ~1% of the endogenous phospholipids. Incorporation into the membrane was instantaneous. Sodium ascorbate (10 mM) was added to the samples at 4°C to assess the spin-label orientation. Chemical reduction reached a plateau in ~5 min. The level of the plateau usually depended upon the time elapsed between incorporation of the spin labels and addition of ascorbate (incubation time). In the Results section, only the levels of the plateau are shown as a function of incubation time, at 4°C.

To control the intracellular calcium concentration, cells were incubated 10 min at 37°C in the following buffer: 9 mM Hepes, 145 mM NaCl, 1 mM EGTA, 5 μM A23187 calcium ionophore, pH 7.4, with various amounts of $CaCl_2$ (23).

Curve fittings corresponding to the evolution of the fraction of nonreducible signal after various periods of incubation were carried out by use of the following equation: $A(t) = A_{eq}[1 - \exp(-kt)]$, where $A(t)$ is the fraction of nonreducible signal, i.e., it corresponds to labels in the inner leaflet; A_{eq} is the fraction of spin label in the inner leaflet at equilibrium; and k is the apparent transverse diffusion constant. A_{eq} and k are determined by a program of nonlinear regression analysis. The initial velocity of the diffusion process is $V_i = kA_{eq}$, while the half time of equilibration is $t_{1/2} = \ln 2/k$.

Data displayed in each of the figures correspond to one set of experiments. All experiments were reproduced at least twice. Experiments with PS* (the most representative label) were repeated from four to six times.

Results

Transverse diffusion of phospholipids in homozygote SS and heterozygote AS red blood cells. The outside-inside diffusion of PC*, PE*, and PS* in sickle cells is shown in Fig. 1 *A*. Corresponding results with AS cells appear in Fig. 1 *B*. AA normal cells behaved as AS cells (results not shown). The parameters obtained by analysis of the data (see Methods) are listed in Table I, which shows that the equilibrium levels and the transverse diffusion rates of PS* and PE* were modified in SS cells compared with AS (or AA) cells. As to PC*, the diffusion was so slow that we could not give any estimate for the equilibrium distribution. In fact no difference of PC* behavior in SS and AS cells could be detected.

Comparison of PS transverse diffusion in various red cells (see Table II and Fig. 2).* Cells were characterized by the type of hemoglobin they contained. The reference was considered to be AA cells, which exhibited, in the presence or absence of oxygen, a fast diffusion rate and a high degree of asymmetric distribution between both halves of the membrane. The same was true with cells containing hemoglobin S and another type of hemoglobin (C or A). SS cells (reversible or 65% enriched in irreversibly sickled type), however, exhibited a lower rate of diffusion and a reduced asymmetric distribution: initial velocity was diminished twofold, the equilibrium distribution was reduced to 3:2 (inner/outer) from 9:1, and the time required for half equilibration increased 50–100%.

The whole heterozygote SC cell population was separated

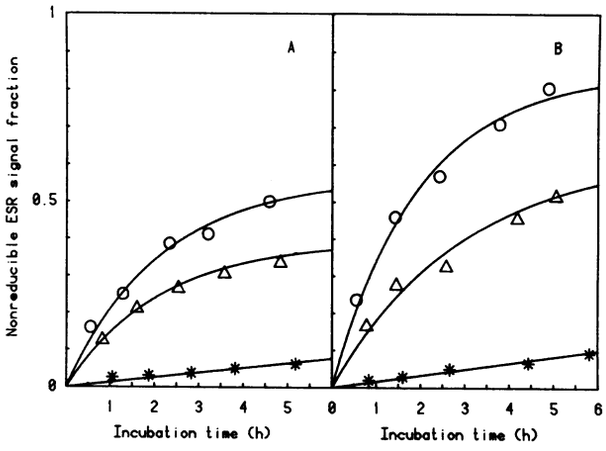


Figure 1. (*A*) Transverse diffusion of PS* (○), PE* (△), and PC* (•) in sickle cells. The nonreducible ESR signal fraction corresponds to the spin-labeled phospholipids that have diffused into the inner leaflet of the membrane. Incubation and measurement were carried out at 4°C as described in Methods and Reference 24. (*B*) Transverse diffusion of PS* (○), PE* (△), and PC* (•) in AS cells.

