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Aminophospholipid translocase in the plasma membrane of Friend erythroleukemic cells can induce an asymmetric topology for phosphatidylserine but not for phosphatidylethanolamine

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The ATP-dependent translocation of phospholipids in the plasma membrane of intact Friend erythroleukemic cells (FELCs) was studied in comparison with that in the membrane of mature murine erythrocytes. This was done by following the fate of radiolabeled phospholipid molecules, previously inserted into the outer monolayer of the plasma membranes by using a non-specific lipid transfer protein. The transbilayer equilibration of these probe molecules was monitored by treating the cells – under essentially non-lytic conditions – with phospholipases A_2 of different origin. Rapid reorientations of the newly introduced aminophospholipids in favour of the inner membrane leaflet were observed in fresh mouse erythrocytes; the inward translocation of phosphatidylcholine (PC) in this membrane proceeded relatively slow. In FELCs, on the other hand, all three glycerophospholipids equilibrated over both halves of the plasma membrane very rapidly, i.e. within 1 h; nevertheless, an asymmetric distribution in favour of the inner monolayer was only observed for phosphatidylserine (PS). Lowering the ATP-level in the FELCs caused a reduction in the rate of inward translocation of both aminophospholipids, but not of that of PC, indicating that this translocation of PS and phosphatidylethanolamine (PE) is clearly ATP-dependent. Hence, the situation in the plasma membrane of the FELC is rather unique in a sense that, though an ATP-dependent translocase is present and active both for PS and PE, its activity results in an asymmetric distribution of PS, but not of PE. This remarkable situation might be the consequence of the fact that, in contrast to the mature red cell, this precursor cell still lacks a complete membrane skeletal network.

Introduction

An asymmetric distribution of the different phospholipid classes over both halves of a plasma membrane is nowadays recognized as a phenomenon that is generally observed in numerous types of normal and healthy cells. This asymmetry is characterized by the two choline-containing phospholipids (phosphatidylcholine (PC) and sphingomyelin) dominating the outer membrane leaflet, whereas the aminophospholipids (phosphatidylethanolamine (PE) and phosphatidylserine (PS)) and phosphatidylinositol are largely confined to the cytoplasmic half of the bilayer. Although the existence of phospholipid asymmetry in the red cell membrane was already established in the early seventies (see Ref. 1 for review), the processes that are involved in the maintenance of this phenomenon are not fully understood.

Although still being subject to much debate and controversial opinions, the following two mechanisms are thought to be responsible for the maintenance of phospholipid asymmetry in the red cell membrane: (i) interaction of the aminophospholipids with proteins from the membrane skeleton [2–5], and (ii) an ATP-dependent translocation of the aminophospholipids from the outer towards the inner monolayer of the membrane [6–11].

Previously, several reports have indicated a specific, though probably rather weak [12], interaction between

Abbreviations: FELC, Friend erythroleukemic cell; ns-LTP, non-specific lipid transfer protein; Pal-116-AMPA, N^{ϵ}-palmitoyl-Lys116 ε-amidinated phospholipase A_2.

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spectrin and PS and PE [4,13–15]. More recently, however, also interactions between PS and band 4.1 have been reported [16–19]. Furthermore, it has been shown that any interference in this interaction between aminophospholipids and skeletal proteins results in an increase in membrane phospholipid mobility, as detected by an increased PC 'flip-flop' rate [20–23]. From these studies it has been concluded that the membrane skeleton is essential for the stabilization of the lipid bilayer, and that it exerts this function through the interaction with aminophospholipids in the inner membrane leaflet.

The second mechanism, an ATP-dependent translocation of aminophospholipids, was discovered by Devaux and co-workers [6] and, since then, has been shown to exist in the membranes of a number of cell types other than the human erythrocyte [24–26].

Next to the question of its maintenance, the biogenesis of phospholipid asymmetry in the red cell membrane during erythropoiesis is another important issue, which has been addressed recently in comparative studies on FELCs and mature murine erythrocytes [27,28]. Though not yet as absolute as in the membrane of the mature erythrocyte, a clearly asymmetric distribution in the plasma membrane of the FELC was observed for PS, of which 80–85% was assigned to be present in the inner monolayer. PE, on the other hand, was, similar to PC, still randomly distributed over both leaflets of the plasma membrane of the precursor cell. As it is known that at this early stage of erythropoiesis some components of the membrane skeleton are not yet synthesized [29,30], which makes the presence of a completely assembled membrane skeleton impossible, one may wonder whether this could play a role in the fact that phospholipid asymmetry is only partly established in this plasma membrane. An additional, or even alternative possibility to consider is that the ATP-dependent translocase functions less efficient (for PS) or not at all (for PE) in the plasma membrane of the FELC. The present studies were undertaken to elucidate this aspect.

