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Maintenance of lipid asymmetry in red blood cells and ghosts: effect of divalent cations and serum albumin on the transbilayer distribution of phosphatidylserine

Jerome Connor, Karen Gillum and Alan J. Schroit

Department of Cell Biology, The University of Texas, M.D. Anderson Cancer Center, Houston, TX (U.S.A.)

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The maintenance of lipid asymmetry in the plasma membrane of human red blood cells (RBC) was investigated by assessing the equilibrium distribution of exogenously inserted NBD-labeled phosphatidylserine (PS) and endogenous PS in RBC and hypotonically lysed ghosts. PS distribution was determined by the ability to 'back-exchange' NBD-lipids into acceptor membranes and bovine serum albumin, and by prothrombinase complex assay for endogenous PS. To maintain the normal asymmetric distribution of PS in RBC, ghosts required Mg^{2+} in the lysis buffer. The inclusion of Ca^{2+}, even in the presence of Mg^{2+} resulted in complete randomization of endogenous and exogenously inserted PS. These results indicate that NBD-labeled PS analogs faithfully monitor the distribution of endogenous PS during ghost preparation. In contrast, treatment of RBC with bovine serum albumin had no effect on the distribution of endogenous PS, although it resulted in a time-dependent movement of NBD-labeled PS from the inner to the outer leaflet (flop). This phenomenon was dependent on continuous incubation in the presence of albumin and could not be duplicated when pure acceptor membranes were used.

Introduction

It is well established that proteins [1,2] and phospholipids [3,4] are asymmetrically distributed in the human erythrocyte membrane. With the exception of the membrane spanning proteins, band 3 and the glycophorins [1,2], all the currently identified erythrocyte proteins are accessible at the cytoplasmic face [5,6]. Similarly, virtually all the PS and most of the PE are found in the inner leaflet of the bilayer, whereas PC and sphingomyelin are preferentially located in the outer leaflet [7,8].

Formation of erythrocyte ghosts by hypotonic lysis does not appear to perturb the native orientation of membrane proteins. In fact, their asymmetric orientation is remarkably stable, since protein 'sidedness' is maintained in right-side-out and inside-out vesicles generated by low ionic strength buffers [5]. Identical procedures, on the other hand, result in a loss of endogenous lipid asymmetry [9] which appears to be dependent on the inclusion of Ca^{2+} [10].

Recent investigations into the maintenance of phospholipid asymmetry in RBC have indicated that an ATP-dependent aminophospholipid translocase [11] specifically transports PS and PE from the outer to the inner leaflet, thereby enabling the cell to adopt and maintain an appropriate equilibrium distribution of aminophospholipids across the bilayer membrane. This phenomenon has been shown by the specific transport of lyso-PS [9] diacyl-PS [12,13] and spin-labeled [11], iodine-labeled [14], and fluorescent-labeled [15] PS analogs. While the use of lipid analogs have proved invaluable in characterizing and identifying the PS transporter [14,16], it is crucial to the interpretation of such experiments that these analogs faithfully monitor the behavior of endogenous lipids.

The present study was initiated to investigate the experimental conditions required to accurately monitor endogenous PS distribution with synthetic fluorescent (NBD-labeled) PS analogs. We used the PS-dependent prothrombinase assay [17–19] which facilitates direct

Abbreviations: ACHase, acetylcholinesterase; BSA, bovine serum albumin; DOPC, dioleoylphosphatidylcholine; G3PD, glycero-3-phosphate dehydrogenase; NBD-PC, 1-oleoyl-2-((*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)caproyl)-phosphatidylcholine; NBD-PS, 1-oleoyl-2-((*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)caproyl)phosphatidylserine; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; 5P8, 5 mM phosphate buffer (pH 8.0).

Correspondence: A.J. Schroit, Department of Cell Biology, The University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030, U.S.A.

comparison of endogenous PS asymmetry with the distribution of PS analogs. We show that endogenous PS asymmetry in ghosts is dependent upon the inclusion of divalent cations in the lysis media and that this phenomena is accurately monitored by fluorescent PS analogs. On the other hand, conditions which induce the rearrangement of NBD-PS from the inner to the outer leaflet (flop) do not result in the redistribution of endogenous PS.

