ATP-Dependent Aminophospholipid Translocation in Erythrocyte Vesicles: Stoichiometry of Transport†

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Received June 18, 1992; Revised Manuscript Received January 19, 1993

ABSTRACT: Vesicles released from human red blood cells by incubation with a suspension of sonicated dimyristoylphosphatidylcholine were purified by gel filtration. Purified vesicles and intact red cells had a very similar composition with respect to phospholipids and integral membrane proteins, but spectrin, the major component of the membrane skeleton, was not found in vesicles. Comparison of red cell and vesicle ATP levels (expressed as micromolar ATP per millimolar hemoglobin) showed a marked difference with a reduced content of only about 30% in vesicles, whatever the initial concentration in the erythrocytes. Spin-labeled aminophospholipids (phosphatidylserine and phosphatidylethanolamine) were translocated to the inner vesicle membrane layer at a comparable rate as in intact red cells provided that vesicles contained enough ATP. The maximum fraction of spin-labeled phospholipids translocated to the inner membrane layer was 84% for phosphatidylserine, 65% for phosphatidylethanolamine, 20–40% for phosphatidylcholine, and below 20% for sphingomyelin. The apparent K_m of translocation, expressed as percent of total membrane phospholipid, was 0.14% for spin-labeled phosphatidylserine and 1.19% for spin-labeled phosphatidylethanolamine. This compares well to values established earlier for intact red blood cells. The fact that no ATP was synthesized in vesicles allowed determination of ATP consumption by aminophospholipid transport. The basic ATP hydrolysis rate was increased upon the addition of labeled aminophospholipids but not of labeled phosphatidylcholine or sphingomyelin. The stoichiometry between lipid translocation and ATP consumption, calculated from the respective initial velocities, was 1.13 ± 0.2 for phosphatidylserine and 1.11 ± 0.16 for phosphatidylethanolamine.

The membrane phospholipids of the red blood cell are distributed asymmetrically with the aminophospholipids phosphatidylethanolamine and phosphatidylserine primarily located at the cytoplasmic side and the choline-containing phospholipids phosphatidylcholine and sphingomyelin at the exterior side of the cell membrane. A similar asymmetric phospholipid organization has also been described for several other membrane systems (Op den Kamp, 1979; Devaux, 1991). Asymmetry is maintained in spite of a transbilayer movement of some of these phospholipids, which is relatively fast with respect to the red cell lifespan. Hence, lipid asymmetry must be maintained by processes that counteract such a long-term randomization.

On the basis of studies with spin-labeled phospholipids (PL*),[1] a rapid translocation of phosphatidylserine and phosphatidylethanolamine from the outer to the inner monolayer of human erythrocytes has been reported (Seigneuret & Devaux, 1984) and has been confirmed by other techniques (Daleke & Huestis, 1985; Tilley et al., 1986; Connor & Schroit, 1987). The phenomenon was not exclusively restricted to red blood cells [for a review, see Zachowski and Devaux (1990)], and it was shown that the translocation was ATP-dependent and that its initial rate and also the steady-state asymmetry were functions of the ATP concentration (Zachowski et al., 1986; Bitbol et al., 1987). The process could be inhibited with SH group specific reagents (Daleke & Huestis, 1985; Morrot et al., 1989) and vanadyl or vanadate ions (Bitbol et al., 1987). From all these studies, it was concluded that a specific carrier protein, the aminophospholipid translocase, must be responsible for the maintenance of cell membrane phospholipid asymmetry (Seigneuret & Devaux, 1984; Zachowski et al., 1986).

Alternatively, it has been proposed that interactions between aminophospholipids and the membrane skeleton (particularly spectrin and band 4.1) were responsible for sustaining phospholipid asymmetry. Such interactions have indeed been suggested to occur (Haest et al., 1978; Cohen et al., 1988) but do not appear to explain lipid asymmetry sufficiently. A study with sickle erythrocytes suggested that both aminophospholipid translocase and the membrane skeleton are required (Middelkoop et al., 1988). On the other hand, glycerophospholipid distribution remained virtually unaffected in heat-treated erythrocytes where spectrin was denatured, suggesting that this component of the membrane skeleton cannot be the major factor in determining the transbilayer phospholipid asymmetry (Gudi et al., 1990).

Erythrocytes release membrane vesicles under a variety of conditions, and different methods to generate these structures in vitro have been described. Phospholipid translocation and steady-state distribution in heat-induced vesicles have been shown to be very similar to the translocation observed in ghosts or intact red cells (Calvez et al., 1988). However, these vesicles still contain about 25% of the red cell spectrin.

† This work was supported by grants from the Swiss National Science Foundation (Grant 31-26629.89), the Hochschulstiftung of the University of Bern, the Centre National de la Recherche Scientifique (UA 526), the Université Paris 7, and the Institut National de la Santé et de la Recherche Médicale (Grant 900104).

