

Lipid Molecular Shape Affects Erythrocyte Morphology: A Study Involving Replacement of Native Phosphatidylcholine with Different Species Followed by Treatment of Cells with Sphingomyelinase C or Phospholipase A₂

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ABSTRACT In a previous report it was shown that the replacement of native erythrocyte phosphatidylcholine (PC) with different PC species which have defined acyl chain compositions can lead to morphological changes (Kuypers, F. A., W. Berendsen, B. Roelofsen, J. A. F. Op den Kamp, and L. L. M. van Deenen, 1984, *J. Cell Biol.*, 99:2260–2267). It was proposed that differences in molecular shape between the introduced PC species and normal erythrocyte PC caused the membrane to bend outwards or inwards, depending on the shape of the PC exchanged. To support this proposal, two requirements would have to be fulfilled: the exchange reaction would take place only with the outer lipid monolayer of the erythrocyte, and the extent of lipid transbilayer movement would be restricted. If this theory is correct, any treatment causing unilateral changes in lipid molecular shape should lead to predictable morphological changes. Since this hypothesis is a refinement of the coupled bilayer hypothesis, but so far lacks experimental support, we have sought other means to change lipid molecular shape unilaterally.

Shape changes of human erythrocytes were induced by the replacement of native PC by various PC species using a phosphatidylcholine-specific transfer protein: by hydrolysis of phospholipids in intact cells using sphingomyelinase C or phospholipase A₂, and by the combination of both procedures. The morphological changes were predictable; additive when both treatments were applied, and explicable on the basis of the geometry of the lipid molecules involved. The results strongly support the notion that lipid molecular shape affects erythrocyte morphology.

The human erythrocyte is surrounded by a lipid bilayer under which a membrane skeleton, composed of proteins, is present. The discoid shape of normal cells is thought to be maintained by this membrane skeleton (1, 2), although recent evidence suggests that the lipid bilayer may also have a role in this respect (3, 4, 5).

The shape of erythrocytes can be modified by treatment of

the intact cell with phospholipase A₂, C, D, and sphingomyelinase C (6, 7). Under nonlytic conditions, only lipids in the outer monolayer of the membrane are hydrolyzed (8). Hydrolysis with phospholipase A₂ leads to formation of crenated cells (echinocytes), whereas the other enzyme treatments result in cup-formed (stomatocytic) cells. The observed changes after phospholipase C treatment have been explained by a

bilayer couple-type of mechanism (3, 6). The diglyceride formed upon hydrolysis was postulated to cross the bilayer to the inner side of the membrane, where it was phosphorylated and expanded this leaflet. This expansion was assumed to cause the membrane to bend inwards (6). Fuji and Tamura (7) suggested that the products of hydrolysis remain in the outer layer of the membrane and directly induce changes in morphology because they differ in structure from the original phospholipids in the polar or apolar region of the molecule. Morphologic changes are also observed when the acyl chain composition of the phosphatidylcholine (PC)¹ of the erythrocyte is modified by use of a PC-specific exchange protein, replacing the normal PC for other PC's with defined acyl chain composition (9, 10). This exchange occurs only with the PC from the outer monolayer (11). Replacement with 1,2-dipalmitoyl PC results in echinocytic cells, whereas 1,2-dioleoyl PC causes a moderate stomatocyte formation (10). It was argued that the morphological changes are related to the molecular structure of the PC introduced since, in contrast to other manipulations of the phospholipids in the erythrocyte, this exchange occurs without any net change in the amount of PC or other lipids. The rate of transbilayer movement of PC in the human erythrocyte is very slow (12). Introduction of a new PC species can therefore be expected to exert its shape-changing effect by disturbance of the outer monolayer only.

The effect that phospholipase treatment and phospholipid exchange might have on the morphological appearance of the cell may be explicable in a unified manner by assuming that the molecular shape of the lipid components in the outer monolayer contribute to the shape of the erythrocyte. Both phospholipase treatment and phospholipid exchange involve changes in the molecular geometry of the membrane lipids. These may lead to the increase of the number of either "cone-shaped" or "inverted cone-shaped" lipids. Packing constraints are important factors in determining lipid aggregation (for review see reference 13). Therefore, a change in the molecular shape of the lipids in the outer monolayer would lead to shape changes in the erythrocyte, and the actual effects that the exchange and phospholipase treatment might have on the morphology of the cells should be predictable. We have tested this hypothesis by a combination of the two approaches. Erythrocytes have been enriched with either cone-shaped or inverted cone-shaped PC species by using the exchange protein. The morphologic changes thus induced, as well as those observed after subsequent treatment of the modified cells with phospholipases, have been studied.

MATERIALS AND METHODS

Erythrocytes: Human blood was collected in standard acid/citrate/dextrose buffer (14) and centrifuged for 5 min at 2,500 g. The supernatant and the buffy coat were removed by aspiration and the erythrocytes were washed three times with 5 vol of a buffer containing 10 mM Tris, 150 mM NaCl, 25 mM glucose, 1 mM EDTA, and 3 mM Na₂S₂O₃, pH 7.4. The buffy coat was removed carefully after each centrifugation. Fresh cells were used for all experiments.

PC-specific Exchange Protein: The protein was purified from beef liver as described by Kamp and Wirtz (15), following the modification of Wirtz et al. (16), and had a specific activity of 5 μmol PC exchanged/mg per min. The exchange protein was stored at a concentration of 2.7 μM in 50% glycerol (vol/vol) at -20°C. Before use, the enzyme was dialyzed overnight against a 1,000-fold volume of the buffer mentioned above. Pretreatment of the dialysate against flake polyethylene glycol was done as described previously (12).

