Outside–Inside Translocation of Aminophospholipids in the Human Erythrocyte Membrane Is Mediated by a Specific Enzyme†

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ABSTRACT: When human erythrocytes are incubated with spin-labeled analogues of sphingomyelin, phosphatidylcholine, phosphatidylserine, or phosphatidylethanolamine, with a short β chain (C5) bearing a doxyl group at the fourth carbon position, the labeled lipids incorporate readily in the outer monolayer. The incorporation is followed in fresh erythrocytes by a selective inward diffusion of the amino derivatives. This observation led us to postulate the existence of a selective ATP-dependent system that would flip aminophospholipids from the outer to the inner monolayer [Seigneuret, M., & Devaux, P. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3751–3755]. This study further examines the nature of this selective transport and demonstrates that it is mediated by a specific membrane protein. By measurement of the initial rate of transverse diffusion of spin-labeled lipids incorporated at various concentrations in the membrane outer leaflet of packed erythrocytes, apparent K_m values were determined for the phosphatidylserine and phosphatidylethanolamine analogues. A ratio of $\simeq 1/34$ was obtained (K_m^{PS}/K_m^{PE}). Using spin-labels bearing either a ^{14}N or a ^{15}N isotope, we have carried out competition experiments allowing us to measure simultaneously the transport of two different phospholipids. By this procedure, we show that phosphatidylserine and phosphatidylethanolamine compete for the same transport site but that phosphatidylserine has a higher affinity, in agreement with a lower apparent K_m. On the other hand, the slow diffusion of the phosphatidylcholine or sphingomyelin analogues has no influence on the transport of phosphatidylserine or phosphatidylethanolamine. Experiments carried out in ghosts loaded with ATP enabled us to determine the activation energies for phosphatidylserine and phosphatidylcholine transverse diffusion. Values of 7.4 and 13 kcal mol^{-1} were found respectively for the former and the latter lipid. This difference argues in favor of a different mechanism of transport. The proteic nature of the aminophospholipid translocation was also demonstrated by the inhibition obtained with SH reagents. Finally, we have shown that the inhibition of the anion carrier (band 3) has no influence on the transport of aminophospholipids, while cytosol Ca^{2+} concentration has an inhibitory effect above 1 μM.

The asymmetric distribution of phospholipids between the two leaflets of the erythrocyte membrane is a characteristic that is constant through the 120 days of the life span of the cell [see the reviews by Op den Kamp (1979), Etemadi (1980), Van Deenen (1981), or Krebs (1982)]. On the other hand, phospholipids are able to diffuse between the two leaflets at an appreciable rate, as shown in human and rat erythrocytes (Rousselet et al., 1976; Renooij et al., 1976; Renooij & Van Golde, 1976; Van Meer et al., 1980). For example, the characteristic time of phosphatidylcholine diffusion is estimated to be approximately 5–20 h. Recently, it was shown that the reorientation of aminophospholipids (phosphatidylserine and phosphatidylethanolamine) proceeds at a much faster rate than the reorientation of phosphatidylcholine (Seigneuret & Devaux, 1984; Bergmann et al., 1984) or sphingomyelin (Zachowski et al., 1985). Thus, the observed asymmetry does not result from an absence of transverse mobility but is the consequence of differential dynamic properties.

Several authors have suggested that selective interaction between aminophospholipids and the cytoskeletal protein spectrin might be responsible for this difference in dynamic properties of the phospholipids in the erythrocyte membrane (Haest et al., 1978; Mombers et al., 1980; Franck et al., 1982; Raval & Allan, 1984). However, we have shown recently that the transbilayer mobility of phosphatidylserine and phosphatidylethanolamine depends upon the presence of hydrolyzable ATP inside the cell and that when ATP is absent, either in ATP-depleted cells or in ghosts, the aminophospholipids diffuse at the same rate as phosphatidylcholine (Seigneuret & Devaux, 1984). These observations suggest the existence in the erythrocyte of an ATP-dependent translocator protein, specific for aminophospholipids ("aminophospholipid translocase").

In this paper, we further characterize the phospholipid diffusion in human erythrocytes as monitored with spin-labeled analogues. These short β-chain (C5) analogues, bearing a doxyl group at the fourth position, incorporate easily and quantitatively in the erythrocyte outer leaflet; their reorientation within the membrane can be quantified by selective chemical reduction of the probe located on one membrane half (Seigneuret & Devaux, 1984; Seigneuret et al., 1984).

Materials and Methods

Freshly drawn human blood was obtained from a local blood bank (Hopital Cochin) and washed 4 times with 20 volumes of 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, and 20 mM Hepes[1] buffer, pH 7.4.

