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Membrane phospholipid asymmetry in DMPC-induced human red cell vesicles

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Vesicles that do not contain spectrin were released from human erythrocytes by incubation with dimyristoylphosphatidylcholine. The transbilayer orientation of membrane phospholipids was subsequently determined by two independent methods. Incubation with phospholipase A_2 revealed that the phospholipid asymmetry observed in red blood cells was essentially preserved in vesicles. By use of the prothrombinase assay a still highly asymmetric distribution of phosphatidylserine could be demonstrated in spite of its slightly increased exposure on the vesicle surface. These results show that membrane phospholipid asymmetry can be maintained in a system that does not contain an intact membrane skeleton or spectrin.

Phospholipid asymmetry; Phospholipase A_2; Prothrombinase assay; Erythrocyte; Membrane vesicle; Membrane skeleton

1. INTRODUCTION

Red blood cell membranes, as well many other cell membranes, have their phospholipids distributed asymmetrically over both leaflets of the bilayer. Whereas the aminophospholipids phosphatidylethanolamine and phosphatidylserine are primarily located at the cytoplasmic side, the choline-containing phospholipids phosphatidylcholine and sphingomyelin are mainly present at the exterior side of the cell membrane [1–3]. Asymmetry is maintained in spite of a transbilayer movement of some of these phospholipids which is relatively fast with respect to the red cell life span. Hence, lipid asymmetry must be maintained by processes that counteract such a long-term randomization.

An ATP-dependent aminophospholipid translocase, that selectively transports the aminophospholipids from the outer to the inner monolayer, has been claimed to be able to generate and maintain phospholipid asymmetry (for review, see [4]). Alternatively, it has been proposed that interactions between aminophospholipids and the membrane skeleton (in particular spectrin and band 4.1) also participate in supporting phospholipid asymmetry. Such interactions have indeed been shown to occur [5–7] but do not appear to be strong enough to explain lipid asymmetry sufficiently [8]. A study with erythrocytes from sickle cell patients suggested that both the aminophospholipid translocase and the membrane skeleton are required [9]. However, recent studies have indicated that the glycerophospholipid distribution remained essentially unaffected in erythrocytes where spectrin was heat denatured [10] or in cells that were partially spectrin-deficient [11], suggesting that this component of the membrane skeleton cannot be the major responsible factor for maintaining transbilayer phospholipid asymmetry.

An approach to examine the importance of the components of the membrane skeleton in preserving phospholipid asymmetry is to prepare and study erythrocyte membrane fragments that lack (parts of) the membrane skeleton. In fact, erythrocytes release membrane vesicles under a variety of conditions and different methods to generate these structures in vitro have been described. So far, determinations of phospholipid asymmetry in these microvesicles have revealed controversial results: in some types of vesicles asymmetry appeared to be preserved [12,13], whereas in others complete scrambling of the phospholipids was observed [14,15].

The vesicles released upon incubation of red blood cells with dimyristoylphosphatidylcholine (DMPC) [16] contain intrinsic membrane proteins but are essentially devoid of spectrin and other elements of the membrane skeleton [17]. Their phospholipid composition is almost identical to the one observed in intact erythrocytes [16]. Recently it was shown that the DMPC-induced vesicles contain an ATP-dependent aminophospholipid translocase activity [18], but the steady-state distribution of the endogenous phospholipids has not yet been characterized.

In the present study, DMPC-induced vesicles were

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Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine; PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; PLA_2, phospholipase A_2; Hgb, haemoglobin; ATP, adenosine 5′-triphosphate; EDTA, ethylene diamine tetraacetic acid; HSA, human serum albumin; AChE, acetylcholinesterase.

used as a model system to examine the orientation of endogenous red cell phospholipids in the absence of an intact membrane skeleton. Phospholipase A_2 and the prothrombinase assay were applied for characterization of asymmetry. The results show that, in spite of a slight destabilization of the membrane, the asymmetric distribution of phospholipids observed in the red blood cell membrane is essentially preserved in the vesicles.

