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SELECTIVE REMOVAL OF LIPIDS FROM THE OUTER MEMBRANE LAYER OF HUMAN ERYTHROCYTES WITHOUT HEMOLYSIS

CONSEQUENCES FOR BILAYER STABILITY AND CELL SHAPE

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(1) Treatment of erythrocytes with phospholipase A_2 from bee venom cleaves about 55% of the phosphatidylcholine in the outer membrane lipid layer without changing the discoid shape of the cells. All of the fatty acids and 80% of the lysophosphatidylcholine produced under this conditions can be sequentially extracted by bovine serum albumin without hemolysis of the cells. (2) The cells remain discoid up to extraction of all of the fatty acids and 15% of the lysophosphatidylcholine. Removal of a higher fraction of lysophosphatidylcholine induces formation of stomatocytes and sphero-stomatocytes, probably going along with an internalization of membrane vesicles. Stomatocytosis can be explained on the basis of the 'bilayer couple hypothesis' (Sheetz, M.P. and Singer, S.J. (1974) Proc. Natl. Acad. Sci. 71, 4457–4461). The shape change will compensate for the differences in surface pressure between the two leaflets induced by selective removal of material from the outer leaf of the bilayer. (3) Increasing the shear modulus of the membrane by diamide prevents this compensatory shape change even after extraction of up to 80% of the lysophosphatidylcholine, which amounts to a loss of 34% of the phospholipids of the outer membrane layer or 22% of its area. This leads to the interesting situation of a membrane possibly having a strikingly diminished ratio of the numbers of phospholipid molecules in the outer to that in the inner lipid layer. A marked difference in surface pressures should arise in this situation, unless other compensatory mechanisms become operative. Evidence for a compensation for outer lipid loss by a constriction of the inner layer has been obtained. A compensation by transbilayer reorientation of phospholipids could not be demonstrated. This latter observation supports the concept of a stabilisation of the asymmetric phospholipid arrangement by proteins such as spectrin.

Introduction

Phosphatidylcholine in the outer lipid layer of the membrane of human eythrocytes can be cleaved by phospholipase A_2 into lysophosphatidylcholine and fatty acids [1]. The degradation products remain attached to the membrane during incubations in saline media. Gul and Smith [2] reported that fatty acids can be extracted quantitatively by albumin without hemolysis, whereas only a small fraction of lysophosphatidylcholine could be removed without hemolysis. The authors concluded that hemolysis at high concentrations of albumin was due to extraction of lysophosphatidylcholine.

Recently, we [3] developed a method to extract a major fraction of the lysophosphatidylcholine produced by the phospholipase treatment without hemolysis. The aim of the present work was to investigate the consequences of such a specific delipidation of the outer lipid layer for bilayer stability and cell shape.

Methods

Preparation of eythrocytes. Human erythrocytes from freshly drawn heparinized blood were isolated

by centrifugation and the buffy coat removed by aspiration. The cells were washed three times with 154 mmol/l NaCl and resuspended in 10 vols. of a medium containing (mmol/l) KCl (90), NaCl (45), Na_2HPO_4/NaH_2PO_4 (10) and sucrose (44) (=Medium A).

After adjustment of the pH to 8.0 iodoacetate was added at a final concentration of 10 mmol/l and the cells were incubated at 37°C for 15 min. This treatment blocks SH-groups of intracellular glutathione [11]. After three washings the cells were incubated (15 min, 37°C) with 10 vols. of Medium A containing 5 mmol/l diamide (diazine dicarboxylic acid bis-(dimethylamide); Calbiochem) and washed three times with Medium A.

Phospholipase A_2 treatment. Freshly washed or chemically modified erythrocytes were suspended in 10 vols. of a medium containing (mmol/l): KCl (90), NaCl (45), Hepes (30), sucrose (44), $MgCl_2$ (0.25) and $CaCl_2$ (0.25) (=Medium B, pH = 7.4). Subsequently, 20 I.U. of phospholipase A_2 from bee venom (Sigma) per ml of erythrocytes were added and the suspension incubated at 37°C. After 1 h of incubation the enzymatic activity was blocked by addition of 1 vol of Medium B containing 10 mmol/l EDTA and the erythrocytes isolated by centrifugation.