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[1] Abbreviations: (0,2)PC, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylcholine; (0,2)PS, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylserine; (0,2)PE, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylethanolamine; (0,2)SM, *N*-(4-doxylpentanoyl)-*trans*-4-sphingenyl-1-phosphocholine; (*m*,*n*), general nomenclature of spin-labeled chains, *m* and *n* being respectively the number of methylene groups after and before the labeled position on the acyl chain; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; NEM, *N*-ethylmaleimide; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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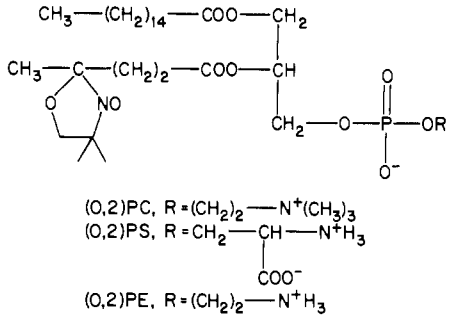
Spin-Labeled Phospholipid Synthesis. The following spin-labeled glycerophospholipids were used:



The sphingomyelin analogue (0,2)SM has the same corresponding structure. For some experiments, the (0,2)PE derivative contained a nitroxide with a ^{15}N isotope. The synthesis of [^{14}N](0,2)PC was described in Keana and La Fleur (1979); [^{14}N](0,2)PS and [^{14}N](0,2)PE were prepared enzymatically from it according to Comfurius and Zwaal (1977). The synthesis of ^{15}N-labeled (0,2) fatty acid was achieved according to Bienvenüe et al. (1978) except that the ceto ester used was 4-oxopentanoic butyl ester. The synthesis of [^{14}N](0,2)SM was described in Zachowski et al. (1985). Lipid extraction was carried out by the method of Rose and Oklander (1964). Analysis of the lipids by thin-layer chromatography was performed on silica gel plates in chloroform–methanol–water (65:25:4).

Spin-Labeling. The desired amount of analogue from a chloroform solution was deposited in a tube and dried under nitrogen. The dried film was resuspended in 1 volume of buffer by vigorously vortexing; then, 9 volumes of packed erythrocytes was added and gently mixed. The incorporation of all the added spin-labels in the outer leaflet of the membrane was completed within 3 min as previously described (Seigneuret & Devaux, 1984). This incorporation is assessed by the absence of narrow lines in the ESR spectra.

Reduction Experiments. At low temperature (4 °C), reduction of the ESR signal arising from the probe located on the outer membrane half was accomplished by adding to the erythrocyte suspension a nonpermeant reducer, i.e., sodium ascorbate (Seigneuret & Devaux, 1984). At 37 °C, signal reduction occurred on the membrane inner leaflet when the paramagnetic moiety was in contact with the reducing erythrocyte cytoplasm (Seigneuret et al., 1984). ESR spectra were recorded with a Varian E109 spectrometer as detailed in a former publication (Seigneuret & Devaux, 1984).

Preparation of ATP-Loaded Resealed Ghosts. Pink ghosts were prepared by the method of Schowch and Passow (1973). Hemolysis was performed by adding 1 volume of erythrocytes (50% hematocrit in 150 mM NaCl) to 20 volumes of 1.2 mM acetic acid, 4.5 mM $MgSO_4$, 0.5 mM EGTA, and 0.5 mM PMSF, pH 3.5 at 0 °C. When the pH stabilized at 6.0, Mg ATP (1 mM), creatine phosphate (10 mM), and creatine phosphokinase (100 IU/mL) were added. After 5 min, isotonicity and a 7.4 pH were reestablished with concentrated solutions of NaCl and phosphate buffer. Then, resealing was accomplished at 37 °C; the ghosts were washed 3 times before use.

Treatment with N-Ethylmaleimide. Washed erythrocytes at a 4% hematocrit were supplemented with various amounts of *N*-ethylmaleimide (NEM) and incubated for 6 min at 37 °C. Then, the suspension was centrifuged and washed twice before spin-labeling.

Treatment with DIDS. Washed erythrocytes were resuspended at a 20% hematocrit in buffer containing 10 μM DIDS.