2. MATERIALS AND METHODS

2.1. *Materials*

Phospholipase A_2 (EC 3.1.1.4) from bee venom (Sigma USA) was dissolved in 100 mM TRIS-HCl, 5 mM $CaCl_2$, pH 7.8, 50% glycerol (v/v) to a concentration of 5 IU/μl, stored at −20°C and used without further purification.

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.

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

2.2. *Erythrocytes*

Erythrocytes were obtained from the ZLB Central Laboratory Blood Transfusion Service SRC (Bern, Switzerland), stored at 4°C and used within 3 days. The cells were washed at least 3 times with isotonic buffer by centrifugation at $1,000 \times g$ for 7 min. After each wash the supernatant and rest of the buffy coat were removed carefully by aspiration.

2.3. *Vesicle preparation*

DMPC-induced vesicles were prepared and purified as described elsewhere [18]. Briefly, freshly washed erythrocytes were incubated in a buffer containing 10 mM TRIS-HCl, 144 mM NaCl, 0.5 mM adenine, 10 mM glucose, 1 mM EDTA, 2×10^5 U/l Penicillin, 1.5×10^5 U/l Streptomycin, pH 7.4, for 4.5 h at 30°C in presence of small unilamellar DMPC-vesicles. After separation from the remnant cells by centrifugation at $1,000 \times g$, the released crude vesicles were pelleted at $34,500 \times g$ and resuspended in buffer A (10 mM HEPES, 144 mM NaCl, pH 7.4) for subsequent purification on a Sephacryl S-1000 superfine (Pharmacia Fine Chemicals) column. After purification the vesicles were stored overnight at 4°C in buffer A.

Yield and recovery of vesicles during the preparation procedure were routinely controlled by determination of the membrane marker enzyme acetylcholinesterase (AChE), measured according to Ellmann et al. [19]. To control the permeability of the vesicle membranes, the cytoplasmic enzyme adenylate kinase was used as a marker. Its activity was measured essentially as described by Beleznay et al. [18], by following the time course of ATP production. Vesicle integrity was expressed as the percent ratio of the activities measured in the absence and presence of 0.01% (v/v) Triton X-100. The relative amount of leaky vesicles did not exceed 2.3% (1.41% ± 0.43, $n = 10$). The total ATP content of the vesicles, determined as described by Beleznay et al. [18], was found to be 11.0 ± 5.4 μM ATP per mM Hgb ($n = 11$), which is 14% of the average value in intact red cells (80 ± 17 μM ATP/mM Hgb, $n = 26$).

2.4. *Phospholipase A_2 assay*

2.4.1. Erythrocytes

Freshly washed red blood cells were resuspended in buffer B (25 mM TRIS-HCl, 30 mM NaCl, 90 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, and 40 mM sucrose, pH 7.8) at a hematocrit of about 8%, equivalent to a phospholipid concentration of 0.35 mM. After addition of 10 IU/ml bee venom PLA_2 the cells were incubated in a rotary shaking waterbath at 110 rpm for 1 h at 37°C. The incubation was stopped by addition of EDTA (pH 7.8) to a final concentration of 10 mM. Aliquots of 2.5 ml were taken for lipid extraction, and hemolysis was estimated by comparing the haemoglobin concentration (measured at 418 nm) of a $1,000 \times g$ supernatant to an equivalent amount of cell hemolysate. Control lipid extracts were made from cell suspensions that had not been exposed to PLA_2.

2.4.2. Ghosts

Open white ghosts were prepared according to Gratzer [20], resuspended in buffer B at a concentration of 0.3 mM total phospholipid and incubated with bee venom PLA_2 as described for erythrocytes.

2.4.3. Vesicles

Vesicles were pelleted by centrifugation at $34,500 \times g$ for 30 min, and resuspended in buffer B to a final concentration corresponding to approximately 1.8 IU AChE/ml, equivalent to a concentration of total phospholipid in the range of 0.29 to 0.35 mM. PLA_2 from bee venom was added to a final concentration of 12 IU/ml and the suspensions with a volume of 3 ml were incubated for increasing time intervals at 37°C in a rotary shaking waterbath at 110 rpm. The incubation was stopped by addition of EDTA (pH 7.8) to a final concentration of 10 mM. Control vesicles were incubated in absence of PLA_2.

