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Transmembrane redistribution of phospholipids of the human red cell membrane during hypotonic hemolysis

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The transmembrane distribution of spin-labeled phospholipids was measured in human erythrocytes before and after hypotonic hemolysis by electron paramagnetic resonance. With a first series of partially water soluble probes a complete randomization of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin analogues was achieved when cells were resealed in the absence of Mg-ATP or when the aminophospholipid translocase was inhibited by vanadate or calcium. If the ghosts were resealed with Mg-ATP inside, the transmembrane asymmetry of the aminophospholipids was reestablished. With long chain insoluble spin-labeled lipids complete randomization was obtained with the phosphatidylcholine analogue but even in the presence of vanadate only a small percentage (approx. 15%) of the spin-labeled phosphatidylserine flopped to the outer monolayer and comparable percentage of the spin-labeled sphingomyelin flipped to the inner monolayer, indicating a hierarchy in the phospholipid redistribution for these water insoluble lipids during hemolysis. The mechanism by which a selective randomization takes place is not known. It may involve phosphatidylserine–protein interactions in the inner leaflet and sphingomyelin–cholesterol or sphingomyelin–sphingomyelin interaction in the outer leaflet.

Introduction

In the intact red blood cell (RBC) it is known that the phospholipids are asymmetrically arranged, with sphingomyelin (SM) and phosphatidylcholine (PC) being predominantly located in the outer leaflet of the bilayer while phosphatidylethanolamine (PE) and particularly phosphatidylserine (PS) are generally confined to the inner leaflet [1,2]. Passive diffusion across the membrane is very slow and the asymmetry is maintained primarily by the action of the aminophospholipid translocase, a Mg^{2+}-ATPase that functions to rapidly restore to the inner leaflet (flip) any PS and PE that may have diffused away (flop) to the outer layer [2–6]. Several laboratories have proposed that the cytoskeleton proteins (spectrin and band 4.1) could also participate in the maintenance of lipid asymmetry [7,8]. The hypotonically lysed RBC membrane preparation – the ghost – has been analyzed to provide considerable information about the content and organization of membrane proteins. The phospholipids of the ghost have also been quantitatively analyzed but for technical reasons, less is known about the orientation of phospholipids in the bilayer of the ghost, in particular the extent of phospholipid reorientation during hypotonic lysis is still debated. Note that if the cytoskeleton proteins were indeed important in the maintenance of lipid asymmetry, one would not expect a priori a lipid scrambling during hemolysis since the cytoskeleton meshwork is preserved in this operation, even if properties of skeletal proteins might be affected by the drop in ionic strength [9].

The early work by Shukla et al. [10] casts some doubt on the use of ghosts for the investigation of lipid asymmetry by the phospholipase technique. Later Dressler and collaborators [11] inferred that ghosts retained the normal asymmetry since lyso-phosphatidylserine slowly accumulated in ghosts unless they were treated with SH oxidizing reagents. In 1985, Williamson et al. [12] claimed from experiments with phospholipase A_2 that ghosts prepared in the presence of Mg as the only divalent cation retained the normal phospholipid asymmetry characteristics of intact erythrocytes. However, in this article the authors reported

Abbreviations: RBC, red blood cell; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; BSA, bovine serum albumin; PLTP, phospholipid transfer protein; EPR, electron paramagnetic resonance.

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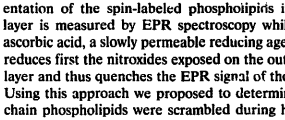
a percentage of PS on the outer monolayer equal to 18% of the PS pool, which is much larger than the values given in the literature for RBCs [1,2].

In a more recent paper, using short chain fluorescent phospholipids NBD-PC and NBD-PS as markers, as well as the prothrombinase assay, Connor et al. [13] claimed to have found a profound reorganization of membrane lipids under similar circumstances. However, the incorporation into the lipid bilayer of these probes depends on the fact that they are slightly soluble in aqueous media. During hypotonic hemolysis such minimally soluble probes could enter the aqueous lysing solution and then be redistributed randomly across the bilayer thereby providing artifactual data about phospholipid reorientation since naturally occurring lipids are totally insoluble. Furthermore, we have recently demonstrated that phospholipids with a short β chain bearing an NBD moiety do not always behave like long chain unlabeled lipids, for example NBD-PE is not transported by the aminophospholipid translocase in red blood cells [14].