Materials and Methods

NBD-PC and DOPC were purchased from Avanti Polar Lipids (Birmingham, AL). NBD-PS was prepared from NBD-PC by phospholipase D-catalyzed base exchange in the presence of L-serine [20] and purified by thin-layer chromatography. Factor V was isolated from bovine plasma [21], activated Factor X (500 U/mg protein) and prothrombin were obtained from Sigma and thrombin and the thrombin-sensitive chromophore, S2238, were purchased from Helena Laboratories (Beaumont, TX). Crystallized, fatty acid-free BSA of the highest purity was obtained from Sigma (product No. A7511). Human RBC were obtained from healthy volunteers by venipuncture into heparinized saline and were washed three times with large volumes of PBS. Steady-state fluorescence was measured at 535 nm (λ_{ex} 468 nm) with a Farrand MK II spectrophotofluorometer at room temperature using 10 nm slit widths.

Insertion of lipid analogs into RBC

To determine whether the asymmetric orientation of membrane phospholipids was maintained upon generation of ghosts, intact RBC were labeled with NBD-PC or NBD-PS in their outer or inner leaflets, respectively. This was accomplished by incubating 5 ml RBC (10% packed) with NBD-labeled lipid (1 μg/10 μl EtOH/ml RBC) for 45 min at 4°C with NBD-PC or at 37°C for NBD-PS. After washing with PBS, the NBD-PS treated cells were incubated for 2 min with 1% BSA to remove residual NBD-PS (lipid not transported to the inner leaflet) from the outer leaflet. These cells, which contained NBD-labeled lipid exclusively in their inner (NBD-PS) or outer (NBD-PC) leaflets were then subjected to hypotonic lysis.

Preparation of ghosts

Ghosts were prepared by hypotonic lysis as described by Steck [1]. Briefly, 50 μl of 50% packed RBC were diluted with 20 volumes of 5 mM phosphate (pH 8) containing 0.1 mM EDTA (5P8) and the indicated cations. The ghosts were incubated on ice for 15 min, collected-by centrifugation (13000 $\times g$ for 5 min) and assessed for their distribution of NBD-labeled lipids. Sealed and unsealed populations were assessed by accessibility of the inner and outer surface markers glyceraldehyde-3-phosphate dehydrogenase and acetylcholinesterase, to their substrates [22].

Maintenance of lipid asymmetry

The ability of ghosts to maintain their initial asymmetric distribution of PS and PC was determined by the ability to remove NBD-labeled lipids in the outer monolayer by back-exchange to BSA. Briefly, after the indicated treatments, aliquots of ghosts were resuspended in 5P8 or 1% BSA in 5P8. The fraction of residual NBD-labeled lipid in the BSA-exchanged population (lipid in the inner leaflet) compared to the total NBD-lipid present in the buffer washed population (lipid in both the outer and inner leaflets) was determined by fluorescence after ghost solubilization in 1% Triton X-100.

Prothrombin-converting activity assay

The rate of conversion of prothrombin to thrombin by the prothrombinase complex assay is an established technique to assess levels of PS expressed on the surface of cells. Briefly, RBC or ghosts (not treated with NBD-lipids) were incubated with appropriate concentrations of $CaCl_2$, Factor Va, Factor Xa and prothrombin as described previously [19]. After 3 min the reaction was stopped by the addition of EDTA. The thrombin-dependent chromophore S2238 was then added, and the rate of chromogen formation was monitored at 405 nm with a Gilford Response spectrophotometer employing appropriate kinetic software. The initial rates of thrombin-converting activity, which is directly proportional to the amount of PS on the catalytic surface (see Ref. 19), were determined from the slopes of absorbance.

Outward translocation of NBD-PS

To determine whether NBD-PS, which resides in the inner leaflet of RBC, could be translocated to the outer leaflet, cells were incubated with NBD-PS for 1 h at 37°C ($2 \cdot 10^7$ cells/100 ng NBD-PS/10 μl EtOH/ml RBC). The RBC were washed to remove unincorporated lipid and then mixed with DOPC acceptor vesicles (0.5 mg/ml RBC) for 45 min at 0°C to remove any residual NBD-PS occupying the outer leaflet. These cells, which contained NBD-PS only in their inner leaflet, were then assessed for the appearance of NBD-PS in their outer leaflet after incubation at 37°C in the presence or absence of 1% BSA or 0.5 mg DOPC acceptor vesicles. To determine whether, under similar conditions, endogenous PS was translocated from the inner to outer leaflet, untreated cells (without NBD-PS) were also incubated with BSA or acceptor vesicles as described. The appearance of endogenous PS in the cells' outer leaflet was then determined by prothrombinase assay.