Aliquots of 0.5 ml were taken for characterization of vesicle integrity, and kept on ice until used. The remaining incubated suspension was used for lipid extraction.

2.5. *Vesicle integrity*

To characterize vesicle integrity after incubation with PLA_2, haemoglobin was used as a cytoplasmic marker protein. The relative amount of leaky vesicles was calculated by comparing the haemoglobin concentration in supernatants and vesicle lysates, measured photometrically using a Molecular Devices Vmax Kinetic Microplate Reader. Supernatants were obtained by centrifugation of a 180 μl aliquot of vesicle suspension for 1 min at $200,000 \times g$ using a Beckman Airfuge Air-Driven Ultracentrifuge. To obtain a vesicle lysate, 500 μl of unincubated vesicle suspension was mixed with 20 μl of an 1% (v/v) Triton X-100 solution (corresponding to the volume of EDTA added after PLA_2 incubation) to yield a final concentration of 0.04% (v/v) Triton X-100. This lysate was further diluted in the range from 5- to 100-fold with buffer B.

2.6. *Phospholipid determination*

Lipids were extracted from erythrocyte suspensions according to the method of Rose and Oklander [21], and from vesicle and ghost suspensions according to the method of Bligh and Dyer [22].

Total phospholipid was quantified by measuring the phospholipid phosphorus according to Rouser et al. [23]. Cholesterol was measured according to Ott et al. [24], using the Monotest for cholesterol (Boehringer Mannheim) with the reagent reconstituted in 0.5% Triton X-100.

Phospholipids were separated by two-dimensional thin layer chromatography according to the method of Broekhuyse [25] using predried silica gel plates (200 by 200 mm; Merck), which were first developed in chloroform/methanol/25% ammonia/water (90:54:5.5:5.5, by volume), followed by drying and subsequent development in the second direction in chloroform/methanol/acetic acid/water (90:40:12:2, by volume). The individual phospholipid spots were detected by iodine vapour and ninhydrin spray (1% ninhydrin in acetone), scraped off and the phospholipid content of each spot was quantified by measuring the phospholipid phosphorus according to Rouser et al. [23].

2.7. *Determination of procoagulant activity (prothrombinase assay)*

The rate of thrombin formation promoted by red blood cells and vesicles was assayed essentially as described by Bevers et al. [26] in a system including purified coagulation factors Xa and Va, prothrombin and a chromogenic substrate specific for thrombin.

A 30 μl aliquot of vesicle or cell suspension (equivalent to a maximum of 0.18 and 0.38 mM total phospholipid, respectively) was added to 295 μl of a buffer containing 10 mM TRIS-HCl, 136 mM NaCl, 2.7

mM KCl, 4 mM $CaCl_2$ and 0.5 mg/ml HSA at pH 7.9. Factors Va and Xa were added and the suspension was incubated for 2 min at 37°C prior to addition of a prewarmed prothrombin solution containing 5.6 mM $CaCl_2$ and 0.5 mg/ml HSA. Final concentrations in the reaction mixture (total 500 μl) were: 3 nM factor Xa, 6 nM factor Va, 4 μM prothrombin, 4 mM $CaCl_2$, and 0.5 mg/ml HSA.

At timed intervals, aliquots of 25 μl were taken from the incubation mixture and transferred to disposable cuvets containing 1 ml buffer (50 mM TRIS-HCl, 120 mM NaCl, 2 mM EDTA, pH 7.5) to stop the thrombin formation. A volume of 75 μl of the thrombin specific chromogenic substrate S2238 (2.2 mM) was added (final concentration 150 μM) and from the rate of absorbance change at 405 nm the concentration of thrombin was calculated.