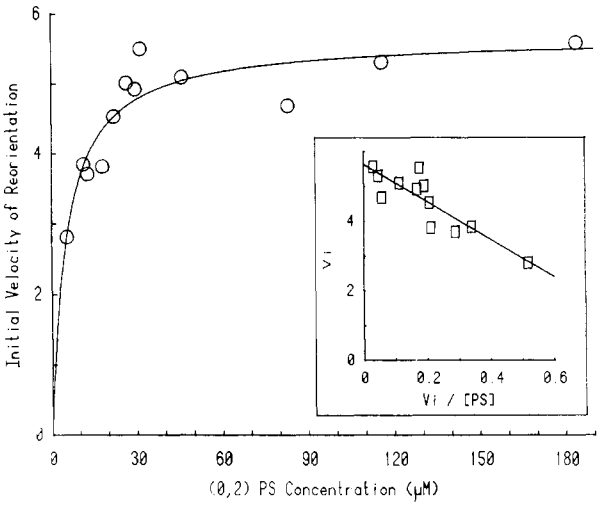


FIGURE 1: Initial rate of (0,2)PS reorientation as a function of the amount added to the erythrocyte membrane. The rate was expressed in nmol of (0,2)PS h^{-1} (mg of protein)$^{-1}$. The curve was drawn by using the Michaelis–Menten equation $v = V_m S(K_m + S)^{-1}$, where S is the probe concentration and V_m and K_m are the parameters deduced from the linearization. (Inset) Woolf–Hofstee plot of the data used to determine the kinetics parameters.

After 50 min at 37 °C, cells were submitted to a standard washing procedure and labeled. A control incubation without DIDS was run in parallel.

Control of Cytoplasmic Calcium Content. Erythrocytes were washed in a 132 mM NaCl, 13 mM KCl, 1 mM EGTA, and 9 mM Hepes buffer, pH 7.4. They were then incubated at a 10% hematocrit in the same buffer in presence of 10 μM A23187 ionophore (from a 500-fold concentrated solution in dimethyl sulfoxide). Various concentrations of $CaCl_2$ were added in order to control the free Ca^{2+} content: 0 mM (0 nM free), 0.5 mM (74 nM free), 0.7 mM (178 nM free), 0.9 mM (643 nM free), 0.95 mM (1.25 μM free), and 1 mM (7.1 μM free). The free concentrations were calculated by assuming an affinity constant of EGTA for Ca^{2+} under these conditions of 1.32×10^7 M^{-1}. After 10 min at 37 °C, cells were centrifuged and spin-labels added.

RESULTS

Concentration Dependence of Spin-Label Translocation Rates. We have studied, in red blood cells, the transbilayer diffusion at 4 °C of (0,2)PS, (0,2)PE, and (0,2)PC in the concentration range 6–180 μM, i.e., 0.2–6% of the total membrane phospholipids. In Figure 1, the initial velocity of the outside–inside translocation is plotted vs. (0,2)PS added. The initial velocity tends to an upper limit, and the graph seems to obey the Michaelis–Menten law. In order to get information on the maximum velocity (V_m) and the apparent affinity (K_m) of the reorientation, data were linearized according to the Woolf–Hofstee transformation.[2] This led to $V_m = 5.7 \pm 0.4$ nmol of (0,2)PS h^{-1} (mg of protein)$^{-1}$ and $K_m = 5.4 \pm 1.8$ μM. When these experiments were performed with (0,2)PE, the same type of conclusions were reached, and the parameter values were $V_m = 7.2 \pm 1.7$ nmol of (0,2)PE h^{-1} (mg of protein)$^{-1}$ and $K_m = 50.4 \pm 20.9$ μM. Note that these latter values were calculated by considering exclusively the solution concentration of spin-labeled aminophospholipids. The correction necessitated by the presence of endogenous aminophospholipids on the outer monolayer can be found under

[2] This transformation was chosen because the concentration range of the substrate was limited by technical constraints and under these conditions this linearization would have magnified departures from linearity.