Extraction of lysophosphatidylcholine and fatty acids. Phospholipase A_2-treated erythrocytes were resuspended in 10 vols. of Medium A, containing 0–400 mg of bovine serum albumin (fatty acid free, Sigma) per ml of erythrocytes. After 5 min at 20 or 37°C the suspension was centrifuged and the erythrocytes washed three times with Medium A.

Quantification of phospholipids. Erythrocyte lipids were extracted without a hemolysis step and phospholipids separated by two-dimensional thin-layer chromatography on silica plates as described before [4]. The amounts of diacyl- and lyso-phospholipids were determined according to Ref. [4]. The amounts of lysophosphatidylcholine and lysophosphatidylethanolamine extracted by albumin were calculated using sphingomyelin, which is not affected by the treatments, as a reference.

Microscopy. Erythrocytes were suspended in Medium A at a hematocrit of 2% and inspected and photographed between two plastic cover slips through an interference contrast microscope (objective 40 X 0.65, Leitz).

Results and Discussion

Extraction by albumin of phospholipid split products from phospholipase A_2-treated erythrocytes

Phospholipase A_2 from *Naja naja* as well as from bee venom have been shown to attack a major fraction of the phosphatidylcholine located in the outer lipid layer of the membrane of human erythrocytes [1]. In the present experiments 55.9 ± 6.0% (n = 15; ±S.D.) of the phosphatidylcholine and 0–5% of the phosphatidylethanolamine could be cleaved by phospholipase A_2 from bee venom. After this cleavage the split products, fatty acids and lysophosphatidylcholine, could be extracted by bovine serum albumin (fatty acid free). As described elsewhere [5], fatty acids are extracted at low amounts of albumin per cell. 6 mg of albumin per ml of cells remove 80% of the fatty acids, but only less than 10% of the lysophosphatidylcholine.

Increasing the amount of albumin from 6 to 35 mg results in extraction of up to 60% of the lysophosphatidylcholine (Fig. 1) by stoichiometric interaction, assuming one binding site for lysophosphatidylcholine per albumin molecule [6]. An additional fraction of 25% of the lysophosphatidylcholine is extracted by overstoichiometric amounts of albumin, while a residual fraction of 15% is not extracted by concentrations of albumin as high as 400 mg per ml

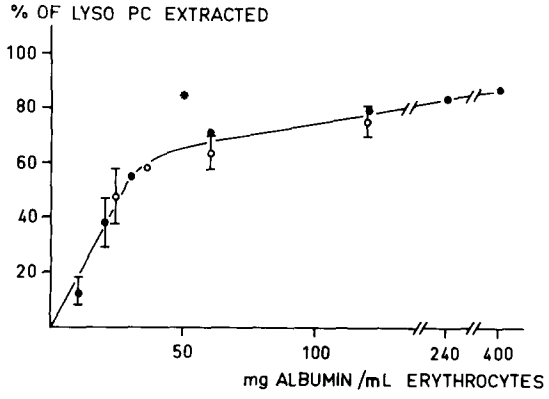

Fig. 1. Extraction of lysophosphatidylcholine from phospholipase A_2-treated erythrocytes by albumin. Erythrocytes were treated with phospholipase A_2 and extracted once at 37°C (open symbols; n = 3–5; ±S.D.) or 20°C (closed symbols) with albumin for 5 min. Sample (*) was extracted three times. See Methods for details.

of cells or by repeated extraction with albumin (Fig. 1). The most obvious reason for this lack of extraction by high amounts of albumin would seem to be a translocation of lysophosphatidylcholine to the inner layer. This interpretation, however, seems to be unlikely in the light of the very slow translocation rates observed for lysophosphatidylcholine [7]. An additional argument against such an interpretation comes from the observation that fatty acids produced by phospholipase A_2 treatment reorient in the bilayer at about the same rates as lysophosphatidylcholine [7], but nevertheless remain completely extractable by albumin under our conditions.

It is proposed therefore that no significant amounts of fatty acids and lysophosphatidylcholine are translocated during the phospholipase treatment and that the fraction of lysophosphatidylcholine not extractable by albumin is tightly bound to protein or lipids in the outer leaf of the bilayer.

