Shape Response of Human Erythrocytes to Altered Cell pH

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Alteration of red blood cell (RBC) pH produces stomatocytosis (at low pH) and echinocytosis (at high pH). Cell shrinkage potentiates high pH echinocytosis, but shrinkage alone does not cause echinocytosis. Mechanisms for these shape changes have not been described. In this study, measured dependence of RBC shape on cell pH was nonlinear, with a broad pH range in which normal discoid shape was maintained. Transbilayer distribution of phosphatidylcholine and phosphatidylserine, measured by back-extraction of radiolabeled lipid, was the same in control and altered pH cells. Possible roles of pH-titratable inner monolayer phospholipids were examined by assessing pH-dependent shape in cells in which their levels had been perturbed. In metabolically depleted cells and calcium-treated cells, which have altered levels of phosphatidic acid, phosphatidylinositol-4-phosphate, and/or phosphatidylinositol-4,5-bisphosphate, low cell pH was stomatocytogenic and high cell pH was echinocytogenic, as in control cells. Thus, neither change in membrane lipid asymmetry nor normal levels of the pH-titratable inner monolayer lipids is necessary for cell pH-mediated shape change.

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Many experimental treatments disturb the membrane curvature of usually smooth, discoid human erythrocytes, transforming them into invaginated (stomatocytic) and evaginated (echinocytic) forms. The shape transformations of red blood cells (RBCs) equilibrated in altered pH buffers require no exogenous compounds and are rapid and reversible. These characteristics suggest a mechanism involving noncovalent, reversible rearrangement of membrane components, but the structures involved have not been identified.

We have shown that cytoplasmic pH is the primary physiological mediator of RBC shape change in altered pH buffers. In vitro, the spectrin-based membrane skeleton contracts at low pH and expands at high pH, as predicted by ionic gel theory. Because the membrane skeleton is physically coupled to the RBC inner membrane, these skeleton changes are expected to cause speculation at low pH and cupping at high pH. Isolated membranes (ghosts) display the predicted shapes, but, paradoxically, intact cells are stomatocytes at low cell pH and echinocytes at high cell pH. Therefore, membrane components in addition to the membrane skeleton must affect shape in intact erythrocytes.

Changes in membrane lipids can affect RBC shape by perturbing the balance in area between the two lipid leaflets. In normal discoid cells, most phosphatidylincholine is located in the outer lipid leaflet, whereas virtually all phosphatidylserine is located in the inner leaflet. Because alteration of this distribution produces abnormal membrane curvature, the possibility that abnormal lipid distribution underlies the shape changes of cells at low and high pH was examined. Another possibility is that charge interactions of lipid headgroups are important in maintaining normal membrane curvature and that pH titration changes lipid headgroup charge and therefore shape. Specifically, the RBC inner monolayer contains three negatively charged phospholipids that titrate in the physiologic pH range, ie, phosphatidylinositol-4-phosphate, phosphatidylinositol-4,5-bisphosphate, and phosphatidic acid. Low cell pH would decrease net negative charge on these lipids, whereas high cell pH would increase net negative charge. Because a 1% change in area of a lipid layer causes significant change in membrane curvature and dephosphorylation of phosphatidylinositols is closely linked with shape change, change in headgroup electrostatic interactions upon pH titration might produce pH-mediated RBC shape change.

The roles of membrane lipids in cell pH-mediated shape change were examined by two approaches. Lipid asymmetry in altered pH cells was assessed by back-extraction of incorporated radiolabeled lipid, and cells with reduced amounts of pH-titratable lipids (metabolically depleted and calcium-loaded cells) were prepared and their shape response to altered pH was assessed.

Materials and Methods

Materials. Chemicals were purchased as follows: 2-[N-morpholino]ethanesulfonic acid (MES), N-[2-hydroxyethyl]piperazineN'-[2-ethanesulfonic acid] (HEPES), and 2-[N-cyclohexylamino]-ethanesulfonic acid (CHES) from Sigma (St Louis, MO), and A12387 from Calbiochem (San Diego, CA). Other chemicals were of reagent grade. [14C]Dilauroylphosphatidylcholine ([14C]-DLPC) and [3H]dioleoylphosphatidylserine ([3H]DOPS) were synthesized as described.

