The Molecular Species Composition of Phosphatidylcholine Affects Cellular Properties in Normal and Sickle Erythrocytes


Fatty Acyl Groups and cholesterol are the major constituents of the hydrophobic interior of all biological membranes. This core provides a permeability barrier to hydrophilic substances at the boundaries of cells and cell organelles and serves as a matrix in which integral membrane proteins are embedded. Each class of phospholipid in any particular biological membrane has a characteristic fatty acyl group composition. Increasing effort has been spent in recent years to understand the rationale for such a specific molecular species composition. Changes in fatty acyl unsaturation and chain length, with the accompanying change in the molecular structure of the phospholipid molecules, will affect membrane properties as shown in several studies of model membrane systems as well as biological membranes. The modification of fatty acids in the phospholipids of the membrane provides an approach to these questions, since the correlation of alteration in fatty acyl group composition and membrane properties can be measured.

Recently, the phosphatidylcholine specific transfer protein (PCTP) isolated from bovine liver was used to manipulate the molecular species composition of phosphatidylcholine (PC) in the membrane of normal (AA) and sickleable (SS) human erythrocytes. Changes in molecular species composition of PC altered morphology as well as cellular deformability and stability as measured with ektacytometry. In normal cells, replacement of native PC with 1-palmitoyl,2-arachidonoyl PC (PAPC) resulted in a decrease in osmotic fragility with no change in hydration, whereas replacement with 1,2-dipalmitoyl PC (DPPC) led to an increased osmotic fragility and cellular hydration. Replacement of native PC by 1-palmitoyl,2-oleoyl PC (POPC) in normal cells had no apparent effect on these parameters. In contrast, replacement of native PC in sickle cells with either PAPC, DPPC or POPC led to cellular hydration. Facilitation of PC exchange between subpopulations of SS cells separated on buoyant density also led to cellular hydration. These observations suggest that the state of hydration of sickle cells can be modified by the fatty acyl composition of PC and illustrate a role for the lipid core in the observed permeability changes in sickle erythrocytes. They also raise the interesting possibility that the state of cellular hydration of sickle cells may be modulated by altering the molecular species composition of the membrane phospholipids.

MATERIALS AND METHODS

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine, 1-palmitoyl,2-oleoyl-sn-glycero-3-phosphocholine, egg phosphatidate, cholesterol,

From the Children's Hospital Oakland Research Institute, CA; the Cancer Research Institute, University of California at San Francisco; and the Laboratory of Biochemistry, State University of Utrecht, The Netherlands.

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Address reprint requests to F.A. Kuypers, PhD, Children's Hospital Oakland Research Institute, 747 52nd Street, Oakland, CA 94609.

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and polyvinylpyrrolidone (PVP) (MW 360,000), were purchased from Sigma (St Louis). 1-Palmitoyl,2-arachidonoyl-sn-glycerol-3-phosphocholine was obtained from Avanti Polar Lipids, Inc (Birmingham, AL). 1-palmitoyl,2,14C]oleoyl-sn-glycerol-3-phosphocholine, and 1-palmitoyl, 2-[14C]palmitoyl-sn-glycerol-3-phosphocholine were purchased from New England Nuclear (Boston). [3H]Glycerol trioleate was obtained from Amersham (Cardiff, UK). Cr was purchased as sodium chromate in saline pH 8.0 from New England Nuclear.

Preparation of vesicles. Vesicles were prepared from a lipid mixture containing one of the three phosphatidylcholine species mixed with an equimolar amount of cholesterol, 6 mol% of egg phosphatidate and trace amounts of [14C]-phosphatidylcholine and [3H]glycerol trioleate, the latter serving as nonexchangeable marker. The lipid mixture was dried from a chloroform/methanol solution (2:1, vol/vol) and vesicles were prepared as described before in a buffer containing 150 mmol/L NaCl, 25 mmol/L glucose, 1 mmol/L ethylenediaminetetraacetate (EDTA), 100 IU/mL penicillin, 100 µg/mL streptomycin and 10 mmol/L tris (hydroxymethyl) aminomethane (TRIS), pH 7.4 (referred to as incubation buffer throughout).

Erythrocytes. After obtaining informed consent, human venous blood was collected in heparinized tubes for normal (AA) individuals and homozygous sickle cell (SS) patients. The erythrocytes were pelleted by centrifugation for five minutes at 2,500 × g and washed three times with a tenfold excess of incubation buffer. Theuffy coat was carefully removed by aspiration after each wash. While most experiments were carried out with unfractionated populations of erythrocytes, some experiments used erythrocytes with well-defined cell densities obtained using discontinuous stractan gradients.