To obtain a reference prothrombinase activity in presence of red cell membrane phospholipids, the assay was performed on cell and vesicle suspensions that had been sonicated for 10 min using a Branson tip sonifier (70 W, duty cycle 50%) at a phospholipid concentration of a maximum of 5 μM.

3. RESULTS

3.1. *Lipid composition*

Table I shows that the phospholipid compositions of red blood cells and vesicles are very similar with the exception of a somewhat increased content in PC that is found in the vesicles as a result of the incubation with DMPC. The fraction of DMPC was calculated to be approximately 30% of the total PC pool of the vesicles (13% of total vesicle phospholipid) by expressing the amounts of the glycerophospholipids relative to the amount of SM. This is in agreement with earlier results [16].

The cholesterol to phospholipid molar ratio was found to be decreased in the vesicles to 0.59 ± 0.10 (n = 20) compared to 0.76 ± 0.06 (n = 15) in red blood cells.

3.2. *Lipid degradation*

3.2.1. Intact vesicles

The time course of the degradation of vesicle phospholipids by bee venom PLA_2 is summarized in Fig. 1. The data shown are expressed as reduction in the amount of undegraded phospholipid. Plateau levels of PC and PE degradation were calculated by fitting the degradation data to single exponential decay curves which resulted in a value of 56.8% for PC and of 7.6% for PE. A calculation based on the relative amounts of the degradation product and the undegraded phospholipid gave a plateau value of 55.5% for PC and 10.6% for PE (corrected for the amount of lysophospholipid present in untreated vesicles). The relative amounts of total PC and SM remained constant during the incubation, whereas the relative amount of PE appeared to increase slightly (data not shown).

Table I

Phospholipid composition of red blood cells and DMPC-induced vesicles, expressed as percent of total phospholipid

Phospholipid	Cells (%)	Vesicles (%)
PC	32.8 ± 1.9	40.7 ± 1.9
PE	25.7 ± 2.9	22.6 ± 0.9
PS	11.4 ± 1.1	11.4 ± 0.8
SM	28.1 ± 2.0	23.7 ± 1.4
PA	1.8 ± 0.5	1.7 ± 0.5

Data are the mean value ± S.D. of 18 cell and vesicle specimens.

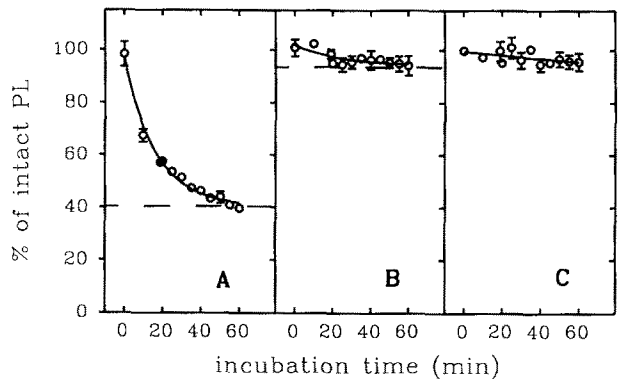

Fig. 1. Time course of phospholipid degradation in intact DMPC-induced vesicles. Phospholipase incubations were carried out as described in Section 2. The plots show the results for the degradations of PC (panel A), PE (panel B), and PS (panel C). The error bars reflect the range of results obtained from multiple measurements (up to 5) performed in 2 independent experiments. For PC and PE, the calculated plateau levels are indicated in the plots as dashed lines.

For the degradation of PS it was not possible to obtain an accurate curve fit, because of the very small decrease in the amount of this phospholipid that was observed during the incubation. The relative amount of PS that was degraded after a one hour incubation period could be estimated to be approximately 4–5%. Moreover, PS degradation could not be calculated from the lysophospholipid formed during the incubation, because a significant fraction of lyso PS was lost during the lipid extraction procedure. This was calculated to be about 70% based on data obtained after complete degradation of ghost lipids (not shown). In all experiments, lysis of the vesicles did not exceed 2.5% during the incubation time.

