Membrane Mechanical Properties of ATP-depleted Human Erythrocytes

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Although the relationships between the metabolic state and the mechanical properties of human red blood cells (RBC) continue to be of current interest, literature reports in this area are not in agreement. The present investigation was designed to determine several intrinsic mechanical properties of human RBC membranes before and after metabolic depletion via incubation at 37°C for 24 hr. Using micropipette and flow channel techniques, three properties were measured: (1) μ, surface shear modulus of elasticity; (2) K, elastic area compressibility modulus; (3) η, shear viscosity in the plastic domain. Our results indicate no significant differences in these parameters between fresh and ATP-depleted human RBC membranes. These present data are thus in disagreement with other literature reports indicating large changes in membrane mechanical properties consequent to metabolic depletion. A brief discussion of the possible reasons for this disagreement is presented.

Although the relationships between the metabolic state and the mechanical properties of human red blood cells (RBC) continue to be of current interest, literature reports in this area are not in agreement. Weed et al. reported the following consequent to metabolic depletion via 24-hr incubation at 37°C: (1) discocyte-echinocyte shape transformation; (2) increased apparent viscosity and more pronounced non-Newtonian flow behavior of RBC-buffer suspensions; and (3) a 12- to 14-fold decrease in membrane deformability as judged by the negative pressure necessary to produce hemispherical deformation of the RBC membrane into a 3-μm micropipette. ATP-calcium-dependent sol-gel changes occurring at the cytoplasmic surface of the membrane were proposed as the basis for the changes in membrane deformability and suspension rheology. Similar ATP-related changes for RBC stored in acid citrate dextrose (ACD) solution have also been obtained: (1) negative pressure for membrane hemisphere deformation into a 2.85-μm micropipette increased threefold after 21 days of storage and nearly ninefold after a 56-day storage period; (2) RBC-buffer suspensions showed a progressive increase in low shear rate viscosity with time of storage that could be returned toward control via adenosine treatment to regenerate intracellular ATP.

Other studies of the mechanical properties of depleted RBC are not consistent with the above-mentioned results. Employing membrane hemisphere deformation into 1.5-μm micropipettes, Leblond reported an identical twofold increase in the negative pressure required for fresh echinocytic RBC produced by shape altering...
agents and for echinocytes produced by incubation at 37°C for 20 hr. Heusinkveld and co-workers, using 1-2.5 μm micropipettes, reported no significant difference in negative pressure for hemisphere formation between fresh RBC and RBC incubated at 37°C for up to 45 hr. Cells partially fixed with low concentrations of glutaraldehyde, however, did show decreased membrane deformability, thus validating his experimental technique. Furthermore, recent studies by one of us indicate that the elevated low shear rate viscosity of RBC suspensions consequent to ATP depletion can be returned to control (i.e., fresh, ATP-rich RBC) levels by restoring RBC morphology to the discocyte form without restoring intracellular ATP levels, thereby indicating that the extrinsic shape of the cell affects bulk rheologic behavior. Additionally, Feo and Mohandas showed that RBC deformability (as measured by a viscometric-diffactometric system) is independent of the intracellular levels of ATP and depends only on the shape of the cells. A completely satisfactory reconciliation between the earlier data and these later studies is not yet possible.

The present investigation was designed to determine three intrinsic material constants of human RBC membranes both before and after metabolic depletion via incubation at 37°C for 24 hr. Our results, which include data on the surface shear modulus of elasticity, elastic area compressibility modulus, and the surface shear viscosity in the plastic domain, indicate no significant differences in any of these parameters between fresh and ATP-depleted human red blood cell membranes.

MATERIALS AND METHODS

Blood and RBC preparation. Blood was collected from hematologically normal adult donors via venipuncture into heparin (5 IU/ml blood) and, unless incubated, was used within 4 hr after collection. Red cells were isolated by centrifugation at 2000g for 10 min, and the plasma and buffy coat were discarded by gentle aspiration. The cells were washed twice via centrifugation-aspiration in a Tris-hydroxymethylaminomethane isotonic buffer (Tris: 0.015 M, CaCl₂: 0.002 M, NaCl: 0.143 M, pH 7.40 ± 0.02 at 25°C, 292 ± 2 mOsm/kg). The Tris buffer contained 0.5% serum albumin. Following the second wash, the packed RBC were suspended in this buffer (at low PCV (0.01-0.05) for measurement of the membrane mechanical properties of fresh RBC.