In designing new experiments to evaluate the orientation of the lipid bilayer during and after hypotonic hemolysis, we synthesized PS, PC and SM with the spin-labeled nitroxide group on the 5th carbon of the palmitoyl chain located at *sn*-2 and referred below as (10,3)PC, (10,3)PS and (10,3)SM. Such long chain fatty acid derivatives of phospholipids are insoluble in aqueous media and, thus, cannot enter the lysing medium during hypotonic lysis. These probes can only be introduced into RBC bilayer by the intervention of a non specific phospholipid transfer protein (PLTP). The orientation of the spin-labeled phospholipids in the bilayer is measured by EPR spectroscopy while adding ascorbic acid, a slowly permeable reducing agent, which reduces first the nitroxides exposed on the outer monolayer and thus quenches the EPR signal of those lipids. Using this approach we proposed to determine if long chain phospholipids were scrambled during hemolysis. For comparison we have carried out also a series of experiments with the partially water soluble spin-labeled lipids: (0,2)PC, (0,2)PS, (0,2)PE and (0,2)SM, where the nitroxide group is on the 4th carbon of the pentanoyl chain located at *sn*-2.

Materials and Methods

The general formula of the spin-labeled glycerophospholipids is the following:



where R is either choline, serine or ethanolamine, (m, n) was respectively (10,3) for the insoluble probes and (0,2) for the soluble probes. The corresponding sphingomyelin molecules were also synthesized.

(10,3)PC was synthesized as previously described [15,16]. (10,3)PS was obtained from (10,3)PC by phospholipase D catalyzed base exchange reaction [17]. The long chain spin-labeled sphingomyelin, (10,3)SM, was synthesized as described by Zachowski et al. [18] except that a palmitoyl chain was used. The (0,2) phospholipids were synthesized as described by Morrot et al. [19].

Non specific transfer protein was purified from maize seedlings [20]. Sodium creatine phosphate, creatine phosphokinase, diisopropyl fluorophosphate, delipidated bovine serum albumin (BSA) and sodium vanadate were obtained from Sigma Chemical Company, St Louis, MO, USA.

Blood was obtained from the 'Fondation Nationale de Transfusion Sanguine'. Experiments were carried out on fresh or one day old stored blood which was washed five times before use with 5-fold volumes of cold buffered saline potassium glucose (BSKG) (145 mM NaCl, 5 mM KCl, and 6 mM glucose, 10 mM phosphate buffer pH 7.4). The spin-labeled (10,3) phospholipid was brought to dryness in a stream of argon and then dispersed into multilamellar vesicles by first vortexing for 20 s in 0.5 ml of BSKG and then sonicating for 5 min at a temperature of 4°C.

Use of spin-labeled phospholipids with one short chain. Prior to labeling, erythrocytes were incubated with 5 mM diisopropyl fluorophosphate to minimize phospholipase-like activity of the deacylase-reacylase complex which hydrolyzes the short chain-phospholipid analogues [14,19,21]. To label the membranes, suitable amounts of (0,2) spin-labels in chloroform/methanol (1:1, v/v) corresponding to approx. 1% of total phospholipids, were dried under vacuum and resuspended by vigorous vortexing in BSKG buffer. Two volumes of the aqueous dispersions of labels were then added to one volume of red cells. The incorporation in red cell membranes was completed within 1 min, as inferred from the EPR lineshape [3]. The transmembrane distribution of the (0,2) phospholipids in the erythrocytes or in the ghosts was assayed by the back exchange technique (incubation in presence of 1% BSA for 1 min) described in Morrot et al. [19]. Cells were either lysed one minute after addition of spin labels at which time all probes were located in the outer leaflet, or after, either one or six hour incubation at 37°C for PS and PE, respectively, allowing the aminolipids to reach their transmembrane equilibrium [19].

Introduction of long chain spin-labeled phospholipids into RBC. Washed RBC were suspended in BSKG to an hematocrit of 50 along with 0.25–0.5 mg/ml of the phospholipid transfer protein and the vortexed soni-

cated multilamellar vesicles of the labeled phospholipid. Typically the spin-label was mixed with the RBC suspension at a concentration representing 25–50% of the total phospholipid content. Direct measurements showed that after a 30 min incubation, (10,3)PS incorporated accounted for 0.5 to 4.1% of total phospholipid, 2.7 to 4.2% for (10,3)SM and 4% for (10,3)PC [22]. The incubation was carried out at 37°C and, at 15-min intervals, aliquots were tested in the EPR spectrometer to monitor the extent of incorporation. Incorporation was stopped by washing RBC five times with large volumes of BSKG to remove PLTP and sonicated vesicles.

