Detection of the Electrical Surface Charge Induced by Treatment of the Membrane Lipid Bilayer of Human Erythrocytes

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ABSTRACT. The lipid bilayer of the membrane of human erythrocytes from which most sialic acid residues on the outer surface had been removed was digested by phospholipase from outside the medium or treated by the incorporation of exogenous amphiphilic lipid, then it was electrophoresed. After treatments that caused the additional exposure of the dissociable polar head group of the amphiphilic lipid to the outer medium, the electrophoretic mobility of the treated cells increased as the pH of the suspending medium rose. This showed that there was an increase in the negative charges in the immediate vicinity of the lipid bilayer surface of the membrane. The possible effect of the dissociation of exposed head groups in the lipids on the morphology of the membrane is discussed.

We reported previously that a change in the shape of human erythrocytes is induced by *in vitro* treatments of the membrane lipid bilayer (1, 8) and that after some treatments which liberate or form anionic head groups in the lipid molecules exposed on the outer surface of the membrane, the degree of the change in shape became markedly dependent on the pH of the medium (8, 9). We assumed that the dissociation of these polar head groups in an appropriate pH range might increase the repulsion among the charged groups or increase the hydration around these groups. This in turn would result in the asymmetric expansion of the outer half of the lipid bilayer, relative to the inner half because the mechanism for the change in the shape of the erythrocytes can be explained by the asymmetric expansion of one half of the bilayer according to the "bilayer couple hypothesis" (7). To prove the validity of our assumption, we have demonstrated by cell electrophoresis that these treatments do cause the increased exposure of dissociable head groups on the bilayer outer surface, and that these groups become negatively charged in the pH range of their dissociation. We used desialyated erythrocytes which had been treated with neuraminidase to remove most of the sialic acid residues on their outer surfaces because the abundant negative charges of these residues must prevent the electrophoretic detection of the relatively weak negative charges contributed by the polar head groups of the membrane lipids.

Abbreviations used: PBS, phosphate buffered saline; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; MES, 2-morpholinoethanesulfonic acid; PLase, phospholipase; PC, phosphatidylcholine; TLC, thin layer chromatography.

MATERIALS AND METHODS

Erythrocytes. Fresh human blood from the same normal donor was used in all experiments. Several milliliters of blood were drawn into a glass syringe containing heparin. The erythrocytes were washed three times with isotonic saline containing 10 mM phosphate buffer solution (PBS), pH 7.4 then resuspended in PBS.

Treatment of erythrocytes with neuraminidase. A 0.25 unit portion of neuraminidase from *Arthrobacter ureafaciens* (Marukin Shoyu Co., Ltd.) per milliliter of cells was added to a 5% erythrocyte suspension. This mixture, at pH 7.4, was incubated at 37°C for 60 min. The treated cells were centrifuged, washed three times with 1% bovine serum albumin (Fr. V, Armour Pharmaceutical Co.) solution in PBS then resuspended in PBS. Sialic acid was determined according to the method of Massamiri *et al.* (4).

Treatment of erythrocytes with phospholipases. Ten volumes of a solution of phospholipase A₂ from bee venom (Sigma Co.), phospholipase C from *Clostridium perfringens* (P.L. Biochemicals Inc.) or phospholipase D from *Streptomyces chromofuscus* (a gift from Toyo Jozo Co.) in Tris-saline containing 5 mM CaCl₂ were added to one volume of a 50% erythrocyte suspension. The mixture, pH 7.4, was incubated at 37°C for 30 min, and the reaction was stopped by adding 11 volumes of isotonic saline containing 10 mM EDTA. Phospholipids were determined after isolation by two-dimensional TLC, and fatty acids were determined by gas chromatography after separation on a TLC plate, as described previously (1, 8).

Treatment of erythrocytes with exogenous lipids. One volume of palmitic acid or dilaurylphosphatidic acid emulsion in PBS was added to one volume of 20% erythrocyte suspension in PBS. This mixture, pH 7.4, was incubated at 37°C for 20 min. The amount of lipid incorporated into the cell was determined as described in the preceding section.

Cell electrophoresis. Isotonic HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid) and MES (2-morpholinoethanesulfonic acid) buffer solutions were prepared by adding NaCl to 100 mM solution of HEPES and MES; isotonicity was determined with an osmometer. Isotonic media for cell electrophoresis at pH 8 and ionic strengths of 0.07, 0.035 and 0.0175 were prepared by mixing one volume of isotonic HEPES buffer, pH 8, with 1.5, 4 and 9 volumes of a 5% (w/v) sorbitol solution. Isotonic media for cell electrophoresis with an ionic strength of 0.0175 at pHs 6, 7 and 8 were prepared by adding one volume of isotonic MES buffer, pH 6 or isotonic HEPES buffer, pH 7 and 8, to 9 volumes of 5% (w/v) sorbitol solution. The electrophoretic mobility of individual cells was measured at 25 ± 0.5°C with a Zeiss cytopherometer. Each cell was allowed to move 16 μm in either direction following the reversal of current in the media. Mobility was determined from separate experiments on 15 cells for each set of conditions and was calculated in μm·s⁻¹·V⁻¹·cm.