Table I. Transverse Diffusion Rates of PC, PS*, and PE* in AS and SS Cells*

Cells	Phospholipid	A_{eq}	k	V_i	$t_{1/2}$
			h^{-1}	h^{-1}	h
AS	PS*	0.85 (0.03)	0.51 (0.05)	0.44 (0.06)	1.36 (0.13)
	PE*	0.64 (0.07)	0.33 (0.07)	0.21 (0.07)	2.07 (0.45)
	PC*	—	—	0.015 (±0.009)	—
SS	PS*	0.57 (0.04)	0.46 (0.07)	0.26 (0.06)	1.51 (0.23)
	PE*	0.40 (0.02)	0.46 (0.05)	0.18 (0.03)	1.50 (0.16)
	PC*	—	—	0.015 (±0.010)	—

Values deduced from curve fitting according to the procedure described in Methods. Mean deviations are in parentheses. A_{eq}, fraction of spin label in the inner leaflet at equilibrium; k, apparent transverse diffusion constant; V_i, initial velocity.

according to Murphy's method (21) to get two subpopulations, one enriched in light cells, the other in dense cells. No differences were noticed in PS* diffusion between these two sets.

Although deoxygenation did not induce sickling of reversible sickle cells at 4°C, we measured PS* transverse diffusion in the presence and absence of oxygen. As already seen with normal cells, these two conditions did not affect diffusion, showing that it is independent from the presence of oxygen in the incubation medium. Nitroxide reduction by the cytoplasm content at 37°C (19) precludes the study of the effect of hemoglobin S polymerization on phospholipid transverse diffusion. Sickle red cells being, on the average, younger than normal red cells, one may wonder whether the reduced phospholipid asymmetry is related to cell age. Thus, we performed these same experiments with a reticulocyte-rich (7%) hemoglobin A containing blood sample from a patient who was recovering from an episode of bleeding. We separated two subpopulations, one containing 21% and the other <1% of reticulocytes; the rest of the population comprised very young normal cells. The results (Table II) show that both samples exhibited a high degree of PS* distribution asymmetry, although kinetics were slower than in an AA cell population. The same was true when PE* behavior was examined (data not shown).

Influence of the cytosolic content on phospholipid transverse diffusion. We have shown previously that the rapid transverse diffusion of aminophospholipids observed in normal red blood cells can be reproduced in ghosts, only if the ghosts are resealed in the presence of 1 mM ATP and contain a regenerating system (phosphocreatine-creatine kinase) (15). Experiments were, therefore, conducted with ghosts from sickle cells. They led to the same conclusion as in normal cells, namely that ATP is necessary for the rapid diffusion of PS* and PE*. Also, the characteristics of PS* transverse diffusion in ATP containing resealed sickle cell ghosts were the same as in the corresponding intact sickle cells (Table II). Because the hemoglobin content was reduced by 95% in the ghosts, differences in diffusion rates noted between normal and sickle cells cannot be attributed simply to the presence of hemoglobin S.

It has been demonstrated that sickle cells exhibit a higher cytoplasmic level of calcium ions than normal cells: 20–60 μmol/liter cells (24). To elucidate a possible role for these ions on the decreased transverse diffusion of aminophospholipids, sickle cells were incubated with a controlled level of calcium (see Methods). Fig. 3 shows that a complete depletion of Ca^{2+} did not enhance PS* transverse diffusion. Very high concentrations of free Ca^{2+} were necessary to completely inhibit PS* transport (3 μM).