EPR measurements. EPR spectra were recorded on a Varian E9 spectrometer on line with a Tektronix computer for storage and analysis and equipped with a temperature control device. Accessibility of the (10,3) spin-label to reduction was assessed by adding to the packed RBC an equal volume of 0.1 M ascorbic acid in 0.1 M $NaHCO_3$ (pH 7.4). An identical aliquot was studied after an equal volume of BSKG was added. Reduction kinetics was followed by recording the spectrum middle-field line at 1-min intervals and plotting its height vs. time. A break in the decay curve indicated when the externally exposed spin-label was totally reduced.

Lysis. Packed RBC were lysed by adding a 20-fold volume of 5 mM phosphate buffer (pH 7.4) at 4°C followed by swirling the suspension on ice for 5 min. A sample containing an identical number of ghosts as RBC used above was then centrifuged and taken for EPR spectrometry with and without ascorbate addition. This measurement provided an assessment of the openess of the lysed RBC during hypotonicity. Then to one aliquot of the lysate 0.25–0.5 mM vanadate was added to totally inhibit aminophospholipid translocase activity, while to another aliquot 3 mM Mg-ATP and an ATP regenerating system consisting of 10 mM creatine phosphate and creatine kinase was added. In the case of experiments using (0,2)PL, two other aliquots were prepared, containing either 0.1 mM or 1 mM $CaCl_2$. After an additional swirling for 5 min at 4°C, isotonicity was restored by adding 1/10 volume of 1.5 M NaCl in 5 mM phosphate buffer (pH 7.4). The suspension was then incubated at 37°C for 60 min. These resealed ghosts were afterwards either incubated with ferricyanide and BSA for the back exchange assay or analyzed in the EPR spectrometer with and without ascorbate addition to determine the orientation of the labeled phospholipids in the ghost bilayer.

Morphology. At each stage (washed RBC, RBC after incubation with labeled phospholipids and phospholipid transfer protein, ghosts during lysis and after resealing), morphology was evaluated by adding 10 μl of suspension to 100 μl 1% glutaraldehyde in BSKG and the RBC or ghosts were viewed by Nomarski interference microscopy.

Results

Transmembrane distribution of (0,2)-spin-labels before and after lysis

Table I indicates the results obtained with the slightly water soluble (0,2)-spin-labeled phospholipids. The transmembrane distribution of four different phospholipids before and after lysis are indicated. The lysis was carried out under different conditions: (i) the aminophospholipid translocase blocked by vanadate; (ii) addition of Mg-ATP, so that the translocase has full activity; (iii) in the presence of a low concentration of Ca^{2+}; (iv) in the presence of a nigh concentration of Ca^{2+} (which blocks the translocase). Furthermore, the aminophospholipid redistribution during hemolysis was tested either immediately after incorporation of probe into the red cells or after transmembrane equilibration of PS and PE (incubation at 37°C of 1 h and 6 h, respectively). The results suggest that when the aminophospholipid translocase is blocked, an extensive redistribution of the four phospholipids tested takes place during hemolysis leading to an almost complete

TABLE I

Percentage of spin-labeled lipids with one short chain on the external leaflet before and after hemolysis and resealing

The percentage of spin-labeled lipids on the external leaflet was determined by back-exchange on BSA (see Ref. 21). Measurements with intact erythrocytes were performed either 1 min after addition of the spin-labeled lipids, or after equilibration of PE and PS between the two membrane leaflet (6 h and 1 h incubation at 37°C, respectively). Measurements with ghosts were performed at the end of the resealing process (see Methods). The experimental error on each value is < 5%.

Phospholipid	(0,2)PC	(0,2)SM	(0,2)PE	(0,2)PE	(0,2)PS	(0,2)PS
Incubation time before lysis:	1 min	1 min	1 min	6 h	1 min	1 h
Intact erythrocyte	100	100	100	21	100	< 5
Ghost + vanadate (50 μM)	60	62	52	33	67	46
Ghost + Mg-ATP (3 mM)	51	42	59	36	10	27
Ghost + calcium (0.1 mM)	52	55	55	32	33	22
Ghost + calcium (1 mM)	44	49	42	52	46	50

transmembrane randomization of the lipids. In particular, in agreement with Connor et al. [13], PS and PC were found almost equally in both leaflets after hemolysis and resealing under conditions of inhibition of the aminophospholipid translocase. In addition we show here that PE and SM are also scrambled under such conditions. In fact we find that the short chain spin-labeled sphingomyelin is redistributed between both leaflets under all our experimental conditions. But one may question whether these amphiphilic probes can leak through the 'holes' in membrane of the red cell produced by hypotonic lysis rather than flip within the bilayer during hemolysis.

