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Phospholipid asymmetry in red blood cells and spectrin-free vesicles during prolonged storage

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Abstract

Erythrocytes and spectrin-free DMPC-induced vesicles released from the cells were incubated for 3 weeks at 6°C under conditions of metabolic ATP-depletion. Phosphatidylserine (PS) asymmetry was monitored during this period by use of the prothrombinase assay. Prothrombinase activities measured at the beginning of the incubation period indicated that approximately 0.06% of PS was located at the outer layer of the red cell membrane, whereas in DMPC-induced vesicles approximately 1.5% the PS was exposed on the outside. After completion of the incubation period PS exposure on the outside of red cells and vesicles was increased by no more than 5-fold. On the other hand, with vesicles prepared with a significantly increased (4-fold) ATP-content to sustain translocase activity, the incubation process resulted in a surprisingly high (20-fold) increase of PS exposure. With vanadate, an inhibitor of the aminophospholipid translocase, included in the incubation medium, the redistribution of PS was even more pronounced. These observations indicate that PS asymmetry in spectrin-free vesicles can not be directly correlated to either ATP content or translocase activity and suggest that besides the aminophospholipid translocase and the membrane skeleton, other mechanisms must be involved in maintaining phospholipid asymmetry.

Keywords: Phospholipid asymmetry; Prothrombinase assay; Erythrocyte membrane vesicle; Membrane skeleton; Aminophospholipid translocase; Vanadate

1. Introduction

The phospholipids in the plasma membrane of many eukaryotic cells are distributed asymmetrically across both halves of the bilayer. In red blood cells, almost all phosphatidylserine (PS) and the major part of phosphatidylethanolamine is located in the cytoplasmic half of the bilayer, whereas the choline-containing phospholipids are mainly present at the outside of the membrane [1,2]. Since the transbilayer movement of some phospholipids is relatively fast compared to the life-span of the red cell, there must be mechanisms available that can counteract long-term phospholipid randomization.

It is widely accepted that an ATP-dependent aminophospholipid translocase is capable of transporting exogenously added aminophospholipids from the outer to the inner monolayer of the membrane (for review, see [3]). However, it is not clear whether this translocase is alone responsible for generating and maintaining phospholipid asymmetry. It has been suggested that it serves rather as a repair mechanism [4], whereas other systems, such as the membrane skeleton [5], play part in retaining PS at the inner monolayer. Recent studies however, have shown that red cell membrane systems that lack (parts of) the membrane skeleton can still remain asymmetric [6–8].

Several investigations have demonstrated that in the erythrocyte membrane phospholipid asymmetry is not immediately lost upon prolonged inactivation of the aminophospholipid translocase, for example due to ATP depletion [9–12]. However, in these studies it could not be excluded that asymmetry was maintained due to the presence of the membrane skeleton. So far, no investigations have elucidated to what extent phospholipid asymmetry can be sustained for a prolonged time period in a membrane system where aminophospholipid translocase activity is abolished, and an intact membrane skeleton is missing.

Abbreviations: DMPC, dimyristoyl phosphatidylcholine; PL, phospholipid; PS, phosphatidylserine; AChE, acetylcholinesterase; Hb, hemoglobin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; HSA, human serum albumin; TCA, trichloro acetic acid.

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DMPC-induced membrane vesicles from red blood cells have been used successfully in studies concerning phospholipid asymmetry. These vesicles contain intrinsic membrane proteins but are completely devoid of spectrin and depleted in other elements of the membrane skeleton [13,14]. With exception of the additional DMPC, their phospholipid composition is almost identical to the one observed in intact erythrocytes. It has been shown that the vesicles contain an ATP-dependent aminophospholipid translocase activity [15], and that the steady-state phospholipid distribution is still highly asymmetric directly after preparation [8].

In the present study, the prothrombinase assay was used as a very sensitive technique to examine the orientation of PS in the membranes of erythrocytes and DMPC-induced vesicles in the course of metabolic ATP-depletion. The investigations were performed on two types of DMPC-induced vesicles with different ATP-levels to obtain more information on the contribution of the translocase to maintaining asymmetry in the vesicles. Vanadate, which can act as an inhibitor of P-type ATPases, and has been shown to inhibit the aminophospholipid translocase [16], was used to further inhibit the translocase activity.

The results suggest that, besides the aminophospholipid translocase and possibly the membrane skeleton, other mechanisms must be considered to contribute to maintaining phospholipid asymmetry.

Part of this work was presented at the FEBS Special Meeting of Biological Membranes in Helsinki, 1994.

2. Materials and methods

2.1. Materials

Purified prothrombin, factor Va and factor Xa were supplied by Dr. R. Wagenvoord, University of Limburg, the Netherlands. The chromogenic substrate S2238 was obtained from Chromogenix AB, Mölndal, Sweden.

Leupeptine was obtained from Boehringer Mannheim, and neuraminidase from Sigma (E.C. 3.2.1.18, type X).

All other reagents used were obtained either from Merck, Fluka or Sigma, and of the highest grade available.

2.2. Prothrombinase assay

The prothrombinase assay was used to obtain semiquantitative information on the orientation of PS in cell and vesicle membranes. The assay, originally designed by Bevers et al. [17], was performed as described earlier [8]. Briefly, cell suspensions (final phospholipid concentrations 1 to 25 μM, dependent on activity) or vesicle suspensions (final phospholipid concentrations 0.2 to 3 μM) that had been diluted freshly with buffer A (10 mM Hepes, 144 mM NaCl, pH 7.4), or buffer as a blank, were assayed with 3 nM factor Xa, 6 nM factor Va, and 4 μM prothrombin (final concentrations) in a buffer containing 10 mM TRIS-HCl, 136 mM NaCl, 1.6 mM KCl, 4 mM $CaCl_2$ and 0.5 mg/ml HSA, pH 7.9. After a 2 min preincubation in presence of factor Va and factor Xa, prothrombin was added and at timed intervals, aliquots of 25 μl were transferred to 1 ml of a buffer containing 2 mM EDTA to stop the reaction. The amount of thrombin formed was assessed using the chromogenic substrate S2238.

