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Phospholipid asymmetry during erythrocyte deformation: maintenance of the unit membrane

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To assess the red blood cell (RBC) membrane's ability to maintain normal phospholipid orientation in the face of deforming stress, we examined RBC subjected to elliptical, tank-treading deformation. As determined by accessibility to phospholipase digestion and by labeling with fluorescamine, normal RBC are able to fully preserve their phospholipid asymmetry despite attaining over 96% of their maximal possible deformation. Phospholipid orientation is unchanged during deformation even for RBC that are ATP-depleted or vanadate-treated and for RBC that already have destabilized phospholipids due to treatment with *t*-butyl hydroperoxide. These data indicate that maintenance of phospholipid organization during marked deforming stress and tank-treading motion of the membrane is ascribable predominantly to the passive stabilizing effect of membrane proteins. This provides additional evidence for the concept of a unit membrane characterized by intimate associations between lipid and protein.

Introduction

The RBC membrane's lipid bilayer is arranged asymmetrically so that 75–80% of its phospholipids containing choline (i.e., PC and sphingomyelin) are found in the outer monolayer, while the inner monolayer retains most of the aminophospholipid (virtually all PS and 80% of the PE). As currently understood, this state is maintained through two mechanisms. Active enforcement of asymmetry appears to depend upon ATP-dependent phospholipid translocating activity ('flipase') [1–3], and passive stabilization is believed to derive from interaction between phospholipids and proteins of the RBC membrane 'skeleton.'

This effect of membrane protein was first suggested by the observation that asymmetry is destabilized by perturbation of the membrane skeletal proteins using diamide [4–6]. This interpretation has been questioned because oxidative damage may adversely affect flipase activity [7,8] and because the interaction between spectrin and phospholipids is somewhat weak [9–11]. However, certain experiments of nature do suggest a role for membrane skeletal proteins. Destabilization of phospholipid asymmetry is evident for RBC that have dysfunctional mutant spectrin [12] or quantitative deficiencies of either spectrin [13,14] or protein 4.1 [14,15]. Moreover, reversible destabilization occurs during sickling-induced deformation of the RBC membrane [16–18], a process that probably involves separation of the lipid bilayer from the underlying skeletal protein network [19].

This stabilizing association of bilayer lipid with skeletal proteins provides an opportunity to test for the existence of a unit membrane in which relationships between lipid and protein are preserved despite changes in cell shape [20]. To do this, we have studied phospholipid asymmetry during elliptical (tank-treading) deformation [21] of normal erythrocytes, ATP-depleted and vanadate-treated RBC, and cells already having destabilized phospholipids by virtue of prior

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; RBC, erythrocyte(s); tBuOOH, *t*-butyl hydroperoxide.

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exposure to an oxidant, *t*-butyl hydroperoxide (tBuOOH). These experiments were performed so as to obtain parallel data on deformation-induced phospholipid translocation and cation leakiness.

Methods

Materials

Reagents were obtained from Sigma Chemical Co., St. Louis, MO.

RBC preparation

Normal RBC from fresh heparinized blood were washed three times with removal of buffy coat using buffer A (10 mM Hepes, 10 mM glucose, 4 mM KCl, NaCl to 290 mosmol/l, pH 7.4). For exposure to oxidant, RBC were then incubated (30 min, 37°C, Hct 10%) in buffer A containing 0.8 mM tBuOOH, after which butylated hydroxytoluene in ethanol was added (0.1 mM final concentration) [22]. To induce ATP depletion, we incubated RBC (60 min, 37°C, Hct 10%) in phosphate-buffered NaCl containing 1 mM iodoacetate [23]. Control RBC were handled identically in all respects except for exposure to the tBuOOH or iodoacetate. RBC were then washed thoroughly using Buffer B (10 mM Hepes, 10 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1 mM ouabain, 1 mM furosemide, NaCl to 290 mosmol/l, pH 7.4). Finally, they were suspended to Hct 10% in Buffer C (same as Buffer B but with 20% dextran having average molecular mass 40 kDa), which has viscosity 0.1 poise at 37°C [22]. For exposure to vanadate, the RBC were suspended in Buffer C containing 200 μM vanadate [24], and they were used directly for deformation studies without washing.

RBC deformation

RBC in Buffer C were placed in a Couette viscometer having a rotating plexiglass outer cylinder and a stationary metal inner cylinder that was perfused to maintain RBC suspension at 37°C [22]. The results described here were obtained at shear stress 220 dyn/cm^2, as well as during parallel static incubation at 37°C.

Phospholipid studies

Phospholipid asymmetry was evaluated by determining accessibility to digestion by phospholipase [6], as well as availability for labeling with fluorescamine [6,25].