¹Abbreviation used in this paper: PC, phosphatidylcholine.

Phospholipases: Phospholipase A₂ (EC 3.1.1.4) from bee venom was obtained from Sigma Chemical Co. (St. Louis) at a specific activity of 1,500 U/mg and was used without further purification. Sphingomyelinase C (EC 3.1.4.12) from *Staphylococcus aureus* was purified according to Zwaal et al. (17), and stored at a concentration of 11 IU/10 μl. The stock solution was diluted 10-fold with a buffer containing 50 mM Tris, 50% glycerol, pH 7.5, before use in the experiments.

Lipids: 1,2-Dipalmitoyl PC, 1-palmitoyl-2-oleoyl PC, and 1,2-dioleoyl PC were synthesized following standard procedures (18), and were a gift from Dr. R. A. Demel (Department of Biochemistry, State University of Utrecht). Cholesterol was purchased from Merck Sharp & Dohme International Div. (Darmstadt) and egg phosphatidic acid from Sigma Chemical Co. Methyl-[¹⁴C] 1,2-dipalmitoyl PC and [³H]-trioleate were obtained from The Radiochemical Center (Amersham International PLC, Amersham, UK). Methyl-[¹⁴C]-egg PC was synthesized according to Stoffel (19).

Preparation of Donor Vesicles: Vesicles were prepared containing a particular species of PC, cholesterol, phosphatidic acid (molar ratio 47:47:6) and trace amounts of [¹⁴C] PC and [³H]glyceroltrioleate (as a nonexchangeable marker). The specific activities of [¹⁴C]PC and [³H]-trioleate were approximately 150 and 700 dpm/nmol PC, respectively. [¹⁴C]-1,2-dipalmitoyl PC was used to label the 1,2-dipalmitoyl PC containing vesicles, whereas [¹⁴C]-egg PC was used in 1-palmitoyl-2-oleoyl PC vesicles. The lipids were mixed from stock solutions in chloroform-methanol 2:1 (vol/vol) and the solvent was evaporated at 35°C under N₂. Residual solvent was removed using a vacuum pump. 2 ml of buffer (see above) was added per 10 μmol of lipid and the mixture was vortexed above the phase transition of the PC. The lipid suspensions were sonicated for 15 min with a Branson B12 sonifier at a power setting of 60 W and the temperature was kept just above the phase transition. After centrifugation at 130,000 g for 30 min to remove larger lipid aggregates and metal particles released from the probe, the supernatant containing unilamellar vesicles was used as the donor system in the exchange experiments. Samples were taken from the supernatant before and after centrifugation in order to determine vesicle recovery by liquid scintillation counting. Recovery varied between 40 and 90%, depending upon the type of PC species involved. Samples were taken for lipid extraction and subsequent determination of the specific activities of the donor vesicle PC and glyceroltrioleate by radioactivity measurements and phosphorus analyses (see *Lipid Analysis* below).

Incubation of Erythrocytes: Washed erythrocytes were incubated at 33% hematocrit together with donor vesicles and exchange protein. The incubations were performed at 37°C in 10-ml screw-capped glass tubes, and rotated at 4 rpm on a clinical blood rotator. A sample of the total incubation mixture was taken immediately upon preparation and was used for determination of the ratio of vesicle PC to erythrocyte PC by phosphorus analysis and liquid scintillation counting. After incubation, the cells were diluted with warm buffer (37°C) and were sedimented by centrifugation for 5 min at 2,500 g. The cells were washed three times with warm buffer without EDTA and Na₂S₂O₃. No hemolysis occurred during the exchange incubations, as judged from the hemoglobin absorbance at 418 nm of the supernatants after the first centrifugation. One aliquot of each incubation mixture was washed separately, lysed with distilled water, and stored frozen at -20°C. These samples were used for lipid extraction and for the determination of the extent of PC replacement in the erythrocytes. 0.25 mM MgCl₂ or 0.25 mM MgCl₂ plus 0.25 mM CaCl₂ was included in the washing buffers for cells intended for further treatment with sphingomyelinase C or phospholipase A₂, respectively. After washing, the cells were resuspended in buffer at a 5% hematocrit and a 1-ml sample was taken, centrifuged, lysed with 1.25 ml of water, and diluted 100 times. The absorbance at 418 nm was read and used to adjust the enzyme addition to equal enzyme/cell ratios for all samples.

For sphingomyelinase C treatment, cells were incubated at 37°C in buffer containing 0.25 mM MgCl₂. The enzyme concentration was 0.28 IU of sphingomyelinase C per ml of packed cells. Samples were taken at intervals of 10 min and the reaction was stopped by addition of one vol of 100 mM EDTA in 0.9% NaCl to 10 vol of incubation mixture. A small portion of the sample was fixed for microscopy (see below) and the remainder was centrifuged at 2,500 g for 5 min. The cells were lysed by adding an equal volume of distilled water to the packed cells and the samples were stored at -20°C until lipid extraction.

Incubation with phospholipase A₂ was performed as described above, except that the buffer contained 0.25 mM MgCl₂ plus 0.25 mM CaCl₂. The enzyme concentration was 32 IU/ml of packed cells. The hydrolysis was stopped by addition of 0.2 vol of 100 mM EDTA in 0.9% NaCl to 10 vol of incubation mixture. The samples were treated exactly as described for sphingomyelinase C-incubated samples. The degree of hemolysis, as judged by the absorbance at 418 nm of the supernatant after centrifugation, was always <3% for all samples.

