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# REDUCED ERYTHROCYTE DEFORMABILITY ASSOCIATED WITH CALCIUM ACCUMULATION

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**Erythrocytes exposed to subhemolytic shear stress in vitro exhibit decreased deformability as determined by a filtration method. Intracellular calcium content of these cells has been measured by atomic absorption spectroscopy and found to be 35 and 55% higher than controls (0.0157 $\mu$mol/ml packed red blood cells) after shear stress levels of 100 and 130 N/cm$^2$, respectively. These alterations occur without significant changes in ATP level, intracellular magnesium content, cell volume, or morphology, and without large associated sodium and potassium fluxes. Results indicate that calcium may be responsible for or associated with changes in the viscoelastic properties of the red cell membrane caused by sublytic mechanical trauma.**

## Introduction

Blood flow through certain medical devices, including prosthetic heart valves and hemodialysis units, is known or suspected to produce shear forces higher than those found in the normal circulation. One consequence of exposure to these forces may be a reduced red cell life span for the patient's erythrocytes. To mimic the effects of this mechanical trauma in the laboratory, cells from normal, healthy individuals have been exposed to similar levels of shear stress in the controlled environment of a viscometer. We and others have previously described a reproducible defect of decreased red cell deformability in normal erythrocytes exposed to sublytic levels of shear stress in vitro [1,2].

The mechanism(s) for this alteration of deformability is not known. Introduction of the model defect occurs without changes in morphology or significant decrease of cell contents and without a large drop in ATP [1,3]. Increased intracellular calcium is now reported for these cells with unaltered cell volume and only small shear-associated sodium and potassium ion fluxes.

The role of calcium in affecting erythrocyte deformability has been a subject of great debate. Reports conflict as to whether or not membrane viscoelastic properties are altered at elevated red cell calcium concentrations. For ATP-depleted red blood cells, Weed et al. [4] found increased calcium to be associated with decreased membrane deformability and filterability. Similar results were reported by Kirkpatrick et al. [5] after calcium-loading of erythrocytes with the ionophore A23187. However, using laser diffraction patterns of red cells in a shear field to measure deformability, Clark et al. [6] reported that increased internal viscosity is responsible for the decreased deformability of the ionophore- and calcium-treated erythrocyte. Dreher et al. [7] recently suggested that the decrease in deformability is rather due to increased membrane rigidity but that the changes

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occurs only if calcium accumulation is accompanied by a decrease in cell volume, loss of potassium, and ATP hydrolysis.

Confusion over the effect of calcium largely arises from the concomitant phenomena which accompany the usual methods of introducing calcium into the erythrocyte [8–12]. Included among these complicating factors are ATP depletion, volume and potassium loss, and changes in morphology, processes which do not occur with exposure to subhemolytic shear stress. This in vitro system thus appears to be an excellent model for investigating the details of the biochemical control mechanism of red cell deformability under conditions where morphology and cell volume changes are minimal.

## Materials and Methods

*Processing of erythrocytes*

Fresh whole blood was drawn by venipuncture from fasting, nonsmoking human donors and added immediately to premeasured 3.8% citrate-anticoagulant (1.1 ml/10 ml whole blood) in 15 ml vacutainers. Separation of erythrocytes was begun by centrifugation at $1000 \times g$ for 10 min. By Pasteur pipette, plasma and buffy coat were removed and discarded. A few red cells were necessarily lost in the process. By resuspension, centrifugation, and removal of supernatant and any pellicle, the red cells were sequentially washed once with saline and twice with Ringer's solution (147.5 mM NaCl, 4 mM KCl, 2.25 mM $CaCl_2$). This washing procedure results in removal of approximately 90% of the platelets and 80% of the leukocytes as determined by successive counting using a hemocytometer. Cells were finally suspended in Ringers/albumin (50 mg/l) and the concentration adjusted to 3.5 $10^6$ cells/$\mu$l with the aid of a Coulter electronic particle counter. Measurement of the packed cell volume by the microcapillary method enabled calculation of the mean cell volume. Size distributions were observed with Coulter Channelizer and *X-Y* Plotter accessories. Micropipette measurements to test for surface area and volume differences were made in 156 mosM saline/albumin with a 1.8 $\mu$m diameter pipette. Hypotonic conditions allow a known, simple cell geometry in the pipette without folding or area dilation of the membrane. Heat treated erythrocytes were produced by incubation of washed red blood cells at 49°C for 60 min. Glutaraldehyde fixation was accomplished by incubation of washed red blood cells in 2% glutaraldehyde (final concentration) for 30 min, with subsequent washing in saline and redispersion in the Ringers/albumin solution.

