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STABILIZING FACTORS OF PHOSPHOLIPID ASYMMETRY IN THE ERYTHROCYTE MEMBRANE

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Transbilayer reorientation (flip) of exogenous lysophospholipids and changes of the transbilayer distribution of endogenous phospholipids were studied in human erythrocytes and membrane vesicles. (1) Exogenous lysophosphatidylserine irreversibly accumulates in the inner membrane layer of resealed ghosts of human erythrocytes. (2) This accumulation even occurs after complete loss of asymmetric distribution of endogenous phosphatidylethanolamine and partial loss of phosphatidylserine asymmetry in diamide-treated cells. (3) Formation of inside-out and right-side-out vesicles from erythrocyte membranes results in a loss of endogenous phospholipid asymmetry as well as of the ability to establish asymmetry of exogenous lysophosphatidylserine. Rates of transbilayer reorientation of lysophospholipids for the vesicles, however, are comparable to those for intact cells. (4) Loss of endogenous asymmetry of phosphatidylserine is also observed in vesicles isolated from erythrocytes after heat denaturation of spectrin. The asymmetry in the residual cells is maintained. (5) In contrast to the loss of asymmetry of phosphatidylethanolamine and of phosphatidylserine, the asymmetry of sphingomyelin is completely maintained in the vesicles. (6) The stability of phospholipid asymmetry in the native cell is discussed in terms of a limitation of access of phospholipids to hypothetical reorientation sites. Such a limitation may either be the result of interaction of phospholipids with the membrane skeleton as in case of phosphatidylserine and phosphatidylethanolamine, or the result of lipid-lipid interactions as in case of sphingomyelin.

Introduction

The asymmetric distribution of phospholipids between the inner and outer layer of the erythrocyte membrane gets lost upon oxidation of membrane SH-groups to disulfide bonds by diamide [1]. Disulfide bond formation is accompanied by crosslinking of the membrane skeletal protein, spectrin [1], and the aggregation of membrane skeletal proteins as well as of intrinsic proteins [2,3]. The loss of asymmetry is not an indiscriminate process, since a redistribution of sphingomyelin was not detectable at all and that of phosphatidylserine was only partial [1]. The induced redistribution of inner layer phospholipids is accompanied by an increase of transbilayer reorientation rates of exogenously incorporated lysophospholipids (zwitterionic lysophosphatidylcholine and negatively charged lysophosphatidylserine [4]) and of endogenous phosphatidylcholine [5]. Inner layer phospholipids, phosphatidylethanolamine and phosphatidylserine become available in diamide-treated cells for transbilayer reorientation in a graded process, depending

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on the time of exposure [4]. The asymmetric arrangement of the inner layer phospholipids was therefore proposed [4] to be due not only to a low rate of the transbilayer reorientation process proper, but also to a limited access of these phospholipids to the reorientation sites. This notion is supported by our observation that exogenous lysophosphatidylserine spontaneously accumulates in the inner layer of the erythrocyte membrane [6]. Suppression of the access of phospholipids to their flip sites may be the result of a direct or an indirect interaction between inner layer phospholipids and membrane skeletal proteins, which becomes disturbed by diamide.

In the present paper we checked the stability of the asymmetric distribution of lysophosphatidylserine, in particular upon an increase of transbilayer mobility of phospholipids by diamide. Moreover, the role of the membrane skeleton in the phospholipid asymmetry was investigated by analyzing the distribution of endogenous phospholipids as well as of exogenous lysolecithin and lysophosphatidylserine, in inside-out vesicles, depleted of the skeletal proteins, spectrin and actin. A preliminary account has been given elsewhere [7].

Materials

Human blood anticoagulated with heparin was obtained from the local blood bank. Erythrocytes were isolated by centrifugation, the buffy coat removed and cells washed three times with 154 mmol · l^{-1} NaCl. L-1-[1-^{14}C]Palmitoyl-lysophosphatidylcholine (spec. act. 45 μCi/μmol) was obtained from Amersham-Buchler. ^{14}C-labelled lysophosphatidylserine was prepared by enzymatic cleavage of L-α-di[U-^{14}C]oleoylphosphatidylserine (spec. Act. 57 μCi/μmol, Amersham-Buchler) with phospholipase A_2 from pig pancreas [4]. Phospholipase A_2 from bee venom and pig pancreas and sphingomyelinase from *Staphylococcus aureus* were obtained from Boehringer, Mannheim. Phospholipase A_2 from *Naja naja*, diamide (diazenedicarboxylic acid bis-dimethylamide) and bovine serum albumin, essentially fatty acid-free, were obtained from Sigma.