Results and Discussion

Perturbation of membrane lipid asymmetry upon hypotonic lysis

The ability of erythrocytes to maintain an asymmetric orientation of phospholipids upon hypotonic lysis was investigated in cells that preferentially contained NBD-PC and NBD-PS in their outer and inner leaflets, respectively. The results shown in Fig. 1 indicate that both NBD-PC and NBD-PS completely randomize between leaflets upon lysis in the absence of divalent cations. This can be seen from the observation that virtually all the fluorescent lipids resided in the appropriate leaflet at the initiation of the experiment. However, after the addition of the 5P8, half of both lipid species was localized in each leaflet. Similar to the data presented by Williamson et al. [10] the inclusion of Ca^{2+} in the lysis media was without effect, whereas Mg^{2+} or Mg-ATP, but not Na-ATP, appeared to partially preserve the initial asymmetric orientation of the fluorescent analogs. These data suggest that Mg^{2+} is required for the maintenance of lipid asymmetry in RBC ghosts.

That the distribution of NBD-PS closely parallels the distribution of endogenous PS was verified by determining the prothrombin-converting activity of ghosts. The results of these experiments (Fig. 1) indicate that proportional amounts of endogenous PS and NBD-PS become exposed at the outer surface of the plasma membrane (Fig. 1).

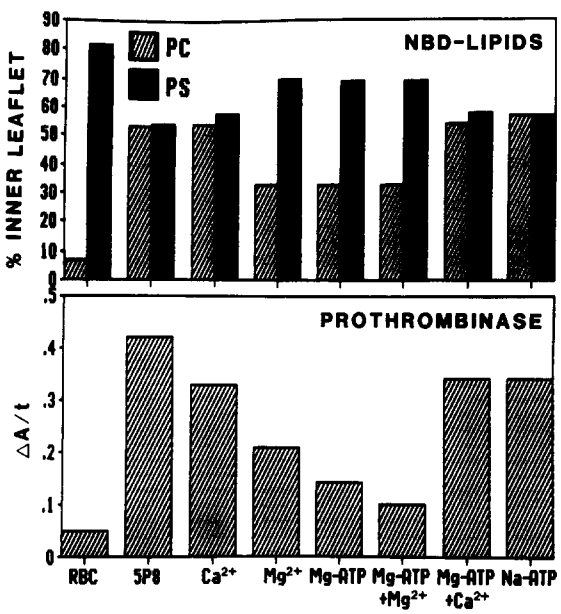

Fig. 1. Effect of divalent cations and ATP on the distribution of endogenous and NBD-labeled lipids after hypotonic lysis. (Top) RBC containing NBD-PS and NBD-PC exclusively in their inner or outer leaflet were prepared as described in Materials and Methods. These cells were then diluted 50-fold with 5P8 containing 0.1 mM EDTA and $CaCl_2$ (1.5 mM), $MgSO_4$ (1.5 mM), Mg-ATP (3 mM) or Na-ATP (3 mM). After centrifugation NBD-labeled lipid distribution was determined by back-exchange. (Bottom) Endogenous PS in the outer leaflet was measured by prothrombinase complex assay.

TABLE I

Assessment of ghost 'sealing' by accessibility of acetylcholinesterase and glycero-3-phosphate dehydrogenase

Treatment [b]	Accessibility (%) [a]	
	ACHase	G3PD
5P8 alone	99	151
Ca^{2+}	107	28
Mg^{2+}	102	114
Mg-ATP	112	89
Mg-ATP + Mg^{2+}	101	12
Mg-ATP + Ca^{2+}	110	8
Na-ATP	98	165

[a] Accessibility of the membrane markers was calculated from the fraction of enzyme activity of intact ghosts in 5P8 compared to identical aliquots solubilized in 0.2% Triton X-100.

[b] Ghosts were prepared exactly as described in Fig. 1.

These findings could conceivably be explained by the possibility that ghost membranes prepared in the absence of Mg^{2+} are unsealed and leaky. If this were the case, then the albumin and the prothrombinase components might have free access to the inner membrane. However, assessment of ACHase and G3PD revealed no correlation between sealed or unsealed populations and loss of lipid asymmetry (Table I). For example, membranes prepared in the presence of Ca^{2+} were essentially sealed (Table I), yet they did not retain membrane lipid asymmetry (Fig 1). Conversely, ghosts prepared in the presence of Mg^{2+} were unsealed, although they appeared to retain some degree of lipid asymmetry. These results suggest, therefore, no relationship between the preservation of membrane lipid asymmetry and whether the ghosts were sealed or unsealed. On the other hand, it does appear that Mg^{2+} is required to preserve membrane asymmetry.