Albumin extraction when carried out at 20°C did not result in any hemolysis of the erythrocytes, even after removal of up to 80% of the lysophosphatidylcholine, corresponding to 13% of the total phospholipids or 26% of the outer layer phospholipids. Gul and Smith [2], on the other hand, noticed hemolysis already before complete extraction of the fatty acids. The authors suggested that hemolysis results from the extraction of lysophosphatidylcholine. The present results disprove this suggestion. The discrepancy between their and our results is most probably due to differences in the extraction procedure, such as the medium and the temperature used for the extraction. For example, hemolysis, while absent when extraction is carried out at 20°C, occurs at 37°C (Table I), although the amount of lysophosphatidylcholine extracted is not affected by the difference in temperature (Fig. 1). Furthermore, extraction in the slightly hypertonic medium B, which increases the surface area to volume ratio of the cells, does not result in lysis, whereas extraction in isotonic media as used by Gul and Smith goes along with hemolysis (Table I). An increased area to volume ratio will enable the cells to withstand a greater loss of membrane material before turning into spherocytes. As will be shown below a loss of membrane material due to internalization in fact occurs upon albumin extraction of phospholipase A_2-treated cells.

TABLE I

HEMOLYSIS BY ALBUMIN OF PHOSPHOLIPASE A_2-TREATED ERYTHROCYTES. DEPENDENCE ON EXTRACTION CONDITIONS

Erythrocytes were treated with phospholipase A_2 and extracted with albumin at 20°C or 37°C in Medium B (see Methods) or in isotonic saline containing 10 mmol/l Hepes (5 min, pH 7.4).

Extraction conditions	% hemolysis upon extraction of cells with albumin (mg/ml cells)		
	100	200	400
Medium B, 20°C	0	0	0
Medium B, 37°C	1	5	10
NaCl-Hepes, 20°C	3	13	–
NaCl-Hepes, 37°C	8	22	–

Albumin-induced shape changes of phospholipase A_2-treated erythrocytes

The biconcave shape of erythrocytes does not change during the phospholipase A_2 treatment. This observation disagrees with a recent report [8] of a transformation of discocytic erythrocytes into echinocytes by phospholipase A_2. The discrepancy may be due to differences in the procedure of the phospholipase treatment or in the microscopic examination of the erythrocytes.

In contrast to the lack of effect of phospholipase A_2 treatment on cell shape, a subsequent extraction of the split products by albumin eventually leads to pronounced changes of cell shape. The shape of normal erythrocytes is only slightly affected by albumin (Fig. 2). At concentrations exceeding 100 mg albumin per ml of cells an early stage of stomatocytosis occurs [9]. Extraction by albumin of 80% of the fatty acids and about 10% of the lysophosphatidylcholine (0.10 μmol/ml of cells) from phospholipase A_2-treated cells does not result in significant shape changes. However, when higher amounts of lysophosphatidylcholine (0.2–0.3 μmol/ml of cells) are extracted stomatocytes are formed. Spherostomatocytes are observed after extraction of more than 0.5 μmol of lysophosphatidylcholine/ml of cells in addition to the complete extraction of fatty acids. In analogy to other stomatocytogenic processes, stoma-