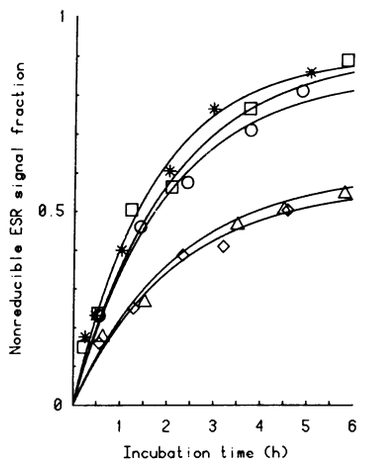


Figure 2. Transverse diffusion of PS* in cells containing various types of hemoglobin: (•), AA cells; (□), SC cells; (○), AS cells; (△; unseparated, ◇, irreversibly sickled cells), SS cells.

Discussion

We have shown that the asymmetric distribution of a spin-labeled PS* and PE* that spontaneously takes place in red blood cell membranes was less pronounced in sickle cells than in normal cells. This result is consistent with the known decreased asymmetry of endogeneous phospholipids in sickle cells (9–12) and suggests that the paramagnetic phospholipids used in this study are valuable reporters of natural phospholipids. The experiments described above showed that the decreased asymmetry at equilibrium is concomitant with a reduced rate of outside-inside aminophospholipid diffusion. As in normal cells this transport was ATP-dependent and may have involved a specific phospholipid carrier (flipase). As these experiments have been done at 4°C, hemoglobin S did not polymerize, and thus sickling did not occur. These experiments detected preexisting membrane changes. They cannot, however, be directly compared with published data describing the effectiveness of sickling (9–12).

The reasons for an abnormal functioning of this specific carrier are not known. However, it has to be noted that red cells containing both hemoglobin S and another hemoglobin (A or C) behaved just as normal red cells do. This and the fact that ghosts prepared from sickle cells gave the same results

Table II. Kinetic Parameters of PS Diffusion in Various Cell Types*

Cell type	A_{eq}	k	V_i	$t_{1/2}$
		h^{-1}	h^{-1}	h
RBC (AA)				
Oxygenated	0.90 (0.04)	0.60 (0.05)	0.54 (0.07)	1.15 (0.10)
Deoxygenated	0.88 (0.04)	0.62 (0.06)	0.55 (0.08)	1.12 (0.11)
RBC (AA)‡ young				
Reticulocyte-rich	0.88 (0.06)	0.30 (0.06)	0.27 (0.07)	2.31 (0.44)
No reticulocytes	0.95 (0.06)	0.29 (0.06)	0.28 (0.07)	2.39 (0.49)
RBC (SC)				
Light	0.85 (0.05)	0.62 (0.08)	0.53 (0.10)	1.12 (0.14)
Dense	0.90 (0.06)	0.50 (0.08)	0.45 (0.10)	1.39 (0.22)
RBC (AS)	0.85 (0.03)	0.51 (0.05)	0.44 (0.06)	1.36 (0.13)
ISC§ (SS)	0.57 (0.04)	0.46 (0.07)	0.26 (0.06)	1.51 (0.26)
RBC (SS)				
Oxygenated	0.60 (0.07)	0.46 (0.08)	0.28 (0.08)	1.51 (0.26)
Oxygenated	0.55 (0.06)	0.33 (0.07)	0.18 (0.06)	2.10 (0.44)
Deoxygenated	0.53 (0.07)	0.36 (0.07)	0.19 (0.06)	1.93 (0.37)
RBC (SS)				
Ghosts + ATP	0.51 (0.07)	0.41 (0.04)	0.21 (0.05)	1.69 (0.16)

Curve fitting followed the equation $A(t) = A_{eq}\,[1 - \exp(-kt)]$, where $A(t)$ is the fraction of PS* on the inner leaflet at a given time and A_{eq} is the fraction of PS* on the inner leaflet at equilibrium. Errors on the parameters are indicated in parentheses. The two values given for SS cells under oxygenated conditions are the extremes obtained over six independent experiments carried out with six different blood samples. V_i, initial velocity; k, apparent transverse diffusion constant; RBC, erythrocyte; ISC, irreversibly sickled cell. ‡ A blood sample from a patient who was recovering from an episode of bleeding. § A fraction containing 65% ISC.