The prothrombinase activity was always expressed per total phospholipid concentration. For a rough quantification of the amount of PS exposed on the membrane surface, the prothrombinase activities measured in presence of sonicated cell and vesicle suspensions, as well as sonicated lipid extracts of both systems were compared. By correlation of the measured activities in presence of these systems [8] to the theoretical fraction of PS that was exposed on the surface of these systems, it could be estimated that under our conditions 100% PS availability on a phospholipid surface would result in a prothrombinase activity in the range of 1300–2400 nM thrombin/min $\cdot$ μM PL. Additionally, completely hemolysed (but not sonicated) cells were obtained by diluting the cells 20 times with distilled water before the assay. The prothrombinase activity in presence of these membranes was found to be 85 ± 19 nM thrombin/min $\cdot$ μM PL.

2.3. ATP-content

The ATP-content of cells and DMPC-induced vesicles was assessed using a luciferin-luciferase bioluminescence assay after TCA precipitation as described earlier [15].

2.4. Vesicle integrity

Adenylate kinase activity was determined as described by Beleznay et al. [15]. Vesicle integrity was expressed as the percent ratio of the enzyme activities measured in absence and presence of Triton X-100.

2.5. Acetylcholinesterase activity

AChE, used as a membrane marker to characterize vesicle concentration, was determined according to Ellmann et al. [18].

2.6. Phospholipid quantification

Lipids were extracted from an aliquot of each cell suspension according to Rose and Oklander [19] and from 0.5 ml of the vesicle stock suspensions using the method of Bligh and Dyer [20]. Total phospholipid was quantified by determination of the phosphorus content according to Rouser et al. [21].

2.7. Erythrocyte storage

Concentrated erythrocytes in standard anticoagulant buffer were obtained from the ZLB Central Laboratory, Swiss Red Cross Blood Transfusion Service (Bern, Switzerland). One volume of cells was washed 3 times in sterile tubes with 2 volumes of sterile filtered buffer B (15 mM sodium phosphate, 2 mM adenine, 45 mM deoxyglucose, 40 mM mannitol and 100 mM NaCl, pH 7.4) by centrifugation at $1000 \times g$ for 5 min and subsequent removal of the supernatant and remaining buffy coat. The cells were resuspended to approximately 20% hematocrit in buffer B, and stored at 6°C for up to 3 weeks.

At given times, an aliquot of cells was washed at least 5 times with 2 volumes of buffer A by centrifugation at $1000 \times g$ for 5 min and aspiration of the supernatant to remove damaged cells. The cells were resuspended in buffer A at the appropriate concentration (20 to 500 μM total phospholipid) and immediately used in the prothrombinase assay. To obtain information on the fragility of the cells, an aliquot of the suspension was taken after gentle re-homogenization, centrifuged at $1000 \times g$, and the supernatant was sampled to determine hemolysis, by comparing the hemoglobin concentration (measured at 414 nm) to an equivalent amount of cell hemolysate. Samples were also taken for the determinations of the total ATP and hemoglobin contents of the cells.

Alternatively, cells were washed and stored in buffer B containing 2.5 mM sodium orthovanadate. In this case, at each given time, 2.5 mM vanadate was also added to buffer A, to wash and resuspend the cells. Otherwise the procedure was the same as with the storage in absence of vanadate. The presence of vanadate did not affect the prothrombinase activity (data not shown).

2.8. Vesicle storage

Membrane vesicles were released both from regular fresh erythrocytes (low-ATP vesicles) and from cells with 3 to 4 times increased ATP-levels (high-ATP vesicles) [15] by incubation with DMPC as described earlier [13]. The vesicles were purified on a Sephacryl S-1000 column [15], resuspended in buffer A at a concentration corresponding to an amount of 10 IU AChE/ml suspension (corresponding to 2 mM total PL) and kept in this buffer at 6°C. Alternatively, vesicles were stored in buffer A containing 2.5 mM sodium orthovanadate. At timed intervals as indicated in the figures, aliquots were diluted 50 to 800 times to obtain the appropriate phospholipid concentrations (verified by comparing the AChE-activities in the stock solution and the assay samples), and immediately used in the prothrombinase assay. Samples were taken for determinations of ATP-content, and vesicle integrity was assessed using the adenylate kinase assay.

When the fraction of opened vesicles exceeded 5%, the vesicles (in a total volume of 1 ml) were subjected to purification on a 4 ml Sephacryl S-1000 column equilibrated in buffer A, by which most of the leaky vesicles could be removed.

2.9. Neuraminidase treatment of erythrocytes

An aliquot (0.5 ml) of packed fresh red blood cells was carefully suspended in 2 volumes of buffer A containing 0.5 mg/l leupeptine and 0.1 IU/ml neuraminidase, and incubated for 1 h at 37°C. The suspensions were centrifuged for 4 min at $1000 \times g$ and the supernatant was used for determinations of sialic acid release and hemolysis. All further manipulations were performed at 4°C. The cell pellets were washed 3 times with 1 ml buffer A containing 0.5 mg/l leupeptine. A 200 μl aliquot of the washed cells was used to prepare white ghosts for sialic acid determination. The remaining cells were washed once again in buffer A and assayed for prothrombinase activity as described for untreated cells.

The sialic acid determination was performed with 30 μl aliquots of packed ghosts and 25 μl aliquots of supernatant according to Skoza and Mohos [22]. Sialic acid release was more than 85% without significant hemolysis (less than 0.5%) of the cells.