Metabolic depletion via incubation was carried out after the above-mentioned washing procedure; the Tris buffer used for both the initial washing and incubation was adjusted to pH 7.70 ± 0.02 at 25°C so that its pH at 37°C would be 7.40. Penicillin (100 U/ml) and streptomycin (0.1 mg/ml) were added to prevent bacterial contamination. Following the second wash, the packed RBC were suspended to a PCV of approximately 0.40, and the suspension was incubated for 24 hr at 37°C in an incubator oven; gentle mixing of these suspensions occurred once at about 12 hr. After the 24-hr period, the RBC were washed twice more in the pH 7.4 Tris buffer, then resuspended in this buffer (at low PCV) for measurement of the membrane mechanical properties of ATP-depleted RBC.

Surface shear modulus and area compressibility measurements. The measurements of the surface modulus of elasticity (μ, dynes/cm) and the elastic area compressibility modulus (K, dynes/cm) were performed using micropipettes. Inasmuch as the details of these techniques have been presented elsewhere, only a brief discussion of each is presented below. All measurements were made at room temperature (22 ± 1°C).

Surface shear modulus of elasticity, μ. After placing the dilute RBC-Tris buffer suspension into a special microchamber on the stage of an inverted light microscope, a small (inside diameter ≤ 1.5 μm) buffer-filled micropipette was positioned in the field near the RBC to be measured. Following proper zeroing of the pressure measuring system attached to the pipette, the central portion of the flaccid discocyte cell membrane was sucked into the pipette such that a decrease in pressure caused a "tongue" of the membrane to extend into the pipette. Since the pipette diameter was much less than the cell diameter, the influence of cell geometry (an extrinsic factor) was eliminated, thus allowing measure-
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ment of the intrinsic membrane material property, termed shear modulus, \( \mu \). This approach is valid provided that no membrane folding or "buckling" occurs. When membrane buckling does occur, the cell folds and moves easily into the pipet (the fold is observable). The aspiration experiments were always conducted such that membrane buckling and folding were avoided.

The data relating membrane deformation to negative pressure inside the pipette were analyzed to obtain the quantity \( \mu \) via a first-order tension-deformation law:9

\[
T_x = \frac{1}{2} \mu (\lambda^2 - \lambda_x^{-2}),
\]

where \( T_x \) is the membrane tension acting in the \( x \) direction and \( \lambda_x \) is the extension or stretch ratio for the cell surface (final length divided by initial length). The extension ratio squared is essentially proportional to the aspirated tongue length divided by the pipette radius.\(^9\) Tongue lengths were observed over a range of one to four pipette radii. In all, 14 fresh and 38 ATP-depleted RBC were measured via this method.

Elastic area compressibility modulus \( K \). In this technique, slightly larger (inside diameter 1.8-2.4 \( \mu \)m) micropipettes were used to suck a "tongue" of cell membrane into the pipette, and measurements were made of changes of tongue length with applied negative suction pressure. Since it was necessary to know the total cell membrane area at any given suction pressure, osmotically preswollen cells were used, thereby resulting in a nearly spherical shape for the portion the the RBC outside the pipette. Thus for this measurement only the cells were suspended in 140-150 mOsm/kg, pH 7.4 Tris-CaCl\(_2\)-NaCl buffer just before the experiments were performed. At this osmolality, the RBC were nearly spherical, so that the aspirated length of the cell projection into the pipette was between one and two pipette diameters. With the portion of the cell outside the pipette in a spherical shape, small movements of the aspirated tongue were proportional to changes in membrane surface area. Precautions were taken to ensure that adhesion to the pipette glass did not occur along the length of the aspirated "tongue" length by careful cleaning of the pipette and careful filtration of the albuminated suspending and pipette-filling buffers.