RESULTS

Human erythrocytes were treated with neuraminidase which removed about 96% of the sialic acid residues in their membranes. The electrophoretic mobility of these desialyated, intact erythrocytes is plotted against the ionic strength of the isotonic suspending medium at pH 8 (Fig. 1). Mobility was very low at an ionic strength of 0.070, but it increased considerably at 0.0175 to a level that was easily measurable by ordinary mobility determination. After further treatment with phospholipase A₂, which hydrolyzed about 40% of the phosphatidylcholine molecules in the outer leaflet of the membrane lipid bilayer, the cells were electrophoresed at various ionic strengths. Fig. 1 clearly shows that a mobility twice as high as that of the original desialyated cells was obtained at ionic strengths of 0.0175 and 0.035, but not at 0.070. In contrast,

Fig. 1. Dependence of the electrophoretic mobility of desialyated and intact erythrocytes with or without phospholipase A₂ treatment on the ionic strength values of the suspending medium. Desialyated (●) and intact (○) erythrocytes, and after further phospholipase A₂ treatment (▲ and △). Electrophoresis was at pH 8. Each point represents the mean value for 15 cells. The length of the vertical bar is the S. D.

intact erythrocytes had very high mobility, and treatment with phospholipase A₂ did not produce any appreciable increase in their mobility. This preliminary experiment showed that an ionic strength of 0.0175 should be used in the following experiments.

Five different types of treatments of the membrane lipid bilayer were applied to the desialyated erythrocytes, after which increases in the fatty acid or phosphatidic acid contents of the treated cells were determined. Incubation with 40 μ M palmitic acid and with 50 μ M dilauroylphosphatidic acid caused the incorporation of an appreciable amount of fatty acid and of phosphatidic acid into the cells (Table I), whereas incubation with phospholipase A₂ or with phospholipase D liberated considerable amounts of fatty and phosphatidic acids as a result of the hydrolysis of 44–48% of the phosphatidylcholine in the outer leaflet of the bilayer (1). Phospholipase C treatment produced neither fatty nor phosphatidic acids (it must split phosphatidylcholine into diacylglycerol and phosphorylcholine), although it hydrolyzed 30% of the phosphatidylcholine in the outer leaflet.

These treated erythrocytes and the control cells (desialyated erythrocytes) then were electrophoresed in isotonic media with an ionic strength of 0.0175 at pH 6, 7 and 8. Results are given in Fig. 2. Cells with increased fatty acid contents, as a result of palmitic acid or phospholipase A₂ treatment, had markedly higher mobility than the control at an alkaline pH, particularly in the medium at pH 8. Similar results were

TABLE 1. INCREASE IN THE CONTENT OF ACIDIC AMPHIPHILIC LIPID IN THE MEMBRANES OF HUMAN ERYTHROCYTES AS A RESULT OF TREATMENT OF THE MEMBRANE LIPID BILAYER

Treatment	Increase in lipid content (nmol/ml cells)		Hydrolysis % of PC in the outer half of the lipid bilayer
	Fatty acid	Phosphatidic acid	
Palmitic acid (40 μ M)	254	—	—
Phospholipase A ₂ *	417	—	44
Dilauroylphosphatidic acid (50 μ M)	—	284	—
Phospholipase D*	—	459	48
Phospholipase C*	—	—	30

* 40, 200 and 0.2 units respectively, of phospholipase A₂, D and C were used per ml of packed cells.

Fig. 2. Dependence of the electrophoretic mobility of desialyated erythrocytes with or without (the control) treatment of the lipid bilayer on the pH values of the suspending medium. Desialyated erythrocytes (the control) (●) and after phospholipase A₂ (Δ), palmitic acid (▲) and phospholipase D (□), dilauroylphosphatidic acid (■) and phospholipase C treatment (○). Electrophoresis was at an ionic strength of 0.0175. Each point represents the mean value for 15 cells. The length of the vertical bar is the S. D.

Fig. 3. Effect of the pH of the suspending medium on erythrocyte shape. Desialyated erythrocytes (the control) (1) were treated with 40 μ M palmitic acid (2), 50 μ M dilauroylphosphatidic acid (3), 200 units per ml packed cells of phospholipase D (4) or 0.2 units per ml packed cells of phospholipase C (5). Treated cells were suspended in a medium of pH 6 (a) or pH 8 (b).

obtained with desialyated cells which had an increased phosphatidic acid content after treatment with dilauroylphosphatidic acid or phospholipase D. In contrast, the mobility of the control cells and cells treated with phospholipase C showed no pH dependence, both had almost identical electrophoretic mobility at all pH values tested.

