Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 3751–3755, June 1984
Cell Biology

ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: Relation to shape changes

(phospholipid asymmetry/transverse diffusion/erythrocyte shape/bilayer couple/electron spin resonance)

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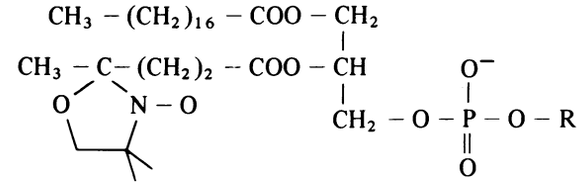
Communicated by Harden M. McConnell, February 24, 1984

ABSTRACT **Spin-labeled analogs of phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine have been used to study phospholipid transverse diffusion and asymmetry in the human erythrocyte membrane. Ascorbate reduction was used to assess the transbilayer distribution of the labels. All three spin-labeled phospholipids initially incorporated into the outer leaflet of the membrane. On fresh erythrocytes at 5°C, the phosphatidylcholine label remained mainly in the outer leaflet. In contrast, the phosphatidylserine and phosphatidylethanolamine labels underwent rapid transverse diffusion that led to their asymmetric distribution in favor of the inner leaflet. The latter effect was reversibly inhibited after ATP depletion of the erythrocytes and could be reproduced on resealed erythrocyte ghosts only if hydrolyzable Mg-ATP was included in the internal medium. It is suggested that an ATP-driven transport of amino phospholipids toward the inner leaflet could be the major cause of the phospholipid asymmetry in the erythrocyte membrane. It is also proposed that the same mechanism could explain the ATP requirement of the maintenance of the erythrocyte membrane discoid shape.**

An asymmetric distribution of phospholipids exists between the two halves of many biological membranes (for review, see ref. 1). The human erythrocyte membrane appears to be the best characterized in this regard. Numerous studies using chemical labeling (2, 3) and phospholipases (4–6) indicate that, in this membrane, phosphatidylcholine and sphingomyelin are distributed in favor of the outer leaflet, while phosphatidylethanolamine and phosphatidylserine are mainly located in the inner leaflet.

These observations raise the problem of how this asymmetry is maintained during the 120-day life span of the erythrocyte. The asymmetric distribution could in principle be maintained if transbilayer diffusion of phospholipids was negligible. However, for phosphatidylcholine, transbilayer "flip-flop" has been shown to occur with a half-time of 8–15 hr in the native membrane (7, 8), although no such measurements exist for the three other major phospholipid species. A possible role of cytoskeletal proteins in maintaining the asymmetry in spite of transbilayer diffusion has been proposed by Haest *et al.* (9, 10), but no direct evidence for such a process has been observed in the intact native membrane.

In the present study, the formation and maintenance of phospholipid asymmetry in the erythrocyte membrane has been addressed by measuring the transverse diffusion of spin-labeled phospholipids bearing different polar head groups. The method, introduced by Kornberg and McConnell (11), is based on the accessibility of membrane-associated spin labels to the nonpenetrating reducing agent ascorbate. Previous studies from our laboratory (7) have indicated that this method can be used successfully in erythrocytes, and the transverse diffusion of phosphatidylcholine has been measured. In the present study, the following phospholipid spin labels have been used.



These phospholipids possess an unmodified polar head group R that can be either choline, serine, or ethanolamine and are termed (0,2)PtdCho [1-palmitoyl-2-(4-doxylpentanoyl) phosphatidylcholine], (0,2)PtdSer [1-palmitoyl-2-(4-doxylpentanoyl) phosphatidylserine], and (0,2)PtdEtn [1-palmitoyl-2-(4-doxylpentanoyl) phosphatidylethanolamine], respectively.

MATERIALS AND METHODS

Preparation of Erythrocytes and Ghosts. Human blood was drawn from healthy donors on citrate/phosphate/dextrose, stored at 5°C, and used within 5 days. Erythrocytes were washed 5 times with 145 mM NaCl/5 mM KCl/1 mM $MgSO_4$/10 mM glucose/20 mM Hepes buffer, pH 7.4 (buffer A). Anaerobic ATP depletion and partial ATP repletion were carried out according to ref. 12. Pink resealed ghosts were prepared by the method of Schwoch and Passow (13) and were washed twice with 120 mM NaCl/7.5 mM KCl/10 mM choline chloride/1.5 mM $MgSO_4$/0.5 mM EGTA/20 mM NaH_2PO_4-Na_2HPO_4 buffer, pH 7.4, containing 0.5 mM phenylmethylsulfonyl fluoride (buffer B).