Materials and Methods

Chemicals

1,2-[^{14}C]Dioleoylphosphatidylcholine, 1,2-dioleoylphosphatidyl[^{14}C]serine and 1,2-dioleoylphosphatidyl-[^{14}C]ethanolamine were purchased from Amersham International, U.K. Cholesterol was obtained from Merck (Darmstadt, F.R.G.). Egg phosphatidylcholine, egg phosphatidic acid, bee venom phospholipase A_2 and *Naja naja* phospholipase A_2 were from Sigma (St. Louis, MO). Sphingomyelinase C was purified from *Staphylococcus aureus* cultures by the method described by Zwaal et al. [31]. Pal-116-AMPA (N^{ε}-palmitoyl-Lys-116 ε-amidinated phospholipase A_2, a chemically modified pig pancreatic phospholipase A_2 containing a palmitic acid residue at position 116) was synthesized according to the procedure described by Van der Wiele et al. [32]. Culture media were from Flow laboratories, Scotland. A constant light signal ATP-determination kit was from Boehringer, Mannheim. All other chemicals were of analytical grade.

Experiments with mature red cells

Erythrocytes. Mouse blood was obtained by decapitation of BALB/c mice and collected in acid/citrate/dextrose. After centrifugation (1500 × *g*, 5 min) erythrocytes were isolated, washed three times and resuspended in the following buffer: 280 mM sucrose, 10 mM NaCl, 20 mM glucose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4), referred to as 'sucrose buffer' throughout [33].

Donor lipid vesicles. Vesicles were prepared from equimolar amounts of phospholipid (egg PC and egg PA in a molar ratio of 10 : 1) and cholesterol, with trace amounts of ^{14}C-labeled dioleoyl-PC, -PS and -PE. The lipids were dried under nitrogen and dispersed into sucrose buffer. Subsequently, the mixture was sonicated under nitrogen and on ice (Branson sonifier, 65W, 10 min) and centrifuged at 19000 × *g* for 10 min to remove any titanium particles. The final phospholipid concentration in the vesicle suspension was about 0.20 mM. The total radioactivity was 0.15 μCi per ml.

Transfer protein. A non-specific lipid transfer protein (ns-LTP) from bovine liver was partially purified, essentially as described in Ref. 34. Before use, the protein was dialyzed extensively against sucrose buffer, to remove glycerol. The final protein concentration was 1 mg/ml.

Incorporation of radioactive phospholipids into erythrocytes. Erythrocytes were incubated at 30% hematocrit in the presence of ns-LTP and donor vesicles for 30 min at room temperature, using a clinical blood rotator operated at 4 rpm. The molar ratio of erythrocyte phospholipid to vesicle phospholipid was about 20 to 1. Per 1 ml of packed cells 1.2 mg of transfer protein was added. Under these conditions, the maximum incorporation of exogenous phospholipid did not exceed 1% of the red cell phospholipid and did not cause any changes in the discoid morphology of the cells.

Determination of transbilayer distribution of radioactive phospholipids in mouse red cells. After incorporation of the radiolabeled phospholipids the cells were washed twice with buffer A (90 mM KCl, 45 mM NaCl, 44 mM sucrose, 20 mM glucose and 10 mM Tris-HCl (pH 7.4), also containing 10^5 IU/l penicillin and 10^5 μg/l streptomycin) to remove the transfer protein and the excess of vesicles.

Subsequently, the cells were resuspended in the same buffer at 3.3% hematocrit and incubated at 37°C. At timed intervals, samples were taken, the cells were spun down (1500 × *g*, 5 min) and resuspended in buffer B (90 mM KCl, 45 mM NaCl, 22 mM sucrose, 10 mM glu-

cose, 10 mM $CaCl_2$, 0.25 mM $MgCl_2$ and 30 mM Hepes (pH 7.4)), again at 3.3% hematocrit. Phospholipase digestion was started by the addition of 10 IU phospholipase A_2 (bee venom) plus 10 IU phospholipase A_2 (*Naja naja*) or 10 IU (20 μg) Pal-116-AMPA. Phospholipase A_2 incubations were terminated after 60 min (37°C) by centrifugation (5 min at $1500 \times g$), followed by the addition of 200 μl 100 mM EDTA in buffer A. Pal-116-AMPA incubations were terminated in the same manner after 5–10 min incubation at 37°C. Erythrocyte lipids were extracted according to Rose and Oklander [35], separation and quantification of the phospholipid classes were achieved by two-dimensional thin-layer chromatography on precoated silica plates (Merck, Darmstadt), using the developing solvents as described by Broekhuyse [36], followed by either a phosphate determination [37] or radioactivity measurements using a Packard Prias TriCarb scintillation counter with 299 TM emulsifier from Packard as scintillation fluid.

Red cell lysis. After the various treatments the extent of cell lysis was determined by measuring the release of hemoglobin from the cells at 418 nm in a $1500 \times g$ (5 min) supernatant and comparing this with a completely lysed preparation. Cell lysis did not exceed 10%.