*Exposure of erythrocytes to shear stress*

Erythrocytes were exposed to subhemolytic shear forces in the Ruska (II) cup-and-bob viscometer. Details of the features and use of this viscometer have been reported [13]. The viscometer was designed and built at Rice University. It has a concentric cylinder geometry with cone-and-plate and cone-and-cone end regions; angles in the end regions and the gap between the cylinders are designed such that a constant shear stress is applied throughout the sample at a given rotational speed. This hybrid geometry minimizes the effects of the air-sample interface. A calibrated tachogenerator and torque transducer indicate the shear rate and shear stress, respectively. The washed red cell suspension was introduced by a valve through the bottom of the cup; the sample was brought to the desired stress level 100 or 130 $N/m^2$ (for this exposure time, the threshold for hemolysis is 150 $N/m^2$ [14]) and left for 2 min. After withdrawal of the sample, the sheared suspension was divided for determination of red cell deformability and assays of calcium, magnesium, sodium, and potassium.

*Deformability measurement*

Erythrocyte deformability was evaluated by a filtration method in which a red cell suspension of low hematocrit (3.5%, 1:9 (v/v) dilution of the original suspension with Ringers/albumin) was forced through the 3 $\mu$m diameter pores of a Nuclepore filter at a constant volumetric flowrate (2 ml/min) [3]. Pressure was monitored with a Statham P23db pressure transducer and recorded as a function of filtration time. After a short transient period, curves became linear with small positive slope. The transient region is attributed to the response time of the low band pass electronic filter in the transducer circuit and the time for the concentration of cells in a small cavity immediately preceding the filter membrane to reach

the concentration of the suspension being tested. The model used to interpret the pressure vs. time curves has been given elsewhere [15]. Briefly, the pressure $P$ at time $t$ is approximated by

$$P = P_0[1-\exp(-t/t_1)][1-\exp(-t/t_2)] + Mt$$

where $t_1$, electronic filter response time constant; $t_2$, mixing time constant for the dead space in the filter holder; $M$, long time slope (due to filter plugging and subsequent reduction in area for flow); $P_0$, the initial pressure rise at the beginning at the filtration experiment ($t = 0$) in the absence of experimental response delays.

The electronic filter time constant was determined by independent voltage jump experiments. $M$, $t_2$ and $P_0$ are determined using a Marquardt algorithm [16] for non-linear parameter estimation. Alternatively, the initial filter pressure drop $P_0$ can be determined by extrapolation of the linear portion of the curve back to time zero. This parameter serves as our indicator of mean deformability of the entire cell population. A small population of rigid cells present in a control suspension would give nearly the same value of $P_0$, but have an increased value of $M$, due to sequential filter plugging.

*Measurement of erythrocyte $Ca^{2+}$ and $Mg^{2+}$*

Red cells were saline-washed and ashed; residue was acid extracted before determination of calcium and magnesium by atomic absorption spectrometry (Perkin-Elmer Model 303) at the 423 and 285 nm bands, respectively. The detailed experimental procedures and precautions necessary for measurement of erythrocyte calcium are described elsewhere [17–19].

*Average sodium and potassium shear-associated fluxes*

Sodium and potassium concentrations were measured with a Coleman Model 21 Flame Photometer. To optimize sensitivity, changes of extracellular potassium and intracellular sodium were determined. Erythrocyte sodium was measured after four isotonic magnesium chloride washes of 0.3 ml packed red cells (90% hematocrit), ashing in platinum crucibles, and acid extraction of residue with 1.00 ml 6 M HCl. This extract was then diluted with radiation buffer and distilled water in a 10.00 ml volumetric flask. Radiation buffers were used to mask contaminant effects and a standard was run between each unknown to provide linear corrections for line pressure variations, aspirator deposits, temperature changes and other problems typical of flame photometry [20]. Verification of values reported in the literature for intracellular sodium and potassium served as a check on our equipment and technique [21].