2.10. SDS-polyacrylamide gel electrophoresis

Open white ghosts were prepared by hypotonic lysis according to Gratzer [23] and stored at −80°C. Vesicle membranes were obtained by incubation of an aliquot of DMPC-induced vesicles (at a concentration of 10 IU AChE/ml) with an equal volume of 3 mg/ml digitonin (Sigma) for 15 min at room temperature. The suspensions were diluted 4 times with buffer A containing 0.5 mg/l leupeptine and centrifuged at $30\,000 \times g$ for 10 min. The pellet was washed with buffer A with leupeptine and subsequently delipidified by a 2-phase lipid extraction as described by Bligh and Dyer [20], with the proteins found at the interface of the phases. The dry protein pellets were stored at −80°C.

Packed ghosts, vesicle suspensions or vesicle membrane extracts were dissolved in a buffer containing 2% SDS and 10% β-mercaptoethanol and applied to a SDS-polyacrylamide gradient gel containing 8 to 15% polyacrylamide. Electrophoresis was performed according to Laemmli [24] and the gel was stained with Coomassie Blue.

3. Results

3.1. Exposure of PS on cell and vesicle surfaces

In presence of freshly prepared DMPC-induced vesicles a higher prothrombinase activity was obtained than in presence of erythrocytes (see Table 1). An estimation of the amount of PS exposed on the outer monolayer could be

obtained by comparing the prothrombinase activity to the activity in presence of sonicated membrane systems as described in Section 2. This revealed that approximately 1.5% PS was exposed on the outside of both types of vesicles, and 0.06% on the outside of intact cells. Removal of sialic acid residues from the cells by treatment with neuraminidase did not result in a significantly increased prothrombinase activity (Table 1).

3.2. Erythrocyte storage

During storage at 6°C in presence of deoxyglucose, the cells were depleted of ATP by more than 94% within 1 week (Fig. 1B). Concomitantly, the prothrombinase activity measured in presence of the erythrocytes increased slightly (Fig. 1A), and the cells became more susceptible to hemolysis (Fig. 1C). However, even with cells stored for up to 3 weeks, the prothrombinase activity did not exceed a level that corresponded to an estimated fraction of 0.3% PS in the outer monolayer. This showed that phospholipid asymmetry is quite stabile at this low temperature in absence of ATP.

When vanadate was present during storage, the prothrombinase activity in presence of cells increased rapidly (Fig. 1A). During 3 weeks of storage PS exposure increased by a factor of 50 to reach approximately 2%. In this case, ATP was also consumed more rapidly than during storage without vanadate (Fig. 1B), and the cells were more fragile (Fig. 1C).

Because of experimental restrictions (low cell concentration) it was not possible to determine hemolysis after having performed the prothrombinase assay. Therefore, hemolysis as measured in the cell suspension (see Section 2) has to be interpreted as an increasing susceptibility of the cells to damage by sample handling, rather than the exact number of leaky cells in the prothrombinase assay medium. Thus, it can not be excluded that the increase in prothrombinase activity observed in Fig. 1A is due to exposure of PS located at the inner monolayer of lysed cells, even though there is no direct quantitative correlation between the patterns of cell fragility (Fig. 1C) and prothrombinase activity (Fig. 1A). As deduced from the assay performed with completely hemolyzed cells (see Section 2) each percent of hemolysis would enhance the prothrombinase activity only by approximately 0.85 nM/min · μM PL, which is significantly lower than the increase in activity shown in Fig. 1A.

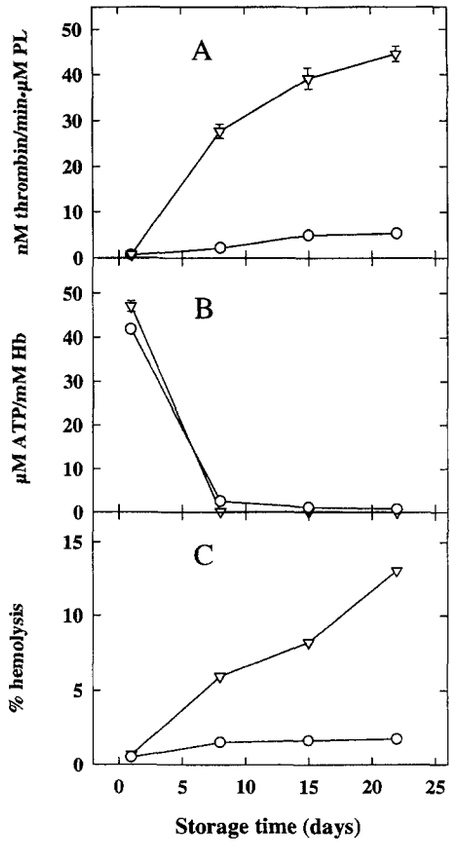

Fig. 1. Prothrombinase activity, ATP-content and cell fragility of red blood cells during storage. Erythrocytes were stored at 6°C in presence of deoxyglucose in absence (circles) and presence (triangles) of 2.5 mM vanadate as described in Section 2. Determinations of prothrombinase activity, ATP-content and hemolysis were performed at the indicated times. The results shown are data from one experiment with blood from one donor. The experiment was reproduced 3 times with different blood specimens. Because of variation in initial values (prothrombinase activity 0.36 to 0.85 nM thrombin/min·μM PL, ATP-content 42 to 61 μM/mM hemoglobin) the data were not included in the figures. However, for each blood sample, the curves displayed the same characteristic dependence with time. Data points without apparent error bars reflect counting errors less than the size of the data symbols. Panel A: prothrombinase activity per μM phospholipid. The vertical bars indicate the S.D. in repeated ($n = 3$) measurements of the same specimen. Panel B: ATP-content of the cells per mM hemoglobin (duplicate measurements). Panel C: percentage of spontaneously hemolysed cells.