RBC were obtained by venipuncture from normal adult volunteers, separated from plasma and Buffy coat by centrifugation, and washed three times in normal saline. Cells were resuspended in phosphate-buffered saline (PBS; 138 mmol/L sodium chloride, 5 mmol/L potassium chloride, 7.5 mmol/L sodium phosphate, 1 mmol/L magnesium sulfate, and 5 mmol/L glucose, pH 7.4). Cells not used immediately were stored at 4°C and used within 12 hours.

Equilibration in altered pH buffers. To adjust cell properties, cells were resuspended (10%) and equilibrated (for 5 minutes) three times in an experimental buffer.

Assay of cell properties. Procedures used to assay morphology and cell properties have been described in detail. Briefly, cell morphology was assayed by fixing cells at 0.7% hematocrit (Hct) in 0.2% glutaraldehyde made in each sample's supernatant solution and examining them using light microscopy.

Morphology was scored on a scale of −4 to +5 as described, with stomatocytes assigned negative scores and echinocytes assigned positive scores. The morphologic index for each sample was the

average score of at least 100 cells. Where absent in figures, error bars are smaller than symbol size. Cell pH was measured directly in packed, freeze-thaw lysed RBC pellets. Membrane potential was calculated from external and cell pH using the Nernst equation. Cell water was measured gravimetrically in packed cell pellets and adjusted for the presence of trapped supernatant.

Assessment of lipid asymmetry. Cells were incubated at 37°C with sonicated vesicle suspensions of [14C]DLPC and [1H]DLPS to steady state as described.1 After equilibration of labeled cells in altered pH solutions, radiolabeled lipids present in the outer monolayer were back-extracted with bovine serum albumin, and radiolabel remaining in the cells was determined by scintillation counting.12 Because lipid-specific activity was high, the amount of incorporation needed for quantitation was small, and the exogenous lipid had no independent effect on cell shape.

Metabolic depletion. Cells were incubated at 37°C in 145 mmol/L NaCl, 20 mmol/L HEPES, pH 7.4, in the presence of 100 μg/mL each of penicillin and streptomycin for the indicated times.

Calcium treatment. Cells were incubated at 37°C (20% Hct) were washed twice in KCl-HEPES (130 mmol/L KCl, 2 mmol/L MgSO4, 20 mmol/L HEPES, pH 7.4) to remove phosphate and then incubated (37°C for 20 minutes) in KCl-HEPES plus 0.5 mmol/L Ca2+ in the presence of 5 μmol/L A23187 (ethanol <1%, vol/vol). Buffer potassium prevented net potassium efflux through calcium-gated channels, preventing cell shrinkage. Control cells were treated identically, except that Ca2+ was omitted.

For recovery from calcium treatment, cells were washed twice (4°C, 5% Hct) in KCl-HEPES buffer plus 4 mmol/L EGTA and then incubated (37°C for 30 minutes) in KCl-HEPES buffer plus 10 mmol/L inosine, 10 mmol/L glucose, and 1 mmol/L adenosine.

RESULTS

Dependence of RBC shape on cell pH. We previously showed that cell pH is the major mediator of RBC shape change in altered pH environments.10 The conclusion was reached by analyzing multiple experiments that assessed cell shape in different combinations of cell physiologic properties.

For a direct demonstration of how erythrocyte shape depends on cell pH, buffer conditions were devised to hold membrane potential and cell water within normal ranges while cell pH was varied widely. Figures 1 and 2 show a representative experiment. Striking nonlinearity in the dependence of shape on cell pH was observed. Normal discoid cell shape was maintained throughout a broad cell pH range (6.3 to 7.9; Figs 1 and 2b through d). Beyond these limits, membrane curvature was abnormal (Figs 1 and 2A and E).

Cell shrinkage at normal cell pH. Cells shrinken in hypertonic saline at normal cell pH were flattened, yet smooth and discoid (Fig 2f). In contrast, whereas some high pH cells were flattened, none was smooth; cells lacking definite membrane spikes had irregular surfaces (Fig 2e).

Altered cell pH and lipid asymmetry. The possibility that shape change in altered pH solutions resulted from transbilayer shifts in lipid distribution was examined by assessing the accessibility to albumin extraction of previously incorporated [14C]DLPC and [1H]DLPS12 in low, normal, and high pH cells. The percentage of labeled phosphatidylcholine present in the inner monolayer was the same in pH 5.5, 7.2, and 9.0 cells (average among conditions, 13.2% ± 0.7%, compared with 12.3% ± 2.6% for control). The percentage of labeled phosphatidyserine in the inner monolayer was also the same among experimental conditions (average, 96.0% ± 0.4%, compared with 94.5% ± 2.3% for control). Thus, a shift in phosphatidylcholine or phosphatidylserine distribution does not accompany and cannot explain pH-dependent shape changes.