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[1] Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; Hgb, hemoglobin; PC*, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylcholine; PE*, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylethanolamine; PL*, 1-palmitoyl-2-(4-doxylpentanoyl)phospholipid; PS*, 1-palmitoyl-2-(4-doxylpentanoyl)phosphatidylserine; SM*, *N*-(4-doxylpentanoyl)-sphingomyelin.

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The vesicles released upon incubation of red blood cells with dimyristoylphosphatidylcholine (DMPC) (Ott et al., 1981) or dilauroylphosphatidylcholine (Takahashi et al., 1983) contain intrinsic membrane proteins but are essentially devoid of spectrin and other elements of the membrane skeleton (Weitz et al., 1982). Their phospholipid composition is almost identical to the one observed in intact erythrocytes (Ott et al., 1981).

In the present investigation, DMPC-induced vesicles were used to study translocation of spin-labeled aminophospholipid analogues in a model system that does not contain spectrin. The results show that the translocation of the aminophospholipid analogues into an asymmetric disposition in vesicles is very similar to the one that has been previously described for intact erythrocytes. On the basis of the observation that vesicles do not have a net production of ATP, it has further been possible to estimate the ratio between aminophospholipid translocation and the concomitant ATP consumption. The stoichiometry thus obtained is close to 1 for both phosphatidylethanolamine and phosphatidylserine.

EXPERIMENTAL PROCEDURES

Vesicle Preparation. DMPC-induced vesiculation was performed essentially as described earlier (Ott et al., 1981). Briefly, DMPC (Sigma) was dispersed at a final concentration of 0.5 mg/mL in a buffer containing 10 mM Tris, 144 mM NaCl, 0.5 mM adenine, 10 mM glucose, 1 mM EDTA, 2×10^5 units/L penicillin, and 1.5×10^5 units/L streptomycin, pH 7.4, and sonicated under nitrogen using a Branson Sonifier at 40 W, 50% duty-cycle, for 30 min. The lipid suspensions were subsequently centrifuged at 92500*g* for 45 min at 20 °C in order to remove deposits from the metal tip and large multilamellar DMPC vesicles. All subsequent steps were performed at room temperature.

Erythrocytes (Central Blood Bank of the Swiss Red Cross) were separated from plasma by centrifugation at 1000*g* for 7 min and the buffy coat and the supernatant carefully removed by aspiration. Cells were then washed 3 times with incubation buffer and pelleted by centrifugation at 1000*g* for 7 min. DMPC-induced vesiculation was started by adding packed red blood cells to the DMPC suspension at a final hematocrit of 10%. Incubation was carried out at 30 °C in a rotary shaking bath for 4.5 h. Erythrocytes were then separated from the incubation mixture by centrifugation at 1000*g* for 7 min, and the erythrocyte vesicles were concentrated by centrifugation of the supernatant at 34500*g* for 30 min. The absorbance at 418 nm of the vesicle-free supernatant was compared to the absorbance of an equivalent cell hemolysate to assess the hemolysis induced by vesiculation. The crude vesicle preparation was resuspended in 3 mL of a buffer containing 10 mM Hepes and 144 mM NaCl, pH 7.4. Vesicle purification was subsequently performed by gel filtration chromatography on a Sephacryl S-1000 superfine (Pharmacia Fine Chemicals) column with a total bed volume of 120 mL. Fractions of 10 mL were collected at a flow rate of 1.5 mL/min. The fractions that contained the vesicles were identified by measuring the activity of acetylcholinesterase, a red cell membrane marker (Ott, 1985), and hemoglobin, using the Merckotest kit (Merck). Purified vesicles were then concentrated by centrifugation at 34500*g* for 30 min.

Vesicles with increased ATP levels were prepared like normal vesicles (see above) except that the red blood cells were first suspended at a hematocrit of 6–7% in a buffer containing 100 mM NaH_2PO_4, 33 mM NaCl, 20 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM glucose, 10 mM pyruvate, 10 mM inosine, 2 mM adenosine, 2×10^5 IU/L penicillin, and 1.5×10^5 IU/L streptomycin, pH 7.4, and incubated for a maximum of 2 h at 37 °C, to increase the ATP levels of the cells. Subsequently, vesicle preparation was carried out with these cells as described above.

For characterizing vesicle integrity, adenylate kinase was used as a cytoplasmic marker enzyme. Its activity was determined by following the time course of ATP production. An aliquot of the vesicle suspensions corresponding to an equivalent concentration of 0.08 mM hemoglobin (Hgb) was added to the assay buffer which consisted of 10 mM Tris-HCl, 144 mM NaCl, 2 mM ADP, 8 mM KH_2PO_4, and 1 mM $MgCl_2$, pH 7.4. The assay was performed at 37 °C for 10 min. At time intervals of 30 s, aliquots of 10 μL were taken for ATP measurement (see below). For determination of the total adenylate kinase activity, vesicle membranes were solubilized by adding Triton X-100 to a final concentration of 0.01% (v/v). Under these conditions, adenylate kinase activity per se was not affected by the detergent. Vesicle integrity was expressed as the percent ratio of the activities measured in the absence and presence of detergent.