Spin Labeling and ESR Experiments. (0,2)PtdCho was synthesized as described (14). (0,2)PtdSer and (0,2)PtdEtn were prepared enzymatically from (0,2)PtdCho according to ref. 15.

Packed erythrocytes or resealed ghosts were resuspended to a final hematocrit of 75% in buffer A or B, respectively, containing the spin label that had been introduced from an ethanol solution. The suspension was then gently homogenized. The spin-label concentration corresponded to 1% of

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Abbreviations: (0,2)PtdCho, 1-palmitoyl-2-(4-doxylpentanoyl) phosphatidylcholine; (0,2)PtdSer, 1-palmitoyl-2-(4-doxylpentanoyl) phosphatidylserine; (0,2)PtdEtn, 1-palmitoyl-2-(4-doxylpentanoyl) phosphatidylethanolamine; (m,n), general nomenclature of spin-labeled chains: m and n are, respectively, the number of methylene groups after and before the labeled position on the acyl chain (see ref. 14).

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endogenous phospholipids. The final ethanol concentration was <0.5%.

For reduction experiments, sodium ascorbate was added to the labeled sample at 5°C at a final concentration of 10 mM from a 10-fold concentrated stock solution in sample buffer (freshly prepared according to ref. 7 and adjusted to pH 7.4) followed by gentle homogenization. ESR spectra were run as described (14).

Optical and Scanning Electron Microscopy. Labeled samples were fixed by dilution to a 5% hematocrit in an ice-cold 2% glutaraldehyde solution in sample buffer, incubated on ice for 1 hr and viewed under a Zeiss WL phase contrast microscope using a magnification of ×800.

In addition, samples for scanning electron microscopy were washed twice, postfixed with 1% osmic acid, dehydrated, and critical point dried using standard procedures (16). After shadowing with Au-Pd, samples were examined with a Jeol JMS 35 scanning electron microscope using a 20-kV tension.

RESULTS

Incorporation of the Spin-Labeled Phospholipids in the Erythrocyte Membrane. In aqueous buffer, each spin-labeled phospholipid yielded an ESR spectrum composed of a narrow triplet and a large wave (not shown). When the label was introduced into an erythrocyte suspension, the spectrum shown in Fig. 1 (*Inset*) was obtained within 3 min at 5°C. No signal arising from the aqueous label was apparent. This suggests a rapid and quantitative incorporation of spin-labeled phospholipids in the membrane. The double integral of the spectrum was stable over 7 hr at 5°C.

Above this temperature, a spontaneous decrease of the signal occurred because of reduction of the nitroxide by the cell suspension. Experiments described below were thus performed at 5°C. On incubation, a narrow ESR signal appeared progressively superimposed on the spectrum of membrane intercalated spin labels. Several lines of evidence indicated that this was due to enzymatic hydrolysis of the labeled chain (unpublished observations). This effect could be taken into account by computer subtraction of the spectrum of the corresponding spin-labeled fatty acid in aqueous buffer at 5°C. All data described below were corrected for this hydrolysis, which was <5% of the total label in 6 hr with cells and resealed erythrocyte ghosts.

Small differences in the ESR line shapes of the membrane-bound component of the various labels were apparent after several hours of incubation (unpublished observations).

Transbilayer Diffusion and Distribution of Spin-Labeled Phospholipids in the Erythrocyte Membrane. The ascorbate reduction kinetics of (0,2)PtdCho and (0,2)PtdSer observed after different incubation times in the presence of freshly prepared erythrocytes at 5°C are shown in Fig. 1. In all cases, the residual ESR signal (i.e., the fraction of nonreduced spin label) reaches a stationary value 5 min after ascorbate addition. This nonreducible label fraction represents that fraction located in the inner leaflet of the membrane (7, 11). Both labels are totally reducible by ascorbate after short incubation times (i.e., a few minutes) at 5°C. On longer incubation, an increasing fraction of each label becomes inaccessible to the reducing agent. This indicates initial incorporation of the spin-labeled phospholipids in the outer leaflet of the erythrocyte membrane and subsequent transverse diffusion across the membrane during the incubation.