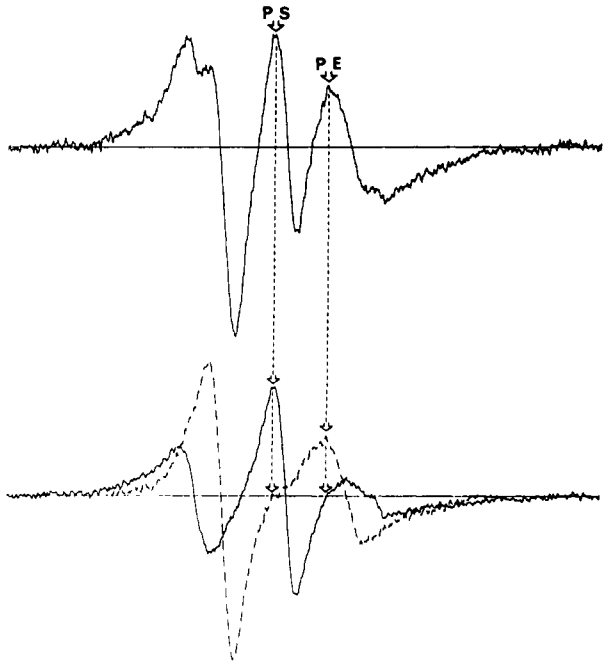


FIGURE 2: (Upper trace) ESR spectrum at 4 °C of a mixture of [^{15}N](0,2)PE and [^{14}N](0,2)PS embedded into the erythrocyte membrane. (Lower trace) ESR spectrum at 4 °C of [^{14}N](0,2)PS (solid line) or of [^{15}N](0,2)PE (dotted line) embedded individually into the membrane. The PS arrow indicates where the PS amount is determined in the spectrum and the PE arrow where the PE amount can be estimated.

Discussion. The influence of membrane concentration on the K_m values of this membrane-bound enzyme is also discussed later.

When (0,2)PC was under study, results were drastically different as no saturation could be evidenced. Instead, the initial velocity varied linearly with the analogue concentration [0.002 nmol of PC h^{-1} (mg of protein)$^{-1}$ μM^{-1}].

Simultaneous Reorientation of (0,2)PE and (0,2)PS, (0,2)PC, or (0,2)SM. Equimolar mixtures of [^{14}N](0,2)PS and [^{15}N](0,2)PE (each representing 1% of the total membrane phospholipids) were incorporated into the erythrocyte membrane.

The reorientation of each component of a pair of labels was followed at 4 and at 37 °C. At low temperature (Figure 2), the residual amount (after reduction) of [^{14}N](0,2)PS was estimated by the height of the upper portion of the middle field line (arrow PS) where no overlap of the ^{14}N signal exists. The amount of [^{15}N](0,2)PE was taken as the height of the upper part of the ^{15}N high-field line (arrow PE) as it can be seen that at this magnetic field the ^{14}N signal is close to the base line. At 37 °C, the residual amount of [^{14}N](0,2)PS was determined by the height of its middle-field line and the residual amount of [^{15}N](0,2)PE by the height of the lower part of its low-field line (Figure 3).

Figures 4 and 5 show the results of various competition experiments carried out with spin-labeled phospholipids. In these experiments, particular attention was given to the initial rates of phospholipid translocation, which can be inferred from the initial slopes of the curves in Figures 4 and 5. Figure 4A corresponds to the inward diffusion at 4 °C of (0,2)PS either alone (open circles) or in the presence of an equimolar amount of (0,2)PE (full triangles). Figure 4B corresponds to (0,2)PE alone (open circles), in the presence of (0,2)PC (full squares), or in the presence of (0,2)PS (full triangles). Figure 5 is a similar investigation carried out at 37 °C. At that temperature, the reduction proceeds spontaneously from the cytosolic side (Seigneuret et al., 1984).

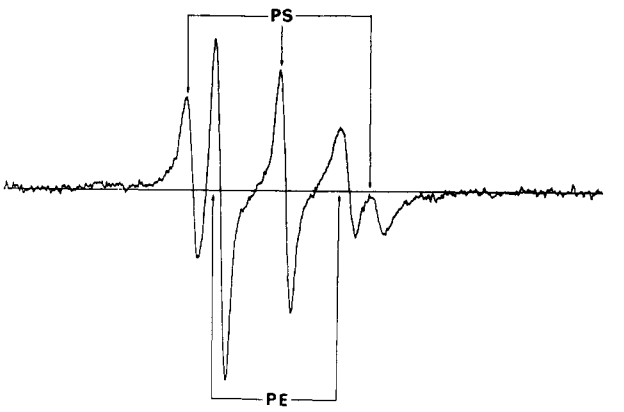


FIGURE 3: ESR spectrum at 37 °C of a mixture of [^{15}N](0,2)PE and [^{14}N](0,2)PS embedded into the erythrocyte membrane. The contributions of each phospholipid into this spectrum are indicated by the arrow manifolds.

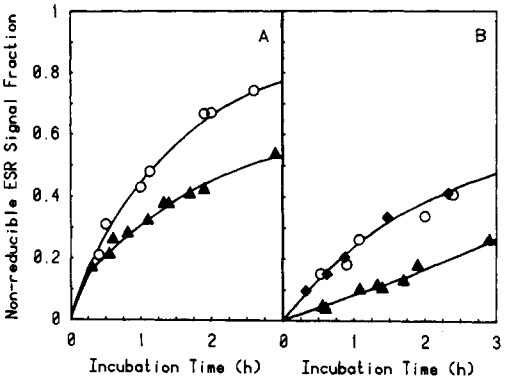


FIGURE 4: Nonreducible spin-label fraction as a function of the incubation time at 4 °C. Each data point corresponds to the level of the plateau of signal intensity reached within a few minutes after addition of 10 mM ascorbate. The incubation time is the time elapsed between addition of the label and addition of ascorbate. (A) (0,2)PS alone (○) or in the presence of an equimolar amount of (0,2)PE (▲). (B) (0,2)PE alone (○) or in the presence of an equimolar amount of (0,2)PC (♦) or of (0,2)PS (▲). Each spin-labeled lipid represents 1% of the total amount of membrane phospholipids.