Microscopy: Samples of enzyme-treated incubated mixtures, supplemented with EDTA, were diluted eightfold with formaldehyde buffer (6 g NaCl, 10 g Na-citrate-2H₂O, 10 ml 40% formaldehyde per liter, pH 7.4). The samples

were allowed to stand for at least an hour for fixation. The cells were examined at a 500× magnification with a Leitz phase-contrast microscope equipped with Normarsky phase-contrast optics and a camera. Micrographs were taken of representative fields. Cells with different shapes were counted and a semi-quantitative expression for the shape changes was calculated. The different shapes of individual cells were assigned arbitrary morphological scores as shown in Fig. 1 of reference 7. A morphological index was calculated which corresponds with reference 7, giving positive scores in the case of echinocyte formation and negative scores in the case of stomatocyte formation. A minimum of 200 cells was counted from each sample. The estimated deviation, when different fields of view of the sample were used to calculate the morphological index, was 10–15% for echinocyte samples and <5% for stomatocytic samples.

Scanning Electron Microscopy: 10 µl of packed erythrocytes were washed three times in 150 mM NaCl, 10 mM Tris, pH 7.4, and fixed for 1 h at 20°C in 1 ml buffer containing 100 mM NaCl, 40 mM Na-citrate, and 0.5% formaldehyde. A second fixation in 1% OsO₄ for 30 min was followed by dehydration in a graded series of ethanol, transfer in isopropanol, and drying at the air. Cells were covered with a thin layer of gold by the sputter process, and morphology was checked in a Cambridge Stereoscan 600 M.

Lipid Analysis: The samples were extracted according to the protocol by Rose and Oklander (20). The solvent was evaporated and the lipids redissolved in chloroform-methanol 2:1 (vol/vol). From samples intended for determination of the extent of phospholipid exchange, aliquots were taken for phosphorus analysis and radioactivity measurements. The latter were transferred to scintillation vials and, after evaporation of the organic solvent, the radioactivity was determined in a Packard Tricarb 3320 liquid scintillation counter using Emulsifier Scintillator 299 (Hewlett-Packard Co., Palo Alto, CA). To determine the lipid composition and the extent of lipid hydrolysis in sphingomyelinase C-treated samples, the lipids were separated on prefabricated thin layer chromatography plates (Merck Kieselgel 60 DC Fertigplatten) using the one-dimensional solvent system of Skipski et al. (21). Phospholipase A₂-treated samples were separated by two-dimensional thin layer chromatography according to the protocol of Broekhuysse (22), on thin layer chromatography plates covered with silica gel HR, containing 7.4% (wt/wt) Mg-silicate (Florisil, Merck Sharp & Dohme International Div., Darmstadt). The lipid spots were visualized by iodine vapor and scraped off the plates. The phosphorous content of the spots was determined as described by Rouser et al. (23). The phosphorous values were corrected for background silica readings. The percentage of sphingomyelin hydrolysis was calculated using the sum of phosphorous in the PC + phosphatidylethanolamine spots as an internal standard. The extent of phospholipase A₂ hydrolysis was expressed as the ratio of lysophosphatidylcholine/lysophosphatidylcholine + phosphatidylcholine.

Calculation of the Exchange in the Erythrocytes: The extent of replacement of native PC by donor PC was calculated from the specific activity of the donor vesicles, the ratio of donor PC to erythrocyte PC and the amount of radioactivity (corrected for vesicle contamination) in the erythrocytes after exchange, by using the formula

$$\% \text{ replacement} = \frac{\text{specific activity of PC in erythrocyte at time} = t}{\text{specific activity of donor PC at time} = 0} \times 100.$$

A detailed description of the calculation procedure is given in reference 12.

RESULTS

Incubation of erythrocytes with donor vesicles of the specified composition in the presence of PC-specific exchange protein does not alter the lipid composition of the erythrocyte, except that of the PC species (9, 10). This is an important point, since a net increase or decrease of lipid molecules would lead to a change in the surface area of the membrane. Changes as small as 1–2% of the area are sufficient to cause shape changes (24). The extraction of cholesterol from the erythrocytes was prevented by incorporation of cholesterol into the donor vesicles (9, 10). Vesicles and erythrocytes incubated in the absence of exchange protein showed a very slow rate of PC exchange and no morphological changes occurred. Thus, the morphological changes following PC exchange were not due to changes in the stoichiometric balance of the erythrocyte lipids. The absence of morphological changes in control cells, incubated in the absence of donor vesicles, indicates that the ATP-levels of the cells were adequate during the experiments.

Metabolic depletion is thus not the cause of the shape changes.

The replacement of the erythrocyte PC with different donor PCs was allowed to proceed until ~35% of the PC had been replaced in the cells that subsequently were to be treated with sphingomyelinase C and to ~25% replacement in those that were to be subjected to the action of the phospholipase A₂. The exact figures are shown in Table I, and were similar for all PC's within one experiment. The extent by which the shape is changed clearly depends upon the degree of replacement (10). In the present series of experiments, the replacement was not extensive and therefore the shapes of the cells were only moderately changed. This is reflected in the values of the morphological indexes (see below), which were not very far from zero. The 1,2-dipalmitoyl PC cells became echinocytic, 1,2-dioleoyl PC cells slightly "cupped," and 1-palmitoyl-2-oleoyl PC cells did not differ from control cells. More extensive shape changes induced by this technique are published elsewhere (10).