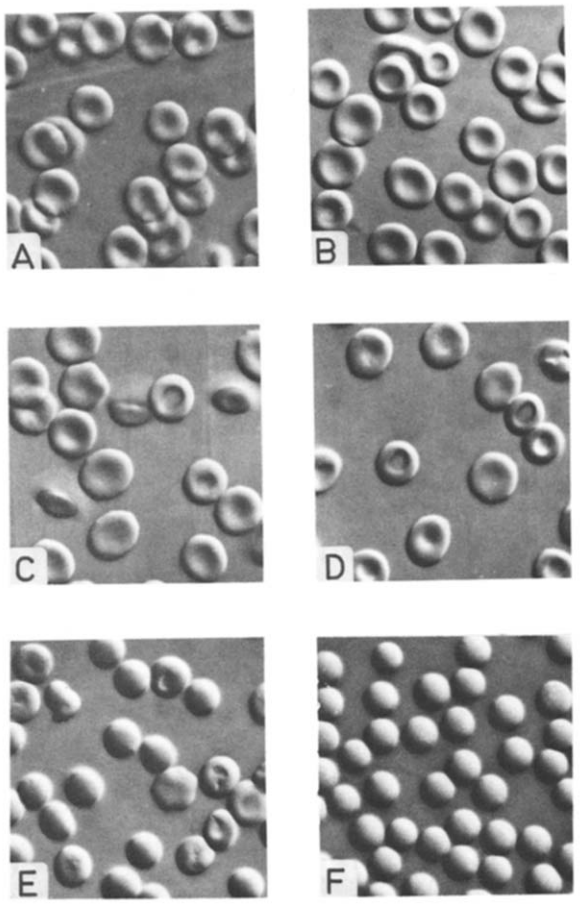

Fig. 2. Shape changes of erythrocytes treated by phospholipase A_2 followed by albumin. Erythrocytes were treated with phospholipase A_2 (C) and extracted with 12 (D), 30 (E) and 60 (F) mg albumin per ml of cells. For reasons of comparison fresh cells (A) and fresh cells with 60 mg albumin per ml of cells (B) are shown. Note the gradual transformation of discocytes into spherostomatocytes.

tocytosis produced by this method may be expected to end up in a formation of endocytotic vesicles.

The observed shape changes can be explained on the basis of the 'bilayer couple' hypothesis of Sheetz and Singer [10]. According to this hypothesis extraction of lipids from the outer membrane layer will result in an inward bending of the membrane, and a subsequent formation of stomatocytes. Inward bending will decrease the area of the outer layer. Due to this decrease, changes of the area available per molecule of phospholipids in the outer layer, to be expected after extraction of 26% of the outer layer phospholipids, will be minimized. Bending can thus be envisaged as a compensatory mechanism in order to keep the interfacial pressures in the two leaflets of the bilayer in equilibrium.

Formation of spherostomatocytes may be responsible for lysis of phospholipase/albumin-treated cells observed under certain conditions (Table I). Spherostomatocytes, which have lost the excess of surface area characteristic for discocytes, will hemolyze upon a small increase of their volume due to swelling or a small loss of membrane area due to formation of endocytotic vesicles from invaginations. That hemolysis under the conditions shown in Table I (37°C) is in fact due to a loss of membrane area may be concluded from our observation that lysis is completely prevented when formation of spherostomatocytes is suppressed by echinocytogenic agents, such as salicylate (data not shown) or by an increase of the membrane shear modulus [11] by diamide (Fig. 3). This increase of the shear modulus is most probably the result of a cross-linking of spectrin via disulfide bridges [11]. Diamide treatment does not affect the amount of lipid extracted (see below).

Swelling of the cells, on the other hand, is unlikely to be responsible for hemolysis since (a) the cells are protected against colloid-osmotic hemolysis under our experimental conditions, and (b) phospholipase treatment followed by albumin extraction does not increase cation leak permeability of the cells (Schwister, K., personal communication). It can be speculated that hemolysis of phospholipase/albumin-treated cells at 37°C occurs when bending forces in the course of endocytotic vesiculation surpass the membrane resistance towards area expansion.

Estimation of the loss of membrane surface area after lipid extraction by albumin

As demonstrated above, the fraction of phospholipids extracted by albumin from phospholipase A_2-treated cells was about 13% of the total phospholipid or 26% of the outer layer phospholipids. On the basis of a molecular area for phosphatidylcholine (in complex with cholesterol) of 52 $Å^2$ [12] and an area loss per extracted fatty acid of 12 $Å^2$ (which is the difference of the molecular area of phosphatidylcholine and lysophosphatidylcholine [13]) it can be calculated that about 17% of the outer surface area are lost