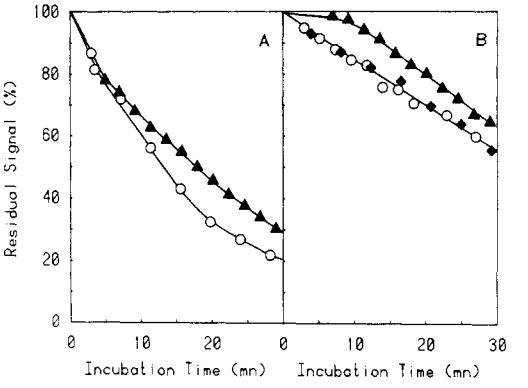


FIGURE 5: Spontaneous reduction at 37 °C of the signal arising from (0,2) phospholipids incorporated into erythrocyte membrane. Each data point is directly related to the actual signal intensity at the time indicated. For symbols used in panels A and B, see Figure 4.

From Figures 4 and 5 one can conclude that under these particular experimental conditions (i) the addition of spin-labeled PC has no effect on the translocation of (0,2)PE [the same conclusion is reached when SM is used instead of PC (not shown)], (ii) the addition of spin-labeled PE has no or little effect on the initial rate of PS translocation but seems to reduce the plateau, and (iii) the addition of PS has a drastic effect on the initial rate of PE translocation (which is reduced

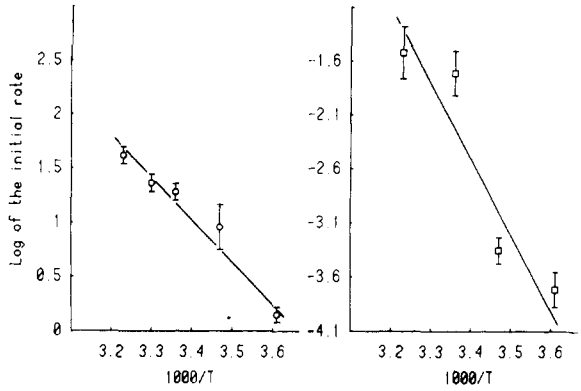


FIGURE 6: Temperature dependence of the intial rate of reorientation of (0,2)PS (left) or of (0,2)PC (right) plotted as an Arrhenius reciprocal plot. Rates were determined by measuring the amount of analogue present on the membrane inner leaflet at five different times after their incorporation into the membrane outer leaflet. These times were inferior to 20 min for PS and 90 min for PC.

to about a third of the initial value) and also probably reduces the plateau.

Reorientation Rates of (0,2)PS and (0,2)PC as a Function of Temperature. The evolution of PS analogue distribution between the membrane leaflets was studied within the 5–37 °C range by using resealed ghosts loaded with ATP. It was previously demonstrated (Seigneuret & Devaux, 1984) that the presence of ATP inside of the ghosts is a prerequisite to observe an aminophospholipid reorientation comparable to the one noticed in whole cells. Ghosts were used instead of intact erythrocytes since, even at 37 °C, they do not exhibit any significant reducing property over the experiment duration. The inital rate of reorientation was determined for both (0,2)PS and (0,2)PC, and the data were analyzed by an Arrhenius plot (Figure 6). For sake of simplicity, we used a single straight line to fit the data of both series of experiments. The energies of acitvation were respectively 7400 ± 900 and 13 000 ± 3400 cal mol^{-1} for PS and PC. The initial rate of (0,2)PE translocation was also measured at 37 °C. For comparison, at this temperature, the rates are respectively 20.9, 13.1, and 0.54 nmol of lipid transferred h^{-1} (mg of protein)$^{-1}$ for (0,2)PS, (0,2)PE, and (0,2)PC.

Influence of Various Cell Treatments on (0,2)PS Redistribution. (A) Incubation with N-Ethylmaleimide. Pretreatment of erythrocytes with increasing NEM concentrations led to a progressive inhibition of the fast transbilayer reorientation of the PS analogue (Figure 7). Above 1 mM NEM, the initial rate of PS translocation was only 5% of its initial value.