Methods

Diamide treatment of erythrocytes

Freshly washed erythrocytes were suspended in 10 vols. of medium A containing (mmol · l^{-1}): KCl (90), NaCl (45), NaH_2PO_4/Na_2HPO_4 (12.5) and sucrose (44). After adjustment of the pH of the medium to 8.0 iodoacetate (5 mmol · l^{-1}) was added and the cells incubated for 15 min at 37 °C. Subsequently, cells were washed three times with medium A, pH 8 and resuspended in 10 vols. of medium A. Diamide was added at a final concentration of 5 mmol · l^{-1}. After an incubation of 15–120 min the cells were spun down and washed three times in medium A.

Preparation of vesicles

Sealed inside-out and right-side-out vesicles were prepared essentially according to Steck and Kant [8]. Membrane sidedness of the vesicles was checked by the accessibility of the inner and outer surface markers glyceraldehyde-3-phosphate dehydrogenase and acetylcholinesterase to their substrates [8].

Vesicles from heat-treated erythrocytes were prepared as follows. Erythrocytes were incubated in 10 vols. of medium A for 15 min at 50 °C. Exocytotic vesicles formed at this temperature [9] were removed from the cells by shear flow during two repetitive aspirations into a 20 ml pipette (orifice diameter 1.8 mm. Cells were removed by low-speed centrifugation (3000 × g for 3 min). The supernatant, which was essentially free of cells, was centrifuged at high speed (40000 × g for 20 min) to pellet the vesicles.

Flip measurements

Intact cells. After incorporation of ^{14}C-labelled lysophospholipid (30 nmol/ml cells) into the outer membrane layer of erythrocytes, transbilayer reorientation of the lysophospholipid to the inner layer was quantified by measuring the increase of the fraction of lysophospholipid inextractable by albumin [4].

Resealed ghosts. Erythrocytes were washed once with 10 vols. of medium A (pH 7.4) containing 1.5% albumin to extract native lysophosphatidylcholine and fatty acids. After two further washings with medium A, 1 ml of cells was hemolysed

by the addition of 1.2 ml of H_2O (0 °C). To saturate the lysophospholipid acylating system membranes were preloaded with 60 nmol of non-radioactive lysophosphatidylserine (15 min). Isotonicity was restored by the addition of 1.2 M KCl and the ghosts resealed by incubation for 30 min at 37 °C. NaF (10 mmol · l^{-1} final concentration) was added to further suppress acylation [10]. Subsequently, ^{14}C-labelled lysophosphatidylserine was incorporated into the outer layer and its reorientation to the inner layer measured as described for intact cells.

Reorientation from the inner to the outer layer was measured in ghosts as follows. 200 µl of the hemolysate were incubated (10–15 min) with 0.05 µCi (1 nmol) of ^{14}C-labelled lysophosphatidylserine prior to reconstitution of isotonicity by the addition of KCl and NaF. Subsequently, membranes were resealed by incubation at 37 °C for 30 min. Outer layer lysophospholipid was extracted by two washings with 10 vols. of 1.5% albumin in medium A, the ghosts were washed twice in medium A and reorientation of the inner layer lysophospholipid to the outer layer measured by the increase of extractability of the lysophospholipid by albumin.