Experiments with FELCs

Friend erythroleukemic cells. Murine erythroleukemic cells, derived from clone 745 A, were grown in polyethylene Costar bottles (150 cm^2) containing 50 ml Leibovitz L15 medium, supplemented with 10 mM Hepes, 10% foetal calf serum and 15 mM sodium bicarbonate, in an incubator at 37°C, 95% humidity and 5% CO_2. Cells were harvested when a bottle contained approx. $(1–2) \cdot 10^6$ cells/ml. Cells were collected by centrifugation at $480 \times g$ for 10 min and washed three times with sucrose buffer. Cells were resuspended in sucrose buffer at a concentration of about $6 \cdot 10^7$ cells/ml, this was achieved by gently pumping the buffer up and down a Pasteur pipet.

Donor lipid vesicles. Vesicles consisted of cholesterol, egg PC and egg PA in the ratio 5.6 : 9.4 : 1, with trace amounts of ^{14}C-labeled dioleoyl-PC, -PS and -PE. The vesicles were prepared as described above. The final phospholipid concentration in the suspension was approx. 0.19 mM, the radioactivity was 0.15 μCi per ml.

Incorporation of radioactive phospholipids into Friend cells. Approximately $6 \cdot 10^7$ cells were incubated in the presence of ns-LTP and donor vesicles for 30 min at room temperature using a clinical blood rotator operated at 4 rpm. The molar ratio of Friend cell phospholipid to vesicle phospholipid was 35 to 1, thereby bringing the ratio of plasma membrane phospholipid to vesicle phospholipid to 13 to 1. About 0.8 mg of transfer protein was added per $6 \cdot 10^7$ cells. Under these conditions approx. 10% of the vesicle phospholipid was incorporated in the Friend cells after 30 min of incubation, this represents less than 0.8% of the plasma membrane phospholipid.

In experiments involving FELCs with a reduced ATP-content, cells were preincubated at 37°C in a glucose-free sucrose buffer containing 10 mM NaF. After an appropriate time of preincubation the cell suspension was supplemented with the transfer protein and the above described donor vesicles which, in these cases, had been prepared in glucose-free sucrose buffer.

Determination of transbilayer distribution of radiolabeled phospholipids in Friend cells. Essentially, the transbilayer distribution of the radioactive phospholipids was determined as described for mouse red cells. Cells were incubated for either 7 min with Pal-116-AMPA (10 IU per $6 \cdot 10^7$ cells) or 60 min with bee venom phospholipase A_2 (15 IU per $6 \cdot 10^7$ cells). In some experiments, sphingomyelinase C (5 IU per $6 \cdot 10^7$ cells) was added to suspensions that had been incubated for 60 min with bee venom phospholipase A_2 and the incubation continued for another 60 min at 37°C.

Lysis of Friend cells. The extent of cell lysis caused by the various treatments was determined by measuring the amount of released lactate dehydrogenase (LDH) (EC 1.1.1.27) in a $760 \times g$ supernatant (10 min) and by comparing this value with that determined in a 10% dilution of a completely lysed cell sample. The reaction mixture for the LDH-test was composed of 42 mM potassium phosphate buffer (pH 7.6), 0.9 mM sodium pyruvate, 0.2 mM NADH and 0.1% Triton X-100. A sample of 0.2 ml of the supernatant was added to 1.0 ml of the reaction mixture and the total volume was adjusted to 3.0 ml. The rate of NADH oxidation was determined by monitoring the change in absorbance at 340 nm for 2 min. Cell lysis never exceeded 15%.

ATP assay. ATP was measured using the constant light signal ATP-kit from Boehringer. Cell suspensions were diluted 10 times in water and kept on ice for up to 1 hour. Samples of 50 μl were added to 50 μl of luciferin/luciferase mixture immediately followed by the determination of the activity in a CA 2000 TriCarb scintillation counter (Packard) using a single photon counting program. The actual ATP concentration in the sample was determined by comparing its light signal with that generated by an ATP calibration solution. Concentrations as low as $5 \cdot 10^{-9}$ M could be detected.

Results

The transbilayer reorientation of radiolabeled phospholipids previously incorporated into the outer membrane leaflet of murine erythrocytes is shown in Fig. 1. The fraction of the radiolabeled phospholipids that could be hydrolysed in the intact cells by exogenous phospholipase A_2 is interpreted to represent that fraction of these phospholipids that was still present in the

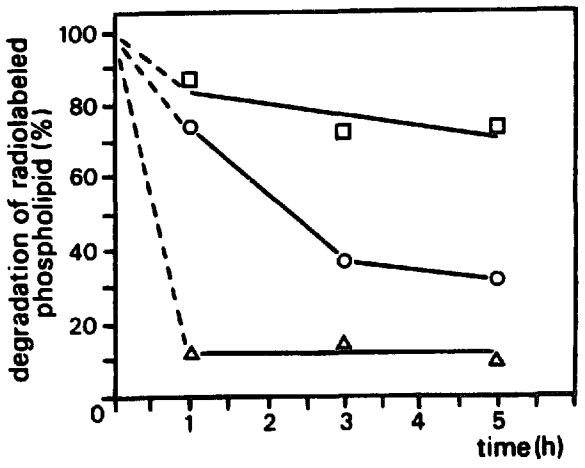

Fig. 1. Transbilayer reorientation of radiolabeled glycerophospholipids, previously inserted into the outer membrane leaflet of intact murine erythrocytes. Ns-LTP-mediated incorporation of probe molecules started at zero time. Treatment of intact cells with phospholipases A_2 was started at the time points indicated. The percentages of PC (□), PE (○) and PS (△) that could be degraded this way were assigned to represent those fractions of the probe molecules still present in the outer membrane leaflet. The figure shows the results of a typical experiment, using Pal-116-AMPA, out of a series of five entirely independent experiments.