Accessibility to ascorbate of the long chain spin-labeled phospholipids

Fig. 1 shows typical reduction curves by ascorbate recorded at 4°C and displayed here in semi-log scale. The black symbols correspond to kinetics obtained before lysis, while the open symbols correspond to results obtained after lysis and resealing. All curves exhibit a discontinuity in their slope after 15–20 min, indicative of the destruction of the signal from the probe on the outer monolayer. This enables one to deduce the fraction of spin-labeled lipid in each monolayer. As an important control, when ascorbate was added to the lysed cells before resealing i.e. when all probes should be accessible to ascorbate, 90 to 100% of the probes were reduced, including the (10,3)PS. Fig. 1 as well as Table II show that in the native erythrocyte, the probes distribute themselves spontaneously as endogenous phopsholipids do, namely with

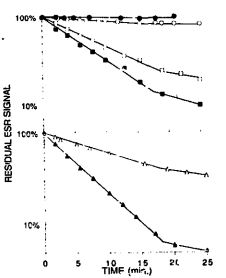


Fig. 1. Kinetics of reduction at 0°C by 0.1 M ascorbate of (10,3)PS (●, ○), (10,3)SM (■, □) and (10,3)PC (▲, △). The black symbols correspond to RBC, the opened symbols to resealed ghosts. Notice the semi-logarithmic scale. The discontinuity in the slope allows one to estimate the percentage of spin label in the outer leaflet.

TABLE II

Percentage of long chain labeled phospholipids reducible by ascorbate at 0°C

Phospholipid	(10,3)PC	(10,3)SM	(10,3)PS
Intact erythrocyte	96±6	90±5	<4
Ghost + vanadate	48±4	64±5	19±4
Ghost + Mg-ATP (immediately after resealing)		50±6	16±3
Ghost + Mg-ATP (after an extra 2 h incubation at 37°C)			<5

most choline containing lipids in the outer monolayer and practically all phosphatidylserine in the inner monolayer. Important redistributions take place during lysis and resealing but these events affect the various probes differently. Indeed, after resealing, (10,3)PC was found to be equally distributed across both leaflets of the bilayer while for the two other lipids there was a degree of persistent asymmetry; about twice as much (10,3)SM was in the outer leaflet as there was in the inner leaflet and only a small amount (approx. 20%) of (10,3)PS had moved to the outer leaflet. With prolonged incubation of the ghosts, and only if they were resealed in the presence of ATP, the small amount of (10,3)PS in the outer leaflet flipped to the inner leaflet.

EPR line shapes

Fig. 2 shows the EPR spectra recorded with either (10,3)SM or (10,3)PS in erythrocytes and in vanadate-treated ghosts. It can be seen that in the case of (10,3)SM, the spectrum obtained with the resealed ghosts has a narrower hyperfine splitting than in erythrocytes (64.1 instead of 65.6 G), indicative of a reduced order parameter. The inverse happens with (10,3)PS (splitting increases from 61.6 to 62.9 G).

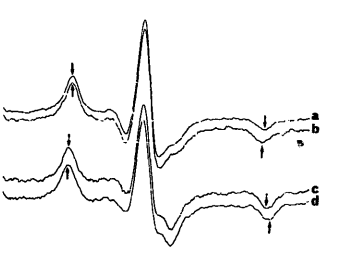


Fig. 2. Change in EPR line shape induced by hemolysis. (a) (10,3)PS in ghosts resealed in the presence of vanadate; (b) (10,3)PS in RBC; (c) (10,3)SM in ghosts; (d) (10,3)SM in RBC. Spectra were recorded at 4°C. Arrows indicate the outer extreme splitting.

Morphology

RBC incubated with (10,3)PC and (10,3)SM promptly became echinocytic being mostly echinocytes II. When RBC were incubated with (10,3)PS half became stomatocytic (stomatocytes I and II) after 10 min, but after 60 min of incubation half were stomatocytes III, the other half were spherostomatocytes containing endocytic vacuoles.