Vesicles. 0.2 µCi of ^{14}C-labelled palmitoyllysophosphatidylcholine was incorporated into the outer layer of vesicles (approx. 1 mg of protein) in 0.25–0.35 ml of medium B containing (mmol · l^{-1}): NaCl (75), NaN_3 (3) and Na_2HPO_4/NaH_2PO_4 (2.5) at room temperature at pH 7.4. After 2 min vesicles were isolated by centrifugation (15 min 30 000 × *g*, 4 °C) and resuspended in 0.8 ml medium B in a new vial at 37 °C. After varying periods of incubation 0.05 ml aliquots were removed and diluted either into 1 ml of medium B to quantify the total amount of incorporated radioactivity, or into medium B containing 5% albumin to quantify the fraction of radioactivity in the inner layer of the vesicles. After 1–2 min at room temperature, vesicles were isolated by filtration of the samples through nitrocellulose filters (2 µm Sartorius) at $0.15 \cdot 10^5$ Pa. The filters containing the albumin-treated vesicles were rinsed with 1 ml of medium B containing 1% albumin, followed by two portions of 1 ml of medium B. Filters containing the buffer-washed vesicles were rinsed with three portions of 1 ml of medium B. The amount of radioactivity retained in the albumin-treated vesicles ($C_{i(t)}$) divided by the amount in the buffer-washed vesicles (C_{tot}) was taken as the fraction of lysophosphatidylcholine in the inner layer of the vesicles.

Acylation and hydrolysis of lysophospholipids

Acylation of lysophospholipids to diacylphospholipids and hydrolysis of lysophospholipids at the ester bond was checked by thin-layer chromatography [6].

Phospholipase treatments

1 ml of a suspension of inside-out or right-side-out vesicles or vesicles from heat-treated cells (approx. 3 mg of protein) were treated with 10 I.U. of phospholipase A_2 or sphingomyelinase in 6 ml of a medium containing (mmol · l^{-1}): NaCl (100) and Hepes (5) at pH 7.4. The treatments with pancreatic phospholipase A_2 and sphingomyelinase were carried out at 37 °C in presence of 0.25 mmol · l^{-1} Ca^{2+}, treatment with bee venom phospholipase A_2 at 25 °C and 0.25 mmol · l^{-1} Ca^{2+}, and treatment with phospholipase A_2 from *Naja naja* at 25 °C and 2 mmol · l^{-1} Ca^{2+}. After different times of incubation the enzymatic activity was blocked by addition of EDTA (Final concentration 1 mmol · l^{-1}) and injection of 4 ml of the suspension into a mixture of 22 ml of isopropanol and 14 ml of chloroform and the mixture stirred for 15 min. Erythrocytes were treated with phospholipase A_2 from bee venom as described before [1]. Phospholipids and their breakdown products were quantified in the extracts as described elsewhere [1].

Results and Interpretation

Studies on resealed ghosts and intact cells

Recently we demonstrated that in analogy to the orientation of endogenous phosphatidylserine, exogenous lysophosphatidylserine introduced into the erythrocyte membrane accumulates in the inner lipid layer of the erythrocyte [6]. The stability of this asymmetric distribution was now checked (Fig. 1) in resealed ghosts by measuring the rate of back reorientation (flop) of lysophosphatidylserine from the inner to the outer membrane layer. Acylation of lysophosphatidylserine, which would

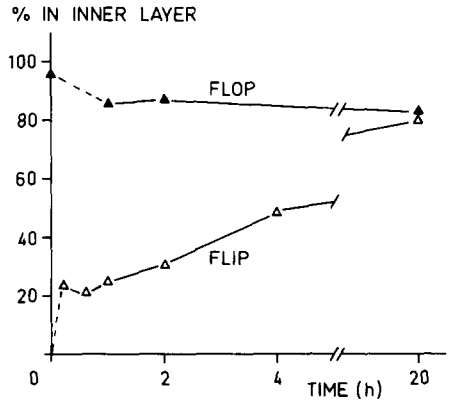

Fig. 1. Stability of accumulation of lysophosphatidylserine in the inner layer of resealed ghosts. ^{14}C-labelled lysophosphatidylserine was incorporated into the outer (△) or the inner (▲) membrane layer of resealed ghosts (0 °C) and reorientation to the opposite layer measured (37 °C) as described in the Methods.

complicate the kinetics of the flop process, could be suppressed completely by preloading ghosts with non-radioactive lysophosphatidylserine prior to resealing (see Methods). In resealed ghosts pretreated in this way labelled lysophosphatidylserine accumulates in the inner membrane layer (Fig. 1) as it does [6] in intact cells. The rate of reorientation is also quite similar to that [6] in intact cells.