(B) DIDS. Binding of DIDS to band III of the erythrocyte membrane had no effect on the reorientation of (0,2)PS, which flipped toward the inner leaflet at a normal rate and accumulated up to 90% on this side of the membrane (not shown).

(C) Cytoplasmic Calcium Content (Figure 8). A calcium depletion of the erythrocyte did not affect the reorientation rate of (0,2)PS embedded into the membrane. However, increasing the cytoplasmic calcium concentration progressively inhibited the transbilayer redistribution of the analogue, which was almost abolished for free calcium concentrations of 1 μM or higher.

Chemical Stability of the Spin-Labeled Phospholipids during Transverse Diffusion. Each (0,2) phospholipid has been incubated at low concentration with ATP-containing ghosts, at 4 °C. After 3-h incubation, approximately 90% (0,2)PS and 60% (0,2)PE have flipped from the outer to the inner monolayer (see above). Lipids were then extracted and

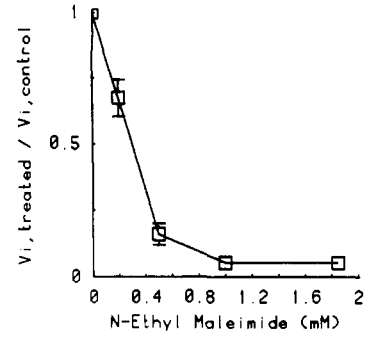


FIGURE 7: Changes in the initial rate (V_i) of phosphatidylserine reorientation following treatment of the erythrocytes by *N*-ethylmaleimide. Data were compared to the initial rate observed in cells that have experienced the same incubation protocol in absence of reagent. Initial rate is either determined graphically or obtained after fitting the data points with a single-exponential law.

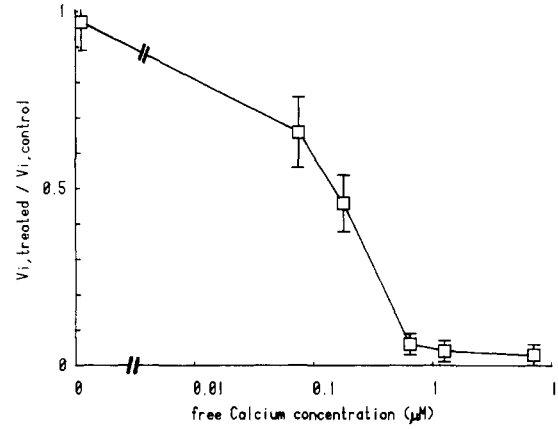


FIGURE 8: Evolution of the initial rate (V_i) of phosphatidylserine reorientation with the free calcium concentration of the cell cytoplasm. These concentrations were obtained by incubating erythrocytes loaded with A23187 ionophore in medium containing defined concentrations of EGTA and calcium ions.

purified on silica plates, and each spot was analyzed for paramagnetism. In all instances, we found that an ESR signal could be detected only in that lipid fraction associated with the spin-labeled phospholipid initially added to the ghost membranes. Thus, there is no evidence that the translocation process involves any chemical modification.

Discussion

In previous papers we have shown that spin-labeled phospholipids, after incorporation in erythrocyte membranes, distribute themselves among inner and outer monolayers according to the nature of their head group: phosphatidylcholine and sphingomyelin analogues stay preferentially on the outer layer while both amino lipid analogues (phosphatidylserine and phosphatidylethanolamine) flip to the inner monolayer (Seigneuret & Devaux, 1984; Zachowski et al., 1985). This selectivity, which enables the spin-labeled analogues to seggregate as endogenous phospholipids do, suggests that these spin-labeled lipids are good tools for the investigation of the mechanisms underlying the asymmetric distribution of lipids in red blood cells. This discussion will emphasize the differences between the various phospholipid analogues. Absolute numbers for translocation rates, apparent K_m, or V_m may be slightly different than the actual corresponding values for endogenous lipids: it will not affect our major conclusions, namely, that a specific protein is involved in the translocation of aminophospholipids.