outer monolayer. Either the naturally occurring phospholipases A_2 from *Naja naja* and bee venom, or the chemically modified pig pancreatic phospholipase A_2, Pal-116-AMPA, have been used in these experiments. The obvious advantage of Pal-116-AMPA over the other two enzymes is that, because of its hydrophobic anchor in the form of the palmitoyl moiety, it has a considerably enhanced membrane penetrating capacity. This feature enables one to achieve a more complete hydrolysis of substrates in the outer leaflet of intact erythrocyte membranes during relatively short incubation times, i.e. 5 to 10 min [38]. Moreover, the chemical modification does not affect the substrate specificity of this enzyme which typically favors negatively charged glycerophospholipids such as PS [32].

Similar to the situation previously observed in human erythrocytes [10], PS reached its stationary transbilayer distribution in the mouse erythrocyte, in which as little as some 10% of the label remained accessible to exogenous phospholipase A_2, within one hour after its insertion into the outer monolayer (Fig. 1). Though appreciably slower, an asymmetric transbilayer distribution in favour of the inner monolayer was also reached by PE. Up to 70% of the labeled PE accumulated in the cytoplasmic leaflet in 5 h at 37°C (Fig. 1), which distribution reasonably resembles that of the endogenous PE in this membrane [28]. Also in similarity with the human red blood cell is the observation that the transbilayer reorientation of PC proceeded very slowly. It should be kept in mind, however, that, in contrast to the situation in the human red cell membrane, the asymmetric distribution of the endogenous PC in the murine erythrocyte is much less pronounced, as only 55–60% of it is present in the outer monolayer [28]. Clearly, this distribution was not yet reached during the five hours that passed after the introduction of the labeled PC into the outer membrane leaflet (Fig. 1). Quite a different situation appeared to exist in the plasma membrane of FELCs. Radiolabeled glycerophospholipids were inserted into the outer monolayer of the plasma membrane of those cells in a way similar to that applied in case of the mature erythrocyte (see Materials and Methods), their fate again being followed by subsequent treatment of the intact cells (at timed intervals) with phospholipase A_2. The results of a typical experiment, involving hydrolysis by Pal-116-AMPA, are shown in Fig. 2. Similar as in the membrane of the mature erythrocyte (compare Fig. 1), the ultimate transbilayer distribution of the radiolabeled PS, which closely resembled the distribution of the endogenous PS in this membrane (15–20% of the PS in the outer monolayer [28]), was reached within 1 h after its insertion into the exofacial leaflet. Quite interestingly, however, an almost instantaneous, i.e. within 1 h, equilibration over both membrane leaflets was now also observed for PE (Fig. 2), an equilibration which truely reflects the symmetric distribution of this phospholipid known to occur in the plasma membrane of the FELC [28]. Furthermore, it appeared that the radiolabeled PC reached its (also symmetric [28]) equilibrium distribution in the plasma membrane after as little as 3 h (Fig. 2), which implies a considerably enhanced transbilayer mobility of this phospholipid when compared to that in the membrane of the mature cell.

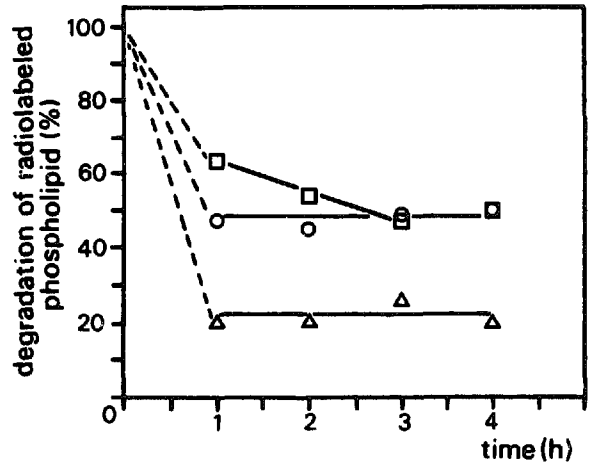

Fig. 2. Transbilayer reorientation of radiolabeled glycerophospholipids, previously inserted into the outer leaflet of the plasma membrane of FELCs. Ns-LTP-mediated incorporation of probe molecules started at zero time. Treatment of intact cells with phospholipases A_2 was started at the time points indicated. The percentages of PC (□), PE (○) and PS (△) that could be degraded this way were assigned to represent those fractions of the probe molecules still present in the outer membrane leaflet. The figure shows the results of a typical experiment, using Pal-116-AMPA, out of a series of 9 entirely independent experiments.