The sequence of shape changes that occur in native erythrocytes which are treated with, respectively, sphingomyelinase C and phospholipase A₂ are as published by Fuji and Tamura (7). To quantify the shape changes, a morphological index was calculated as described by those authors. The shape changes were time dependent and occurred in parallel with the hydrolysis of the substrates (Fig. 1). However, the relationship between morphological index and the degree of hydrolysis was not linear. To obtain slow rates of hydrolysis and concomitant shape changes, the sphingomyelinase concentration was kept very low, and a batch of bee venom phospholipase A₂ with low activity toward the membranes of intact erythrocytes was used.

The time course for the sphingomyelinase C-induced hydrolysis of sphingomyelin in natural and PC species-modified erythrocytes is shown in Fig. 1A. The hydrolysis proceeded more or less at a similar rate in all samples, and 10–20% of the sphingomyelin was degraded within 40 min. This equals the hydrolysis of ~4% of the total phospholipids in the erythrocyte. Since the removal of the polar headgroup constitutes a pronounced change in the optimal surface area of sphingomyelin, it is not surprising that this low degree of hydrolysis gives rise to morphological changes. Upon this hydrolysis, the sequence of shape changes in modified and

TABLE I. Exchange Conditions and the Extent of Phosphatidylcholine Replacement

PC in donor vesicles	Molar ratio vesicle/erythrocyte PC	Incubation time h	% Replacement*	Further treatment
di 16:0 PC†	1.9	6	34	Sphingomyelinase C
di 18:1 _c PC	1.5	5	39	Sphingomyelinase C
16:0/18:1 _c PC	1.5	3	33	Sphingomyelinase C
di 16:0 PC	2.5	6	20	Phospholipase A ₂
di 18:1 _c PC	1.8	5	25	Phospholipase A ₂

Erythrocytes were incubated in buffer at 37°C together with exchange protein and vesicles containing different donor phosphatidylcholines. Vesicles and cells were prepared as described in Materials and Methods. The cells were incubated at a hematocrit of 33%. The exchange protein concentration was 2–3 µM.

* Calculated as described in Materials and Methods.

† The numbers denote the number of carbon atoms, and double bonds of the fatty acids.

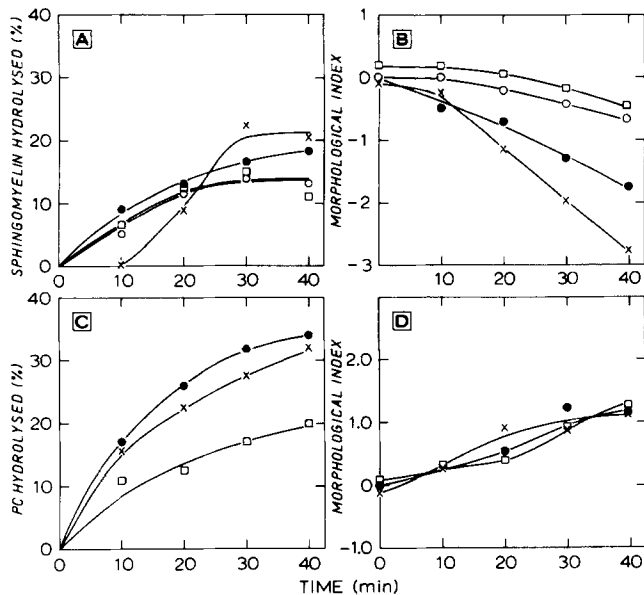


FIGURE 1 Phospholipid hydrolysis and changes in morphology of erythrocytes modified in PC composition and treated with sphingomyelinase C or phospholipase A₂. Control cells (●) and erythrocytes in which part of the native PC had been replaced with 1,2-dipalmitoyl PC (□), 1,2-dioleoyl PC (x), or 1-palmitoyl,2-oleoyl PC (○) were incubated with sphingomyelinase C (A and B), or phospholipase A₂ (C and D) as described in Materials and Methods. The extent of sphingomyelin (A) and PC hydrolysis (C) can be correlated with the concomitant changes in morphology, expressed as the morphological scores (B and D, respectively).