The amount of spin label diffusing to the inner leaflet after a given incubation time is dependent on the polar head group. This is shown in Fig. 2, in which the stationary fraction of nonreducible ESR signal is plotted as a function of the

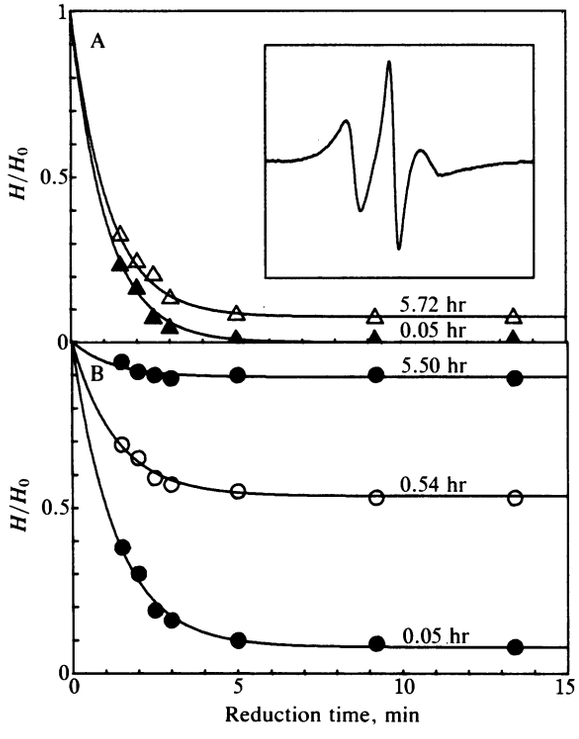

FIG. 1. Kinetics of ascorbate reduction of (0,2)PtdCho (*A*) and (0,2)PtdSer (*B*) after different incubation times (indicated near each curve) with intact erythrocytes at 5°C. After each incubation time, sodium ascorbate was added to the sample, which was then transferred to an ESR flat cell and introduced into the ESR cavity thermostatted at 5°C. First the central ESR line and then the whole spectrum were scanned repetitively. The central line height H of the ESR spectrum of spin-labeled erythrocytes, normalized to its initial value H_0, is plotted as a function of the time elapsed after ascorbate addition. H_0 was obtained from the spectrum of the sample without ascorbate after correcting for dilution. In a few experiments, the double integral of the whole spectrum was plotted as a function of time. This latter analysis gave results practically identical to the former one, showing that the change in line shape has a negligible effect on H/H_0. (*Inset*) ESR spectrum of (0,2)PtdCho after a 3-min incubation with intact erythrocytes at 5°C.

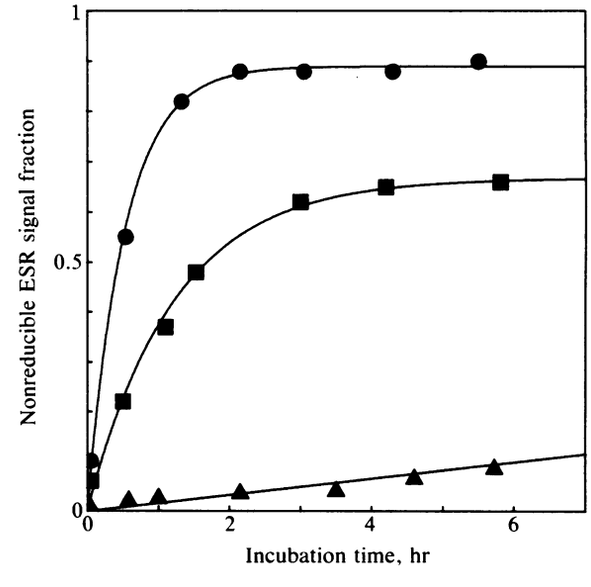

FIG. 2. Nonreducible spin-label fraction as a function of the incubation time at 5°C for (0,2)PtdCho (▲), (0,2)PtdSer (●), and (0,2)PtdEtn (■) after addition to intact erythrocytes. The values for data points were obtained from plots similar to those in Fig. 1.

incubation time of the labeled erythrocytes for the three head groups. The transverse diffusion of (0,2)PtdCho is slow, the amount of label located in the inner leaflet reaching only 10% in 6 hr. On the other hand, for (0,2)PtdSer and (0,2)PtdEtn, a rapid transverse diffusion occurs, the initial rate of which is more than one order of magnitude larger than for (0,2)PtdCho. Furthermore, transverse diffusion leads to a stationary transbilayer distribution of the two amino phospholipids, which is asymmetric and favors their location in the inner leaflet of the membrane. The final fraction of label in the inner leaflet amounts to 90% and 65%, respectively, for (0,2)PtdSer and (0,2)PtdEtn at 5°C.