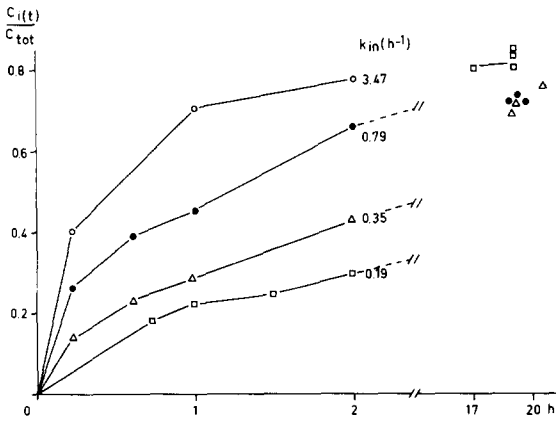

Fig. 2. Accumulation of lysophosphatidylserine in the inner membrane layer of diamide-treated erythrocytes. Erythrocytes were treated with diamide for 0 (□), 20 (△), 30 (●) and 120 (○) min at 37 °C and washed. ^{14}C-labelled lysophosphatidylserine was incorporated into the outer layer and its reorientation to the inner one measured (see Methods). $C_{i(t)}/C_{tot}$ represents the fraction of lysophosphatidylserine in the inner layer. k_{in} represents the rate of unidirectional reorientation from the outer to the inner layer calculated as described before [4].

The back reorientation to the outer layer, however, is at best a very slow process for the major fraction of the inner layer lysophosphatidylserine. It follows from these results that exogenous lysophosphatidylserine has the same preference for the inner layer in resealed ghosts as in native cells [6], and that its preferential orientation to the inner surface represents a stable equilibrium.

In Fig. 2 we present evidence that this stable asymmetry of lysophosphatidylserine does not depend on a low transbilayer mobility, since its asymmetric distribution is even maintained after a 15-fold increase of its transbilayer mobility by diamide.

These findings can be interpreted in terms of a general model considering factors which affect the transbilayer distribution and reorientation of phospholipids (and other amphiphilic constituents intercalated between membrane lipids). According to this model (Fig. 3) distribution and reorientation rates are governed by three conceptually independent parameters: (1) the probability of the formation of a structural defect acting as a flip site (A_1), (2) the probability of a phospholipid to undergo transverse reorientation within such a flip site (A_2), and (3) the probability of a phospholipid to get access to the flip site (B). Since flip sites actually occur in the erythrocyte membrane and the capability of aminophospholipids to reorient in such sites is indicated by our results with lysophosphatidylserine, the stable asymmetry of lysophosphatidylserine and its native analogue may be assumed to arise from a low probability to approach flip sites from the inner face of the membrane. Various reasons for a limitation of access can be envisaged, among which interactions between lipids and proteins, notably membrane skeletal proteins, are particularly intriguing. The

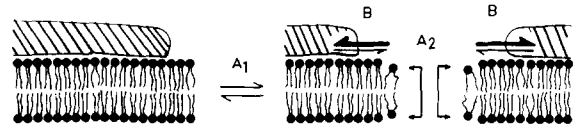

Fig. 3. Schematic drawing showing the features that may limit transbilayer reorientation of phospholipids. A_1, probability of flip site formation; A_2, probability of translocation of a phospholipid within the flip site; B, probability for a phospholipid to gain access to the flip site. Low probability is symbolized by the thick arrow pointing away from the flip site.

role of such interactions is dealt with in the following section.

Studies on membrane vesicles

Membrane skeletal proteins have previously been invoked in the stabilization of phospholipid asymmetry and the limitation of flip rates [1,3–5]. The skeletal proteins are lost or perturbed in various types of membrane vesicles. Such vesicles are therefore a suitable object to check the relationship between the stability of phospholipid asymmetry, their transbilayer mobility, and the state of the skeletal proteins. In the present studies we tested inside-out vesicles lacking spectrin and actin, right-side-out vesicles still containing most of their skeletal proteins, and vesicles prepared by heat denaturation of the membrane skeletal protein spectrin.

(a) Vesicles prepared from lysed cells

The transbilayer movements of lysophospholipids can be followed for many hours in inside-out and right-side-out vesicles, as shown in Fig. 4. The half-times for the flip of lysophosphatidylcholine are somewhat decreased, those for the flip of lysophosphatidylserine not altered significantly as compared to intact erythrocytes (Table I). In spite of this unaltered transbilayer mobility the final distribution attained by exogenous lysophosphatidylserine is essentially symmetric, i.e. the asymmetry observed in intact cells and still present in resealed ghosts gets lost upon formation of both types of vesicles (Table II). Since right-side-out vesicles still contain most of their spectrin and actin [8], the removal of skeletal proteins is obviously not a prerequisite for the loss of asymmetry.