Our original studies (Seigneuret & Devaux, 1984; Zachowski et al., 1985) suggested the involvement of a specific

process in the aminophospholipid flip-flop. We speculated that a protein, a specific translocase, was involved. The results obtained with NEM inhibition of translocation sustain this view. If the protein is specific, a restricted number of sites should be detected. Our studies of the reorientation rates in the presence of variable concentrations of probes show this to be the case. The rates obtained with (0,2)PS and (0,2)PE tend to an upper limit at high probe concentration. If the curve of Figure 1 and the corresponding curve for (0,2)PE (not shown) are interpreted as resulting from a Michaelian enzymatic activity, the apparent K_m for (0,2)PS is 9-fold lower than for (0,2)PE. On the other hand, the maximum velocities are close together, arguing for common reorientation sites for both aminophospholipids. Since the final distribution of PE and PS between the membrane halves should reflect the catalytic efficiencies (V_m/K_m) of inward and outward transport, our data on the inward process indicate that much of the asymmetric distribution can be explained by consideration of V_m/K_m for the two compounds. One could object that such a saturability of the transport rate might be that the quantity of exogenous lipids the erythrocyte can take is limited. Thus, the apparent transmembrane transport rate might reach a plateau because the substrate concentration has reached a plateau. But, as recalled previously in this paper, all the analogue added to the cell is incorporated rapidly (less than 3 min) into the membrane outer leaflet as shown by the ESR spectra. Thus, in the concentration range used herein, the substrate concentration in the membrane *does not* reach a plateau. Consequently, the plateau of the transmembrane rate is not related to any plateau in the substrate concentration. Besides, all three spin-labeled lipids incorporate with the same efficiency in the membrane; thus, a saturation related to a substrate concentration cannot explain the *selective* mutual inhibition observed.

The above constants were determined by considering only the labeled amino lipids. Since practically all endogenous phosphatidylserine molecules are located in the inner monolayer, the kinetics constants of PS can be reckoned indeed by using solely the concentration of spin-labeled PS. On the other hand for PE, it might be useful to take into consideration the fraction of endogenous phosphatidylethanolamine exposed on the outer layer, which, according to Verkleij et al. (1973), corresponds to approximately one-fifth of the total PE. This correction would shift the apparent K_m of PE to approximately 170 μM. In principle, an additional correction should be made due to the fact, demonstrated in this paper, that both amino lipids are transported by the same protein. However, this latter correction would be negligible for PS because of the low affinity of PE; as for PE, there is in reality no additional correction to be made because of the negligible amount of PS on the outer layer. Finally, the relative affinity of aminophospholipids for this translocase is given by the ratio of the effective K_m values, which turns out to be

$$K_m^{PS}/K_m^{PE} \simeq 1/34$$

It should be pointed out that only this latter number is really meaningful. Indeed, the K_m's calculated above would have different values if the experiments were performed at different membrane concentrations. This is because the binding between enzyme and substrate takes place in the plane of a membrane; reaction kinetics applicable to soluble molecules cannot be applied directly (Parry et al., 1976).

The assumption that PS and PE are transported by the same system is sustained by the reciprocal competition between (0,2)PS and (0,2)PE. In our experiments, each analogue was introduced at a concentration of 30 μM in the membrane. This represents about 5 times the K_m for (0,2)PS and half the K_m for (0,2)PE. Under these conditions, it was shown that (0,2)PS greatly affects (0,2)PE translocation and that (0,2)PE has only a slight effect on that of (0,2)PS. If it is assumed that for each analogue its inhibition constant is equal to its K_m and that these compounds are competitive inhibitors, the changes in the initial rates of reorientation at 4 °C can be explained by respective affinities for the systems of about 5.5 [(0,2)PS] and 30 μM [(0,2)PE]. These values are in good agreement with those determined from the saturation curve. If one takes into account the unlabeled PE, then it is not surprising that the addition of labeled PE has a moderate effect on the initial rate of translocation of labeled PS since the concentration of PE on the outer layer is increased only by $\simeq$25%. Nevertheless, the very clear effect of labeled PS on the rate of labeled PE translocation proves unambiguously that they compete for the same site. A simple calculation (as above) taking into account the unlabeled aminophospholipids leads to respective affinities of ca 4.2 (PS) and 114 μM (PE), values comparable again to those deduced from the saturation curves.

As for PC, its lack of effect on PE reorientation is consistent with the fact that it simply diffuses from one monolayer to the other. Similarly, SM does not affect the PE transmembrane translocation.

A basic assumption for our model is the absence of lateral seggregation of the phospholipids in the plane of the outer monolayer. No data argue for such a phenomenon. On the contrary, ESR spectrum analysis favors a homogeneous distribution of all the phospholipids in the outer monolayer. Indeed, ESR spectra arising from (0,2)PC, (0,2)SM, (0,2)PE, and (0,2)PS incorporated in the outer half of the membrane are identical (Seigneuret et al., 1984; Zachowski et al., 1985).