Shape Changes of the Labeled Erythrocytes. On addition of any of the three labels, erythrocytes immediately became crenated (Fig. 3*A*). With (0,2)PtdCho, echynocytes persisted for 6 hr at 5°C. On the other hand, with (0,2)PtdSer and (0,2)PtdEtn, echynocytes transformed gradually to discocytes and stomatocytes (type I) on incubation (Fig. 3*B*). For both labels, the kinetics of this latter shape change (Fig. 3*C*) is similar to that of transverse diffusion (Fig. 2).

Influence of ATP on the Transverse Diffusion and Transbilayer Distribution of Spin-Labeled Phospholipids in the Erythrocyte Membrane. Pink ghosts resealed in isotonic buffer containing Mg^{2+} ions (13) had a crenated morphology, similar to that of echynocytes (not shown). As revealed by the ascorbate assay, transverse diffusion of the three spin-labeled phospholipids in the membrane of these ghosts was identical and very slow (Fig. 4). When Mg-ATP was included in the hemolysis and resealing medium, the resealed ghosts thus obtained had a discoid or cup-shaped morphology (not shown). As shown in Fig. 4, the rapid transverse diffusion of (0,2)PtdSer and (0,2)PtdEtn and their asymmetric distribution favoring the inner leaflet of the membrane could be recovered in resealed ghosts prepared in the presence of Mg-ATP. The nonhydrolyzable analog adenylyl imidodiphosphate could not substitute for ATP. Sodium orthovanadate (1–2 μM) strongly inhibited the effect of ATP on transverse diffusion, resulting in asymmetric transbilayer distribution of spin-labeled phospholipids, as well as on ghost shape. On the other hand, ouabain (0.5 mM) had no effect.

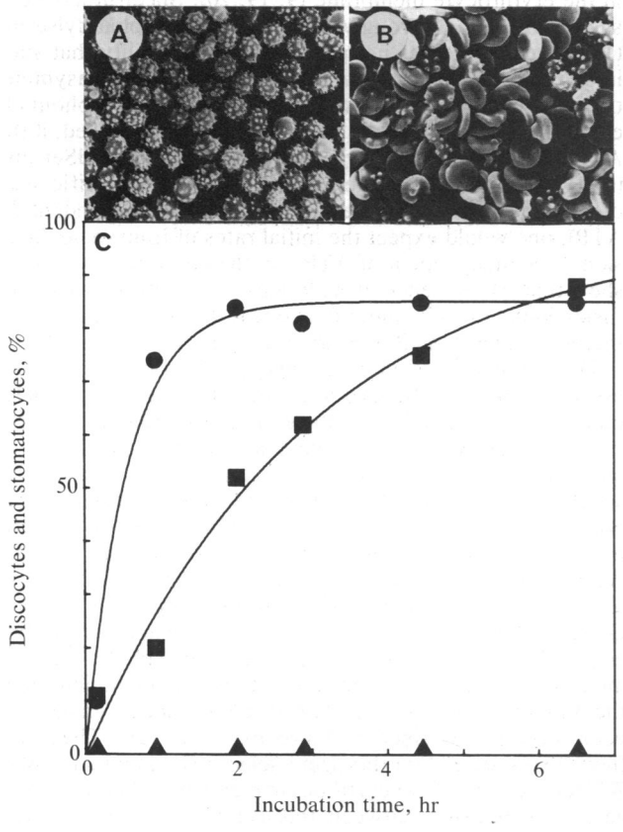

FIG. 3. Scanning electron micrographs of erythrocytes incubated for 3 min (*A*) or 6.5 hr (*B*) at 5°C after labeling with (0,2)PtdSer. (*C*) Percentage of discocytes and stomatocytes as a function of the incubation time at 5°C for erythrocytes labeled with (0,2)PtdCho (▲), (0,2)PtdSer (●), and (0,2)PtdEtn (■). For each data point, at least 300 cells distributed in five fields selected at random were counted, using the phase contrast microscope.