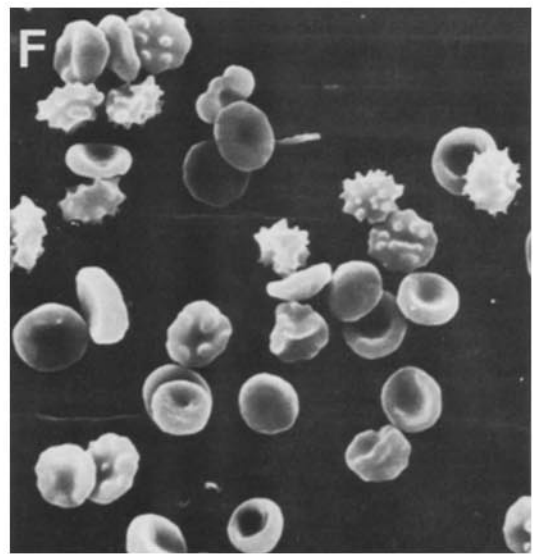
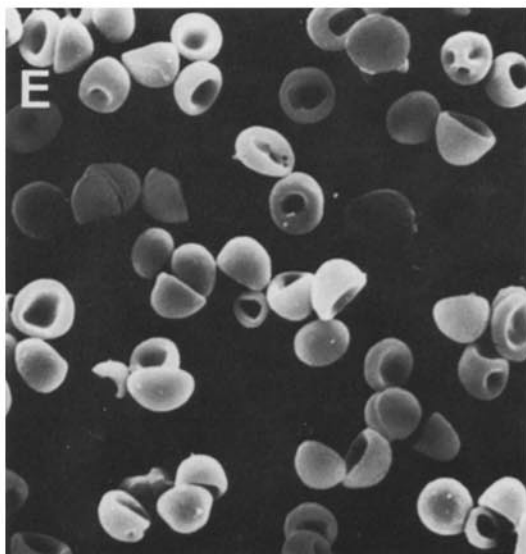
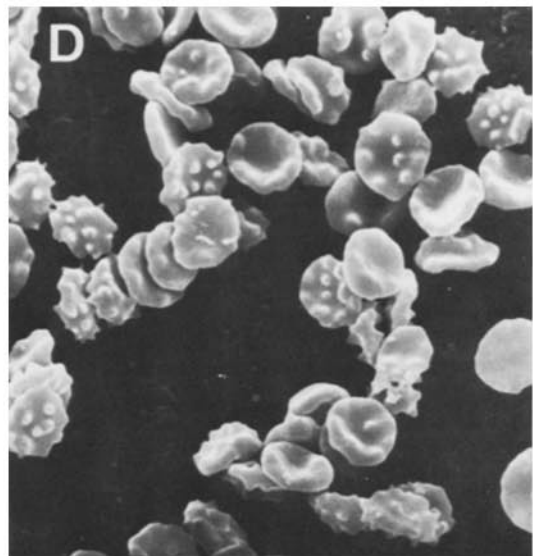
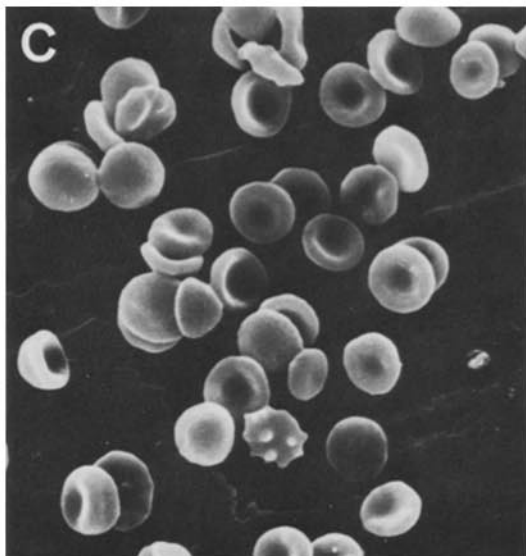
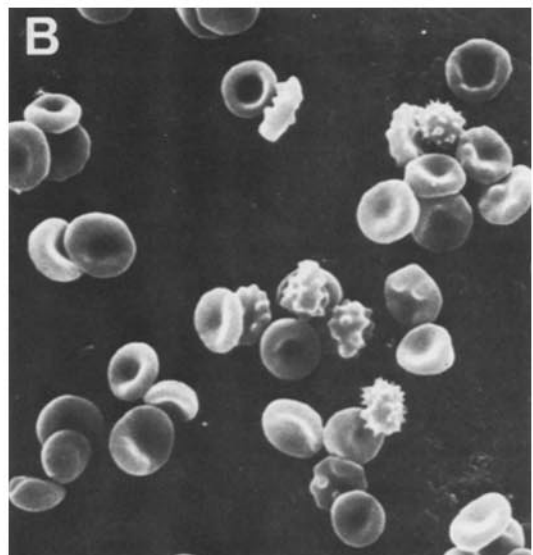
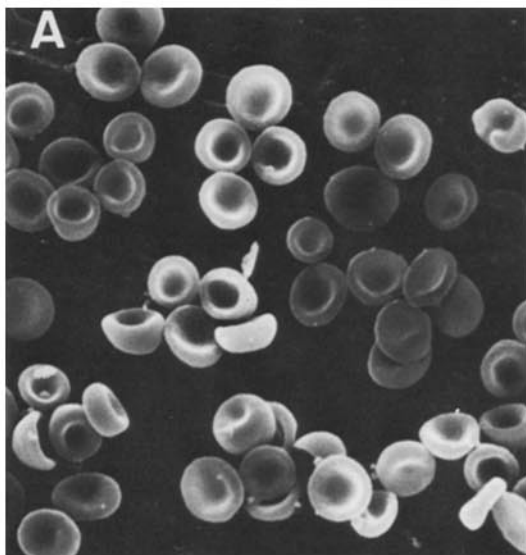
nonmodified cells was the same. Despite the similar extent of hydrolysis, however, the shape changes occurred at quite different rates in cells containing different PC species (Fig. 1 B). Erythrocytes enriched with 1,2-dipalmitoyl PC exhibited a slow rate of shape changes compared to the control cells, whereas the 1,2-dioleoyl PC cells changed shape more rapidly. 1-Palmitoyl-2-oleoyl PC cells, like 1,2-dipalmitoyl PC cells, opposed shape changes induced by the degradation of sphingomyelin in the outer monolayer. Figs. 2, A, C, and E show scanning electron micrographs of the different samples after 20 min of hydrolysis, when ~12% of the total sphingomyelin in the erythrocyte was hydrolyzed.