After desialyated erythrocytes had been treated with exogenously added PLases or amphiphilic lipids, they were placed in a medium at pH 6 or pH 8 (Fig. 3). The shape of the palmitic acid or dilauroylphosphatidic acid-treated cell was altered at pH 6 from that of a weak echinocyte (2a or 3a) to a spheroechinocyte at pH 8 (2b or 3b). A similar shape change was observed for PLase A₂-treated cells (data not shown). But, stomatocytes observed at pH 6 (4a) in the PLase D-treated cells became echinocytes at pH 8 (4b). In contrast, the morphology of untreated (biconcave disc, 1a and 1b) and PLase C-treated cells (stomatocyte, 5a and 5b) was only mildly dependent on the pH.

DISCUSSION

The electrophoretic mobility of human erythrocytes estimated in a commonly used medium of relatively high ionic strength reflects almost exclusively the negative charges of the sialic acid residues in the peripheries of the polypeptide chains protruding from the outer surface of the membrane. An increase in the electrophoretic mobility of the cell as the ionic strength of the measuring buffer solution decreases has been observed in erythrocytes and tumor cells. The internal ionized groups on the cell surface have a greater effect at low ionic strengths (2, 3). With decreasing ionic strength, the effective thickness of the double ionic layer at 25°C is $3.06 \times (\text{ionic strength})^{-1/2}$ Å, as calculated from Debye-Hückels equation (5). In our present electrophoretic study, we wanted to compare the electric charges of the dissociable polar head groups of the membrane lipids exposed to external medium at various pH values, thus we used human erythrocytes deprived of most (ca. 96%) of their sialic acid residues and an isotonic medium with a low ionic strength, 0.0175. Under these conditions, when we used the thickness of the ion atmosphere as an approximate estimation of the electrokinetic plane of shear, theoretically we could detect the

ionized groups in the peripheral 0–23 Å zone of shear. The dependency of electrophoretic mobility on ionic strength suggests that the polar head groups of the membrane lipid may exist in a zone deeper than 20 Å in desialydated erythrocytes.

The desialydated erythrocytes were treated as follows; incubation with palmitic acid or a medium-chain phosphatidic acid which is easily incorporated into the membrane (8); incubation with added phospholipase A₂ or D which hydrolyzes phospholipid molecules (mainly PC) in the outer leaflet of the lipid bilayer and liberates the fatty acid carboxyl group or the second OH group of phosphoric acid (1, 9); and incubation with phospholipase C which removes the phosphorylcholine moiety from the phospholipids in the outer leaflet. The first two treatments were to increase the number of anionic groups of the amphiphilic lipids exposed to the external medium, whereas the last treatment served as a control in the sense that it produced no dissociable polar head group *in situ* when the membrane lipid was hydrolyzed.

A definite increase in electrophoretic mobility was observed on the specimen after treatment 1 or 2 in a medium of pH 7 or 8, but not in medium of pH 6. In contrast, at all the pHs tested, after treatment 3 the erythrocytes showed mobility that was almost identical with that of the control cells which had had no treatment. These facts indicate that the increased mobility at these alkaline pH values reflects an increase in the negative charges produced by dissociation of the anionic head groups of the amphiphilic lipid molecules added or formed in the outer half of the lipid bilayer in the pH range of 6 to 8 because the COOH group of a long-chain fatty acid and the second OH of the phosphoric acid in the phosphatidic acid molecules in a monolayer or bilayer state dissociated in this pH range (6).

We demonstrated that the shape of desialydated erythrocyte becomes markedly dependent on the pH of the suspending medium after treatment of the cell membrane lipid bilayer to expose additional anionic groups of lipids. This confirms similar results obtained for native erythrocytes with intact sialic acid residues (8, 9). The treated cells became crenated to a greater extent at pH 8 than at pH 6 when treated with PLase A₂, palmitic acid or dilauroylphosphatidic acid. But, cells which were invaginated at pH 6 by PLase D treatment became crenated at pH 8. In contrast, the normal biconcave disc shape of the controls and the invaginated shape of the PLase C-treated cells remained about the same at pH 6 and 8. These results support our previous assumption (8, 9) that the negative charges of dissociable head groups of membrane lipids exposed to the external medium effect the membrane conformation and shape to a considerable extent, probably due to expansion of the outer half of the bilayer by increased repulsion among the charged groups or by increased hydration around these groups. Interestingly, removal of sialic acid residues, which contribute the majority of negative charges on the outer surface of the membrane, has no effect on the shape of the erythrocytes (9). Electrical charges in the immediate vicinity of the membrane bilayer surface may be what is important for the regulation of the membrane conformation and, hence, cell shape.

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