Table 1
Prothrombinase activities obtained with regular erythrocytes, neuraminidase-treated cells, and with low- and high-ATP DMPC-induced vesicles directly after preparation

Membrane system	Prothrombinase activity (nM thrombin/min·μM PL)	n
erythrocytes	1.06 ± 0.54	12
erythrocytes, neuraminidase treated	1.47 [a]	1
low-ATP vesicles	19.6 ± 4.7	6
high-ATP vesicles	31.9 ± 10.7	5

Data for erythrocytes and low ATP vesicles were in part published in [8]. Values are given as mean ± standard error, n is the number of different cell or vesicle populations.

[a] The concomitant value for untreated cells of the same blood population was 1.62 nM/min·μM PL.

3.3. Storage of DMPC-induced vesicles

During 22 days of storage at 6°C the prothrombinase activity in presence of low-ATP vesicles (Fig. 2A) in-

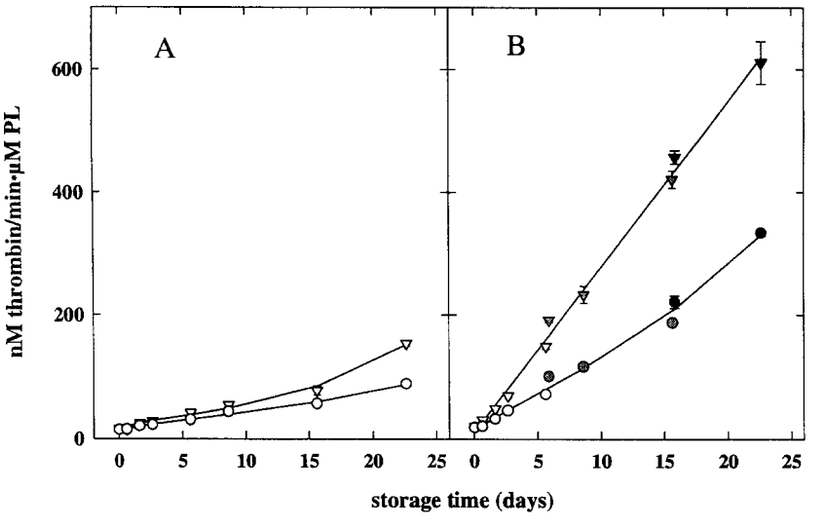

Fig. 2. Prothrombinase activity in presence of DMPC-induced vesicles during storage. DMPC-induced vesicles were prepared from either regular erythrocytes with an ATP-level of 65 μM/mM hemoglobin (low-ATP vesicles), or from cells with an increased ATP-level of 184 μM/mM hemoglobin (high-ATP vesicles), and stored at 6°C. Panel A shows the prothrombinase activity in presence of low-ATP vesicles upon storage in absence (circles) and presence (triangles) of 2.5 mM vanadate. Panel B shows the same in presence of high-ATP vesicles. The results presented are data from one experiment representative of 4 storage experiments with different blood specimens in absence of vanadate. The experiment in presence of vanadate was reproduced once with a different vesicle specimen in presence of 1 mM vanadate, with consistent results. Because of variation in the initial values (14.2 to 45.2 nM thrombin/min · μM PL), the data from the other experiments could not be included in the figures. However, for each vesicle preparation the curves indicated the same characteristic dependence with time. The vertical bars indicated in the figures represent the variation between triplicate measurements of the same specimen. Data points without apparent error bars reflect counting errors less than the size of the data symbols. A change in symbol filling indicates an in-between purification of the vesicles as described in Section 2.

creased by a factor of 5, which reflected an estimated final distribution of 5% PS exposed on the outside. The ATP-level, as shown in Fig. 3A, had decreased at this point to 15% of the ATP-content of freshly prepared low-ATP vesicles, which corresponded to a level of less than 5% of the ATP-content of regular erythrocytes.

In presence of high-ATP vesicles the prothrombinase activity was in a similar range as the activity in presence

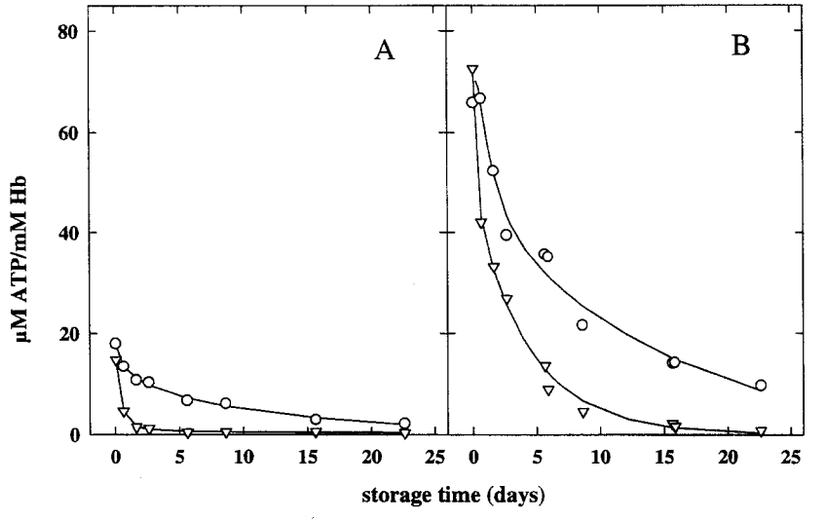

Fig. 3. ATP-depletion during storage of DMPC-induced vesicles. Panel A shows the ATP-content of low-ATP vesicles upon storage in absence (circles) and presence (triangles) of 2.5 mM vanadate. Panel B shows the same for high-ATP vesicles. The results shown are data from one representative experiment (same blood specimen as in Fig. 2) of 4 storage experiments with different blood specimens in absence of vanadate. The experiment in presence of vanadate was reproduced once in presence of 1 mM vanadate, which showed a slightly lower ATP-hydrolysis rate. Because of variation in the initial values (ATP-content of low-ATP vesicles 6.9 to 18.0 μM/mM hemoglobin, of high-ATP vesicles 51.6 to 95.3 μM/mM hemoglobin), the data from the other experiments were not included in the figures. However, for each vesicle specimen the curves showed the same characteristic dependence with time.

of low-ATP vesicles when both populations were measured directly after the preparation (see Table 1). However, as shown in Fig. 2B, an 18-fold increase in prothrombinase activity was seen after 22 days, in spite of the fact that the ATP-level of this system (Fig. 3B) was 4 times higher than that of the low-ATP vesicles. This shows that the prothrombinase activity in presence of the vesicles does not correlate to their ATP-content. According to our estimation approximately 20% PS was found on the outside of the high-ATP vesicles after 22 days.