3.2.2. Open ghosts

A first series of control experiments was performed with open white ghosts to assess the relative rate and the extent of degradation by PLA_2 for the phospholipids of the red blood cell membrane. As shown in Fig. 2, all glycerophospholipids were degraded by more than 95% within 15 min, reflecting very high reaction rates with all phospholipids, although the degradation of PS appeared to be slightly slower than that of PC and PE.

3.2.3. Sonicated vesicles

A second series of control experiments was performed to examine to what extend bee venom PLA_2 was

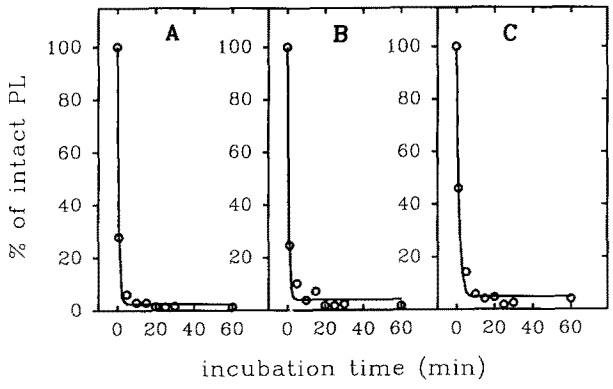

Fig. 2. Time course of phospholipid degradation in open white ghosts. Phospholipase incubations were performed as described in Section 2. The plots show the time dependence of the degradations of PC (panel A), PE (panel B) and PS (panel C).

able to hydrolyze the different phospholipids in a membrane system similar to the vesicles, but with all phospholipids randomized across both monolayers of the membrane. To obtain such a system, a vesicle suspension was sonicated for 12 min (80 W, 50% duty cycle) using a Branson tip sonifier. Subsequently the suspension was treated with PLA_2 under the same conditions as described for intact vesicles.

The results summarized in Fig. 3 show that 69% of PC, 54% of PE and 50% of PS were degraded after an incubation time of 10 min. This initial phase of rapid degradation was followed by a second phase with slower degradation rates. Even if the reaction did not go to completion during a 3 h incubation period the results still confirm that all vesicle glycerophospholipids are susceptible to PLA_2 action.

3.2.4. *Intact cells*

For comparison with earlier work, control experiments were also carried out with intact red blood cells. Degradation was complete within 1 h and reached (corrected for the amount of lysophospholipid present in untreated cells) 64 ± 2.5% for PC, and 8.6 ± 3.6% for PE (n=8) while no PS degradation could be observed, which is in close agreement with previous studies (data not shown). Hemolysis never exceeded 2%.

3.3. *Prothrombin converting activity*

The prothrombinase assay was used as a second method for the characterization of PS asymmetry. The results summarized in Table II show that the prothrombinase activity obtained with intact vesicles is considerably higher (19-fold) than with intact red blood cells. On the other hand, the ratio of activities obtained with intact and sonicated structures is low for both red cells and vesicles. The activity in presence of sonicated vesicles was only 55% of the value in presence of sonicated cells.

4. DISCUSSION

DMPC-induced vesicles are very similar to red blood cells in their composition with respect to phospholipids and integral membrane proteins, but they are completely devoid of spectrin [17]. They can therefore be considered as a membrane model system without an intact membrane skeleton and have been used in the present study to give insight into the mechanism by which phospholipid asymmetry is maintained. Transbilayer distribution of the endogenous phospholipids of the vesicles was assessed by two different, independent approaches, namely by use of phospholipase A_2 and by use of the prothrombinase assay.

The results of the experiments carried out with PLA_2 (Fig. 1) show a similar phospholipid degradation pattern for DMPC-induced vesicles and red blood cells. It has been shown that in an intact membrane system, PLA_2 is able to hydrolyse primarily the phospholipid of the outer membrane monolayer. Hence, the fraction of degraded phospholipid corresponds to the fraction of phospholipid located in that leaflet. This would suggest that the steady-state transbilayer distribution of endogenous phospholipids is comparable in the erythrocyte and vesicle membrane.