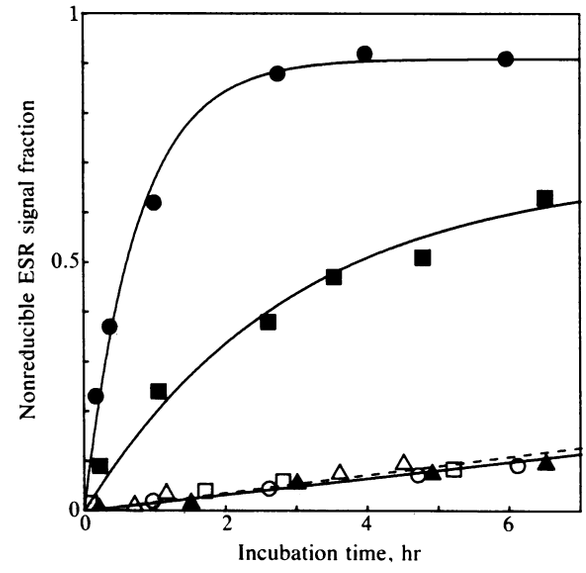

FIG. 4. Nonreducible spin-label fraction as a function of the incubation time at 5°C for (0,2)PtdCho (△, ▲), (0,2)PtdSer (○, ●), and (0,2)PtdEtn (□, ■) after addition to ghosts resealed in the absence (open symbols and dashed line) and in the presence (closed symbols and solid lines) of 1 mM Mg-ATP. Hemolysis was carried out by adding the erythrocyte suspension (50% hematocrit in 155 mM NaCl) to 20 vol of 1.2 mM acetic acid/4.5 mM $MgSO_4$/0.5 mM EGTA/0.5 mM phenylmethylsulfonyl fluoride, pH 3.5, at 0°C in the presence or absence of 1 mM Mg-ATP. The pH increased to 5.9–6.1 on addition of erythrocytes. In experiments with Mg-ATP, an ATP-regenerating system composed of 10 mM creatine phosphate and 100 international units of creatine kinase per ml was added at this stage. After a 5-min agitation at 0°C, tonicity was brought to 120 mM NaCl/20 mM NaH_2PO_4-Na_2HPO_4, pH 7.4, using concentrated stock solutions. After a 10-min agitation at 0°C, the suspension was incubated for 50 min at 37°C for resealing.

The influence of ATP could also be observed on whole cells. On ATP depleted erythrocytes, which are echynocytes, the transverse diffusion of (0,2)PtdSer and (0,2)PtdEtn slowed down considerably and did not lead to an asymmetric distribution favoring the inner leaflet. The inhibition could be partially reversed on ATP repletion, which also led to the recovery of the discoid shape.

DISCUSSION

The present work is based on the use of spin-labeled analogs of naturally occurring erythrocyte phospholipids. Our basic assumption is that these labels can be considered as good reporters of phenomena involving endogenous phospholipids of the intact membrane, provided that the differences ob-

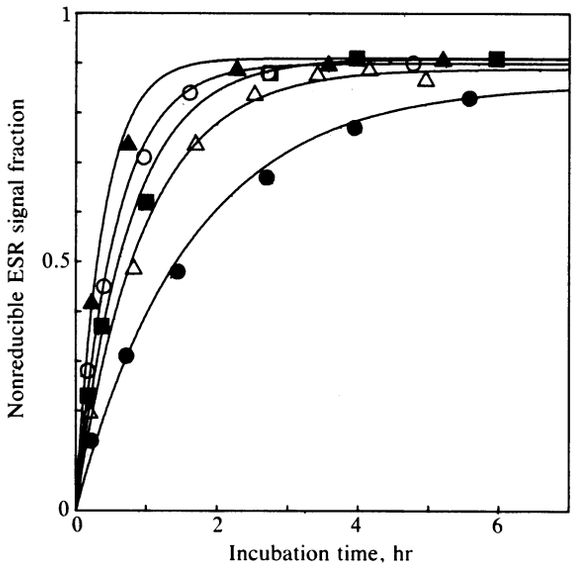

FIG. 5. Nonreducible spin-label fraction as a function of incubation time at 5°C for (0,2)PtdSer after addition to ghosts resealed in presence of 0.3 mM (●), 0.6 mM (△), 1 mM (■), 2 mM (○), and 5 mM (▲) Mg-ATP. An ATP-regenerating system was present in all samples (see legend to Fig. 4).