as whole cells, eliminate the presence of hemoglobin S per se as the determining factor of phospholipid behavior. Experiments carried out with very young AA red cells mixed with more or less reticulocytes showed that cell age did not influence the asymmetric distribution of aminophospholipids between both membrane leaflets. Thus the abnormality detected with sickle cells cannot be related to their shorter life time. Free calcium ions, present in sickle cell cytoplasm at a relatively high level, also cannot be responsible for the observed phenomenon. We must stress that the free Ca^{2+} concentration (3 μM) that inhibited the PS* diffusion (Fig. 3) was about two orders of magnitude greater than the normal free calcium concentration in red cells (25). However, in normal erythrocytes we observed the same inhibition of PS* diffusion, only at 100 μM free Ca^{2+} concentration (data not shown). This means that sickle cells have a significantly greater sensitivity to calcium ions. At present, it is difficult to speculate about the mechanism of this sensitivity, but we think the effective concentration values argue for a protein mediator. On the other hand, it was shown that the spontaneous oxygen radical generation in sickle cells is twice that in normal cells (26), and that the enzymatic and nonenzymatic antioxidant mechanisms are impaired (27). These facts were related to the perturbed spectrin-actin lattice (28), cation (29), or nonelectrolyte (30) transport. The present findings about aminophospholipid decreased kinetic and equilibrium characteristics in sickle cells may be considered another consequence.

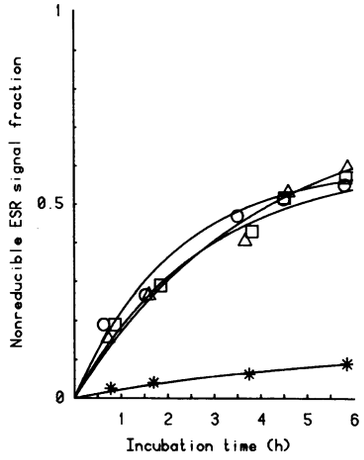


Figure 3. Transverse diffusion of PS* in sickle cells containing various concentrations of free calcium ions: 3 μM (•); 11 nM (□); no free ions (△). Control experiment without ionophore (○).

We want to emphasize the importance of a decreased rate of aminophospholipid transport on the mechanical and hydrodynamical properties of red blood cells. As stated in the Introduction, cell deformability is necessary for red cell physiology. Membrane-extreme bendings, such as those experienced in capillaries, impose a partial redistribution of lipids from the inner to the outer layer of the cell. Thus, it is clear that rapid recovery of the functional discocyte shape requires an efficient outside-inside transport. We have previously demonstrated (15) that the time course of spontaneous phospholipid diffusion in normal red blood cells is identical to that of shape changes leading to discocyte or stomatocyte red blood cells after drug induction of the echinocyte shape. Consequently, one may

infer that the decreased efficiency of the active diffusion of aminophospholipids toward the inner leaflet will lead to a slowing down of the aptitude of the sickle cell to recover its shape. Recent observations on sickle cells by Evans et al. (31) and Nash et al. (32) may be consistent with such a hypothesis.

The time course for rapid elastic recovery reported in references 31 and 32 is 1 s, not 1 h as in our experiments. However our experiments have been carried out, for technical reasons, at 4°C, resulting in a considerable slowing down of the transverse diffusion process. Experiments carried out at 37°C with normal red blood cells have shown that the transverse diffusion of PS* is too fast to be measured by a spin-label reduction technique (19).

In the light of the present results, we suggest that altered deformability of sickle cells may not be entirely due to the hemoglobin content or to cytoskeletal proteins, but also in part to the efficiency of the selective transport of aminophospholipids that has been recently discovered in red blood cells.

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