The data relating total membrane area (as determined from simultaneous measurements of "tongue" length, pipette diameter, and diameter of spherical portion of cell outside of pipette) and negative suction pressure were analyzed to obtain \( K \) via a first-order linear form of an elastic constitutive relation as given by Evans et al.:\(^9\)

\[
\bar{T} = K \Delta \alpha,
\]

where \( \bar{T} \) is the locally isotropic membrane tension and \( \Delta \alpha \) is the fractional change in membrane area (dilation) \( \Delta A/A_0 \). \( K \) was measured for 46 fresh and 90 ATP-depleted RBC; all measurements were made with the cells in the special microchamber on the stage of an inverted microscope as described above for the shear modulus technique.

Shear viscosity in plastic domain, \( \eta_p \). The technique used for measurement of the plastic growth of RBC membrane "tethers" was that described by Hochmuth et al.\(^13\) Briefly, RBC adhering to the surface of a parallel-plate flow chamber form membrane tethers when subjected to fluid shear stress at the wall in excess of approximately 1.5-2 dynes/cm\(^2\).\(^{14-18}\) Tether growth, as indicated by the motion of the cell along the surface, was recorded on video tape and subsequently graphed. The slope of the line for cell position versus time yields the tether growth rate (\( \dot{L}, \mu\text{m/sec} \)), where, at a given shear stress in the channel, the tether growth rate \( \dot{L} \) is inversely proportional to the plastic viscosity \( \eta_p \), which represents frictional energy dissipation during irreversible membrane extension (plastic deformation).

The relation between \( \dot{L} \) and \( \eta_p \) has been given as \(^{14,15}\)

\[
\eta_p = \tau_{\text{crit}} A \dot{L}/8\dot{L} \pi.
\]

where \( A \) is the area of the cell in contact with the flowing fluid and \( \dot{G}_i \) is a dimensionless "tether growth parameter" that is a function only of the ratio of \( \tau \) (the constant fluid shear stress acting on the cell during tether growth) to \( \tau_{\text{crit}} \) (the critical fluid shear stress required to form tethers). Previous reports\(^{14-16} \) of \( \tau_{\text{crit}} \) for fresh biconcave RBC indicated that it is on the order of 1.5-2.0 dyne/cm\(^2\), and three measurements performed for this experimental series gave values for \( \tau_{\text{crit}} \) of 1.85-2.2 dynes/cm\(^2\) for ATP-depleted RBC. Therefore in the present study, where the fluid shear stress at the wall (\( \tau \)) was held constant at 3.5 dynes/cm\(^2\), \( \tau/\tau_{\text{crit}} = 1.75 \) (for \( \tau_{\text{crit}} = 2.0 \) dynes/cm\(^2\)), yielding a value of \( G_i = 2.0. \) The tether growth rate and thus \( \eta_p \) was determined for 19 ATP-depleted RBC; all measurements were carried out at room temperature (22° ± 1°C).
RESULTS AND DISCUSSION

Three intrinsic mechanical properties of the RBC membrane were measured for this study: (1) shear modulus of elasticity, \( \mu \), an index to the forces necessary to "stretch" or deform a portion of the membrane without changing the surface area of the membrane; (2) elastic area compressibility modulus, \( K \), a measure characterizing the resistance to membrane area expansion or compression; and (3) shear viscosity in plastic domain, \( \eta_p \), a mechanical property that characterizes the rate at which the membrane undergoes plastic, permanent deformation once the applied forces exceed a critical level. Note that the measurement of the shear modulus of elasticity, \( \mu \), is qualitatively analogous to the earlier measures of negative pressure for hemisphere formation in micropipettes in that similar basic data are obtained; calculation of \( \mu \) from these data involves application of a first-order tension deformation law. However, experimental errors and small initial membrane tensions can compromise the validity of the single hemisphere—pressure calculation (data for progressive aspiration lengths are essential for reliable analysis). Note also that for all three mechanical properties (\( \mu, K, \eta_p \)) an increase above the fresh control RBC levels would indicate "increased rigidity" or reduced "deformability" of the RBC membrane.