Calibration of the instrument preceded any set of determinations; standards contained the same quantity of radiation buffer (and acid for sodium) as was used for unknowns. Measurements were made in triplicate with each followed by Flaminox detergent (Fischer Scientific Co.) and a distilled water rinse.

Area and time average shear flux across the cell membrane was calculated from:

$$K^+ \text{ flux} = \frac{(\Delta C_{K^+})(1-H)(1\text{ ml})}{N(10^3\,\mu\text{l/ml})(145\,\mu\text{m}^2)(120\text{ s})}$$

where $\Delta C_{K^+}$, change in supernatant potassium concentration ($\mu$mol/ml); $H$, cell volume fraction; $N$, cell count per $\mu$l
and

$$Na^+ \text{ flux} = \frac{(\Delta C_{Na^+})\,H(1\text{ ml})}{N(10^3\,\mu\text{l/ml})(145\,\mu\text{m}^2)(120\text{ s})}$$

with $\Delta C_{Na^+}$, change in intracellular sodium concentration ($\mu$mol/ml).

## Results

After washing and concentration adjustment of the erythrocytes, coincidence corrected mean red cell count was $(3.76 \pm 0.04) \cdot 10^6$ cells/$\mu$l (S.D.). Over the shear stress range of interest in this study, apparent viscosity of these suspensions was constant at 2.5 cP (Fig. 1). Cells exposed to subhemolytic shear stresses in Ringer's solution exhibited an increased intercept pressure drop $P_0$ or reduced erythrocyte deformability. The magnitude of this effect increased with the level of shear stress applied in the viscometer (Fig. 2).

Changes in mean red cell volume were not responsible for the decreased deformability result-

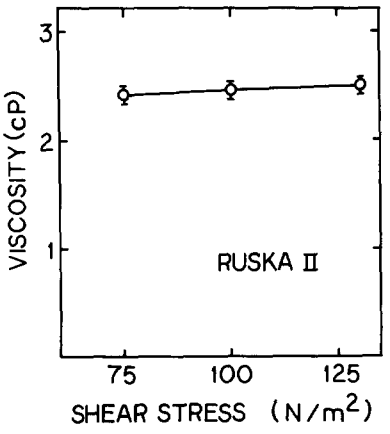


Fig. 1. Apparent viscosity as a function of shear stress. Red cell suspensions (35% hematocrit) were exposed to subhemolytic shear stresses for 2 min in a concentric cylinder viscometer.

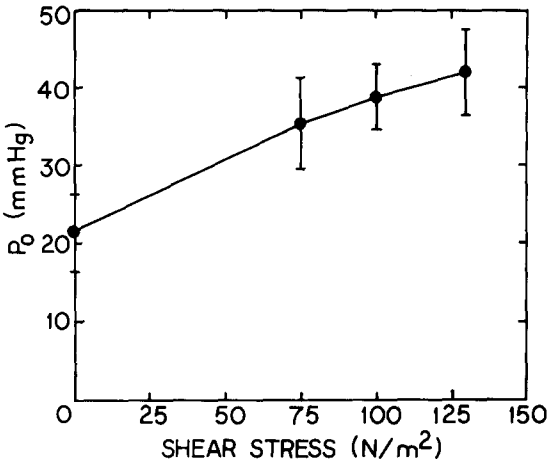


Fig. 2. Effect of shear stress level on the deformability indicator. Pressure was monitored as dilute cell suspensions were forced through 3-$\mu$m diameter pores of a Nuclepore filter at a constant volumetric flowrate. Normal values for the initial pressure drop as shown by the $y$-intercept are 22 mmHg (or 0.029 atm).

ing from mechanical trauma (Table I). This was verified by scanning electron microscopy, Coulter channelizer size distributions, and by micropipette measurements of surface area and volume (data not shown).