ATP Determination. A bioluminescence (luciferin–luciferase) assay was used for ATP determination (1243-102 ATP monitoring kit, LKB). Aliquots of vesicle or red cell suspensions were added to an equal volume of a solution of 10% trichloroacetic acid containing 4 mM EDTA and kept on ice for at least 10 min. The specimens were subsequently centrifuged in a Heraeus Biofuge A at 13 000 rpm for 5 min to precipitate the denatured proteins. The supernatant which contained ATP was diluted 1000–5000-fold with a buffer containing 100 mM Tris–acetate and 2 mM EDTA, pH 7.75, to adjust the final ATP concentration to the nanomolar range. The samples were subsequently measured in a BioOrbit 1250 LKB luminometer. ATP standard solutions in the concentration range between 0.5 and 1000 nM were used for calibration.

Lipid Analysis. Lipids were extracted from red cells and vesicles (Rose & Oklander, 1965) and quantified by measuring the phospholipid phosphorus (Rouser et al., 1970).

Protein Analysis. Red cells for protein analysis were lysed in 10 volumes of cold 10 mM Tris-HCl, pH 7.4, and the membranes pelleted by centrifugation at 34500*g* and 4 °C for 20 min. The pellet was resuspended in the above buffer and recentrifuged. This crude preparation was washed at least 4 more times in the same buffer, and the resulting white ghosts were frozen immediately and stored at −70 °C until used. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out in a discontinuous buffer system (Laemmli, 1970) using 8–18% acrylamide gradient gels containing 0.1% sodium dodecyl sulfate.

Characterization of Phospholipid Translocation in Vesicles. Purified vesicles were resuspended in a buffer containing 30 mM Hepes and 124 mM NaCl, pH 7.5. Preparation of the spin-labeled lipid analogues [1-palmitoyl-2-(4-doxylpentanoyl)-phospholipids] and labeling were performed as described previously (Morrot et al., 1989). Experiments to establish the kinetic parameters of translocation were carried out by mixing a vesicle suspension (corresponding to 3 mM vesicle phospholipid) with a solution that contained between 15 and 60 μM spin-labeled phospholipid analogues (corresponding to 0.5–2% of the total endogenous vesicle phospholipids).

When lipid translocation was characterized as a function of vesicle ATP concentration, spin-labeled phospholipid

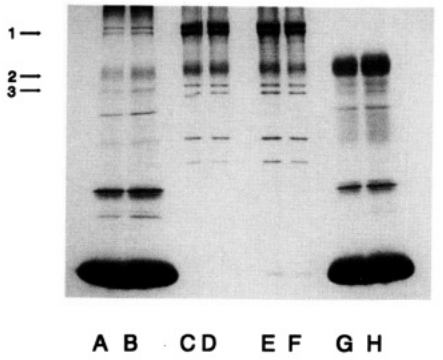

FIGURE 1: SDS–polyacrylamide gel electrophoresis of vesicles and red cell membrane proteins. The numbers indicate the proteins mentioned in the text. 1, Spectrin α and β; 2, band 3 protein; 3, protein 4.1. Lanes A and B represent the vesicles before gel filtration; lanes G and H show the vesicles purified on Sephacryl S-1000. Lanes C and D are ghosts prepared from intact red blood cells; ghosts from remnant cells after vesiculation are shown in lanes E and F. In each lane, 150–200 μg of protein was applied.

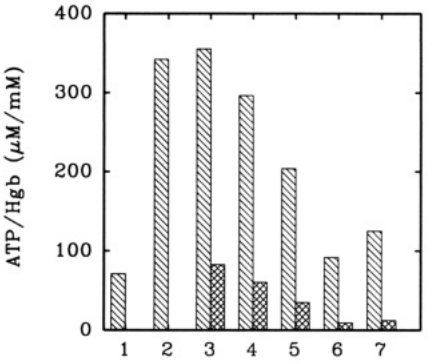

FIGURE 2: ATP levels during DMPC-induced vesiculation. DMPC-induced vesiculations were performed as described under Experimental Procedures. Hatched bars represent high ATP conditions; cross-hatched bars represent normal conditions. Bars 1 and 2 show the ATP loading of intact red cells; bars 3–7 show the vesiculation experiment. 1, Red cells at zero time; 2, red cells after 2 h of incubation for ATP loading; 3, red cells at zero incubation time in the presence of sonicated DMPC vesicles; 4, red cells after 4.5 h of incubation in the presence of sonicated DMPC vesicles; 5, remnant red cells after vesiculation; 6, crude vesicles; 7, vesicles purified on Sephacryl S-1000.

analogues were used in an amount corresponding to 1% of the endogenous vesicle phospholipids, while the ATP concentrations in the suspension were varied between 2 and 20 μM, corresponding to 55–550 nmol of ATP/mL of packed vesicles.