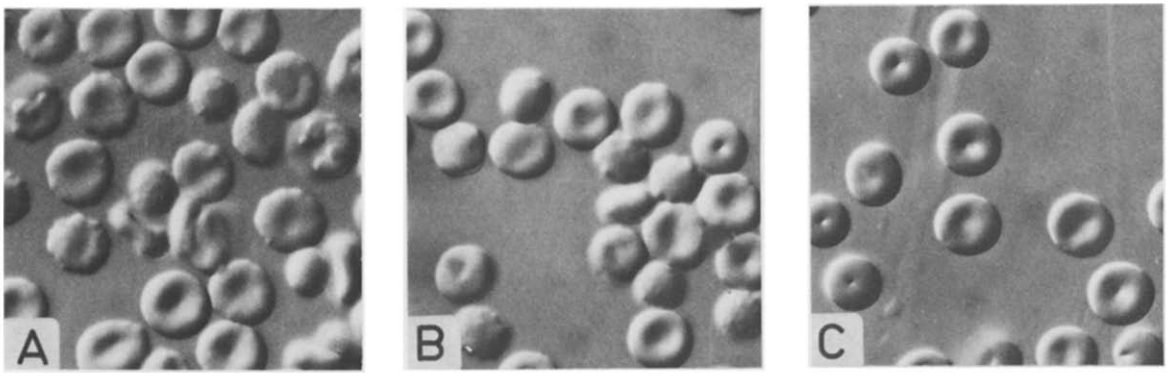

Fig. 3. Suppression by diamide of shape changes of erythrocytes treated with phospholipase A_2 followed by albumin. Erythrocytes were treated with iodoacetate (10 mmol/l, 15 min, pH 8.0, 37°C) followed by 5 mmol/l diamide (15 min, pH 8.0, 37°C). After washing the cells were treated with phospholipase A_2 (A) followed by extraction with 60 (B) and 120 (C) mg albumin/ml of cells. Note the suppression of spherostomatocytosis by diamide. The observed atypical shape effects of albumin (plate C) are presently not understood.

upon the extraction with albumin (Table II). The extent of this loss is not changed when the compensatory bending of the membrane is prevented by increasing the shear modulus with diamide. As becomes evident from Table II, pretreatment of cells with 0.5 mmol/l of diamide followed by phospholipase A_2 treatment and albumin extraction also leads to a decrease of the outer surface area by 16%. Higher concentrations (5 mmol/l) of diamide result in a cleavage of a larger amount (26%) of phospholipid due to hydrolysis of phosphatidylethanolamine in addition to phosphatidylcholine [4] and subsequent extraction of more lipid. This demonstrates that lysophosphatidylethanolamine formed by the enzyme treatment can also be extracted by albumin.

Effect of removal of lipids from the outer layer on the transverse orientation of inner layer phospholipids

In previous studies [3] we could obtain no evidence that a removal of outer layer lipids is compensated in the normal, shear-deformable membrane by a translocation of inner layer phospholipids to the outer layer. We now investigated possible consequences of a selective delipidation of the outer lipid layer for the orientation of inner layer lipids in cells

TABLE II

LOSS OF MEMBRANE SURFACE AREA BY AN ALBUMIN TREATMENT OF PHOSPHOLIPIPASE A_2 TREATED ERYTHROCYTES

Erythrocytes were treated with phospholipase A_2 and extracted with 120 mg of albumin per ml of cells, which extracts all of the fatty acids [5]. The amounts of lysophospholipid extracted were determined as described in the Methods. The fraction of phospholipid extracted was calculated assuming a value of 5 μmol of phospholipids per ml of packed cells. PC, phosphatidylcholine; PE, phosphatidyl ethanolamine; n.d., not determined.

	% of each phospholipid cleaved by phospholipase A_2		% of lysophospholipid extracted by albumin		% of total phospholipid extracted	Estimated loss of outer surface area (%)
	PC	PE	Lyso PC	Lyso PE		
Control (n = 15)	55.9 ± 6.0	<5	76.0 ± 5.7	n.d.	13	17
0.5 mmol/l diamide (n = 2)	60	16	69	n.d.	12	16
5 mmol/l diamide (n = 4)	67.0 ± 2.9	24.3 ± 2.5	68.0 ± 5.4	40.8 ± 14.8	17	22

shear-stiffened by diamide and therefore unable to undergo compensatory shape changes.