3.4. Storage of DMPC-induced vesicles in presence of vanadate

To obtain information on the influence of vanadate on the vesiculation process, both low- and high-ATP vesicles were prepared in presence of 1 mM vanadate. The time dependence and quantity of vesicle release, monitored by AChE-activity, was not altered. The presence of vanadate during the vesiculation process did not influence the ATP content of the released vesicles, and the composition of the obtained vesicles with respect to phospholipid and protein content was not different from normal DMPC-induced vesicles (data not shown). On the other hand, in high-ATP vesicles the initial velocity of translocation as measured with a spin-labelled PS-analogue (according to [15]) was inhibited by 75%, independent of the time point of addition of vanadate (data not shown). Based upon these observations, vesicles were prepared without vanadate (as described above) and subsequently stored in presence of 2.5 mM vanadate.

With low-ATP vesicles stored in presence of vanadate, the prothrombinase activity (Fig. 2A) was approximately 20% higher than in absence of vanadate. After 22 days of storage, 6% PS was estimated to be located in the outer monolayer. On the other hand, in presence of high-ATP vesicles a much more pronounced effect of vanadate was observed (Fig. 2B) with a 2-fold increase in activity compared to storage without vanadate, leading to exposure of approximately 40% of the total PS-pool after 22 days. Furthermore, in both types of vesicles this was accompanied by a more rapid loss of ATP (Fig. 3).

A parallel investigation of vesicle membrane asymmetry with phospholipase A_2 turned out to be unreliable, because the high-ATP vesicles immediately lysed upon treatment with the enzyme, suggesting a decreased membrane stability.

3.5. Vesicle integrity during storage

In the high-ATP vesicle preparations the fraction of leaky vesicles was always higher than in the low-ATP preparations, indicating that the high-ATP vesicles were more fragile (Fig. 4). This fragility was markedly increased in presence of vanadate. To make sure that increased prothrombinase activity was not due to exposure of PS in lysed vesicles, high-ATP vesicles were subjected to several purification steps during the storage period. A comparison of Fig. 2B with Fig. 4B shows that a marked reduction of the fraction of leaky vesicles after purification (indicated by a change in symbol filling) did not result in a concomitant reduction of the prothrombinase activity, indi-

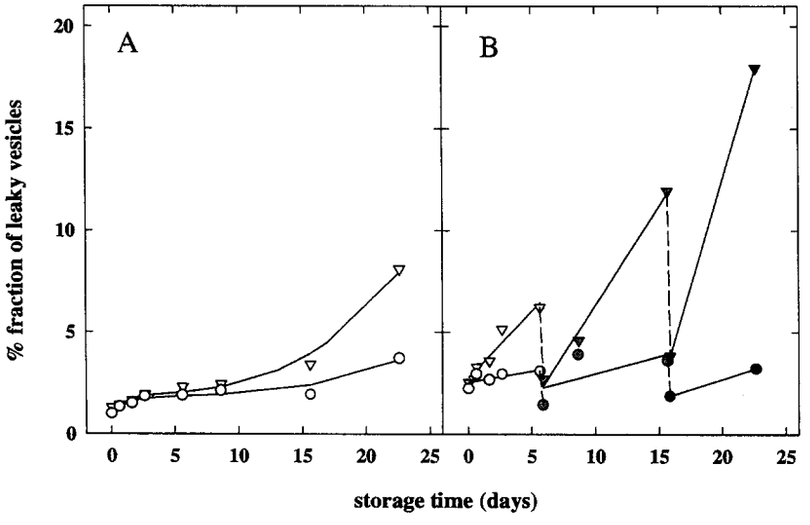

Fig. 4. Adenylate kinase index of DMPC-induced vesicles during storage. Values are expressed as the percent ratio of adenylate kinase activity in absence and in presence of Triton X-100. Panel A shows the integrity of low-ATP vesicles during storage in absence (circles) and presence (triangles) of 2.5 mM vanadate. Panel B shows the same for high-ATP vesicles. The results shown are data from one storage experiment (same blood specimen as in Fig. 2 and 3) representative of 4 experiments with different blood specimens in absence of vanadate. The experiment in presence of vanadate was reproduced once in presence of 1 mM vanadate, with consistent results. A change in symbol filling indicates an in-between purification of the vesicles as described under Section 2.

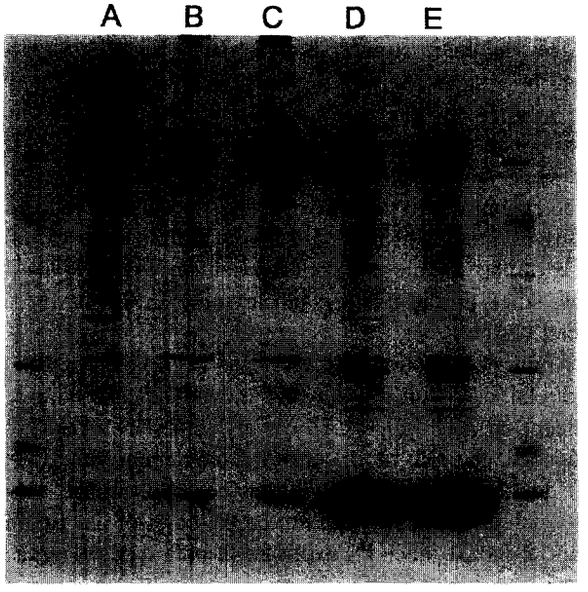

Fig. 5. SDS-polyacrylamide gel electrophoresis of the proteins from red cell membranes, vesicles, and isolated vesicle membranes. Proteins were stained with Coomassie blue. Subsequent silver staining did not reveal any further differences between the vesicle types (data not shown). Lane A: red cell membranes; lane B: delipidified low-ATP vesicle membranes; lane C: delipidified high-ATP vesicle membranes; lane D: low-ATP vesicles; lane E: high-ATP vesicles. The fractions were prepared as described under Section 2. The outer lanes show molecular weight standards (94, 67, 43, 30, 20.1, and 14.4 kDa).