Preparation of the transfer protein. The phosphatidylcholine specific transfer protein was purified from bovine liver according to Kamp et al and had a specific activity of five µmol PC exchanged per milligram protein per minute. It was homogeneous on SDS-disc gel electrophoresis and was stored at a concentration of 75 µg/mL in 50% glycerol at −20°C. Before use, glycerol was removed by overnight dialysis at 4°C against 600 vol of incubation buffer. After dialysis, the volume of the protein solution was reduced by concentrating against polyethylene glycol (Aquacide III, Calbiochem, San Diego), until a protein concentration of 150 µg/mL was reached.

Replacement of erythrocyte PC by different PC species from donor vesicles. Cells were preincubated at 37°C for one hour with gentle shaking under room air or nitrogen. The replacement reaction was started by the addition of a mixture of the PC vesicles and PCTP. The incubation of the resulting 30% cell suspension, containing a final concentration of 0.6 to 0.9 mmol/L vesicle PC and 3 µmol/L transfer protein, was continued under either air or nitrogen.

Aliquots were taken at timed intervals and added to 5 mL of incubation buffer at 37°C. Cells were isolated by centrifugation for five minutes at 2,500 × g and the residual vesicles and transfer protein were removed by two additional washes with a 50-fold volume of incubation buffer. Lipids were extracted from the erythrocytes according to the procedure of Rose and Oklander. The extent of PC replacement was calculated from the specific radioactivity of the phosphatidylcholine fraction as described above. A correction was made for the extent of contamination of erythrocytes with vesicles by measuring the amount of nonexchangeable marker [3H]glycerol trioleate present in the extracted lipids. 14C POPC was used as a marker for the transfer of PAPC for practical reasons. Radioactively labeled PAPC was not readily available and POPC satisfactorily reflects the actual exchange process of PAPC from vesicles to red cell membranes.

PC exchange between subpopulations of sickle cells. Subpopulations of sickle cells with a defined range of cell densities were

Fig 1. Replacement of native erythrocyte PC in normal RBC (x) and oxygenated (O) or deoxygenated (0) sickle RBC. (A) Replacement of native PC by DPPC, (B) replacement of native PC by POPC, and (C) replacement of native PC by PAPC. The extent of replacement was determined by using tracer amounts of [14C]palmitoyl PC (A) or 1-palmitoyl, 2-[14C]oleoyl PC (B and C), respectively, in the donor vesicles. The resulting specific radioactivity of the erythrocyte PC was analyzed and data are expressed as percentage of total erythrocyte PC replaced by donor vesicle PC. During the exchange incubations, cells were kept under room air (x, O) or nitrogen (0). Oxygenated cells were preincubated at 37°C for one hour under room air; deoxygenated cells were preincubated at 37°C for one hour under humidified nitrogen.
harvested from a discontinuous stractan gradient and washed three times with 10 vol of incubation buffer. Erythrocytes with cell densities in the range of 1.0 to 1.0880 g/mL were incubated for two hours at 37°C under gentle shaking with vesicles containing egg PC and (14C)-1-palmitoyl,2-oleoyl PC, prepared as described above, in the presence of 2 μmol/L PCTP. The ratio of vesicle PC to erythrocyte PC was one to one. This incubation resulted in a partial replacement of erythrocyte PC by vesicle PC. The cells were subsequently separated from the vesicles and exchange protein as described above. These cells, now labeled with 14C in the PC fraction, were incubated for one hour at 37°C at a hematocrit of 40% in the presence of 0.5 μCi15Cr, and subsequently washed twice with buffer. Samples were taken to determine the specific radioactivity of 51Cr per gram of hemoglobin and 14C per gram of lipid phosphorus.

Aliquots of this double radio-labeled subpopulation were mixed with aliquots of the unlabeled high density subpopulations (cell density > 1.0880 mg/mL) from the stractan gradient to reconstitute the original density distribution of the whole sickle cell population. This mixture of cells was incubated at 37°C overnight (14 hours) in presence or absence of PCTP. After this incubation cells were separated on a stractan gradient and the specific radioactivity of 15Cr and 14C was determined in each subpopulation.