Ghost morphology was virtually the same for all probes used. Ghosts resealed with Mg-ATP became discocytic and cup shaped whereas ghosts resealed without Mg-ATP and with vanadate remained echinocytic.

Discussion

The observation of lipid reorientation during hypotonic lysis could provide information concerning mechanisms of disruption of the membrane during hypotonic shock. When disrupted does the bilayer undergo total disorganization or does a degree of organization persist in the form of a partially scrambled bilayer with variations in the movement of each of the phospholipid classes studied? In carrying out these studies the nature of the probe is critical if it is to be interpreted as revealing the movements of the endogenous phospholipids. The phospholipids with one short chain are partially water soluble which enables their easy incorporation into the RBC outer leaflet. However, because of the partial water solubility it is conceivable, although not certain, that during cell lysis the probes redistribute themselves between both leaflets via the aqueous phase through the membrane pore formed during hemolysis. The long chain spin-labeled phospholipids, on the other hand, are faithful replicas of endogenous phospholipids since the latter molecules are virtually insoluble in aqueous media. Their insertion into RBC bilayer requires the use of a purified phospholipid transfer protein (PLTP).

The data obtained with the water soluble probes, (0,2)PL (see Table I) indicate that the probes underwent a close to total scrambling during hypotonic hemolysis, followed by the selective movement of the aminophospholipids from the outer leaflet inward, when Mg-ATP is present in the cytosol. If vanadate or calcium is used to block the aminophospholipid translocase activity, the lipid scrambling persists. These results raise some difficulties. If applied to endogenous lipids, a total randomization would mean that a large fraction of PC and SM flips to the inner leaflet during hemolysis. Not only does this contradict the observation of Dressler et al. [11] who found that SM asymmetry was stable even in vesicles made from red cells, but also, it would contradict our own data on ghost shapes. Indeed, after resealing in the presence of Mg-ATP, it would be necessary that PC and SM flopped to the outer layer as rapidly as the aminophospholipids flipped to the interior leaflet, in order to explain the discoid shape obtained with such ghosts. If that did not happen, the inner leaflet would be overpopulated and extreme stomatocytosis would take place. Yet, such a rapid transmembrane movement of PC and SM is unlikely.

However, the results obtained with long chain spin-labeled (10,3) phospholipid are significantly different from the results with the short chain lipids. Hypotonic hemolysis followed by resealing in the presence of Mg-ATP completely randomized the distribution of (10,3)PC representing a movement of 40% of the probe. By contrast, only approximately 25% of (10,3)SM moved to the inner leaflet and 15% of (10,3)PS moved outward. In making these measurements there was a concern that any outward flop of PS would be immediately counteracted by the aminophospholipid translocase. As can be seen in Table II use of vanadate in concentrations that completely block the translocase gave initially similar results. However, prolonged incubation of the ghost preparation in the presence of Mg-ATP reveals that the aminophospholipid is eventually pumped back to the inner leaflet. This additional incubation had no effect on the distribution of (10,3)PC and (10,3)SM. These data on PC and SM also demonstrate that the amount of PS accessible to ascorbate just after ghost resealing cannot be attributed to an artefact, such as an incomplete resealing, which would disappear during the additional incubation.

It is interesting to note that the incorporation of 0.5–4% (10,3)PC or (10,3)SM into the membrane of the intact RBC produced prompt echinocytosis while (10,3)PS incorporation produced stomatocytosis. In a recent article, we have shown that the non specific exchange protein from plant is capable of lipid exchange [22]. However, similar proteins were reported to catalyse lipid addition. Thus, the simpler interpretation of the observed shape changes would be a net lipid addition, localized into the outer leaflet in the former case ((10,3)PC and (10,3)SM) and eventually accumulated into the inner leaflet in the latter case ((10,3)PS). But in fact the substitution of (10,3)SM or (10,3)PC for the endogenous SM or PC may suffice to induce the shape change due to the presence of the bulky doxyl ring close to the headgroup. On the other hand (10,3)PS exchanged into the outer leaflet of the membrane for PC or SM is rapidly flipped by the translocase to the inner leaflet, faster than PE or PC can possibly flop to the outer leaflet. This imbalance may produce inner leaflet expansion, buckling and stomatocytosis. In fact when large amounts of (10,3)PS were exchanged into the RBC membrane, the inner leaflet expansion produced by the translocase action was such that endocytic vacuoles appeared.