However, several aspects of phospholipid degradation have to be considered. PLA_2 has been widely used as a tool to study phospholipid asymmetry in various membrane systems, and the potential as well as the pitfalls of the assay have been described extensively [2,27]. It is well known, that phospholipases from various sources have different substrate specificities which might provoke incorrect interpretations of degradation experiments. To account for this possibility, open ghosts, in which phospholipids from both membrane leaflets are accessible to degradation, were treated with bee venom PLA_2. The degradation patterns (Fig. 2) confirmed that the enzyme was able to hydrolyse the glycerophospholipids of the erythrocyte membrane at

Table II

Prothrombinase activities obtained with red blood cells and DMPC-induced vesicles expressed as nM formed thrombin per min per μM total phospholipid

	Prothrombinase activity		Intact/sonicated
	Intact	Sonicated	Ratio
Erythrocytes	0.96 ± 0.17 (n = 4)	1505 ± 384 (n = 5)	0.064%
Vesicles	18.2 ± 3.2 (n = 3)	824 ± 179 (n = 2)	2.2%

Assays were performed as described in Section 2. The value 'n' corresponds to the number of different cell or vesicle populations. For each population up to 30 individual measurements were performed to achieve the mean value ± S.D. shown in the table.

comparable rates with 95% of total PS converted within only 15 min. This makes it appear unlikely that the low amount of this lipid degraded in DMPC-induced vesicles is due to a poor specificity of the enzyme for this particular substrate.

Furthermore, phospholipase activity is also dependent on the packing density of lipids in the membrane [28,29], which means that phospholipid hydrolysis rates in ghosts and intact vesicles are not necessarily comparable. Therefore, vesicles were sonicated to scramble the phospholipids across the bilayer before treatment with bee venom PLA_2. The observed degradation patterns (Fig. 3) show in the first place that all glycerophospholipids species can be degraded in the sonicated structures and the extent of degradation point at a more symmetric distribution due to the sonication of the vesicles. Furthermore, the biphasic degradation behavior suggests a fast degradation of the readily accessible outer monolayer phospholipids within approximately 10 min, followed by a slower degradation of the phospholipids originally located at the inner monolayer, that become accessible to the enzyme by lipid transbilayer movement or by disintegration of the vesicles. The extent of degradation after 10 min (69% for PC, 54% for PE and 50% for PS) can be explained by the observation that sonicated vesicles have an unequal distribution of phospholipids across outer and inner monolayer, with up to 70% of the lipids of these small vesicles located in the outer monolayer [30] due to packing constraints induced by the strong curvature.

Taken together, the considerations made above strongly suggest that the steady-state transbilayer disposition of the phospholipids in DMPC-induced vesicles closely corresponds to their distribution in intact red blood cell membranes. As in the intact red cells, more than 56% of the PC but only some 9% of PE and less than 5% of PS could be degraded by PLA_2, after completion of the reaction. This would reflect a localization of PC primarily in the outer layer and of PE and PS predominantly in the inner membrane leaflet.

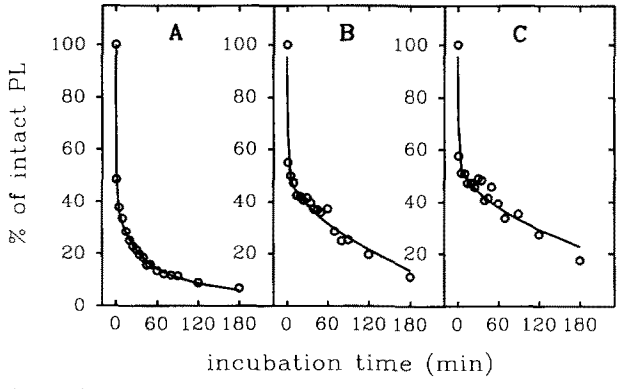

Fig. 3. Time course of phospholipid degradation in DMPC-induced vesicles after sonication. Phospholipase incubations were carried out as described in Section 2. The plots show the time dependence of the degradations of PC (panel A), PE (panel B), and PS (panel C).