Osmotic gradient ektacytometry. A Technicon ektacytometer was used to measure whole cell deformability as a continuous function of the suspending medium osmolality at a constant applied shear stress of 170 dynes/cm². For these studies, the deformability index (DI) of erythrocytes was continuously recorded as the suspending medium osmolality was linearly increased from 50 to 500 mosm/kg. As previously shown, the curve relating the variation of DI with suspending medium osmolality can be analyzed to provide information about initial cell surface area, surface area-to-volume ratio, and cell water content.16

Cell morphology. Samples of 10 μL of washed packed cells were fixed for one hour at 20°C in 1 mL of buffer containing 100 mmol/L NaCl, 40 mmol/L Na citrate, and 0.5 mmol/L formaldehyde. After washing, the cells were post-fixed with 1% OsO₄ during 30 minutes and subsequently dehydrated in a graded series of ethanol, transferred into isopropanol, and the air dried. Cells were covered with a thin layer of gold by the sputter process. Microscopy was performed with a Cambridge Steroscan 600 M electron microscope.

RESULTS
Incubation of normal (AA) and sickle (SS) erythrocytes at 37°C in the presence of donor vesicles and the PC-specific transfer protein resulted in replacement of the native PC species of the erythrocyte membrane by the PC species from the donor vesicles (Fig. 1). Despite similar incubation conditions, a clear difference was seen in the PC replacement profiles for different PC species. The disaturated PC molecules (Fig 1A) were incorporated at a slower rate and to a lower extent than 1-saturated,-2-unsaturated species in both sickle and normal erythrocyte membranes (Fig 1B, C). The time course of replacement of PC species in sickle cells was very similar to that observed in normal erythrocytes except for the replacement reaction of POPC (Fig 1B). In this case, native PC of deoxygenated sickle cells was replaced by this particular species to a greater extent than in oxygenated sickle cells, indicating an increased pool of exchangeable PC

Fig 2. Electromicrographs of sickle red cells after partial replacement of their native PC. (I) Cells after four hours of incubation during which their native PC was partly replaced by POPC (A), DPPC (B), or PAPC (C) to an extent of 30%, 15%, or 20%, respectively. (II) Cells after 16 hours of incubation during which their native PC was partly replaced by POPC (A), DPPC (B) or PAPC (C) to an extent of 45%, 25%, and 40%, respectively. (III) Cells as depicted in II, after incubation under nitrogen for one hour. After reoxygenation, cells readopted their shape as shown in II.
in sickle cells under deoxygenated conditions. Deoxygenation had no effect on the replacement reaction of normal cells (data not shown). Figure 1 documents the exchange process as well as the level of replacement for each molecular species of PC. Using this measurement, we are able to relate the level of replacement with the observed effects on other cellular parameters.

**Cell-shape.** Replacement of the native PC in normal human RBCs by either DPPC or PAPC led to dramatic changes in morphology, whereas replacement of the native PC by POPC had no effect on the shape of the cell. Similar changes in morphology were observed in sickle cells under oxygenated conditions. Figure 2 shows that even when 45% of native PC was replaced by POPC, morphology was unchanged (Fig 2, IIA), while replacement of native PC with either DPPC or PAPC resulted in echinocytic transformation. Replacement of only 15% of the native PC with DPPC led to the generation of a fair number of echinocytes (Fig 2, IB), while replacement of 25% of native PC with this molecular species resulted in almost complete conversion to the echinocytic shape (Fig 2, IIB). The most marked shape change was observed during replacement of native PC by PAPC. Replacement of 20% of native PC with this species led to a complete conversion into a crenated morphology with dimples (Fig 2, IC). After eight hours of PC exchange incubation, the spikes began to disappear, although the dimples were still present (Fig 2, IIC, and Fig 3).

Reversible sickle cells (RSC) in which the native PC had been replaced by either of the above mentioned species sickled following incubation under nitrogen (Fig 2, III). Cells in which part of the PC had been replaced by either DPPC or PAPC almost completely lost their echinocytic spikes, especially those cells that adopted the most pronounced sickled morphology (Fig 2, IIIIB, and C). This process appeared to be completely reversible since these cells immediately readopted their earlier crenated shape upon reoxygenation.