The (10,3) phospholipids gave us further indication

that some lipid scrambling takes place during hypotonic hemolysis. Indeed the change in EPR line shapes reveals that after hemolysis, the more rigid leaflet (the outer one) becomes less rigid (decrease in hyperfine splitting), while the less rigid leaflet (the inner one) becomes more rigid (increase in hyperfine splitting). These results resemble those of Ohnishi and collaborators [23,24] who reported that the difference in line shape of specific spin probes of the inner (i.e. PS) and outer (i.e. PC) leaflets is reduced after the formation of white ghosts. Their experiments as well as ours can be interpreted by assuming that the difference in viscosity between RBC inner and outer leaflets is due to the difference in composition (fatty acid chains and headgroups [25]) and that the scrambling, or at least partial scrambling, of the endogenous lipids cancels or diminishes the difference in physical characteristics.

Overall, the results with the (10,3) phospholipids show that in confirmation of the above (0,2) phospholipids data and of the previous C6-NBD data of Connor et al. [13] hypotonic lysis is accompanied by a redistribution of phospholipids. However, the (10,3)PL which are better analogs of endogenous lipids because of their insoluble character indicate that there is a hierarchy of redistributive movements during hemolysis. The data with long chain spin-labeled lipids fit very well with the data published in 1978 by Shukla et al. [10] who found, by the phospholipase C assay, about the same proportions of endogenous PC, SM and PS on the outer layer after ghost formation as we do with spin-labels (they found respectively 50, 20 and 30% in the absence of ATP). They are also consistent with data reported by Haest et al. [26] who found 57% of PC and 15% of PS on the outer leaflet of ghosts prepared in the absence of Mg^{2+}.

What is the origin of the selectivity? Because the long chain phospholipids are totally insoluble in water, the pathway for the redistribution of the (10,3) phospholipids must be membranous. Yet because the scrambling is limited, the passage between both leaflets must be opened only temporarily and likely corresponds to the instable situation associated with the formation of the pore(s) through which hemoglobin and cytosol leak out. One might implicate specific lipid–protein interactions to explain the differences between PS and PC; for example PS–spectrin interaction [7–9] or PS interaction with any other protein such as band 3 or glycophorin. However, it should be kept in mind that the lateral diffusion of fluorescent PS on the inner leaflet of RBC ghosts was reported to be rapid, more rapid than that of PC on the outer leaflet [27]. One then must postulate that the domain of fast diffusion does not include the whole surface area of the RBC inner leaflet. An alternative, yet very speculative, explanation could be that the opening of the pore involves localized charges which repell the negatively charged PS. As for SM, there are many reports in the literature suggesting its restricted mobility is due to SM–SM and SM–cholesterol interactions [28,29].

Analysis of our data using two virtually complete sets of phospholipid probes with varying degrees of analogy to the endogenous phospholipids leads to the following proposals. Hypotonic hemolysis provides a powerful impetus for rapid transmembrane movement in the direction of scrambling of the bilayer. The short chain phospholipid probes in fact are completely scrambled in confirmation of previous reports [13]. However, these probes, while very useful, are partially water soluble and thus subject to artifact as noted above. We propose that the (10,3) phospholipids, while technically difficult to use, provide a more accurate reflection of the movements undertaken by the endogenous phospholipids under the impetus of hypotonic hemolysis. It would have been preferable to have tracked all of the major phospholipids, unfortunately, for technical reasons we were not able to follow the redistribution of (10,3)PE. It would have required 4–5 h, at 37°C to allow (10,3)PE to equilibrate in the inner monolayer [19]. During that time period, the (10,3)PE crossing to the inner leaflet would have been subjected to the continuous reducing power of the cytosol milieu which would have eventually destroyed the EPR signal. However, our experiments show different degrees of scrambling for each of the three (10,3) phospholipids studied, and the possible explanations of this hierarchy of scrambling have been outlined above.

The final problem is to reconcile the net movements of the (10,3) phospholipids we have studied with the fact that isotonically resealed ghosts are echinocytic which, according to the bilayer couple hypothesis [3,4,30,31], means that there is a slight excess of lipids in the outer leaflet. However, with the (10,3) phospholipids probes used, we find that hypotonic lysis produces a net transfer to the inner leaflet. It is therefore necessary to postulate that the necessary outward compensation producing the echinocytic shape change comes from PE.

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