To assess the correlation between phospholipid translocation and ATP consumption, vesicles (corresponding to 6 mM phospholipid) were preincubated for 20 min at 37 °C before the labeled lipid analogues were added in an amount corresponding to 0.2–2% of the endogenous phospholipids. In all cases, vesicle suspensions and solutions of labeled lipids were mixed together in equal volumes. After mixing of the vesicles and the labeled lipid, aliquots of 100 μL were removed from the incubation mixture at the time points indicated in the figures and added to 50 μL of 3% fatty acid-free bovine serum albumin in 30 mM Hepes/124 mM NaCl, pH 7.4. The samples were kept on ice for 1 min to allow the reextraction by bovine serum albumin of the labeled lipid analogues from the outer membrane leaflet. The vesicles were then pelleted by centrifugation for 20 s at 200000*g* using a Beckman Airfuge air-driven ultracentrifuge. The supernatants which contained the extracted lipids were stored at −20 °C until measured. Reoxidation of any reduced label prior to analysis by electron paramagnetic resonance spectroscopy was obtained by adding 10 mM potassium ferricyanide. In parallel, samples of 10 μL were taken for ATP measurements. The experimental points were fitted with exponential equations using the SigmaPlot Scientific Graphing System Version 4.10.

RESULTS

Characterization of DMPC-Induced Red Cell Vesicles. Gel filtration chromatography was used as a final step to obtain a homogeneous vesicle population that was not contaminated with ghost membrane fragments. The result of sodium dodecyl sulfate–polacrylamide gel electrophoresis showed that crude vesicles contained small amounts of spectrin that was completely absent in the final preparation (Figure 1). It is also obvious from the figure that the integral band 3 protein was still present in vesicles while the content of band 4.1 was markedly reduced. The patterns obtained with untreated cells and remnant cell membranes after vesiculation were similar to each other. Phospholipid analysis of red cells, vesiculated cells, and vesicles showed no significant difference, with only a moderate increase of the phosphatidylcholine content from 32% in control cells to 38% in vesicles and remnant cells, due to the incubation with DMPC (data not shown).

In the study of transverse phospholipid diffusion, the intactness of the vesicles is most important. Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), a marker enzyme to determine ghost permeability (Steck & Kant, 1974), was not detected in either crude or purified vesicles, which indicates that this assay is not sensitive enough to probe vesicle integrity. Among several other markers tested, adenylate kinase (EC 2.7.4.3) was found to be sufficiently sensitive. In the crude vesicle preparations, some 5–35% of the total adenylate kinase activity could be attributed to leaky vesicles, depending on the extent of hemolysis that occurred during the DMPC-induced vesiculation. After gel filtration, this fraction was reduced to 0.8–10% of total adenylate kinase activity. For translocation experiments, preparations were used where less than 2% adenylate kinase activity could be attributed to leaky vesicles. This value did not increase upon incubation with spin-labeled lipids.

The ATP content of DMPC-induced vesicles expressed as the ATP/Hgb ratio (micromolar ATP per millimolar Hgb) was reduced to less than 30% with respect to intact red blood cells. It was not possible to increase this level by incubation of the vesicles in the presence of metabolites used in red cell ATP production. Therefore, the enzymatic activities involved in the pathways of red cell ATP synthesis were determined after solubilization of membranes by Triton X-100. When compared to red blood cells, vesicles exhibited most of the required activities with the exception of hexokinase activity (EC 2.7.1.1) that was completely missing, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) that was reduced to one-third, and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) that was reduced to one-sixth of the original activity measured with red cells. These reductions can explain why vesicles do not resynthesize ATP. On the other hand, it was possible to increase ATP levels in red cells up to 4-fold by incubation in an appropriate buffer (see Experimental Procedures). When these red cells were used as a starting material for DMPC-induced vesiculation, a final vesicle preparation was obtained with ATP levels that were as high as the levels observed in normal red cells (Figure 2). In all experiments, the vesicle ATP concentration will be expressed as nanomoles of ATP per milliliter of packed vesicles.

Translocation of Phospholipids across Vesicle Membranes. The addition of low concentrations of the four phospholipid analogues to vesicles with high ATP levels revealed translo-

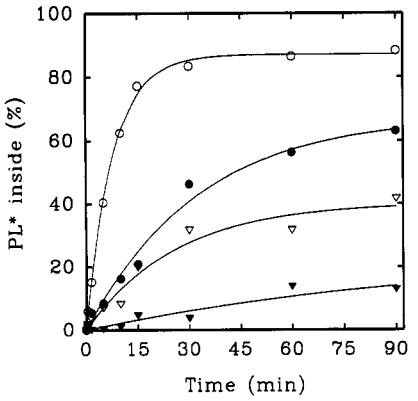

FIGURE 3: Translocation of spin-labeled phospholipid analogues in vesicles. Translocation experiments were carried out as described under Experimental Procedures using vesicles with an ATP content of 635 nmol/mL of packed vesicles. To an amount of vesicles equivalent to 3 mM phospholipid were added the following lipids (amounts given in percent fraction of vesicle phospholipid): 0.2% PS* (open circles); 0.7% PE* (closed circles); 0.7% PC* (open triangles); 0.7% SM* (closed triangles).

cation kinetics that were similar to those observed in red cells (Morrot et al., 1989). Spin-labeled phosphatidylcholine (PC*) and sphingomyelin (SM*) diffused at a moderate rate to the inner leaflet. On the other hand, the spin-labeled aminophospholipids phosphatidylserine (PS*) and to a lesser extent phosphatidylethanolamine (PE*) accumulated quickly in the inner layer (Figure 3). The maximum fraction of the added labeled lipids that was translocated to the inner membrane leaflet during an incubation time of 90 min was 87% for PS*, 64% for PE*, 39% for PC*, and 14% for SM*.