The time course of hydrolysis after bee venom phospholipase A₂ treatment of PC-modified cells is shown in Fig. 1 C. The PC in the cells in which part of this phospholipid had been replaced by the dipalmitoyl PC species was hydrolyzed at a slower rate when compared to that of the PC in control cells or in those modified with dioleoyl PC. After 40 min, ~20% of the PC was hydrolyzed in the 1,2-dipalmitoyl PC cells, compared to >30% in the other cells. These amounts equal, respectively, 6 and 9% of the total phospholipid content. The amount of lipid hydrolyzed, as well as the amount of PC needed to be exchanged in order to give rise to shape changes, are larger than those needed upon sphingomyelinase

C treatment. This is not surprising, since upon phospholipase A₂ treatment the polar headgroup of PC remains in the lysophosphatidylcholine molecule. The change of optimal surface area due to the liberation of the fatty acyl moiety is probably not as drastic as in the case of sphingomyelinase C treatment. In the case of PC exchange, the shape alterations involve only small changes in the areas occupied by the apolar acyl chains. Therefore, a larger percentage of lipids must be exchanged, or PC molecules be hydrolyzed, in order to obtain shape changes. In addition to PC, a minor fraction of the phosphatidylethanolamine was also hydrolyzed, which is in agreement with the preferential distribution of phosphatidylethanolamine in the inner monolayer of the human erythrocyte (8). No hydrolysis of phosphatidylserine occurred. The rates of shape changes were similar to the three samples (Fig. 1 D). On the basis of the morphological scores, it appeared to be impossible to obtain morphological indexes that were accurate enough to discriminate between the rates of shape change in control and 1,2-dioleoyl PC cells, in both of which the PC hydrolysis proceeded at a similar rate. However, the 1,2-dipalmitoyl PC-modified cells turned echinocytic already at a lower degree of PC hydrolysis when compared to the control and 1,2-dioleoyl PC-modified cells. After 30 min, when the PC hydrolysis in 1,2-dipalmitoyl PC cells was similar to that in the control cells after 10 min (~16% PC hydrolysis), the morphological indexes were +1.0 and +0.3, respectively. Figs. 2, B, D, and F show the scanning electron micrographs of those samples after phospholipase A₂-induced hydrolysis of ~16% of the PC.

It should be noted that the 1,2-dipalmitoyl PC cells, prior to phospholipase A₂ treatment, were hardly echinocytic. This agrees with the moderate degree of 1,2-dipalmitoyl PC replacement (Table I). Nevertheless, the 1,2-dipalmitoyl PC-modified cells were markedly more echinocytic than the other cells after hydrolysis (Fig. 2 D). It could be argued that the echinocytosis was due to the uptake of lysophosphatidylcholine liberated by degradation of residual donor vesicles adhering to the erythrocytes after washing. However, the degree of contamination, as measured by the presence of unexchangeable marker in the samples, was usually <4% of the erythrocyte PC. This small amount (less than 1.10⁶ molecules/cell) could hardly have been a major cause of shape changes. Secondly, di-oleoyl PC-enriched erythrocytes turned echinocytic at the same rate as control cells (with no contaminating vesicles) (Fig. 1 C). This would not have been the case if lysophosphatidylcholine was liberated from the contaminating vesicles. It can be concluded that the modification of the phospholipids in the outer monolayer of the erythrocyte membrane, subsequently achieved by the exchange of part of the native PC with well-defined species and the nonlytic treatment of the cells with phospholipases, gives rise to morphological changes which appear to be governed by the changes in the geometry of the phospholipid molecules thus induced. A tentative model explaining these shape changes is shown in Fig. 3.

FIGURE 2 Scanning electron micrographs of modified erythrocytes treated with sphingomyelinase C and phospholipase A₂. This figure shows control cells after sphingomyelinase (A) and phospholipase A₂ (B) treatment. In addition, cells are shown in which part of the native PC was replaced by 1,2-dipalmitoyl PC, followed by limited hydrolysis of sphingomyelin (C) and PC (D). Finally, cells which were enriched in 1,2-dioleoyl PC and subsequently treated with sphingomyelinase (E) and phospholipase A₂ (F). After sphingomyelinase treatment, ~12% of the sphingomyelin was hydrolyzed in all cases, whereas 16% of the PC was hydrolyzed during the phospholipase A₂ incubations.