For the analyses using phospholipase, viscometer rotation was allowed to proceed for 15 min, at which point bee venom phospholipase A_2 was added in the ratio of 15 units enzyme per 0.25 ml RBC. After 60 additional minutes of deformation, viscometer rotation was stopped, EDTA (to final concentration 5 mM) was added immediately to terminate enzyme activity, and aliquots of RBC suspension were taken for lipid analysis. RBC were then washed with isotonic NaCl containing 5 mM EDTA, and lipids were extracted using isopropanol and chloroform [26]. After drying under N_2, lipid was resuspended in chloroform/methanol (2:1, v/v) and analyzed by two-dimensional thin-layer chromatography: first dimension, $CHCl_3/CH_3OH/NH_3(25\%)/H_2O$ (90:54:5.5:5.5, v/v); second dimension, $CHCl_3/CH_3OH/CH_3OOH/H_2O$ (90:40:12:2, v/v). Developed spots were compared to authentic standards, and their phospholipid content was quantitated by measurement of lipid phosphorus.

For the analyses using fluorescamine, 1.25 ml of RBC suspension taken before and at the end of 75 min of deformation was added to 50 ml Buffer D (20 mM Hepes, 5 mM glucose, 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM $NaHCO_3$, pH 8.0) and rapidly centrifuged. Packed RBC were suspended to Hct $\approx$ 1% in Buffer D, and we then added 14 μl of 1 M fluorescamine in dimethylsulfoxide to 15 ml of this RBC suspension to provide 20 μmol fluorescamine per μmol phospholipid. The mixture was immediately vortexed for 30 s, and reaction was stopped by addition of 30 ml Buffer D containing 30 mM glyclglycine. After 15 min at room temperature, lipids were extracted and analyzed by TLC as above. Percent labeling with fluorescamine was determined by measurement of lipid phosphorus content of scraped spots representing PE and PS and their fluorescamine-modified forms. In the alternative experiment, RBC were suspended in Buffer E (20 mM Hepes, 10 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20% dextran, NaCl to 290 mosmol/l, pH 8.0), and labeling was actually done during deformation by addition of fluorescamine directly to the rotating viscometer. Viscometer rotation was then stopped after 30 s, and the glyclglycine solution was added directly to the RBC suspension; samples were otherwise handled as described above.

Measurement of cation leak

The development of deformation-dependent leakiness to potassium was monitored over the period of RBC deformation as described [22]. For this, triplicate 1 ml aliquots of RBC suspensions were added to 2 ml ice-cold 10 mM Tris-buffered $MgCl_2$ (pH 7.4), and 1 ml of this supernatant was added to 1 ml of 6 mM LiCl. Potassium concentration was determined by flame photometer (Radiometer model FLM3, Louisville, KY). Based on suspension hematocrit, results were converted to mequiv. K leak per liter RBC. Since these studies were done in presence of ouabain and furosemide, results reflect net passive K leak. As described previously [22], results were corrected for the trivial contribution from RBC lysis which was $< 0.7\%$ over the period of deformation in these experiments.

Results

The accessibility of PC, PS, and PE for digestion by phospholipase A_2 over 60 min is summarized in Table I. For the normal RBC, hydrolysis of phospholipid did not change significantly during deformation compared to parallel static incubation. Likewise, deformation of tBuOOH-treated RBC did not adversely affect phospholipid availability to digestion, even though these cells manifested somewhat abnormal susceptibility to phospholipase to begin with (i.e., even under static conditions). RBC also were able to maintain normal phospholipid status during deformation despite severe ATP depletion (to 0.22 nmol/mg Hb compared to 3.53 nmol/mg for control RBC). Even the presence of vanadate (200 μM) had no adverse impact on either normal or tBuOOH-treated RBC subjected to deformation (Table I).

In separate experiments, we also used fluorescamine to probe for any actual loss of asymmetry, as shown in Table II for labeling performed at the very end of the deformation period. tBuOOH-treated RBC were slightly abnormal in this respect even under static conditions, but application of deforming stress had no effect whatsoever on aminophospholipid labeling with this agent. Results were similar in other experiments (not shown) performed using either phospholipase digestion at other shear stresses or fluorescamine labeling by direct addition to deforming cells.

The cells used for these studies manifested the typical K leak previously documented for RBC undergoing elliptical deformation in this experimental system [22]. That is, normal RBC manifested a minimal amount of K leak during the experiment (< 0.5 mequiv./l RBC), and tBuOOH-treated RBC showed an abnormal exaggerated deformation-induced leak (> 3 mequiv./l RBC). In comparison to the results presented above, sickle RBC undergoing deoxygenation-induced deformation in our hands manifested the typical enhanced susceptibility of PE and PS to phospholipase already amply documented in the literature [16–18]: for two patients the 60 min PE digestion increased from 7.5 to 14.3% and from 14.1 to 24.1% during sickling, while PS digestion increased from 0.7 to 5.5% and from 2 to 13.6%.