Determination of phospholipid translocation in vesicles with variable ATP concentrations showed for PE* and PS* that the initial translocation rate and the maximum extent of translocation obtained within 90 min were dependent on the ATP content of vesicles showing a saturation behavior with respect to ATP requirement. This correlation was not seen with PC* and SM* (Figure 4). It can also be seen that the steady-state equilibrium obtained with the aminophospholipids depended on the amount of ATP present in the vesicles. However, it is difficult to establish a clear quantitative correlation between ATP levels and translocation, because PS* and PE* translocation resulted in vesicular ATP exhaustion (see next paragraph).

Determination of aminophospholipid translocation in vesicles with a high ATP level and variable amounts of spin-labeled phospholipids showed for PS* that with increasing concentrations of added lipid analogue the percent fraction of spin-labeled aminophospholipids translocated per time unit decreased (Figure 5A), a correlation that was less pronounced with PE* (Figure 5C), while the absolute amount of translocated lipid increased (Figure 5B,D). Linearization of these data according to Lineweaver–Burk led to an apparent Michaelis constant, $K_{m,app}$, of 0.14% ± 0.01% (4.69 ± 0.61 μM) for PS. As for PE, the data permitted only a rough estimation of $K_{m,app}$, which was 1.19% ± 0.61% (39.2 ± 23.9 μM). These numbers indicate that, under the experimental conditions used, the transport system exhibited a higher affinity for PS*.

ATP Consumption during Phospholipid Translocation. Translocation of aminophospholipid analogues in vesicles was accompanied by ATP consumption. Figure 6A,B shows that the vesicle ATP content decreased at a constant rate when no PL* was added. With PC* and SM*, this basal rate was hardly changed. However, addition of either PS* or PE* to

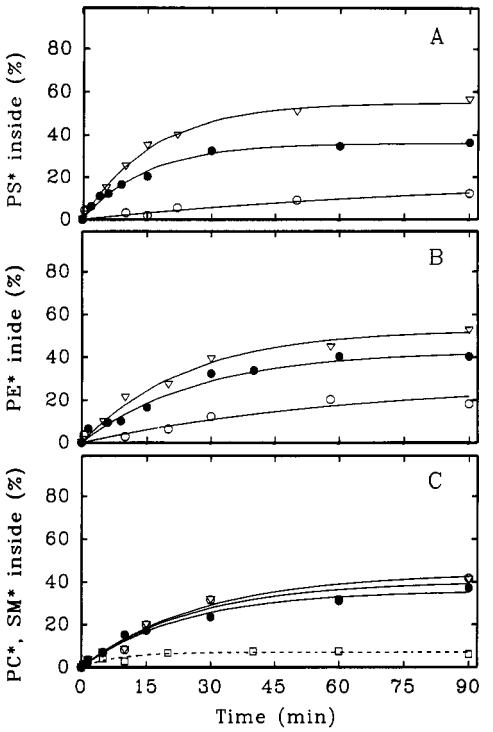

FIGURE 4: Translocation of spin-labeled phospholipid analogues in vesicles at various ATP concentrations. Translocation of PS* (panel A), PE* (panel B), and SM* and PC* (panel C) was measured as described under Experimental Procedures. The ATP content of vesicle was (in nanomoles of ATP per milliliter of packed vesicles); 72 (open circles), 313 (closed circles), and 476 (open triangles). SM* translocation (panel C, open squares) was measured in the presence of 440 nmol of ATP/mL of packed vesicles. Spin-labeled analogues were introduced as 1% of the endogenous phospholipids.

vesicles resulted in a significantly increased ATP consumption which started immediately after the addition of the lipid and lasted for at least 15 min, depending on ATP and PL* concentrations. After that time period, it returned to the basal rate. The rate of ATP consumption observed with PS* was higher than that observed with PE*. However, the difference reflects also the saturability of transport by the aminophospholipids. In the experiments summarized in Figure 6A, the analogues were introduced at a concentration corresponding to 0.7% of the endogenous phospholipids, which was 5 times the apparent K_m for PS* and 0.6 times the apparent K_m for PE*. Under these conditions, the translocase was working at its maximum rate for PS* but not for PE*, and the difference in ATP hydrolysis is evident. In the experiments shown in Figure 6B, the analogues were used at a concentration corresponding to 2% of the endogenous phospholipids (which is almost twice as much as the apparent K_m for PE*). Under these conditions, the rate of ATP consumption during translocation of PE* was almost as high as during PS* translocation, because the translocation rate of PE* was closer to its maximum.