**Osmotic deformability profiles of normal cells.** The relationship between the deformability index (DI) and the osmolality of the suspending medium for normal red cells is shown (Fig 4A, curve 1). The features of such a profile for a normal red cell are that a maximum value of DI is reached around 290 mosm/kg, while an increase or decrease in tonicity lowers the deformability and therefore the DI of the cell. The decrease in tonicity below 290 mosm/kg lowers the DI until a minimum value around 135 mosm/kg is reached. The osmolality at which this minimum is reached is directly related to the osmolality at which 50% of the cells have lysed in a classical osmotic fragility assay. The effect of replacement of native PC on the osmotic deformability profile of normal cells is shown in Fig 4A. Replacement of native PC by POPC did not significantly change the osmotic deformability profile of normal red cells (Fig 4A, curve 2), whereas a shift in the deformability profile was observed after replacement of native erythrocyte PC by either PAPC or DPPC (curves 3 and 4, respectively). The profile obtained after replacement with PAPC showed a decrease in the osmolality at which DI reached a minimum in the hypotonic region, while the hypertonic arm of the profile remained unchanged. The maximum attainable value of DI remained constant, but this value was noted over an osmolality range that was broader than that for control cells. Replacement of native PC by DPPC resulted in a profile that showed an increase in the osmolality at which the DI reached its minimum in the hypotonic region. In addition, the hypertonic arm of the curve was also displaced to higher osmolality.
Osmotic deformability profiles of native and PC-modified sickle erythrocytes are shown in Fig 4B. The profile of unmodified SS cells (Fig 4B, curve 1) is clearly different from that of normal erythrocytes with much of this difference being accounted for by the heterogeneity in water content of sickle cells. While normal cells were unaffected by substitution of POPC for native PC (Fig 4A, curve 2), similar treatment of SS cells showed a right shift of the hypertonic arm, indicating increased cell hydration (Fig 4B, curve 2). A similar shift in the osmotic deformability profile was observed with each of the PC species tested (Fig 4B, curves 2, 3, 4) suggesting that PC replacement resulted in increased hydration of sickle cells. While the shifts in osmotic deformability profile found in normal cells seemed to be directly related to the extent of replacement of native PC by PAPC or DPPC this was clearly not the case for sickle cells. Very low levels of replacement of native PC by DPPC (5%) gave a virtually identical shift in the deformability profile as that seen in cells in which 20% of native PC had been replaced by DPPC. Furthermore, while incubation of red cells with PCTP (with or without POPC donor vesicles) had no effect on the osmotic deformability profile of normal RBC (Fig 5A), SS cells showed a shift in their osmotic deformability profile consistent with cellular hydration when incubated with PCTP, with or without POPC donor vesicles (Fig 5B). These results indicate that the presence of PCTP in itself can induce cellular hydration within the heterogeneous sickle cell population.

The concentration dependence and time course of cell hydration following incubation with PCTP is shown in Fig 6. This figure demonstrates the results obtained with red cells from three different patients. Sickle cells become more hydrated with time when incubated in the presence of PCTP (Fig 6A). Maximal hydration induced by 1.1 μmol/L PCTP appeared to occur after 5 to 8 hours incubation (Fig 6B). For a fixed period of time (three hours) the increase in cell hydration, as measured by the shift of the osmotic deformability profile, was found to be dependent on the concentration of PCTP (Fig 6C). Due to the large variations in the initial state of red cell hydration among patients, it is difficult to quantitatively compare the extent of cell hydration induced by PCTP from one patient to another. However, red cell hydration was increased in blood samples from all patients studied.

Further evidence for the increased hydration of SS cells was provided by analysis of the buoyant density of sickle cells following incubation with PCTP (Fig 7). Incubation of sickle cells with PCTP led to a decrease in cell density in unseparated sickle cell samples (Fig 7A) as well as in dense sickle cells (Fig 7B).

In order to document whether this hydration of sickle cells induced by PCTP was accompanied by an exchange of PC species between subpopulations of cells, we performed the experiment depicted in Fig 8. Cells with density up to 1.0880 mg/mL were labeled with 14C-PC and 51Cr, as described in the method section. Following incubation in the presence or absence of PCTP, the cells were again separated by buoyant density, and the relative distribution of specific radioactivity over the subfractions was determined (Fig 8). The distribution of 51Cr over the gradient, indicating the buoyant density distribution of the originally labeled cells after the incubation, was virtually identical in the presence (Fig 8A) or absence (Fig 8B) of PCTP. Approximately 60% of the 51Cr label was found in the original position within the gradient (fractions 1 and 2) while the remainder was distributed in higher density fractions (fractions 3 to 6). The distribution of 14C-labeled PC in the density gradient following the incubation in the presence of PCTP showed an even distribution over all cell fractions (Fig 8A), while in the absence of PCTP the distribution of 14C-labeled PC followed the 51Cr distribution (Fig 8B). These results document that exchange of PC species composition and RBC properties.
between red cell membranes is facilitated by PCTP and that the observed cell hydration in sickle cells is directly related to this exchange of PC molecules between subpopulations of sickle cells.