Cells with depleted inner membrane pH titratable lipids. To test the hypothesis that the pH titratable inner membrane lipids phosphatidic acid, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-bisphosphate have roles in cell pH-mediated shape change, cells with altered levels of these lipids were prepared and their shape response to altered cell pH was observed.

Metabolic depletion has been shown to induce dephosphorylation of phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol and of phosphatidic acid to diacylglycerol.7 Maximally depleted cells contain approximately 30% of normal phosphatidylinositol-4,5-bisphosphate levels, unchanged phosphatidylinositol-4-phosphate levels, and 80% of normal phosphatidic acid levels. Accompanying these changes in lipid levels is a progressive echinocytosis to morphologic index of +3 (stage I spherocytosis) in maximally depleted cells.

Based on the reported data, metabolically depleted cells were used as a model in which to assess involvement of phosphatidylinositol-4,5-bisphosphate and phosphatidic acid in cell pH-mediated shape change. Progressively metabolically depleted cells with initial levels of echinocytosis as shown in Fig 3A (solid symbols) were placed at low cell pH, and their shape responses were compared with those of normal, replicate control cells (Fig 3A, open symbol). In all cases, low pH treatment caused morphologic indices to become more negative (ie, morphologic index as a function of decreasing pH was uniformly negative). The magnitude of
the shape response (the slope of the plot) was at least as great in depleted samples as in controls.

Calcium-treated RBCs crenate severely, reportedly because a calcium-activated phospholipase C depletes inner membrane lipids. In calcium-treated cells, the levels of phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate are about 20% of those of untreated cells, but the levels of phosphatidic acid are normal. After incubation with supplements to restore the metabolically replete state, cells assume a more discoid morphology. Phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate levels remain 20% of normal; phosphatidic acid levels increase because of phosphorylation of accumulated diacylglycerol. Figure 3B shows the effects of pH on the morphologies of untreated, calcium-treated, and calcium-treated, metabolically replete cells. Low cell pH again made the morphologic index more negative in each preparation, whereas high cell pH produced a more positive morphologic index in the conditions examined.

In summary, these experiments altered the populations of those inner monolayer phospholipids expected to protonate/deprotonate in the pH range examined. In some specimens, phosphatidylinositol-4,5-bisphosphate was expected to be deeply depleted, whereas phosphatidylinositol-4-phosphate levels remained normal; in other conditions, depletion of both phosphatidylinositol-4,5-bisphosphate and phosphatidylinositol-4-phosphate was expected. Phosphatidic acid levels were predicted to be mildly depleted, normal, or elevated. In each combination, the direction and magnitude of cell shape response to lowered or elevated cell pH was essentially the same as those of controls.

**DISCUSSION**

Response of RBC shape to change in cell pH. The membrane skeleton is expected to exert pH-dependent effects on membrane curvature in intact cells. Because skeleton gel tension increases steadily throughout the studied pH range, the morphologic index would be expected to decrease as cell pH increases, and the decrease should be approximately linear. In intact cells, not only does the morphologic index increase as the cell pH increases, but the transformation is strikingly nonlinear, with a plateau of normal morphology extending well below and above physiologic cell pH (Fig 1). Combination of membrane skeleton effects with effects of other pH-sensitive membrane structures could produce this complex dependence of cell morphology on cell pH.

Possible roles of membrane lipids in pH-mediated shape change. Changes in membrane lipids might be important
in pH-mediated shape change. At room temperature, this shape change occurs immediately upon titration of cytoplasmic pH and is rapidly reversed. Thus, it is unlikely that degradation or synthesis of lipid, which require more extended incubation at 37°C, are involved. However, alteration of cell pH might allow rapid transbilayer movement of lipid by creating pH-dependent "flip-flop" sites. Alternatively, pH titration of charged inner monolayer lipids might affect headgroup electrostatic interactions sufficiently to change bilayer balance and shape. These two possible mechanisms of lipid involvement were examined.

Distributions of radiolabeled phosphatidylcholine and phosphatidylethanolamine were determined in normal and altered pH cells as a measure of overall membrane lipid disposition. These two lipids were distributed identically in normal and altered pH cells. Thus, the change in lipid asymmetry does not account for the shape change in these cells.