As shown in Figure 6, the experimental data points obtained in measurements of ATP utilization could be fitted to a single-exponential curve during either PC* or SM* translocation, or in the absence of any phospholipid analogue. On the other hand, a clearly increased ATP consumption was observed during translocation of either PS* or PE*. In this case, double-exponential decay curves were necessary to provide an optimal fit to the experimental data points. This indicates that different rates of ATP consumption can be discerned. One is similar to that observed without added lipid or with PC* and SM* and therefore has to be considered as the basal ATP hydrolysis

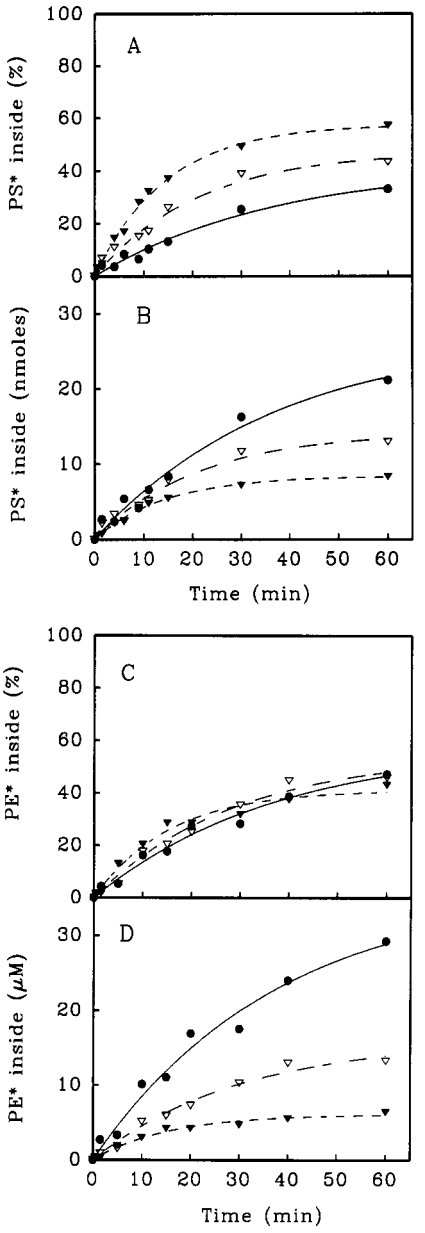

FIGURE 5: Translocation of different amounts of spin-labeled phospholipid analogues in vesicles. Experiments were carried out as described under Experimental Procedures. Panels A and B shows the translocation of PS* and panels C and D the translocation of PE*. In panels A and C, the amount of lipid analogue translocated is indicated as the percent fraction of vesicle phospholipid and in panels B and D as an absolute amount (calculated for an incubation volume of 1 mL). The labeled lipids were added to vesicles in the following concentrations (indicated as percent fraction of vesicle phospholipid and as concentrations): 0.5% (15 nmol/mL) (closed triangles); 1% (30 nmol/mL) (open triangles); 2% (60 nmol/mL) (closed circles). The ATP concentration was 352 nmol/mL of packed vesicles.

rate. As soon as either PE* or PS* is added, this rate is increased during the time period that is required to translocate the phospholipid analogues into their asymmetric disposition. Including ouabain (0.1 mM) in the incubation medium had no effect on the relocation of PL*; the basal ATP hydrolysis rate was reduced by 41%, but the increased consumption, noted after the addition of either PS* or PE*, was not affected (data not shown). Both phenomena remained unaffected by calcium chelators. The difference between the two ATP consumption rates must therefore be due to aminophospholipid translocation. Figure 7 illustrates this phenomenon: for the sake of clarity, data points were omitted, and the differences

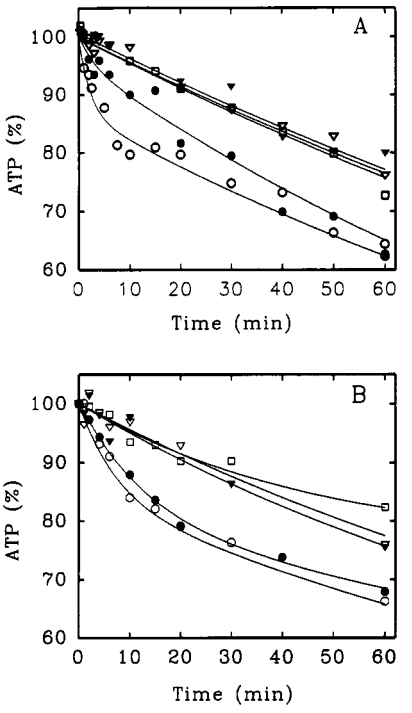

FIGURE 6: ATP consumption during phospholipid translocation in vesicles. Experiments were carried out as described under Experimental Procedures. Lipids are represented by the following symbols: PS* (open circles); PE* (closed circles); PC* (open triangles); SM* (closed triangles); controls without PL* (open squares). The spin-labeled analogues represented either 0.7% (panel A) or 2% (panel B) of the endogenous phospholipids. Experiments were carried out with 463 (panel A) and 760 (panel B) nmol of ATP/mL packed vesicles.