**DISCUSSION**

The PC specific transfer protein from bovine liver is a powerful tool to alter red cell membranes in a well-defined manner. Under proper conditions this protein facilitates exchange of PC molecules between two membranes without altering other aspects of the membrane composition. The present study demonstrates that this exchange process is not limited to PC movement between donor PC vesicles and cells, but that exchange of PC between cell membranes also takes place. In sickle cells as well as normal cells, the rate and extent of PC replacement facilitated by PCTP depends strongly on the PC species involved. These results are in agreement with previously reported results on model systems and erythrocytes.

In contrast to normal cells, deoxygenated sickle cells showed a clear difference in the time-dependent replacement of native PC by POPC when compared to oxygenated sickle cells. This observed increase in size of the exchangeable pool of PC in the membrane of sickle cells under nitrogen is in agreement with our earlier observations, and is also an indication that the transbilayer movement of PC in these membranes is accelerated under these conditions.

Since the apolar part of the PC molecules in the membrane can be directly responsible for changes in red cell morphology, retailoring of the molecular species composition of PC might be expected to lead to dramatic changes in cell morphology. The morphologic changes observed in oxygenated sickle red cells, after replacement of the native PC by DPPC or PAPC, were identical to those observed in normal cells. Sickle cells in which PC was modified changed to a sickle morphology under deoxygenating conditions and at the same time lost their echinocytic appearance. When reoxygenated, the echinocytic shape instantaneously reappeared. This observation is similar to the morphologic changes as a consequence of sickling and desickling of phospholipase A$_2$-treated RSC's. This loss of echinocytic projections during sickling may indicate a local reorganization of membrane lipids during the sickling process or mechanical stretching of the membrane by the polymerized hemoglobin.

The most striking effect we found in this study was that the ektacytometric deformability profile and state of hydration of the red cell can be affected by retailoring its PC
molecular species. Incubation of normal cells with PCTP in the absence of vesicles or with vesicles in the absence of PCTP had no significant effect on the ektacytometric profile. Replacement of up to 40% of native PC in normal cells by POPC also did not alter the osmotic deformability profile. Replacement of up to 40% of native PC in normal cells by PCTP had no significant effect on the ektacytometric profile.

However, replacement of native PC by DPPC led to a shift in the osmotic deformability profile indicating a decrease in osmotic fragility as measured by ektacytometry. In contrast to normal cells, sickle cells demonstrated a shift in the ektacytometric profile, indicating hydration after incubation with PCTP and vesicles with each type of PC species tested. Moreover, incubation of sickle cells with PCTP in the absence of vesicles also induced a change in buoyant density accompanied by an exchange of PC species between individual cells in the sickle cell population. This effect was concentration and time dependent and was found with all patient samples despite individual variations in initial osmotic deformability profile. Our hypothesis for this phenomenon is that PCTP, by facilitating PC molecular species exchange between membranes or domains within a membrane, induces changes in packing of the molecules in the lipid core, which in turn affects the permeability characteristics of the membrane. Moreover, the replacement of native PC by vesicle PC is not an isolated process since PC exchange between cells takes place as well. The shift in the ektacytometric pattern as shown in Fig 4B is a result of both of these processes. Our results suggest that dehydrated sickle cells lose or gain certain PC species during the exchange process. As a consequence, cell permeability is altered, facilitating cell hydration. Another possibility, one that is less likely but cannot be ruled out at present, is that the PCTP has a unique yet undefined physical interaction with the most dense cells in the SS population and induces a direct change in permeability in these cells. This effect could take place independent of the exchange of PC between subpopulations of sickle cells.

From this study, it can be concluded that changes in the species composition of phosphatidylcholine in normal as well as in sickle cells can lead to an altered cell morphology, membrane permeability, and can affect membrane properties that govern cellular deformability and stability.

A better understanding of the role membrane lipids play in the state of hydration of the sickle cell could be of physiologic significance, since the rate of S hemoglobin polymer formation is extremely dependent on the state of hydration of the cell.

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The molecular species composition of phosphatidylcholine affects cellular properties in normal and sickle erythrocytes

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