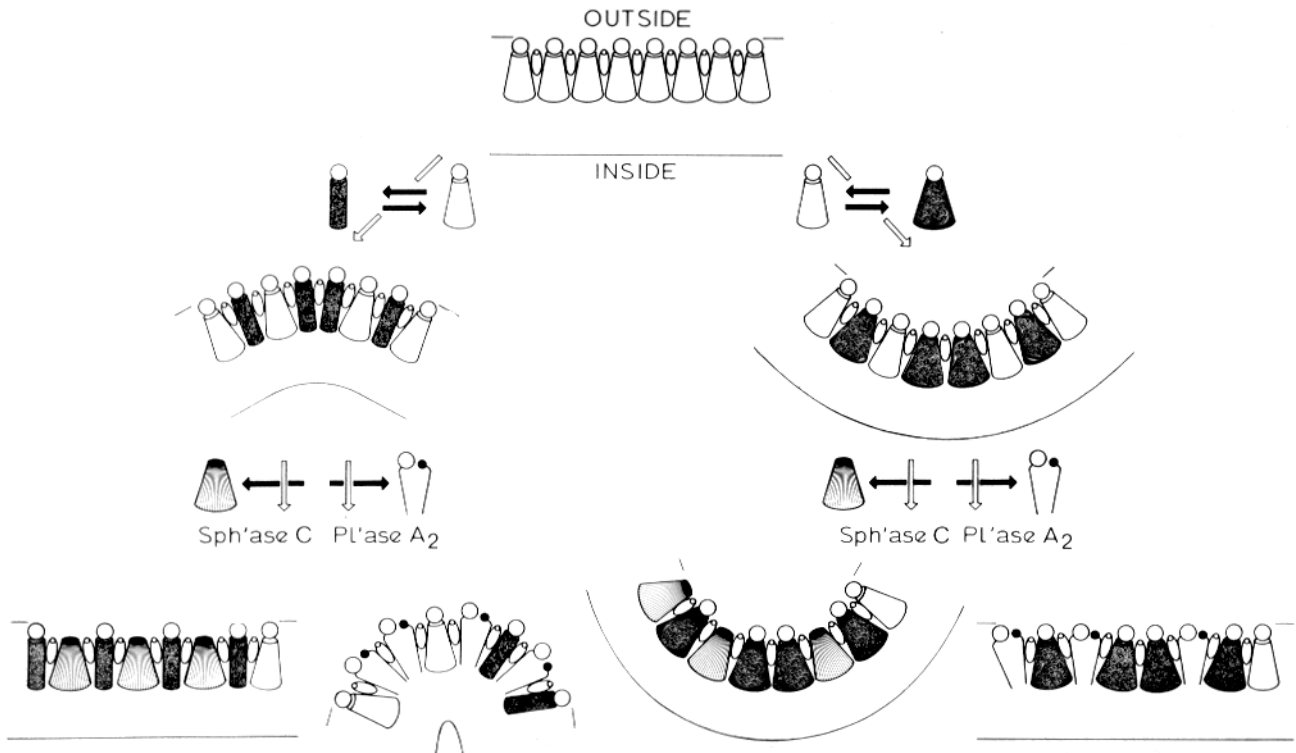


FIGURE 3 Model to explain shape changes of erythrocytes induced by changes in lipid molecular shape. PC exchange is depicted in the first step of the scheme: on the left side the replacement with dipalmitoyl PC; on the right the replacement with dioleoyl PC. Subsequent treatments with phospholipase A₂ (PL'ase A₂) and sphingomyelinase C (Sph'ase C) are depicted below. The small figures between the phospholipid molecules represent cholesterol. For sake of clarity they are depicted this way, although they actually occupy a cone shape (32).

DISCUSSION

The physical basis for assembly of lipids into bilayers or other configurations, e.g., micelles, has been described by a theory that links thermodynamics, interaction-free energy, and molecular geometry (13, 25, 26). Each lipid molecule can be assigned a molecular shape on the basis of attractive and repulsive interactions that occur between polar and apolar parts of the molecules. A simple example showing that molecular shape is important to the shape of the aggregate formed, is the fact that the shape properties of PC's with different acyl chain composition cause them to pack upon sonication into unilamellar vesicles of different radii (25). The above theory can be applied in a qualitative way to our results. PC exchange as well as hydrolysis of phospholipids in the outer monolayer of the erythrocyte will alter the geometry of the lipids and thus disturb the packing in the outer monolayer. This results in an energetically unfavorable situation, and a new packing with minimal interaction free energy for all lipid molecules and other membrane components will appear. A redistribution of the lipid components over the two bilayer halves could be one possible way to minimize the energy, as shown to occur in model systems (27–29). However, for reasons discussed below, we postulate that a redistribution does not occur in the erythrocyte, at least not extensively and rapidly. An alternative way to minimize the energy would be to allow the lipids, while remaining in the outer layer, to pack together in a way which is determined by their molecular shape. As a consequence, PC replacement should result in the formation of either stomatocytes in case of dioleoyl PC, or

echinocytes in case of dipalmitoyl PC, or no change in morphology at all in case of palmitoyl-oleoyl PC because this species closely resembles the most abundant native PC molecules. It was shown previously that these predicted modifications indeed occur (10).

Enzymatic treatment of phospholipids in the membrane of intact erythrocytes also results in morphology changes (6, 7), which can be explained with the same model. Sphingomyelinase C from *S. aureus* attacks sphingomyelin by removal of the polar headgroup, thereby producing the cone-shaped ceramide. In case the ceramide would remain in the outer monolayer, this shape change already would explain the observed change in cell morphology. However, an alternative explanation is that the ceramide, by analogy with the diacylglycerol formed upon treatment of intact cells with phospholipase C, flipped over to the other half of the bilayer, and caused cupping by accumulation in the inner monolayer (6). Since the membrane phospholipids are obviously restricted in their ability to redistribute between the two halves of the bilayer (8, 12, 30), a net transfer of a substantial amount of ceramide to the inner monolayer would be energetically unfavorable. Phospholipase A₂-treatment of intact erythrocytes produces lysophosphatidylcholine and free fatty acids and although the exact molecular shape of these products will depend on their acyl composition (31), it can be assumed that products of hydrolysis, taken together, have a more inverted cone shape than the original PC molecules. In our experiments both PC exchange and phospholipase treatment are combined and it is obvious that the morphology changes which are induced by the individual treatments are additive.