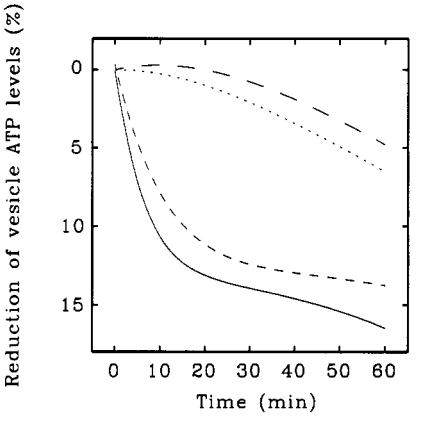

FIGURE 7: Specific ATP consumption during translocation of spin-labeled phospholipids. Net ATP consumption during phospholipid translocation was deduced from the results shown in Figure 6B during translocations of PS* (solid line), PE* (medium dashed line), PC* (long dashed line), and SM* (dotted line) added as 2% of the endogenous phospholipids. The curves shown here are the differences between the fits of the experimental points obtained in the presence and absence of lipid analogues.

between the curve fits obtained from the data points of the experiments reported in Figure 6B are plotted.

From the fitted curves established for net ATP consumption (Figure 7) and for phospholipid translocation (Figures 3–5), the corresponding initial velocities could be calculated. The results of these calculations from eight experiments carried out under different conditions of both ATP and PL* concentrations are shown in Figure 8. The stoichiometry of ATP-dependent phospholipid translocation thus established was 1.11 ± 0.16 mol of PE* or 1.13 ± 0.21 mol PS* translocated per mole of ATP hydrolyzed.

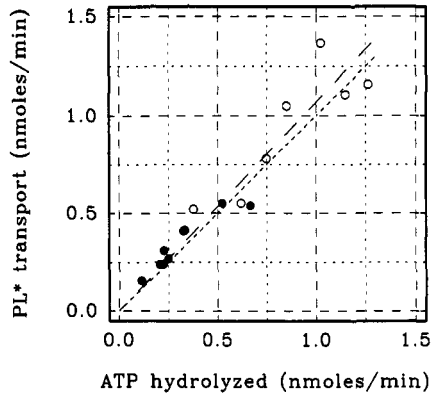

FIGURE 8: Aminophospholipid transport rate as a function of net ATP hydrolysis induced by lipid. Translocation of PS* (open circles) and PE* (close circles) was assayed in vesicle populations containing different amounts of ATP, the spin-labeled lipid being introduced as 0.2–2% of the endogenous phospholipids. The rates correspond to the initial velocities of either PL* transport or ATP hydrolysis.

DISCUSSION

In DMPC-induced vesicles, there is no detectable increase in ATP concentration even if the incubation medium contains all the metabolites that are needed for ATP synthesis in intact erythrocytes. As stated above, this must be due to the absence of hexokinase activity in the vesicles. Therefore, the ATP content of the vesicles can only be varied by changing the ATP content of the red cells from where they bud. In other words, it is possible to obtain vesicles with a defined ATP content, but it is not possible to restore their ATP levels. Thus, it has to be taken into account that any ATP-dependent process, such as aminophospholipid translocation, slows down with time because ATP is hydrolyzed.

In red blood cells, the steady-state distribution of exogenously added spin-labeled phospholipid analogues (Morrot et al., 1989) and the asymmetric distribution of endogenous phospholipids determined by various other methods (Devaux, 1991) have been shown to be essentially the same. It is fair to assume that the situation is the same in the case of vesicles. On the basis of the translocation observed for the different phospholipid analogues (Figure 3), this would suggest that also in spectrin-free vesicles phospholipids are distributed asymmetrically with approximately 82–87% of phosphatidylserine, 56–68% of phosphatidylethanolamine, 25–40% of phosphatidylcholine, and less than 20% of spingomyelin located in the inner membrane leaflet. This asymmetry is somewhat less pronounced than in the intact red cells. It must be considered, however, that in the vesicle system a real steady-state asymmetry probably can never be obtained because of the decrease of ATP levels during long-term incubation. This would imply that only PS*, that is translocated most rapidly, would tend to reach its equilibrium distribution. The arrangement of PE* would be affected by decreasing ATP levels because this lipid is always translocated more slowly than PS*. A reason for the slower PE* translocation is the fact that the aminophospholipid translocase has a higher affinity for PS* than for PE* (K_m^{PS}/K_m^{PE} ratio = 1/9 in red cells; Zachowski et al., 1986).