Competition experiments carried out at 37 °C confirm that PS and PE share the same system of reorientation. However, no constants can be drawn from the spontaneous reduction at high temperature, as they reflect not only the presence of the probe on the inner membrane half but also the reduction rate of the nitroxide by the cytoplasmic content, which in some case may be the limiting step (Seigneuret et al., 1984). To avoid this difficulty, resealed ghosts must be used to study the reorientation as a function of the temperature. These ghosts were prepared in the presence of Mg^{2+} as the only divalent cation and of EGTA to chelate any Ca^{2+} ions. Under these conditions, it was established that the ghosts retained the normal phospholipid asymmetry characteristic of intact erythrocytes (Williamson et al., 1985). With such systems, we found that (0,2)PS always accumulates on the membrane inner leaflet (90%) and that (0,2)PC remains predominantly on the outer one (70%). These equilibrium situations are close to the ones found in the intact erythrocyte membrane by phospholipase treatment (Verkleij et al., 1973; Op den Kamp, 1979). They are also in good agreement with the values found by Bergmann et al. (1984) using lysoderivatives as phospholipid analogues. The activation energy for PS reorientation is approximately half that for PC. This difference argues in favor of two different mechanisms for phospholipid translocation. A similar difference was also reported by Bergmann et al. (1984); however, the values given by these authors were somewhat higher than ours. The different chemical nature of the analogues used to monitor the reorientation (respectively, lysoderivatives and spin-labeled phospholipids) is probably the cause of this discrepancy. This remark holds when the characteristic times of the phenomenon are compared: the latter constants are roughly 20-fold lower in our hands but the ratio PS constant/PC constant is in both cases approximately 3.

Under the experimental conditions, *N*-ethylmaleimide reacts principally with three membrane proteins, namely, spectrin, band 3, and band 4.2 (Haest et al., 1978). In order to test a possible involvement of the anion carrier (band 3) in the phospholipid flip-flop, the reorientation of (0,2)PS was studied in cells treated by DIDS in such conditions that the majority of band 3 has reacted (Lepke et al., 1976). This did not affect (0,2)PS diffusion, ruling out that band 3 is the aminophospholipid translocase.

When the erythrocyte was calcium-depleted by addition of the ionophore A23187 and suspension of the cells in an EGTA-containing buffer, (0,2)PS reorientation remained comparable to what it is usually. Thus, the transport of the lipid through the bilayer is not controlled, under physiological conditions, by the calcium present within the cell. When the free cytoplasmic calcium was increased, a progressive fall in the rate at which PS is translocated was evidenced. This could be the consequence of the ATP loss in the cell due to Ca^{2+}-ATPase stimulation by calcium ions as the curve of Figure 8 closely resembles the one displaying the ATP concentration after a very similar treatment [Figure 2 of Taylor et al. (1977)]. Moreover, Willamson et al. (1985) reported that treatment of intact erythrocytes with ionophore and millimolar levels of Ca^{2+} (i.e., similar to the more drastic conditions used here) abolished the membrane phospholipid asymmetry. The inhibition of the aminophospholipid translocase might be one of the clues of this phenomenon.

Haest, Deuticke, and their collaborators (Haest et al., 1978, 1980; Bergmann et al., 1984; Dressler et al., 1984) attribute the asymmetry of phospholipid distribution to the interaction of phosphatidylserine and phosphatidylethanolamine with the membrane skeleton. However, we have shown previously that the initial rate of (0,2)PS translocation from the outer to the inner monolayer increases with ATP concentration inside the cell. This does not seem compatible with a mere stabilization from within the cell; rather, it implies an active transport through the membrane. In addition, data reported here show that these aminophospholipids do not flip by diffusion, as they exhibit a lower activation energy than phosphatidylcholine that diffuses passively. Thus, the reorientation of aminophospholipids must use another route to be established. This does not preclude all interactions between lipids and the cytoskeleton, which secondarily might stabilize the asymmetry.

Finally, there is evidence in the literature of lipid metabolism in the human red cell membrane (Mulder & Van Deenen, 1965; Marinetti & Cattieu, 1982); some authors have concluded that lipid metabolism could be involved in the selective translocation of phospholipids (Hirata & Axelrod, 1978; Ferrell & Huestis, 1984). Such mechanisms may indeed concern a fraction of the phospholipid redistribution in the erythrocyte membrane. We have shown that although some hydrolysis of the spin-labeled lipids can be detected (Seigneuret et al., 1984), the major fraction of the spin-labels used in this study preserves its chemical identity after translocation. Thus, it seems unlikely that the putative aminophospholipid translocase could be a metabolic enzyme.

Recently, Daleke and Huestis published an article showing that the selective transport of long-chain aminophospholipids from the outer to the inner monolayer is an ATP-dependent process in erythrocytes (Daleke & Huestis, 1985).

Registry No. Calcium, 7440-70-2; aminophospholipid translocase, 101077-55-8.

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