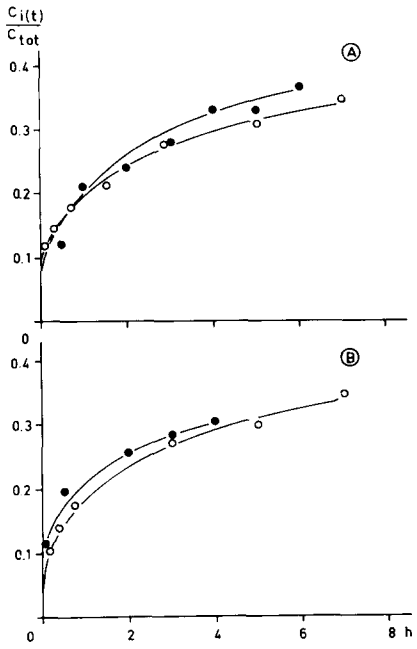

Fig. 4. Reorientation of lysophosphatidylcholine (○) and lysophosphatidylserine (●) from the outer surface layer to the inner membrane layer of inside-out vesicles (A) and of right-side-out vesicles (B). ^{14}C-labelled lysophospholipids were incorporated into the outer surface layer of the vesicles and their reorientation to the inner layer measured as described in Methods.

In further experiments we could also obtain evidence for a loss of the asymmetry of endogenous glycerophospholipids in inside-out and right-side-out vesicles using three types of phospholipase A_2 from different sources and with different substrate specificity to quantify phospholipids exposed to the outer surface. Lysis of the vesicles was circumvented by selecting adequate conditions of temperature and Ca^{2+} concentration. Nevertheless, the treatment of the vesicles with the lipases had to be limited to 30 min, since onset of lysis occurred upon longer exposition times. In inside-out vesicles a saturating cleavage of about 50% was obtained for phosphatidylcholine with phospholipase A_2 from bee venom (Fig. 5 and Table II), for phosphatidylethanolamine with all three phospholipases and for phosphati-

TABLE I

HALF-TIMES OF REORIENTATION OF LYSOPHOSPHOLIPIDS IN INSIDE-OUT AND RIGHT-SIDE-OUT VESICLES

^{14}C-labelled lysophosphatidylcholine (LysoPC) or lysophosphatidylserine (LysoPS) were incorporated into the outer lipid layer of inside-out (IOV) and right-side-out (ROV) vesicles and the reorientation to the inner layer measured (see Methods). Half-times of flip were calculated as described in Refs. 4 and 6.

Object	$t_{1/2}$ (h) of flip	
	LysoPC	LysoPS
IOV	5.8 ± 1.4 ($n = 6$)	5.1 ± 1.8 ($n = 4$)
ROV	2.9 ($n = 2$)	2.8 ($n = 2$)
Native cells (from Ref. 6)	11.0	3.4

TABLE II

CHANGES OF THE PHOSPHOLIPID DISTRIBUTION IN ERYTHROCYTE MEMBRANE VESICLES

The mean values (±S.D.) of the maximal fractions of phosphatidylcholine (PC), -ethanolamine (PE) and -serine (PS) cleavable by phospholipase A_2, as obtained on four or five different preparations of inside-out vesicles (IOV, 71–79% inside-out) and heat-induced vesicles (HIV); and on two preparations of right-side-out vesicles (ROV, 74–80% right-side-out) are compared to those in whole cells (taken from Ref. 18). The fraction of sphingomyelin in the layer originating from the outer layer of the intact cell was calculated. The maximal fractions of extractable ^{14}C-labelled lysophosphatidylcholine (lysoPC) and lysophosphatidylserine (lysoPS), representing the fraction in the outer layer of the vesicles, are compared to those in native cells, taken from Ref. 6.