served among labeled phospholipids can be solely attributed to the influence of the head group. The different stationary transbilayer distributions of the labeled lipids observed here are consistent with what is known concerning those of endogenous phospholipids of the erythrocyte membrane. It may be noted that, in 1976, Tanaka and Onishi found that spin-labeled phospholipids incorporated in the erythrocyte membrane yield ESR spectra with line shapes dependent on the polar head group (17). In this study, phospholipids bearing a short labeled chain were selected because of their higher water solubility, which allows easy incorporation in the outer leaflet of the membrane (18). Our data may possibly not reflect the exact quantitative behavior of endogenous erythrocyte lipids. In particular, transverse diffusion rates may be influenced by the presence of a short acyl chain bearing a nitroxide, as well as by the use of a nonphysiological temperature (5°C). On the other hand, our approach allows the observation of biologically important phenomena that would be difficult to detect with endogenous phospholipids.

Our data indicate that, with intact erythrocytes, (0,2)PtdCho remains predominantly located in the outer leaflet of the membrane upon incubation. This agrees with reports indicating that transverse diffusion of phosphatidylcholine in the erythrocyte membrane is a slow process (7, 8). However, it must be noted that our experiments were conducted at 5°C to avoid reduction and extensive hydrolysis of the labels. (0,2)PtdCho may possibly reach a less asymmetric transbilayer distribution at higher temperature.

In contrast, transverse diffusion of (0,2)PtdSer and (0,2)-PtdEtn in the membrane of intact erythrocytes is more than one order of magnitude faster than that of (0,2)PtdCho. Furthermore, this process ultimately leads to a stationary transbilayer distribution in which both spin-labeled amino phospholipids are predominantly located in the inner leaflet. Thus, an asymmetric transbilayer distribution comparable to that observed for endogenous phospholipids appears spontaneously.

Experiments using resealed ghosts and ATP-depleted cells indicate that formation of this asymmetry requires internal ATP. This appears to be related to the enzymatic cleavage of the nucleotide, since adenylyl imidodiphosphate is ineffective.

To explain the data, we propose that the phospholipid asymmetry in the erythrocyte membrane is promoted and maintained by an ATP-energized active transport of amino phospholipids toward the inner leaflet. Such a mechanism is indeed expected to selectively facilitate transverse diffusion of phosphatidylserine and phosphatidylethanolamine from the outer leaflet and to lead to their accumulation in the inner leaflet, as shown here for spin-labeled analogs. An asymmetric stationary distribution may then occur when the inward transport is counterbalanced by an outward leakage process. The leakage process is the passive transverse diffusion of amino phospholipids in the direction opposite to the transport. This model explains the Mg-ATP concentration-dependent transverse diffusion rate of (0,2)PtdSer and (0,2)PtdEtn. It can also account for the much slower and ATP-independent transverse diffusion of (0,2)PtdCho, which only occurs passively. This model is not contradictory with reports indicating that the asymmetry of endogenous phospholipids in the erythrocyte membrane is barely modified in ATP-depleted cells or in ghosts resealed without ATP (4, 6). As shown in Fig. 4, transverse diffusion is slow for all phospholipids in the absence of ATP. Unpublished experiments from our laboratory indicate that the asymmetry of spin-labeled phospholipids formed on intact erythrocytes is not destroyed when resealed ghosts without ATP are prepared from the labeled cells. Thus, the inactivation of the selective transport of amino phospholipids does not lead to immediate loss of asymmetry.