The experimental results are shown in Table 1, where the mean (± SD) values of \( \mu, K, \) and \( \eta_p \) are listed for fresh and ATP-depleted RBC. The \( \eta_p \) data for the 48 fresh RBC were obtained in a previous study using experimental conditions identical to those employed for the present investigation. Inspection of the data indicates no significant changes in these three intrinsic mechanical properties of the RBC membrane consequent to ATP depletion via incubation at 37°C for 24 hr. Our results for the shear modulus of elasticity, \( \mu \), are thus in agreement with the measurements of Heusinkveld et al. In addition, the information indicates that the membrane's resistance to area expansion (\( K \)) as well as its rate of plastic permanent deformation (\( \eta_p \)) are unaffected by metabolic loss of ATP.

In discussing these results, it is important to note that whereas the calculation of \( \mu \) and \( K \) from the raw experimental data does not involve a priori determination of cell morphology, the calculation of \( \eta_p \) is based upon an estimated cellular area \( A \) in contact with the flowing fluid. That is, a value for \( L \) for each cell is substituted into Eq. (3) and a value of \( \eta_p \) is calculated using the estimated area \( A \). The choice of \( A \) affects the product of \( \tau_{crit} \times A \), which is the estimate for the "true" critical force on the cell. For example, a flat disk with radius \( R \) has an exposed area of \( A = \pi R^2 \), whereas the exposed area of a hemisphere of the same dimension is twice as much. Because of the spherical morphology of the ATP-depleted

<table>
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<tr>
<th>Condition</th>
<th>Shear Modulus of Elasticity (( \mu ), dynes/cm)</th>
<th>Elastic Area Compressibility Modulus (( K ), dynes/cm)</th>
<th>Shear Viscosity in Plastic Domain (( \eta_p ), dynes sec/cm)</th>
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<tbody>
<tr>
<td>Fresh</td>
<td>0.018 ± 0.004 ( (n = 14) )</td>
<td>353 ± 121 ( (n = 46) )</td>
<td>0.010 ± 0.004* ( (n = 48) )</td>
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<tr>
<td>ATP-depleted via 24-hr incubation</td>
<td>0.021 ± 0.004 ( (n = 38) )</td>
<td>402 ± 127 ( (n = 90) )</td>
<td>0.0068 ± 0.0036 ( (n = 19) )</td>
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* From Reference 13.
echinocyte, the exposed area used in the drag calculation is 100 μm² instead of the value of 50 μm² appropriate to the cross section.

Our results indicate no change in the shear modulus of elasticity following ATP depletion, clearly in disagreement with the earlier studies of Weed et al. The basis for this discrepancy appears to be the methods of analysis and instrumentation used for these earlier studies. It can be shown that the original design of the pressure-measuring manometer and the use of mouth suction to draw the hemisphere of RBC membrane into the micropipettes produces overestimates of the actual negative pressures involved, particularly for the shape-transformed ATP-depleted RBC. Furthermore, the use of large-bore (2.5–3.5 μm) micropipettes in these earlier studies may have produced overestimates of this negative pressure because the magnitude of the pressure associated with the application of large pipettes depends on the intrinsic membrane shear modulus as measured in our study convolved with the extrinsic shape or geometry of the whole cell. Thus whole-cell deformability includes both factors and has been estimated by micropipette transit pressure or by filtration methods. This has been shown to decrease (entire cell less deformable) consequent to ATP depletion via incubation. Inasmuch as the later studies of Heusinkveld et al. indicated no change in shear modulus consequent to ATP depletion, this experimental apparatus hypothesis appears tenable. Also, initial membrane tensions associated with shape transformations may greatly affect the hemisphere pressure but not the “slope” of the pressure versus aspirated length data, which determines the intrinsic membrane shear modulus. It has been shown theoretically that very small curvature elastic energy changes (induced bending moments) can produce membrane shape transformations without changing the membrane surface elastic properties (area compressibility and resistance to stretch at constant area).

REFERENCES


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