Outer layer fraction of	Native cells	IOV	ROV	HIV
PC	55	48±8 (n = 6)	n.d.	52±5 (n = 8)
PE	5	46±2 (n = 7)	n.d.	26±8 (n = 6)
PS	0	54±5 (n = 11)	46	35±7 (n = 7)
Sphingomyelin	85	82	n.d.	80
^{14}C-LysoPC	70	54±5 (n = 7)	65±5 (n = 4)	n.d.
^{14}C-LysoPS	19	48±5 (n = 4)	46	n.d.

dylserine with pancreatic phospholipase. The much lower cleavage of phosphatidylcholine by *Naja naja* and pancreas enzyme and of phosphatidylserine by *Naja naja* enzyme is not unexpected in the light of the substrate specificity of the latter enzymes [11,12].

Since in leaky membranes phospholipase A_2 rapidly (5–10 min) degrades more than 85% of the three glycerophospholipids (data not shown), our results demonstrate the inaccessibility of about 50% of the phosphatidylethanolamine and the phosphatidylserine to phospholipase A_2 in inside-out vesicles.

It may be concluded therefore that the asymmetric distribution of phosphatidylethanolamine and phosphatidylserine in the intact cell [13] is lost in inside-out vesicles. This observation is at variance with conclusions reached by other investigators [14]. These authors, however, only used phospholipase A_2 from *Naja naja* and did not consider the substrate specificity of the enzyme. Furthermore, the kinetics of degradation were not studied.

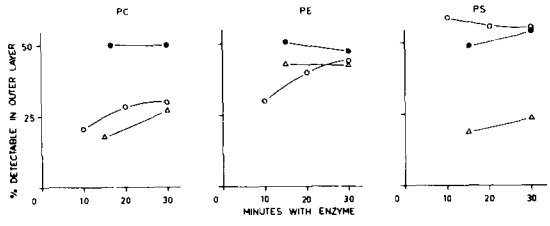

Fig. 5. Distribution of phospholipids over the membrane layers of inside-out vesicles. Phospholipids in the outer layer were detected by their cleavability by phospholipase A_2 from bee venom (●), pancreas (○) and *Naja naja* (△) (see Methods). No lysis of the vesicles, as quantified by measuring of the increase of accessibility of acetylcholinesterase, could be detected. Abbreviations: PC, PE and PS are resp. phosphatidyl-choline, -ethanolamine and -serine.

In right-side-out vesicles endogenous aminophospholipids also lost their asymmetric distribution, in line with the data for exogenous lysophosphatidylserine. In a typical experiment 46% of the phosphatidylserine could be cleaved (30 min, 37 °C) by pancreatic phospholipase A_2 in a preparation containing 87% right-side-out vesicles. In addition, 36% of the phosphatidylethanolamine and 26% of the phosphatidylcholine were degraded. Previously, evidence has been obtained that pancreatic phospholipase A_2 is only able to attack phospholipids in the outer layer of erythrocytes, when this layer contains negatively charged phosphatidylserine [1].

It seems conceivable that the loss of asymmetry occurring in both types of vesicles is related to a perturbation of the membrane skeleton during vesicle formation (see also Refs. 15 and 16). In contrast to the glycerophospholipids, sphingomyelin maintains its asymmetry in membrane vesicles. Treatment with sphingomyelinase for 60–90 min resulted in a non-lytic cleavage of 33% of the sphingomyelin in preparations containing 80% inside-out vesicles. The maximal amount of cleavable sphingomyelin to be expected if no reorientation of sphingomyelin occurs in the vesicles can be

calculated (considering that 85% of sphingomyelin is in the outer layer of the native cell; Ref. 13) to be 29%. The essential agreement between the predicted and the observed (33%) cleavable fraction of sphingomyelin indicates that sphingomyelin orientation is stable (Table II) and not dependent on an unperturbed configuration of skeletal proteins.

(b) Vesicles prepared from heat-treated cells

In order to obtain further support for the role of membrane skeletal proteins in the stabilization of the aminophospholipid asymmetry we investigated changes of phospholipid orientation in heat-treated (50 °C) erythrocytes. This treatment is known to result in a denaturation of spectrin [17] and the formation of exocytotic vesicles [9]. In such vesicles, separated from the cells, a saturating fraction of about 35% of phosphatidylserine was degraded by bee venom and pancreas phospholipase A_2 (Fig. 6 and Table II), indicating a substantial loss of asymmetry.