That Mg^{2+} is indeed required for the preservation of membrane lipid asymmetry is shown in Fig. 2. It can be seen that increasing the concentration of Mg^{2+} to approx. 1.5 mM resulted in increased stabilization of membrane lipid asymmetry, as assessed for endogenous (prothrombinase assay) and NBD-labeled PS. On the other hand, Ca^{2+} destroyed lipid asymmetry, irrespective of the inclusion of Mg^{2+}. This can be seen from the observation that increasing amounts of Ca^{2+} added to lysis media containing Mg^{2+} resulted in a reduction in the fraction of NBD-PS remaining in the ghosts' inner leaflet and a concomitant increase in prothrombinase activity (Fig. 3).

These observations can best be explained in terms of a general and transient perturbation in the transbilayer distribution of membrane phospholipids upon hypotonic lysis. This conclusion is supported by the observa-

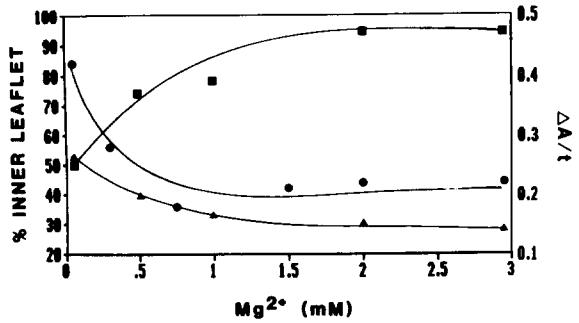

Fig. 2. Stabilization of membrane lipid asymmetry by Mg^{2+}. Ghosts were prepared from RBC in 5P8 containing increasing concentrations of Mg^{2+}. The fraction of NBD-labeled lipid remaining in the inner or outer leaflet was determined by back-exchange. The distribution of endogenous PS was determined by prothrombinase assay. ▲, NBD-PC; ■, NBD-PS; ●, prothrombinase.

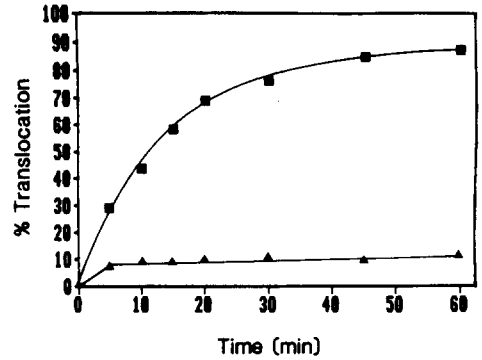

Fig. 4. Translocation of NBD-PS from the outer to inner leaflet of RBC. RBC were incubated with NBD-PS (■) or NBD-PC (▲) for 2 min at 0°C. The cells were washed with ice-cold PBS and incubated for the indicated times at 37°C. The fraction of NBD-labeled lipid in the inner leaflet was determined by the inability to remove the lipid by back-exchange to BSA.

tion that lipid sidedness does not seem to be determined by the formation of sealed/unsealed ghosts but rather by the inclusion of Mg^{2+}. The finding that 50% of the total NBD-PC and NBD-PS can be removed from the ghosts in 'asymmetric' preparations suggests that the appearance of these lipids in their opposing leaflets is the result of an extremely rapid and transient reorganization of membrane lipids. This conclusion is supported by related experiments that have indicated that once the NBD-labeled lipids are removed from the outer monolayer, residual lipid remains stably associated with the ghosts' inner leaflet (not shown).

Stability of lipid asymmetry in intact cells

Recent studies designed to ascertain whether endogenous PS can be translocated to the outer leaflet of intact RBC have yielded conflicting results. It has been reported that an ATP-dependent outward movement of spin-labeled PS analogs occurs in RBC [23,24], a phenomenon which we have not been able to show using NBD-labeled PS [25]. In an effort to define conditions that facilitate the inside-to-outside movement of PS, RBC containing NBD-PS exclusively in the inner leaflet were subjected to various treatments. The inner leaflet of RBC was selectively-labeled by incubating the cells with NBD-PS at 37°C. Since maximum accumulation of the lipid occurred within 30 min (Fig. 4) RBC were incubated for 45 min to ensure optimal inner leaflet labeling. Residual (outer leaflet) lipid was then removed from the cells by back-exchange before assessing inside-to-outside movement of NBD-PS. The rationale behind these experiments was that if 'flop' were significant, long term incubation of RBC with acceptor membranes (or BSA) at 37°C would force a shift in the equilibrium distribution of PS from the RBC to the acceptor population. The results presented in Table II indicate that significant movement of NBD-PS from the inner to outer leaflet of the cells could be detected only after long-term incubation of the cells in the presence of BSA. If, however, DOPC vesicles were employed as the acceptor population or RBC were incubated with BSA for short periods of time (a 2 min centrifugation time, which completely removes all NBD-labeled lipid in the cells' outer leaflet), no movement could be detected. This finding suggests that this result was an anomaly of BSA and/or NBD-PS. Indeed, identical conditions failed to induce any detectable redistribution of endogenous PS, as determined by prothrombinase activity (Table II).