The predicted changes in morphology are shown in Fig. 3 and are indeed observed. Treatment of dipalmitoyl PC-enriched cells with sphingomyelinase C results in a relatively slow and limited morphology change when compared with control cells, because the shapes of ceramides and dipalmitoyl PC are complementary. On the other hand, a rapid and extensive invagination of erythrocytes is observed in case of sphingomyelin-treatment of dioleoyl PC-enriched cells, because two types of cone-shaped molecules are present. As expected, a behavior in between these extremes is observed for cells enriched with palmitoyl-oleoyl PC. Phospholipase A₂-treatment of dipalmitoyl PC-enriched cells gives a more rapid and extensive echinocyte formation than palmitoyl-oleoyl PC-treated and control cells. Small changes in morphology were observed when dioleoyl PC-modified cells were treated with phospholipase A₂, due to the fact that the cone shape of this species counteracts the crenation of the cells as a consequence of the production of the inverted cone-shaped lysophosphatidylcholines and free fatty acids.

Our model can thus explain in a qualitative way the shape changes that occur upon exchange and phospholipase treatment. Additional support is given by the results of Fuji and Tamura (7). They observed that the crenation caused by phospholipase A₂-treatment could be counteracted by phospholipase C-treatment, and vice versa. A similar qualitative use of the concept of lipid molecular shape was found useful for explaining the regulation of lipid composition in the wall-less prokaryotic *Acholeplasma laidlawii* (32). That localized changes in phospholipid structure within the membrane may lead to overall shape changes of intact erythrocytes was postulated recently also by Ferrell and Huestis (33). They correlated the conversion of phosphatidyl 4,5-bisphosphate to phosphatidylinositol as induced by ATP depletion with crenation.

As mentioned above, the model does apply only in the case that rapid redistribution of newly introduced PC and hydrolysis products over both membrane layers does not occur.

The fact that the observed changes in morphology of the erythrocyte were predictive and additive may in itself be an argument in favor of our assumption that the molecules which are responsible for the alterations remained in the outer monolayer of the membrane. Other data support this postulation. The rate of transbilayer movement of PC is slow in the human erythrocyte compared to the timescale of our experiments. The halftimes for 1,2-dipalmitoylphosphatidylcholine- and egg PC translocation are 27 and 13 h, respectively (12). The shape changes obtained after PC exchange are most probably caused by the effect of PC in the outer monolayer only. Upon hydrolysis of intact erythrocytes with phospholipase A₂ and sphingomyelinase C, the resulting hydrolysis products remain in the membrane (7). The rate of translocation of lysophosphatidylcholine in intact cells is similar to that of PC (34). In phospholipase A₂-treated cells the re-allocation of lysophosphatidylcholine might proceed even slower since prolonged incubations in the presence of the enzyme (up to 24 h) does not result in exchange of the lysophosphatidylcholine with intact PC from the inner leaflet. Lysophosphatidylcholine would thus affect the outer monolayer only. After hydrolysis of almost all the phospholipid in the outer monolayer, phosphatidylserine and phosphatidylethanolamine from the inner monolayer do not appear on the outside (8). This indicates that the translocation of these lipids is severely restricted.

The rates of translocation of free fatty acids and ceramides are not known and might be faster. Cholesterol is able to redistribute rapidly over the membrane of the intact cell in certain circumstances (35). We cannot exclude that a certain redistribution of cholesterol occurred during our experiments. This might explain why the shape changes after PC exchange did not occur until after a certain percentage of PC had been replaced (10). Likewise, a limited initial redistribution of cholesterol may be the reason why the morphological index scale is nonlinear (Fig. 1B), i.e., the largest changes in lipid composition are required to produce the first detectable shape changes, whereas later changes come more easily. However, although the rate of redistribution of cholesterol, free fatty acids, and ceramides might be faster than that of PC, the amount translocated is probably restricted.

Although no definite proof is available yet, evidence is accumulating that interactions between the cytoskeleton and the cytoplasmic side of the lipid bilayer are important for the maintenance of the lipid asymmetry in the erythrocyte membrane (1, 36, 37). The asymmetry as well as the rate of transbilayer movement of lipids are affected by manipulations that disturb the organization of the cytoskeleton (36, 38, 39). An energetically favorable way to translocate a lipid molecule from one side of the membrane to the other is probably to couple the transport with a translocation of a second molecule in the other way (28). If the cytoskeleton restricts the possibility to translocate lipids to the outer monolayer, then the transport of one molecule to the inner monolayer will require additional energy. Possibly this is the reason why the rates of PC and lysophosphatidylcholine translocation are slow. If the energy requirement for translocation is high compared to the energy needed to bend the membrane, then bending is the most probable event that occurs. The energy dissipation necessary to obtain a stable lipid packing is then obtained by the bending of the membrane. This line of reasoning implies that the membrane skeleton does not itself determine the cell shape in these circumstances. Similar conclusions have been reached concerning the role of the cytoskeleton in the echinocytic shape of isolated ghosts (40). Yet, the presence of an interaction between the membrane skeleton and the inner monolayer lipids seems to be a necessary requirement to explain our findings. This interaction might still be possible if the membrane skeleton conforms to the shape changes (40). The observed changes would be difficult to explain in a unified manner if the lipids were able to translocate freely across the membrane.

In conclusion, the results of this investigation give further strength to our proposal that lipid molecular shape affects erythrocyte morphology. We have shown, using two different approaches to alter lipid molecular geometry, that, in human erythrocytes, morphological changes due to different lipid manipulations (other lipid molecules, increase in number of molecules, complete modifications by loss of polar headgroup, etc.) are additive and predictable, and can be explained on the basis of molecular shape. These morphological changes seem to be due to the restricted transbilayer mobility of the lipids.

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