cating that this activity is not correlated to leakiness of the vesicles (at least below the level of 10% leaky vesicles).

3.6. Protein compositions

SDS-polyacrylamide gel electrophoresis of the protein of membranes isolated from DMPC-induced vesicles, as depicted in Fig. 5, showed that vesicle membranes contain the main intrinsic membrane proteins of the red cell membrane. On the other hand, spectrin was completely lacking and other peripheral (skeleton) proteins such as band 4.1 were partly absent. This confirms previous studies performed with intact vesicles, and, furthermore, demonstrates that both high- and low-ATP vesicles show the same membrane protein composition, which in turn suggests that protein composition can not be the reason for the increased fragility of the high-ATP vesicles.

4. Discussion

The rate of conversion of prothrombin to thrombin by the enzyme complex factor Xa-factor Va has been widely used as a convenient, rapid, and sensitive method to monitor in a semiquantitative way the extent of exposure of PS at the cell surface [8,17,25,26]. By this method the fraction of PS at the outer monolayer of erythrocyte membranes was estimated to be less than 0.1% under normal conditions (see Table 1, and [8]). No increase in prothrombinase activity was observed in presence of neuraminidase treated cells, indicating that the very low activity in presence of the cells is not due to reduced accessibility of the phospholipid surface to the factors of the prothrombinase complex, through shielding by sialic acid residues.

Several studies have reported that phospholipid asymmetry is not immediately lost upon ATP-depletion [9–11], or during prolonged storage of red blood cells [12]. In the present study, it is accordingly shown that PS asymmetry is relatively stabile during storage of the cells at 6°C in presence of deoxyglucose, even when ATP-depletion is complete. The maximum fraction of PS exposed to the outside after 3 weeks of storage was 0.3%, which is definitely too low to be recognized by conventional techniques, like by the assay with phospholipase A_2 [12]. Increasing cell fragility, reflected by enhanced spontaneous hemolysis, might have lead to the slight increase in prothrombinase activity observed during the storage time (Fig. 1C). This suggests that ATP-depletion induces secondary events that affect cell fragility, rather than that it has a direct effect on phospholipid redistribution. Indeed, the intracellular level of ATP influences the degree of phosphorylation of membrane skeletal proteins, which has been suggested to alter the stability of the membrane [27]. Apart from ATP-depletion, several abnormalities have been reported to occur in red cells during in vitro storage, presumably due to oxidative damage [28].

In presence of low-ATP DMPC-induced vesicles the prothrombinase activity is significantly higher than the activity in presence of the cells they are derived from, even though the distribution of PS is still highly asymmetric [8]. As stated before, treatment of the cells with neuraminidase has revealed that this difference is probably not due to increased accessibility of the vesicle phospholipid bilayer to the enzymes of the prothrombinase complex.

The observed enhanced exposure of PS on the outer monolayer of the vesicles has to arise in the relatively short period of approximately 5 h that pass between the onset of vesicle release and the first assay. Spontaneous outward movement (flop) of spin-labelled PS analogues in presence of Mg-ATP has been reported to have a half-time of 1 h at 37°C [29], but with synthetic dilauroyl-PS this was reported to be 15 h [30]. In analogy to phosphatidylcholine-flop [31], PS-flop is likely to be dependent on acyl chain length, thus slower with the endogenous long fatty acyl chain phospholipids. Moreover, the temperature has not exceeded 30°C during preparation and purification of the vesicles. Thus, the observed redistribution can hardly be attributed to spontaneous outward movement of PS alone.

As discussed before in the same context [8], increased transbilayer movement of the phospholipids could have occurred at fusion sites during vesicle budding [2,32,33]. Our present results however, do not support this model. In

the first place, asymmetry would have been restored more efficiently in the high-ATP vesicles because of the higher aminophospholipid translocase activity in these vesicles [15], whereas low- and high-ATP vesicles showed similar initial prothrombinase activities (see Table 1), which were dependent on the blood specimen they were derived from, rather than on the ATP-content (unpublished observations). Moreover, the presence of vanadate during the preparation of vesicles did not influence the vesiculation process and the phospholipid distribution in the vesicle membranes, since the initial prothrombinase activities were similar in presence and absence of vanadate, showing that the aminophospholipid translocase does not play a role in the vesiculation process.

A more likely explanation for the observed loss of asymmetry is therefore, that structural differences between cells and vesicles are responsible for the new steady-state transbilayer distribution in the vesicles. As discussed earlier in this context [8], increased accessibility of PS in the vesicles to the enzymes of the prothrombinase complex might have originated from an increased transbilayer mobility induced by the slightly changed lipid composition of the vesicle membrane [34], from a difference in the ratio outer versus inner monolayer due to curvature of the membrane, or from a difference in relative membrane surface versus total phospholipid content, which affects the PS-density [26]. Also, it can not be excluded at present, that the absence of an intact membrane skeleton is responsible for the observed difference in PS-asymmetry between vesicles and red blood cells [4,35].