Role of pH titratable inner membrane lipids. Lipids whose net charge changes in the pH range in which shape change occurs might be important in the shape change mechanism. Although there is uncertainty about the in situ dissociation constants of titratable RBC phospholipid protons, some clearly titrate outside the pH range in which shape effects occur. The phosphate group protons of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol dissociate below pH 3; the carboxyl group on phosphatidylserine has a pKa around 4; and the amines on phosphatidylethanolamine and phosphatidylserine titrate have a pKa around 10.

In contrast, the phosphate groups on phosphatidic acid, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-bisphosphate bear protons that dissociate around pH 7. Each site is expected to be fully protonated at pH 5.5 and fully deprotonated at pH 9.0. These three phospholipids might participate in a membrane structure that counterbalances membrane skeleton shape effects.

This hypothesis was tested by depleting these inner membrane phospholipids by metabolic depletion and by calcium treatment. If the presence of these phospholipids was important in opposing membrane skeleton pH effects, depletion would reverse the direction of cell pH-mediated shape change toward that predicted by ionic gel theory. However, cell preparations with a range of degrees of depletion of phosphatidylinositol-4-phosphate, phosphatidylserine, and phosphatidic acid all had normal responses to altered cell pH (Fig 3). Thus, these experiments do not support the hypothesis that pH titratable inner membrane lipids are involved in cell pH-mediated shape change.

Role of cell shrinkage in crenation. When RBCs are shrunken or swollen at normal cell pH, they become either flattened or plump, but the smoothness of the cell surface is not disturbed (Fig 2 and Gedde et al16). In contrast, early RBC studies linked shrinkage and echinocytosis; for example, an authoritative study of RBC membrane mechanics used preparations of hypercontracted erythrocytes. Also, cell water content strongly modulates high cell pH echinocytosis, with cell shrinkage intensifying the crenation and cell swelling relieving it. These observations should be reconciled with the finding that membrane curvature is not affected by cell water change alone.

From the perspective of membrane mechanics, cell water content is expected to affect cell shape through its effects on cell deformability, because the cell surface area to volume ratio (SA:V) restricts the range of geometrically allowed shapes. At maximum cell water, when SA:V is at a minimum, the only allowed shape is a sphere. As the cell shrinks, the SA:V increases and more shapes are permitted.

Covariance of cell water content and cell deformability appears to explain the impact of cell water content on high cell pH echinocytosis. A swollen high pH cell would be less deformable than one with normal water content, so the most strongly crenated shapes would be disallowed. Conversely, a high pH cell that is shrunken would have an increased cell deformability, allowing a degree of echinocytosis otherwise not permitted.

The relationship between cell water content and deformability may also explain the report of crenation in RBCs shrunken in hypertonic saline. In that study, drops of whole blood were mixed with several volumes of saline on glass slides. Cells mixed with isotonic saline had normal biconcave shape on glass, whereas cells mixed with hypertonic saline were shrunken and crenated.

Washed RBCs placed on glass become echinocytic (glass crenation). An increase of pH of solutions in contact with glass is involved in this shape change, and cells on glass may have elevated cell pH. The albumin in whole blood, which binds to the RBC outer glycocalyx, stabilizes discocytes against glass crenation. The cells in whole blood diluted in isotonic or hypertonic saline on glass were exposed to identical pH and albumin concentrations; they differed only in their cell water content. The shrunken cells had higher SA:V and greater deformability. Thus, crenation of the shrunken cells may represent a degree of glass crenation that is preventable by albumin binding to cells with normal water content but is not preventable in the more deformable shrunken cells.
Significance of cell pH-induced shape change mechanism.
The membrane skeleton is expected to exert pH-dependent
effects on cell shape, but in a direction opposite to the ob-
served shape responses of intact cells. This suggests that a
homeostatic, pH-sensitive mechanism counteracts pH shape
effects of the membrane skeleton in the cell pH range 6.3
to 7.9. Such a mechanism would be important in the RBC,
which must maintain its smooth shape in hypoxic as well as
well-perfused tissues. Because many types of cells have
spectrin-based membrane skeletons, a process that balances
membrane skeleton effects on cell shape during pH shifts
could be of general importance.

This study has shown that membrane lipids do not play a
major role in membrane shape homeostasis in altered pH
conditions. However, preliminary observations indicate that
association of a 36-kD cytoplasmic protein with the RBC
inner membrane correlates with pH-dependent shape change
in intact erythrocytes. Details of structures involved in this
process remain to be clarified.

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