TABLE I

Phospholipid status probed using phospholipase

Percent phospholipid hydrolysis was determined during a 60 min exposure of RBC to bee venom phospholipase A_2 during elliptical deformation and parallel static incubation. Results of three experiments for normal and tBuOOH-treated RBC are shown (mean ± S.D.); other results are for single experiments.

	Phospholipid hydrolysis (%)		
	PC	PE	PS
Normal RBC			
static control	64.6 ± 1.3	8.8 ± 1.4	0 ± 0
+ deformation	64.8 ± 0.7	9.4 ± 0.9	0 ± 0
tBuOOH-treated RBC			
static control	65.6 ± 1.1	13.5 ± 1.9	0 ± 0
+ deformation	65.7 ± 0.9	14.1 ± 2.7	0 ± 0
ATP-depleted RBC			
static control	63.9	7.8	0
+ deformation	64.6	8.5	0
Vanadate-treated RBC			
static control	64.3	8.9	0
+ deformation	65.2	9.2	0
tBuOOH plus vanadate RBC			
static control	64.9	12.8	0
+ deformation	63.9	12.5	0

TABLE II

Phospholipid status probed using fluorescamine

Normal and tBuOOH-treated RBC were pulse labeled with fluorescamine before and at the end of 75 min of deformation or parallel static incubation.

	Phospholipid labeling (%)					
	normal RBC			tBuOOH RBC		
	zero time	plus deformation	static control	zero time	plus deformation	static control
PE	13.2	13.6	13.4	16.2	16.9	16.8
PS	0	0	0	0	0	0

Discussion

To assess the RBC membrane's ability to preserve normal phospholipid asymmetry during cellular deformation, we examined RBC during application of shear stress in a viscous medium. Under these conditions RBC become prolate ellipsoids, and the cell membrane exhibits a tank-treading motion with rotation around the cytoplasm along the ellipsoid's long axis [21]. The present experiments were conducted at shear stresses that are far below the established hemolytic threshold, so our system induces minimal hemolysis; the benign nature of this experimental system has been amply documented [22]. Nonetheless, based on our prior direct analysis of RBC deformation in this system [27], normal RBC at shear stress 220 dyn/cm^2 achieve > 96% of their maximal possible deformation. Because tBuOOH-treated RBC are somewhat less deformable in this shear stress range, they achieve approx. 84% of their maximal deformation. RBC depleted of ATP and examined in this time frame remain discocytic and normally deformable [23].

Thus, this experimental system presents a significant deforming stress, despite which normal constraints on phospholipid asymmetry are preserved. Significantly, even normal RBC that are severely (> 90%) ATP-de-

pleted or treated with 200 μM vanadate preserve normal phospholipid organization during deformation. The latter cells are particularly informative because they clearly are unable to utilize flipase to enforce phospholipid orientation [24]. Thus, maintenance of asymmetry during elliptical deformation appears to be wholly dependent upon stabilizing effects of membrane skeletal proteins. Consistent with this dominant effect of protein-derived stabilization, tBuOOH-treated RBC that actually already have a somewhat destabilized bilayer maintain unchanged phospholipid status during deformation, even if their flipase is inhibited with vanadate. This implies that their static, partial loss of asymmetry is the result of tBuOOH impairing protein-derived phospholipid stabilization; yet this does not deteriorate further during deformation.

Although the period of observation in our experiments is short (1 h), the extreme membrane disruption (formation of elongated spicules with actual separation of bilayer from skeleton [19]) that occurs during RBC sickling easily and dramatically promotes transbilayer movement of aminophospholipids in this period. In striking contrast, our results thus indicate that even marked elliptical deformation with tank-treading motion of the membrane does not cause such disassembly of putative protein/lipid associations. The original observation of tank-treading membrane behavior established that the underlying membrane skeleton tank treads as well [21], and it recently has been observed that tank-treading does not seem to disrupt normal associations between band 3 protein and the cytoskeleton [28]. In further arguing against occurrence of shear-induced mixing of membrane components or separation of integral membrane from underlying skeleton during significant cellular deformation, our data support the notion that the RBC membrane moves as a unit during cell shape change [20] without disruption of skeleton/membrane associations.

Of secondary interest, our data on tBuOOH-treated RBC illustrate that even if an oxidative membrane perturbation is severe enough to detectably destabilize phospholipid orientation and promote cation leak, additional application of deforming stress affects these functions independently (increases leak but not phospholipid flip-flop). Thus, these two membrane properties are not inextricably linked, at least under physiologic degrees of perturbation.

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