Several authors have suggested that selective interaction between amino phospholipids and the cytoskeletal protein spectrin might be the cause of the phospholipid asymmetry in the erythrocyte membrane (9, 19, 20). Spectrin has been shown to interact strongly *in vitro* with phosphatidylserine (19). Our model does not preclude the possibility that such interactions might contribute to stabilization of the asymmetry. However, our results with spin-labeled phospholipids cannot be explained solely by this mechanism. Indeed, if the ATP-dependent asymmetric distribution of (0,2)PtdSer and (0,2)PtdEtn were only due to interaction with specific sites of the inner leaflet (whose affinity would be dependent on ATP), one would expect the initial rates of transverse diffusion to be independent of ATP. On the contrary, our results show that the presence of ATP does increase transverse diffusion initial rates by more than one order of magnitude and, thereby, suggest ATP-dependent transport.

The hypothesis of an amino phospholipid transport process also appears to be able to account for the effect of ATP on erythrocyte membrane shape. The ATP requirement for the maintenance of the discoid shape on both erythrocytes and ghosts is well known (21–23). Hypotheses of a role of spectrin (24) or lipid (25) phosphorylation in mediating this effect have been recently dismissed (26–28). In our experiments on both cells and ghosts, the presence of internal ATP, in addition to its effect on phospholipid asymmetry, also engenders a discoid shape. Furthermore, with resealed ghosts, micromolar concentrations of orthovanadate inhibited the ATP effect on both spin-labeled phospholipid asymmetry and membrane shape. The orthovanadate inhibition of the ATP-induced recovery of the discoid shape on ghosts has been recently described by Fairbanks *et al.* (28). These authors also showed that this agent selectively inhibits the Mg-ATPase activity of the erythrocyte membrane. Our data suggest a relationship between this ATPase activity and the ATP-dependent phospholipid transport postulated here.

To understand how an active transbilayer transport of phospholipids may account for the effect of ATP on membrane shape, it should be noted first that the crenated shape (i.e., the echynocyte) observed in the absence of ATP has a higher ratio of external to internal surface than the discoid shape (16, 29). The effect of an ATP-driven transport of ami-

no phospholipids could be to induce a net transfer of phospholipids from the outer to the inner leaflet. At the steady state, this would maintain a decreased outer-membrane surface and an increased inner-membrane surface. Such an effect could conceivably produce and maintain a discoid shape of the membrane.

In this study, the phospholipid spin labels are shown to induce shape changes on erythrocytes. These shape changes are consistent with the bilayer couple hypothesis of Sheetz and Singer (16) in that incorporation of the three spin labels in the outer leaflet induces the formation of echynocytes by increasing the outer-membrane surface area. For (0,2)-PtdSer and (0,2)PtdEtn, transverse diffusion to the inner leaflet abolishes and reverses (5) this surface-area increase, and the shape reverts to discocyte or stomatocyte. The fact that the membrane shape is changed during transverse diffusion thus supports the proposal that a net transbilayer transport of phospholipid occurs. This provides an understanding of how regulation of the phospholipid asymmetry might also maintain cell shape.

Our interpretation does not preclude the possibility that the erythrocyte cytoskeleton might contribute to the determination of the erythrocyte shape as proposed by other workers (24, 30, 31). On the other hand, it does not seem that the selective diffusion of the amino phospholipids or the shape changes that accompany this phospholipid migration can be explained by an endocytosis process (32). Indeed, lateral segregation followed by selective internalization of the spin-labeled lipids in vesicles would lead to strong spin–spin interaction, which are never observed.

Note Added in Proof. Haest *et al.* (9) have observed that, in erythrocytes, oxidative crosslinking of spectrin by diamide allows partial destruction of the phospholipid asymmetry. In two recent reports (33, 34), the same group showed that this treatment creates discontinuities in the membrane and increases up to 150-fold transverse diffusion rates of both lysophosphatidylcholine and lysophosphatidylserine. Their results suggest that spectrin crosslinking destroys the asymmetry by a nonspecific increase of phospholipid transverse diffusion. In such a situation, the amino phospholipid transport process proposed in our model would indeed become ineffective due to the increased rate of the counteracting leakage process.

We thank Drs. Edith Favre and Paulette Hervé for the gift of the spin labels and Dr. Andreas Hermann, with whom preliminary experiments were carried out. We acknowledge helpful discussions with Drs. Didier Bonnet, Marc Le Maire, and Pieter Cullis. This work was supported by grants from the Centre National de la Recherche Scientifique, the Délégation Générale à la Recherche Scientifique et Technique, and the Université Paris VII.

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