To this end cells treated with 0.5 mmol/l diamide * were first delipidated by the usual phospholipase/albumin procedure as outlined in the previous section and in Table II. A possible reorientation of inner layer phospholipids (phosphatidylserine and phosphatidylethanolamine) to the outer layer was then checked using phospholipase A_2 as a tool for the assessment of phospholipid distribution in the bilayer of the membrane [1,4]. As shown in Table III, extraction of 16 and 24% of the outer layer phospholipids does not lead to a relevant decrease of the content of inner layer phospholipids.

This observation demonstrates the stability of the asymmetric arrangement of phospholipids even in a situation, where the number of phospholipid molecules differs markedly between the inner and the outer lipid layer. This, in turn, supports the view that constraints such as those imposed by spectrin, a membrane protein located at the inner surface of the membrane, are responsible for the stability of the orientation of aminophospholipids in the inner lipid layer.

TABLE III

EFFECT OF A REMOVAL OF PHOSPHOLIPIDS FROM THE OUTER MEMBRANE LAYER ON THE ORIENTATION OF INNER LAYER PHOSPHOLIPIDS OF RIGIDIFIED ERYTHROCYTES

Erythrocytes rigidified by diamide (0.5 mmol/l) were treated with phospholipase A_2, extracted with 60 mg of albumin/ml cells at 22°C for 5 min, washed once and treated again with phospholipase A_2 for another hour (see Methods and Ref. 4 for details). Data from two representative experiments. PE, phosphatidylethanolamine; PS, phosphatidylserine, respectively.

% of outer layer phospholipids removed by phospholipase albumin	Inner layer phospholipds (μmol/ml of cells)			
	Before phospholipase/albumin treatment		After phospholipase/albumin treatment	
	PE	PS	PE	PS
16	1.40	0.80	1.30	0.70
24	1.55	0.75	1.50	0.65

Inner membrane surface area changes as a result of variation of outer membrane surface area

The phospholipid analyses presented above indicate a decrease of the area of the outer membrane layer in phospholipase/albumin-treated erythrocytes. This decrease may or may not go along with a decrease of the 'effective surface area' of the erythrocyte, whcih determines, e.g., the critical hemolytic volume of the cell. The critical hemolytic volume, which corresponds to the volume of a sphere having the surface area of the original discoid erythrocyte can be deduced from osmotic fragility curves obtained in hypoosmolar solutions [14]. The effective surface area of phospholipase/albumin-treated erythrocytes was determined after extraction of small amounts of split products since extraction of higher amounts resulted in a loss of the sigmoidal form of the osmotic fragility curve, due to the partial transformation of biconcave cells into spherostomatocytes. phospholipase A_2-treated cells were extracted with 10 and 20 mg of albumin per ml of cells, which results in the removal of all of the fatty acids (=0.85 μmol/ml cells) and about 15 and 35% of the lysophosphatidylcholine (=0.13 and 0.30 μmol/ml of cells). This treatment gave rise to a definite increase of the osmotic fragility of these cells (data not shown).

From the shift of the osmotic fragility curves a loss of 'effective surface area' of 6 and 7% could be calculated by a procedure described in Ref. 15. The decrease of the outer layer calculated from the loss of lipid is somewhat higher, namely 7 and 10%, respectively. A close similarity between a change of the effective surface area deduced from fragility measurements and a change of the area of the outer membrane layer was also observed in cells expanded by incorporation of small amounts of lysophosphatidylcholine (0.10 μmol/ml of cells, Table IV).

Experiments with higher amounts of exogenous lysophosphatidylcholine could not be evaluated as a result of the cation leak induced by exogenous lysophosphatidylcholine (Ref. 16 and Schwister, K.,

* As shown earlier [4], extensive treatment with diamide per se results in a loss of the phospholipid asymmetry. For this reason cells were stiffened by treatment with low concentrations of diamide for a short time.