Once the new steady-state situation is established, the low-ATP vesicle asymmetry remains fairly stabile. The prothrombinase activity in presence of low-ATP vesicles after 22 days of storage reflected a still highly asymmetric distribution of PS (estimated maximally 5% outside). Because DMPC-induced vesicles lack the capability of regenerating ATP [15], storage is accompanied by loss of ATP, independent of the amount of nutrients in the storage medium. The final ATP-level after 22 days of storage was below 5% of the normal ATP-content of erythrocytes, which is not sufficient for full activity of the aminophospholipid translocase [2].

Paradoxically, the high-ATP vesicles lost their PS-asymmetry 3 to 4 times more rapidly than the low-ATP vesicles, although the ATP-level in this system was approximately 4 times higher during the entire storage period (Fig. 3). This controversial relation between the (re)distribution of PS and the ATP-content of the vesicles suggests that other mechanisms than the aminophospholipid translocase play a role in maintaining phospholipid asymmetry.

It has been reported [31,36] that in a bilayer system consisting of long acyl chain phospholipids in presence of cholesterol, spontaneous transbilayer diffusion is very slow. This would be able to explain the extreme stability observed in the low-ATP vesicles during storage at low temperature. However, this does not explain the observed loss of PS-asymmetry in the high-ATP vesicles, since there are no apparent structural differences between the two types of vesicles, neither in phospholipid composition, nor in cholesterol to phospholipid ratio (unpublished observations) or in protein composition (Fig. 5). This means that the same structure is disturbed in case of the high-ATP vesicles, but not in low-ATP vesicles, or in red blood cells where asymmetry is more efficiently maintained. An influence of the membrane skeleton can be excluded in this case, since this is not present anymore in the DMPC-induced vesicles.

Aminophospholipid transport has been suggested to be bi-directional, with the outward movement also being dependent on ATP [29,37]. However, with such an ATP-dependence, the rate of PS outward movement would actually decrease when more ATP has been consumed. Therefore such a mechanism can not be the basis of our findings (Figs. 2 and 3), since PS exposure in both types of vesicles increases progressively with time and does not appear to be dependent on ATP concentrations.

Another mechanism that has been shown to induce scrambling of phospholipids, is increasing the intracellular Ca^{2+} concentration to millimolar levels [38], provided that the aminophospholipid translocase and the Ca^{2+}-pump are inhibited. Since the prothrombinase assay medium contains 4 mM Ca^{2+}, it can not be excluded from our experiments that increased permeability to this ion, leading to a sudden rise in the internal Ca^{2+} concentration during the assay, might have induced the observed loss of PS asymmetry in the vesicles. However, Ca^{2+}-induced scrambling has been reported to take 1 h to complete in erythrocytes [32,38,39], whereas in our experiments the prothrombinase activity expressed per minute was independent of the time interval of measurement up to 3 min (data not shown). This implies that either scrambling of the vesicle phospholipids was completed within the 2 min of preincubation in Ca^{2+}-containing buffer, or that Ca^{2+} internalization simply has not occurred.

Even when asymmetry would be lost due to the presence of Ca^{2+} in the assay rather than to a defect initiated upon storage of the vesicles, it is not clear what mechanism would have made high-ATP vesicles more fragile and thus more permeable to Ca^{2+} than low-ATP vesicles. In particular, removal of that fraction of vesicles that were known to be leaky did not lead to a decrease in prothrombinase activity (compare Fig. 2B and 4B), implying that even in presence of the population of permeable vesicles the prothrombinase activity was not increased. Moreover, it could be expected that both the Ca^{2+}-pump and aminophospholipid translocase are more active in high-ATP vesicles, making them better able to maintain low internal Ca^{2+} concentrations and to restore membrane asymmetry, which is clearly not the case. Sulpice et al. [40] have proposed that the presence of PIP_2 in the membrane is correlated to Ca^{2+}-induced scrambling, what would agree with the content of this phosphorylated lipid being higher

in high-ATP vesicles, thereby inducing more scrambling, whereas it would be diminished in the low-ATP vesicles. However, as earlier mentioned, high-ATP vesicles lose their ATP content during storage, what would accordingly result in a decrease in PIP_2 content [40], and thus a decrease in the ability to scramble. Since scrambling continuously increased with time of storage (Fig. 1B), any mechanism related to the transiently increased presence of a phosphorylated compound can not agree with our results.

The loss of asymmetry observed in presence of vanadate in low-ATP vesicles and even more pronounced in high-ATP vesicles (Fig. 2), as well as in red blood cells (Fig. 1A), might be attributed to an inhibition of the aminophospholipid translocase. However, here again, it should be expected that with decreasing ATP-content of the vesicles the effect of vanadate decreases, since in a situation of ATP depletion the contribution of the translocase to maintaining asymmetry is diminished as well. Yet, the scrambling effect of vanadate did not decrease upon prolonged storage, even though ATP was actually hydrolysed much more rapidly. Therefore, a direct perturbing effect of vanadate on membrane asymmetry has to be considered. This would be in line with an observation of Bitbol et al. [16] who have proposed that, after reduction of vanadate to vanadyl [41], this ion interacts directly with the phospholipids. Since the intracellular reduction of vanadate is related to processes that require energy, this might also explain the enhanced ATP decrease in presence of vanadate [41].

A valid explanation for the different behaviors of the two vesicle populations can not be provided yet. Several ATP-dependent processes may contribute, such as the modulation of either protein and lipid phosphorylation [42] or transmembrane pH or ion gradients [43,44]. Further investigations will have to reveal the exact nature of the underlying phenomenon.

In conclusion, we have shown that the asymmetric distribution of PS is well preserved in low-ATP DMPC-induced vesicles, in spite of the fact that these membrane vesicles do not contain an intact membrane skeleton, and under conditions where the aminophospholipid translocase is not able to contribute to PS inward transport. Moreover, in identical vesicles with an elevated ATP-content PS-asymmetry is perturbed more rapidly, which indicates that this phospholipid redistribution is independent of ATP and membrane composition. We therefore postulate that other mechanisms must be involved in maintaining phospholipid asymmetry.