Some discrepancy is obvious with respect to previous studies on phospholipid asymmetry in vesicle membranes. This can be attributed to the fact that vesicle ATP levels were well characterized in the present study while no information on this point is available from earlier work (Dressler et al., 1984; Scott et al., 1984; Raval & Allan, 1984).

The influence of ATP concentration on phospholipid translocation can be estimated from the initial velocities of PS* or PE* translocation at different ATP levels. It appears from Figure 4 that at any initial ATP concentration PE* translocation is slower (Figure 4B) than PS* translocation (Figure 4A). However, the continuous basal decrease of the ATP content makes it difficult to obtain a reliable quantification of the ATP dependency of aminophospholipid translocation in terms of apparent affinity.

The fact that neither PC* nor SM* diffusion is affected by changes in vesicle ATP content indicates that inward movement is ATP-dependent exclusively for aminophospholipids. The relatively fast PC* translocation can be explained by the modifications observed in the vesicle bilayer where the cholesterol to phospholipid molar ratio is decreased to approximately 0.6 whereas it is 0.8 in the intact red cell membrane (P. Ott, unpublished observation). As previously demonstrated (Morrot et al., 1989), such a decreased cholesterol level leads to an increased transbilayer diffusion rate of phosphatidylcholine.

From the data presented in Figure 5, kinetic parameters for translocation of spin-labeled aminophospholipid analogues could be deduced. It has to be considered, however, that the Michaelis–Menten-type kinetics applied to the vesicle system are strictly valid only for a homogeneous (water)-phase one-substrate enzyme reaction. Furthermore, the endogenous aminophospholipids present in the outer monolayer of the vesicle membrane are also substrates of the translocase that dilute and compete with the spin-labeled analogues, and it is not possible to exclude that the affinity of the carrier for its substrates might (slightly) vary with the molecular species. Therefore, all kinetic parameters deduced from the measured results have to be considered as apparent parameters. Moreover, technical reasons (sensitivity of the method, membrane lysis) limit the range over which the PL* amount can be varied and thus make it difficult to obtain very accurate parameters. Their ratio, however, will reflect the relative affinities of the translocase system for PS* and PE*. Furthermore, the values calculated for the vesicle system ($K_{m,app}^{PS}$ = 0.14% ± 0.01 of the endogenous phospholipids and $K_{m,app}^{PE*}$ = 1.19% ± 0.61 of the endogenous phospholipids) and the published numbers for intact red cells (0.18% of the endogenous phospholipids for PS* and 1.68% of the endogenous phospholipids for PE*; Zachowski et al., 1986) are in good agreement. Taken together, all the above considerations clearly indicate that not only in red cells but also in spectrin-free vesicles an ATP-dependent aminophospholipid accumulation in the inner leaflet can be observed. The similarity of the apparent kinetic translocation parameters observed makes it furthermore fair to assume that in both systems the same translocation mechanism is active. This then indicates that the observed asymmetric disposition of the (amino-) phospholipid analogues is not dependent on the presence of an intact membrane skeleton and that the aminophospholipid translocase is able, by itself, to create the asymmetry noticed between the phospholipids, as already suspected after studies using partially spectrin-depleted vesicles (Calvez et al., 1988) or red cells with a heat-denatured skeleton (Gudi et al., 1990). It cannot be precluded, however, that in situ components of the membrane skeleton (such as band 4.1) might help to maintain this asymmetry.

Because several enzymatic activities involved in red cell ATP production were either absent or significantly reduced in the release vesicles, these structures did not show a net ATP production but rather a slow and continuous decrease

of their ATP content. The fact that this ATP hydrolysis was significantly increased upon the addition of aminophospholipids and persisted during the time period that was needed to translocate these lipids to the inner leaflet of the vesicle membrane (Figures 3–5 and 7) strongly suggests that the two processes are directly correlated. This idea is supported by the observation that the ratio between the respective rates of lipid translocation and specific ATP hydrolysis is the same over a considerable range (Figure 8). Furthermore, it appears unlikely that other activities, such as Na,K-ATPase or Ca-ATPase, are directly stimulated by the added lipid, because, on one hand, 0.1 mM ouabain neither affected the increased ATP consumption triggered by the spin-labeled aminophospholipids nor changed the kinetics of translocation while reducing basal ATP consumption by approximately 41% and, on the other hand, the presence of calcium chelators in the incubation medium had no effect on any measurement (not shown). Therefore, it appears justified to consider the ATP hydrolysis observed after lipid addition as a direct consequence of lipid translocation. As detailed above, the stoichiometry of this transport process is approximately 1 aminophospholipid moved per ATP molecule hydrolyzed.

The close correlation between ATP consumption and aminophospholipid translocation described in this paper further supports the model of an aminophospholipid translocase as a two-substrate enzyme or a highly coupled multicomponent system that includes a Mg-ATPase (Devaux et al., 1988).

ACKNOWLEDGMENT

We thank the Central Blood Bank of the Swiss Red Cross for supplying fresh human red blood cells. Ms. P. Hervé is acknowledged for providing us with the various spin-labeled molecules.

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