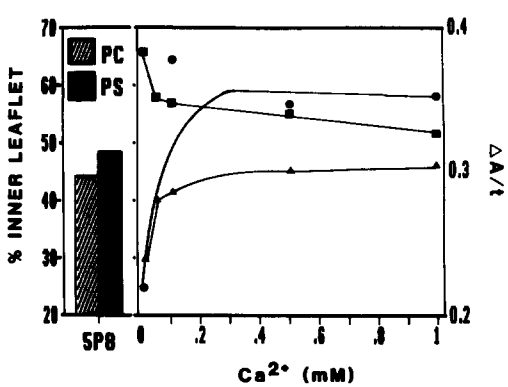

Fig. 3. Destabilization of membrane lipid asymmetry by Ca^{2+}. The fraction of NBD-labeled lipid remaining in the inner or outer leaflet was determined by back-exchange. The distribution of endogenous PS was determined by prothrombinase assay. (Left) Ghosts prepared in the absence of divalent cations. (Right) Ghosts prepared in the presence of 1.5 mM Mg^{2+} with increasing Ca^{2+} concentrations. ▲, NBD-PC; ■, NBD-PS; ●, prothrombinase.

In conclusion, our results indicate that, contrary to the stable asymmetric organization of erythrocyte membrane proteins after hypotonic lysis, membrane phospholipids undergo a rapid and transient reorganization. The results presented here extend the initial observations of Dressler et al. [9] and Williamson et al. [10] and verify that, under appropriate conditions, NBD-labeled lipid analogs accurately monitor the distribution of

TABLE II

Inside-to-outside movement of NBD-PS and endogenous PS in RBC [a]

Time (h)	NBD-PS % lipid in inner leaflet [b]				Prothrombinase $\Delta A/t$ [c]	
	DOPC		BSA		Control [f]	BSA
	wash [d]	incubation [e]	wash [d]	incubation [e]		incubation [e]
0	100		100		0.02	0.02
1	99	89	83	70		
2	100	87	82	47		
3	93	89	75	27	0.02	0.02

[a] RBC were incubated with NBD-PS for 45 min at 37°C. Residual NBD-PS in the outer leaflet (lipid not transported to the inner leaflet) was removed by back-exchange with either BSA or DOPC vesicles. These cells were then washed and treated as described.

[b] Cell associated NBD-PS after back-exchange.

[c] Thrombin activity was determined spectrophotometrically at 405 nm after the addition of the chromogenic substrate S2238.

[d] After the indicated incubation period at 37°C, the RBC were spun through a 1% BSA solution or incubated for 45 min at 0°C with 0.5 mg DOPC acceptor vesicles. Residual NBD-PS was then determined as described in Materials and Methods.

[e] RBC were incubated for the indicated time at 37°C in the presence of 1% BSA or 0.5 mg DOPC vesicles.

[f] Cells incubated with PBS only.

endogenous lipids. However, data obtained using the prothrombinase assay indicated that NBD-PS may not always be indicative of endogenous PS distribution. Conditions that promoted the inside-to-outside movement of NBD-PS (long-term incubation with BSA but not with DOPC vesicles) did not induce the movement of endogenous PS. Although lipid analogs have proven to be invaluable in the study of lipid translocation, whether their patterns of behavior actually reflect the behavior of endogenous lipids should be carefully ascertained.

Note added in proof: (Received 26 April 1990)

Recent experiments indicate that the 'flop' of NBD-PS observed in RBC incubated for prolonged periods with BSA is ATP-dependent. This suggests that the observed inside-to-outside translocation is indeed a protein-mediated event (see Bitbol and Devaux [23]), which because of affinity and kinetic considerations, requires the presence of BSA and not acceptor membranes to be measurable.

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