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References

[1] Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71.
[2] Devaux, P.F. (1991) Biochemistry 30, 1163–1173.
[3] Zachowski, A. and Devaux, P.F. (1990) Experientia 46, 644–656.
[4] 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.
[5] Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32.
[6] Gudi, S.R.P., Kumar, A., Bhakumi, V., Gokhale, S.M. and Gupta, C.M. (1990) Biochim. Biophys. Acta 1023, 63–72.
[7] Kuypers, F.A., Lubin, B.H., Yee, M., Agre, P., Devaux, P.F. and Geldwerth, D. (1993) Blood 81, 1051–1057.
[8] De Jong, K. and Ott, P. (1993) FEBS Lett. 334, 183–188.
[9] Tilley, L., Cribier, S., Roelofsen, B., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1986) FEBS Lett. 194, 21–27.
[10] Henseleit, U., Plasa, G. and Haest, C. (1990) Biochim. Biophys. Acta 1029, 127–135.
[11] Sugihara, T., Sugihara, K. and Hebbel, R.F.A. (1992) Biochim. Biophys. Acta 1103, 303–306.
[12] Geldwerth, D., Kuypers, F.A., Bütikofer, P., Allary, M., Lubin, B.H. and Devaux, P.F. (1993) J. Clin. Invest. 92, 308–314.
[13] Ott, P., Hope, M.J., Verkleij, A.J., Roelofsen, B., Brodbeck, U. and van Deenen, L.L.M. (1981) Biochim. Biophys. Acta 641, 79–87.
[14] Weitz, M., Bjerrum, O.J., Ott, P. and Brodbeck, U. (1982) J. Cell. Biochem. 19, 179–191.
[15] Beleznay, Zs., Zachowski, A., Devaux, P.F., Puente Navazo, M. and Ott, P. (1993) Biochemistry 32, 3146–3152.
[16] Bitbol, M., Fellmann, P., Zachowski, A. and Devaux, P.F. (1987) Biochim. Biophys. Acta 904, 268–282.
[17] Bevers, E.M., Comfurius, P., Van Rijn, J.L.M.L., Hemker, H.C. and Zwaal, R.F.A. (1982) Eur. J. Biochem. 122, 429–436.
[18] Ellmann, G.L., Courtney, D.K., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88–95.
[19] Rose, H.G. and Oklander, M. (1965) J. Lipid Res. 6, 428–431.
[20] Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
[21] Rouser, G., Fleischer, S. and Yamamoto, A. (1970), Lipids 5, 494–496.
[22] Skoza, L. and Mohos, S. (1976) Biochem. J. 159, 457–462.
[23] Gratzer, W.B. (1982) Meth. Enzymol. 85, 475–480.
[24] Laemmli, U.K. (1970) Nature (London) 227, 680–685.
[25] Bevers, E.M., Comfurius, P. and Zwaal, R.F.A. (1982) Eur. J. Biochem. 122, 81–85.
[26] Connor, J., Bucana, C., Fidler, I.J. and Schroit, A.J. (1989) Proc. Natl. Acad. Sci. USA 86, 3184–3188.
[27] Boivin, P. (1988) Biochem. J. 256, 689–695
[28] Wolfe, L.C. (1989) Semin. Hematol. 26, 307–312.
[29] Bitbol, M. and Devaux, P.F. (1988) Proc. Natl. Acad. Sci. USA 85, 6783–6787.
[30] Daleke, D.L. and Huestis, W.H. (1989) J. Cell Biol. 108, 1375–1385.
[31] Middelkoop, E., Lubin, B.H., Op den Kamp, J.A.F. and Roelofsen, B. (1986) Biochim. Biophys. Acta 855, 421–424.
[32] Comfurius, P., Senden, J.M.G., Tilly, R.H.J., Schroit, A.J., Bevers, E.M. and Zwaal, R.F.A. (1990) Biochim. Biophys. Acta 1026, 153–160.
[33] Lucy, J.A. (1993) Biochem. Soc. Trans. 21, 280–283.
[34] Morrot, G., Hervé, P., Zachowski, A., Fellmann, P. and Devaux, P.F. (1989) Biochemistry 28, 3456–3462.
[35] Franck, P.H.F., Op den Kamp, J.A.F., Roelofsen, B. and Van Deenen, L.L.M. (1986) Biochim. Biophys. Acta 857, 127–130.
[36] Daleke, D.L. and Huestis, W.H. (1985) Biochemistry 24, 5406–5416.
[37] Connor, J., Pak, C.H., Zwaal, R.F.A. and Schroit, A.J. (1992) J. Biol. Chem. 267, 19412–19417.
[38] Williamson, P., Algarin, L., Bateman, J., Choe, H.-R. and Schlegel, R.A. (1985) J. Cell. Physiol. 123, 209–214.

[39] Williamson, P., Kulick, A., Zachowski, A., Schlegel, R.A. and Devaux, P.F. (1992) Biochemistry 31, 6355–6360.
[40] Sulpice, J.-C., Zachowski, A., Devaux, P.F. and Giraud, F. (1994) J. Biol. Chem. 269, 6347–6354.
[41] Macara, I.G., Kustin, K. and Cantley Jr., L.C. (1980) Biochim. Biophys. Acta 629, 95–106.
[42] Ferrell Jr., J.E. and Huestis, W.H. (1984) J. Cell Biol. 98, 1992–1998.
[43] Eastman, S.J., Hope, M.J. and Cullis, P.R. (1991) Biochemistry 30, 1740–1745.
[44] Redelmeier, T.E., Hope, M.J. and Cullis, P.R. (1990) Biochemistry 29, 3046–3053.