The observation of an essentially maintained asymmetry with a slightly increased exposure of PS on the outer monolayer of the vesicles is further confirmed by the results obtained with the prothrombinase assay. The prothrombin converting activity of the coagulation factors Va and Xa is supposed to be promoted by phospholipids and in particular by PS. In various studies it has been shown that exposure of PS on the surface of platelet or red cell membranes results in an increased prothrombinase activity [26,31] which makes it fair to assume that prothrombinase activity reflects exposure of PS on the outer monolayer of an intact membrane system. According to the results shown in Table II, the exposure of PS on the outer monolayer of DMPC-induced vesicles is increased with respect to intact erythrocytes. The percent ratio of the activities measured with intact and sonicated vesicles (2.2%) however points at a still considerably asymmetrical phospholipid distribution. To allow an estimation of the relative amount of PS located at the exterior side of the membrane, it has to be assumed that sonicated cells expose about 70% of their phospholipid on the outer monolayer because of the curvature of the small vesicles [30]. Comparing the prothrombin converting activities measured in presence of intact and sonicated cells, it can be estimated that the relative amount of PS in the outer monolayer of intact red blood cell membranes does not exceed 0.05% of the total PS fraction. A raise in activity expressed per total phospholipid as was found with intact vesicles would, according to this calculation, still reveal an asymmetric distribution with only about 0.85% PS exposed on the outer monolayer. On the other hand, prothrombinase activity was significantly lower with sonicated vesicles than with sonicates cells. Because no difference was found in the relative content of PS between cells and vesicles (see Table I), this is must be either due to a difference in vesicle size or in the extent of phospholipid scrambling after sonication. Based on the phospholipase degradation of sonicated vesicles, it can be assumed that in sonicated vesicles maximally 50% PS is located in the outer monolayer. Thus, comparing the prothrombinase activities in presence of intact and sonicated vesicles, the fraction of PS exposed on the outer leaflet would be approximately 1.1%. Note, that values in this order of magnitude are just around the detection limit of the phospholipase assay.

Several factors can contribute to the observed slightly increased exposure of PS in vesicles. First, some of the vesicles have been shown to be fragmented (2.5%, see Section 3) with possibly both membrane layers accessible to phospholipase and to the coagulation factors of the prothrombinase assay. Second, the increased accessibility of PS may originate from an increased transbilayer mobility, due the absence of a membrane skeleton [9,32] with a concomitant destabilization of the bilayer. Furthermore, the changed composition of the vesicle membrane with respect to the cholesterol to phospho-

lipid ratio and DMPC-content might affect membrane fluidity and lipid packing [33,34] which in turn may affect PS mobility [35] and accessibility.

Finally, a scrambling of phospholipids has been suggested to occur at the fusion sites during vesicle budding [3,36,37]. In spite of the fact that an aminophospholipid translocating activity has been demonstrated in DMPC-induced vesicles by use of spin-labelled phospholipid ana'log'ues [18], it is not known at what rate the endogenous PS can be transported back to the inner monolayer. It appears from the present study that a (small) fraction of PS remains exposed in the outer monolayer, which might be correlated to the low ATP content of the vesicles.

In conclusion, the two methods by which phospholipid asymmetry was determined are in perfect agreement and the results presented in this study show a significant asymmetry in aminophospholipid distribution in spectrin-free vesicles derived from red blood cell membranes by incubation with DMPC. Together with the data of other authors [12,13] the present study suggest that asymmetry is preserved in vesicles in spite of the absence of major parts of the membrane skeleton. The finding is further supported by observations made on red cells where spectrin was either denatured or partially absent [10,11].

Vesicle membrane phospholipid asymmetry is maintained in spite of a destabilization of the membrane that is reflected by a slightly increased exposure of PS at the outer monolayer, possibly due to a somewhat increased transbilayer mobility of all phospholipids in the vesicles. It is well possible, that in vesicles the aminophospholipid translocase helps to maintain the asymmetrical distribution. In view of the low ATP content of the vesicles, however, it has to be considered that additional factors may support phospholipid asymmetry in vivo.

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