The above studies on FELCs were performed using either: (i) bee venom plus *Naja naja* phospholipase A_2, (ii) these two enzymes in combination with sphingomyelinase C (to ensure a complete degradation of the substrates in the outer monolayer by the phospholipases A_2 [31]), or (iii) Pal-116-AMPA in, respectively, 5, 1 and 3 independent experiments. The results obtained in all of those cases are essentially similar to those depicted in Fig. 2. It is also worth noting that all of the radioactivity that was introduced into the FELCs via the probe molecules, was recovered from the same diacylglycerophospholipid fractions plus the corresponding 1-acyl lyso-derivatives that had been produced from them by the action of the phospholipase(s) A_2. This indicates that under the conditions and in the time scale of these experiments no probe molecules had been metabolized into other lipid components.

To study whether the rapid inward translocation of (amino)phospholipids in the plasma membrane of the FELC was ATP-dependent, as it is in mature erythrocytes and (at least) a number of other cells [24–26], similar experiments were performed with FELCs, previously depleted of their ATP. It appeared, however, that this could not be achieved by starvation of the cells, due to severe cell lysis during prolonged incubations in a glucose-free buffer. This problem could be overcome by incubating the cells in the presence of 10 mM (sodium)fluoride, an anion known to inhibit glycolysis. As ATP-consumption in a metabolically active cell as the Friend cell is usually rather high, it took only 60 min of incubation in the presence of NaF to lower the ATP-content of the cell to approx. 5% of the original level (Fig. 3). But also in control cells, incubated at 37 °C in a buffer containing 10 mM glucose, the ATP-level appeared to drop after 30 min and was reduced during the next 30 min by almost 50% (Fig. 3). Providing the system with additional glucose simply prevented a further drop in cellular ATP (Fig. 3), indicating that the observed fall in ATP had been caused by a shortage of this energy source.

FELCs were treated with 10 mM NaF for various (short) periods of time, prior to incorporation of the radiolabeled phospholipids into the outer leaflet of their plasma membrane. After insertion of the labeled compounds and subsequent removal of the ns-LTP and donor vesicles, cells were immediately exposed to the action of Pal-116-AMPA, which incubation was terminated after 7 min. Fig. 4 shows the relative fractions of each of the three radiolabeled phospholipids that were degraded this way in intact FELCs containing different levels of ATP. It is quite obvious that the extent to which either PE or PS were accessible to the Pal-116-AMPA, representing those fractions of the probe molecules still present in the outer membrane leaflet, is inversely related to the relative amount of ATP in the cell. In other words, the inward translocation of both aminophospholipids slowed down as the ATP-content of the cells was reduced. Interestingly, changes in the ATP-content of the cells did not at all affect the relatively rapid equilibration of the radiolabeled PC over both halves of the bilayer (Fig. 4).

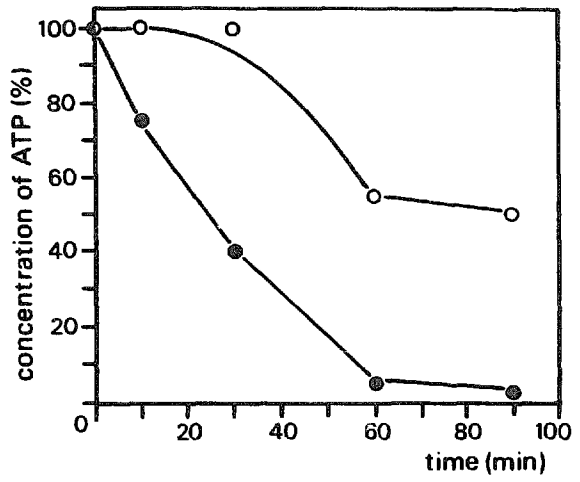

Fig. 3. Time dependence of the ATP-content of FELCs incubated in the presence of 10 mM NaF in glucose-free sucrose buffer (●). Control cells (○) were incubated in glucose-containing sucrose buffer without NaF. After 60 min, control cells were supplemented with additional glucose (10 mM). ATP-levels were determined by the luciferin/luciferase method as described under Materials and Methods (p. 243).