Cleavage of phosphatidylethanolamine was also somewhat enhanced. Since, however, the total fraction of exofacial phosphatidylethanolamine may be as high as 20%, of which three fourth are not cleavable by phospholipase A_2 under ordinary conditions [1]., a net transverse reorientation of phosphatidylethanolamine has probably not occurred. As in case of the inside-out vesicles no reorientation of sphingomyelin could be detected in heat-induced vesicles (Table II). Interestingly, no reorientation of phosphatidylserine was detected in the residual cells remaining after removal of the vesicles. The exofacial fractions of glycerophospholipids correspond to those in untreated cells (phosphatidylcholine 57–60%, phosphatidylethanolamine 4–10%, phosphatidylserine 0%, $n = 3$).

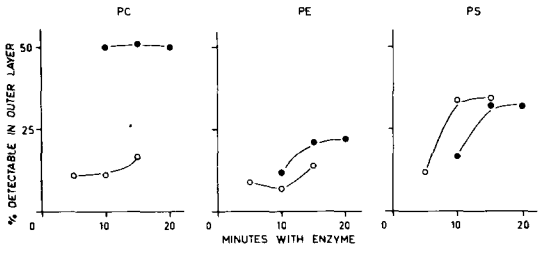

Fig. 6. Distribution of phospholipids over the membrane lipid layers of vesicles from heat-treated erythrocytes. Erythrocytes were incubated at 50 °C for 15 min, exocytotic vesicles isolated by shear flow followed by differential centrifugation, and outer layer phospholipids analysed by their cleavability by phospholipase A_2 from bee venom (●) and pancreas (○) (see Methods). Lysis detectable by hemoglobin release was negligible. For abbreviations see Fig. 5.

Conclusions

The transbilayer mobility of aminophospholipids in the erythrocyte membrane and their asymmetric distribution over the two lipid layers can be altered independently by treatments perturbing the membrane skeletal proteins.

The experiments supporting this statement and data presented previously [1,3–6] allow a number of conclusions and hypotheses concerning the mechanisms underlying the maintenance of asymmetry of phospholipids.

(a) Stable asymmetric orientation of aminophospholipids to the inner membrane surface is not the consequence of a low transbilayer mobility. Asymmetry can be maintained in spite of a marked increase of transbilayer phospholipid mobility in diamide-treated cells (see above and Ref. 4) and in cells subjected to dielectric breakdown [18]. Parameters others than immobility must therefore be involved in the maintenance of asymmetry.

(b) Transmembrane asymmetry of endogenous aminophospholipids and the ability to establish asymmetry of their exogenous lyso-derivatives are lost upon certain perturbations of the membrane skeleton going along with the formation of membrane vesicles. These changes are summarized in Table II. Transbilayer phospholipid distribution is proposed to be controlled by a low probability (B in Fig. 3) of inner layer aminophospholipids to gain access to the hypothetical flip sites, presumably due to constraints imposed by the membrane skeleton.

(c) The stable asymmetric distribution of sphingomyelin (see also Refs. 4, 18 and 21), on the other hand, cannot be explained by such constraints. Stabilization is also unlikely to result from specific interactions between intrinsic proteins and sphingomyelin for two reasons. Firstly, such interactions are unknown for the erythrocyte membrane. Secondly, according to present concepts

[22,23], they could, if existing, only immobilize a minor fraction of the sphingomyelin, namely about 25 sphingomyelin molecules per copy of intrinsic protein, out of a total of about 150 (calculated from data in Refs. 24 and 25). The asymmetry of sphingomyelin has therefore to be considered either to result from a limitation of its access to flip sites due to lipid-lipid interactions, e.g. strong hydrogen bonding [19], or to an intrinsic inability to reorient within a flip site.

(d) The asymmetry of phosphatidylserine is lost in vesicles released from heat-treated erythrocytes, but not in the residual cells. This is a remarkable finding if one considers that spectrin is probably denaturated all over the heat-treated membrane' [20], while the transbilayer mobility of phospholipids is likely to be essentially unaltered [3]. Denaturation of spectrin as such is thus not sufficient to induce loss of asymmetry. Additional processes, restricted to the membrane domain involved in vesicle formation, must be postulated.

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