TABLE IV

CHANGES OF MEMBRANE AREA AS A RESULT OF AN EXTRACTION OF LIPIDS FROM THE OUTER MEMBRANE LAYER

		Changes of membrane area (% increase; ±S.D.; n = 4) calculated from	
		(A) the amount of lipid extracted or incorporated	(B) the change of the critical hemolytic volume
Incorporation of lysoPC (μmol/ml of cells)			
0.10 ± 0.02		+1.3 ± 0.2	+0.9 ± 0.2 [a]
Extraction (μmol/ml of cells)			
Fatty acids	Lyso PC		
0.85	0.15	−7.4	−6.3
0.85	0.30	−9.6	−7.0

[a] Value calculated according to Ref. 15 from a shift of the NaCl concentration at 50% hemolysis by 2.3 ± 0.2 (±S.D.; n = 3) mmol/l.

personal communication). The results demonstrate that area changes of the outer lipid layer result in a change of the effective surface area. This change, however, implies that the area of the inner layer has changed, too. A decrease of the inner surface area, on the other hand, requires compression of the inner layer unless compensatory mechanisms become operative.

Bilayer stability after diamide-induced changes of the asymmetric distribution of phospholipids

A reorientation of 30% of the aminophospholipids from the inner to the outer surface of the membrane has been observed after extensive treatment of erythrocytes with diamide [4]. This reorientation could not be shown to be accompanied by a translocation of significant amounts of outer layer choline-phospholipids to the inner layer. Consequently, the reorientation of the aminophospholipids represent a net shift of material between the two leaflets of the bilayer, increasing the fraction of phospholipids in the outer layer from 50 to 65% and decreasing the fraction of inner layer phospholipid from 50 to 35%.

Echinocytosis, as a compensatory shape change predicted by the bilayer couple hypothesis [10] for this type of lipid reorientation, cannot occur and compensate for the differences of surface pressure resulting from the uneven distribution of phospholipid between the two layers. Nevertheless, the membrane is obviously stable in this situation, a surprising observation in view of the phospholipid imbalance. Thus, other compensatory mechanisms have to be discussed. One such mechanism might be a translocation of cholesterol from the outer to the inner layer. Transbilayer movement of cholesterol in fresh erythrocytes has recently been demonstrated to be very fast [17]. Transbilayer mobility of cholesterol may be expected to become highly enhanced by a diamide treatment in line with the observed [7,18] strong increase of transversal mobility of other lipids. The half-time for the movement of lysophosphatidylcholine from the outer to the inner membrane layer is decreased from 16 h to 1 h after a diamide treatment that leads to a loss of phospholipid asymmetry (Bergmann, W. and C.W.M. Haest, unpublished data). Another possible compensation for the loss of inner lipids in diamide-treated membranes may be an increase of the area per inner phospholipid molecule due to a loss of interation of aminophospholipids with spectrin. This loss of interaction may even be the reason for the translocation of aminophospholipids to the outer layer.

Conclusions

A remarkably high fraction of outer layer phospholipids can be removed from the erythrocyte membrane without the loss of membrane integrity. This disturbance of the material balance between the two leaflets of the bilayer is accompanied by a transformation of shear-deformable discoid erythrocytes via stomatocytes into spherostomatocytes. This shape change will at least partly compensate for the loss of outer layer lipid by keeping normal the surface pressure of that layer. A decrease of the total (effective) membrane surface area due to a compression of the inner lipid layer (by increased intramolecular attractive forces becoming operative in the outer layer and coupling between the two layers of the bilayer) provides a further means of compensation.

Additional compensatory mechanism might result from secondary changes in the mass balance between the leaflets. A reorientation of inner layer phospholipids to the outer layer could be excluded. Reorientation of cholesterol (see above) remains a serious alternative, which would also account for the decrease of the effective surface area.

After suppression of shape changes by an increase of the membrane shear modulus due to spectrin modification by low concentrations of diamide, only transbilayer reorientation of material (i.e. cholesterol) could compensate for the outer layer 'delipidation'. If no such reorientation occurs, we are faced with the possibility of an uncompensated imbalance of material between the two lipid monolayers in shear-stiff erythrocytes. The balance of forces acting at the two interfaces is established by modified spectrin, the major component of the membrane skeleton.

A similar situation probably prevails in cells treated with concentrations of diamide high enough to produce reorientation of amino phospholipids from the inner to the outer membrane layer uncompensated by oppositely directed choline phospholipid movements. Experimental delipidation of erythrocytes by phospholipase/albumin, and diamide-treatment thus provide interesting tools to study bilayer properties and bilayer-protein interplay in the erythrocyte membrane.

Acknowledgements

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