From the data presented in Fig. 4 one can easily calculate the relative apparent rate at which the individual phospholipid classes were translocated from the

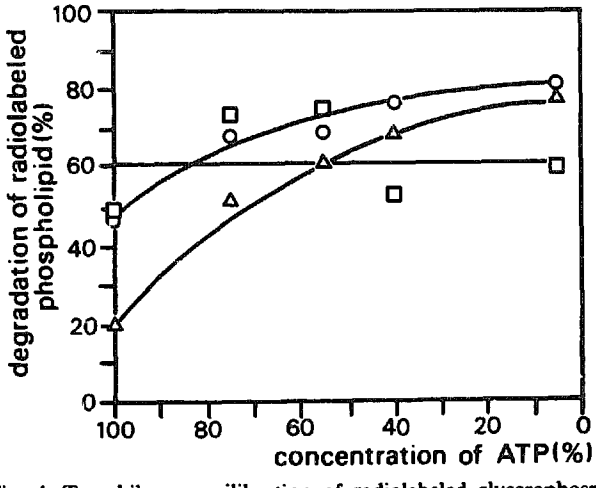

Fig. 4. Transbilayer equilibration of radiolabeled glycerophospholipids, newly introduced into the plasma membrane of FELCs in relation to the ATP-content of the cells. ATP-levels had been reduced by incubating the cells in the presence of 10 mM NaF, and are expressed as percentage of the ATP-content of FELCs before addition of NaF. After subsequent incorporation of the probe molecules and appropriate washes (see Materials and Methods), cells were immediately treated with Pal-116-AMPA for 7 min at 37 °C. Percentages of PC (□), PE (○) and PS (△) that could be degraded this way were assigned to represent those fractions still present in the outer membrane leaflet.

TABLE I

Relative apparent rate for the inward translocation of radiolabeled phospholipids in the plasma membrane of FELCs in relation to their ATP content

ATP content * (%)	Relative apparent rate of inward translocation		
	PC	PE	PS
100	1.00	1.00	1.00
75	0.54	0.61	0.62
55	0.50	0.59	0.48
40	0.95	0.45	0.40
5	0.80	0.37	0.29

* ATP levels are expressed as percentage of those in untreated control cells.

outer towards the inner monolayer of the plasma membrane of FELCs containing different amounts of ATP (Table I). It appears that the inward translocations of PE and PS in this membrane were equally dependent on the ATP-content of the cells, whereas that of PC did not show any correlation with this parameter.

Discussion

Recently, comparative studies involving both FELCs and mature murine erythroctyes have been performed to gain some insight into the biogenesis of the marked asymmetric distribution of phospholipids over the red cell membrane [27,28]. These studies showed that, in contrast to the situation in the membrane of mature mouse erythrocytes, which appeared to be essentially identical to that in human erythroctes, there is no asymmetry in the distribution of PE in the plasma membrane of the FELC, whilst only 80% (versus 100% in the mature erythrocyte) of the PS is found in the inner leaflet.

During the last few years, ample experimental evidence has been obtained to show that the erythrocyte membrane is equipped with an ATP-dependent translocase that plays a major role in maintaining the asymmetric distribution of both aminophospholipids in this membrane [6–11,39,40]. It has even been stated that, in addition to this energy-dependent translocation system, no other factors or mechanisms are needed to maintain the asymmetry [39]. Therefore, the present study was undertaken to determine whether the lack of any asymmetry in the transbilayer distribution of PE, and the presence of only a partial asymmetry for PS, in the plasma membrane of the FELC could be the consequence of an impaired functioning of the ATP-dependent aminophospholipid translocation system in this membrane.

The ns-LTP from bovine liver was used to incorporate trace amounts of the radiolabeled phospholipids (PC, PE and PS) into the outer membrane layer of mature murine erythrocytes, as well as into the plasma membrane of FELCs. Essentially identical conditions were applied as had been used in a previous study [10], which avoid the occurrence of appreciable changes in lipid composition, or even total lipid content, of the plasma membranes.

The transbilayer equilibration of each of the three radiolabeled glycerophospholipids in the membrane of fresh, ATP-containing, mouse erythrocytes (Fig. 1) proceeds similar to that previously observed for human red cells [10], be it that the translocation of the PC in the murine cell seems to be somewhat faster. Whether this difference is due to the fact that, in contrast to the situation in the human red cell, more of the endogenous PC is located in the inner monolayer [28], and/or the PC in the murine cell experiences a transbilayer movement that is faster than that in the human erythrocyte membrane, cannot be answered at present. Important to note is, however, that the transbilayer equilibration of the PC in the membrane of the murine erythrocyte is still very much slower than that of the two aminophospholipids. There are no reasons to assume that the inward translocation of PE and PS in the murine red cell membrane would proceed by a mechanism different from that in the human erythrocyte. Unfortunately, however, definitive proof of its ATP dependence could not be obtained, as murine erythrocytes showed considerable lysis upon ATP depletion.

The translocation of the radiolabeled phospholipids in the plasma membrane of the FELC (Fig. 2) showed some marked differences when compared to that in the mature mouse erythrocyte (Fig. 1). The ultimate transbilayer equilibration of the probe molecules in the plasma membrane of the FELC, reflecting the distribution of the corresponding endogenous phospholipid, was reached within one hour after their insertion, not only for PS, but also for PE. Even the reorientation of the radiolabeled PC in the FELC (Fig. 2) was considerably enhanced when compared to that in the red cell membrane (Fig. 1), and reached its equilibrium distribution within as little as 3 h. This very rapid equilibration of PC indicates that, again in contrast to the situation in the membrane of the red cell, PC experiences rapid transbilayer movements in the plasma membrane of the FELC. This is at variance with previously published data derived from PC-exchange experiments [28], for which discrepancy we have no plausible explanation yet. On the other hand, a markedly enhanced transbilayer mobility of PC in the plasma membrane of the precursor cell, which not yet contains a completed skeletal protein network, is in accordance with previous studies on sickled erythrocytes [22], hereditary pyropoikilocytes [21] and tetrathionate- or diamide-treated red cells [20], which invariably showed that a disturbance in the interaction of the lipid bilayer with the

membrane skeleton was accompanied by a pronounced acceleration of PC flip-flop in the bilayer.

Experiments with FELCs containing reduced levels of ATP showed a positive correlation between this parameter and the relative rate of inward translocation of both radiolabeled aminophospholipids (Fig. 4, Table I). The data suggest that the ATP-dependence of the translocation processes for PS and PE are very similar. However, even when the ATP content of the FELCs was as low as 5% of its original level, the radiolabeled PC still exhibited a very rapid transbilayer reorientation, as it almost reached its equilibrium distribution in the plasma membrane within one hour after its insertion. Hence, it can be concluded that, in obvious contrast with the passive (but fast) transbilayer equilibration of PC in the plasma membrane of the FELCs, the inward translocation of both aminophospholipids in this membrane is mediated by an ATP-dependent translocase.

Comparing the data for PE as shown in Fig. 2 with those in Fig. 1, it will be clear that the radiolabeled PE introduced into the plasma membrane of the FELC reached its ultimate distribution over both halves of the bilayer significantly faster than when inserted into the membrane of the mature erythrocyte. Although our data do not allow a more detailed quantification of the actual translocation rates, the conclusion seems to be justified that an ATP-dependent translocation system for aminophospholipids is already present and active in the plasma membrane of the proerythroblast, and that this system mediates the inward translocation of PS as well as of PE. Moreover, it appeared that in the plasma membrane of the precursor cell this translocation of PE proceeds at least as fast as that of PS (Fig. 2). This then raises the question why the ultimate distribution of the two aminophospholipids in the plasma membrane of the FELC is so much different from that in the mature erythrocyte.

Processing data that had been published by Devaux and colleagues [6], two independent groups arrived at entirely opposite conclusions regarding the mechanism(s) that provide(s) a major – if not exclusive – contribution to the maintenance of phospholipid asymmetry in the red cell membrane. Herrmann and Müller [39] concluded that the ATP-dependent translocase is fully capable of maintaining this asymmetry, by the combined processes of ATP-dependent unidirectional inward translocation of aminophospholipids and passive backward diffusion, and that – if at all – the membrane skeleton plays only a secondary role. Williamson and co-workers [40], on the other hand, arrived at the conclusion that the translocase only 'represents the activity of an ATP-dependent lipid flip/flop catalyst' (essentially a bidirectional process), but that the actual asymmetry is established and maintained by interactions of the aminophospholipids with (components of) the membrane skeleton. Our observations regarding the situation in the plasma membrane of the Friend cell are not in full support of either of the above views. It is quite obvious that, despite the fact that it mediates a very fast inward translocation of both PE and PS (Fig. 2), the translocase is not capable of maintaining an asymmetric distribution of these aminophospholipids to the same extent as is found in the mature erythrocyte. Notwithstanding this, an appreciable extent of asymmetry, favouring the inner half of the plasma membrane of the FELC, is already observed for PS. As the Friend cell does not yet contain a complete membrane skeleton [29,30], this asymmetric distribution of PS can only be ascribed to the action of the ATP-dependent translocase. Consequently, this is at variance with the view that phospholipid asymmetry would be established by interactions between aminophospholipids and the membrane skeleton only [40]. On the other hand, it seems plausible to conclude that an incomplete asymmetry in transbilayer distribution of aminophospholipids is the consequence of the absence of such a skeletal protein network in the Friend cell. The present studies therefore support our previous conclusion [34] that phospholipid asymmetry in the red cell membrane is maintained by both a unidirectional, ATP-dependent translocation of aminophospholipids and their interaction with the membrane skeleton.

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References

1 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71.
2 Haest, C.W.M. and Deuticke, B. (1976) Biochim. Biophys. Acta 436, 353–365.
3 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32.
4 Haest, C.W.M. (1982) Biochim. Biophys. Acta 694, 331–352.
5 Williamson, P.J., Bateman, J., Kozarsky, K., Mattocks, K., Hermanowicz, N., Choe, H.R. and Schlegel, R.A. (1982) Cell 30, 725–733.
6 Seigneuret, M. and Devaux, P.F. (1984) Proc. Natl. Acad. Sci. USA 81, 3751–3755.
7 Daleke, D.L. and Huestis, W.H. (1985) Biochemistry 24, 5406–5416.
8 Connor, J. and Schroit, A.J. (1988) Biochemistry 27, 848–851.
9 Schroit, A.J., Madsen, J.W. and Tanaka, Y. (1985) J. Biol. Chem. 260, 5131–5138.

10 Tilley, L., Cribier, S., Roelofsen, B., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1986) FEBS Lett. 194, 21–27.
11 Zachowski, A., Favre, E., Cribier, S., Hervé, P. and Devaux, P.F. (1986) Biochemistry 25, 2585–2590.
12 Maksymiw, R., Sui, S., Gaub, H. and Sackmann, E. (1987) Biochemistry 26, 2983–2990.
13 Mombers, C.A.M., Verkleij, A.J., De Gier, J. and Van Deenen, L.L.M. (1979) Biochim. Biophys. Acta 551, 271–281.
14 Sikorski, A.F., Michalak, K. and Bobrowska, M. (1987) Biochim. Biophys. Acta 904, 55–60.
15 Cohen, A.M., Liu, S.-C., Derick, L.H. and Palek, J. (1986) Blood 68, 920–926.
16 Sato, S.B. and Ohnishi, S. (1983) Eur. J. Biochem. 130, 19–25.
17 Cohen, A.M., Liu, S.-C., Lawler, J., Derick, L. and Palek, J. (1988) Biochemistry 27, 614–619.
18 Shiffer, K.A., Goerke, J., Düzgüneş, N., Fedor, J. and Shohet, S.B. (1988) Biochim. Biophys. Acta 937, 269–280.
19 Rybicki, A.C., Heath, R., Lubin, B.H. and Schwartz, R.S. (1988) J. Clin. Invest. 81, 255–260.
20 Franck, P.F.H., Roelofsen, B. and Op den Kamp, J.A.F. (1982) Biochim. Biophys. Acta 687, 105–108.
21 Franck, P.F.H., Op den Kamp, J.A.F., Lubin, B., Berendsen, W., Joosten, P., Briët, E., Van Deenen, L.L.M. and Roelofsen, B. (1985) Biochim. Biophys. Acta 815, 259–267.
22 Franck, P.F.H., Chiu, D.T.-Y., Op den Kamp, J.A.F., Lubin, B., Van Deenen, L.L.M. and Roelofsen, B. (1983) J. Biol. Chem. 258, 8435–8442.
23 Franck, P.F.H., Bevers, E.M., Lubin, B.H., Comfurius, P., Chiu, D.T.-Y., Op den Kamp, J.A.F., Zwaal, R.F.A., Van Deenen, L.L.M. and Roelofsen, B. (1985) J. Clin. Invest. 75, 183–190.
24 Zachowski, A., Herrmann, A., Paraf, A. and Devaux, P.F. (1987) Biochim. Biophys. Acta 897, 197–200.
25 Sune, A., Bette-Bobillo, P., Bienvenue, A., Fellman, P. and Devaux, P.F. (1987) Biochemistry 26, 2972–2978.
26 Martin, O.C. and Pagano, R.E. (1987) J. Biol. Chem. 262, 5890–5898.
27 Nijhof, W., Van der Schaft, P.H., Wierenga, P.K., Roelofsen, B., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1986) Biochim. Biophys. Acta 862, 273–277.
28 Rawyler, A., Van der Schaft, P.H., Roelofsen, B. and Op den Kamp, J.A.F. (1985) Biochemistry 24, 1777–1783.
29 Hanspal, M. and Palek, J. (1987) J. Cell Biol. 105, 1417–1424.
30 Pfeffer, S.R. and Redman, C.M. (1986) J. Cell Biol. 103, 103–113.
31 Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83–96.
32 Van der Wiele, F.Chr., Atsma, W., Dijkman, R., Schreurs, A.M.M., Slotboom, A.J. and De Haas, G.H. (1988) Biochemistry 27, 1683–1688.
33 Franck, P.F.H., De Ree, J.M., Roelofsen, B. and Op den Kamp, J.A.F. (1984) Biochim. Biophys. Acta 778, 405–411.
34 Middelkoop, E., Lubin, B.H., Bevers, E.M., Op den Kamp, J.A.F., Comfurius, P., Chiu, D.T.-Y., Zwaal, R.F.A., Van Deenen, L.L.M. and Roelofsen, B. (1988) Biochim. Biophys. Acta 937, 281–288.
35 Rose, H.G. and Oklander, M. (1965) J. Lipid Res. 6, 428–431.
36 Broekhuyse, R.M. (1969) Clin. Chim. Acta 23, 457–461.
37 Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496.
38 Van der Wiele, F., Chr., Atsma, W., Roelofsen, B., Van Linde, M., Van Binsbergen, J., Radvanyi, F., Raykova, D., Slotboom, A.J. and De Haas, G.H. (1988) Biochemistry 27, 1688–1694.
39 Herrmann, A. and Müller, P. (1986) Biosci. Rep. 6, 185–191.
40 Williamson, P.J., Antia, R. and Schlegel, R.A. (1987) FEBS Lett. 219, 316–320.