The mean concentration of calcium for (unsheared) control erythrocyte suspensions was normal at $0.0157 \pm 0.0026$ $\mu$mol/ml red cells (S.D., $n = 11$). After application of shear stresses of 100 and 130 N/m$^2$, intracellular calcium increased by

TABLE I

MEAN CELL VOLUME ($\mu$m$^3$)

$\sigma$, standard deviation; $n$, number of independent determinations.

| | Mean cell volume | | |
|---|---|---|---|
| Shear stress (N/m$^2$) | 75 | 100 | 130 |
| Control | 94($\sigma$ = 2.5, $n$ = 6) | 97($\sigma$ = 4.8, $n$ = 38) | 96($\sigma$ = 6.7, $n$ = 22) |
| Sheared cells | 92($\sigma$ = 2.3, $n$ = 6) | 96($\sigma$ = 5.4, $n$ = 38) | 95($\sigma$ = 6.5, $n$ = 22) |

TABLE II

CALCIUM AND MAGNESIUM CONTENT OF SHEARED ERYTHROCYTES

$\sigma$, standard deviation; $n$, number of independent determinations.

| Shear stress (N/m$^2$) | $\mu$mol/ml packed red cells | |
|---|---|---|
| | [$Ca^{2+}$] | [$Mg^{2+}$] |
| 0, controls | 0.0157($\sigma$ = 0.0026, $n$ = 11) | 2.16($\sigma$ = 0.48, $n$ = 11) |
| 100 | 0.0214($\sigma$ = 0.0049, $n$ = 7) | 2.06($\sigma$ = 0.40, $n$ = 7) |
| 130 | 0.0244($\sigma$ = 0.0039, $n$ = 6) | 2.02($\sigma$ = 0.41, $n$ = 6) |

TABLE III

SHEAR-ASSOCIATED $Na^+$,$K^+$-MEMBRANE FLUXES

$\sigma$, standard deviation; $n$, number of independent determinations.

| | $J$ ($\mu$mol·$\mu$m$^{-2}$·s$^{-1}$) | |
|---|---|---|
| Shear stress (N/m$^2$) | 100 | 130 |
| $J_{Na^+}$ ($\times 10^{15}$) | $-1.9(\sigma=2.6, n=4)$ | $-4.1(\sigma=2.8, n=4)$ |
| $J_{K^+}$ ($\times 10^{15}$) | $4.8(\sigma=1.8, n=13)$ | $11.6(\sigma=5.1, n=5)$ |

35% and 55%, respectively (Table II). These differences were statistically significant by the $t$-test for the difference of means ($p<0.01$). On the other hand, red cell magnesium was essentially unaffected by shear stress (Table II). Unsheared erythrocytes had a magnesium concentration at $2.16 \pm .48$ $\mu$mol/ml red blood cell ($n=11$). Average shear-associated sodium influx and potassium efflux were both small. The magnitude of these fluxes increased with the level of shear stress applied in the viscometer (Table III).

Experiments were performed to test the sensitivity of the deformability index ($P_0$) to a small contaminating population of rigid cells. Table IV shows values of $P_0$ and $M$ for a control sample and samples containing 0.3% and 1% (by cell count) glutaraldehyde-fixed red blood cells. $P_0$ is essentially unchanged but $M$ increases dramatically as the percent glutaraldehyde-treated cells is increased. Heat treated cells have a $P_0$ of 63 mmHg at our standard cell count. Addition of up to 10% heat-treated cells to control red blood cells had little effect on the erythrocytes $P_0$, though the slope $M$ did increase slightly. Above 10% heat-treated cells, $P_0$ and $M$ both increased (data not shown).

TABLE IV

EFFECT OF A SMALL POPULATION OF RIGID RED BLOOD CELLS ON $P_0$ AND $M$

| | %Glutaraldehyde-treated RBCs (by cell count) | | |
|---|---|---|---|
| | 0 | 0.3% | 1.0% |
| $P_0$ (mmHg) | 25.1 | 24.2 | 26.1 |
| $M$ (mmHg/s) | 0.041 | 0.246 | 0.60 |

## Discussion

We have demonstrated that the marked alteration of erythrocyte deformability resulting from subhemolytic shear stress is associated with an increase in intracellular calcium. This calcium increase is much smaller than that produced by ionophore loading or other commonly used methods and occurs without the concomitant morphology, volume and ATP level changes associated with these techniques.

Cell deformability has been ascribed to three physical properties: (1) surface-to-volume ratio, (2) internal viscosity or mean corpusular hemoglobin concentration (MCHC), and (3) membrane viscoelastic properties [22,23]. With no morphologic, volume or surface area changes evident in our experimental model, the decreased deformability resulting from subhemolytic shear stress is likely to be caused by altered membrane viscoelastic properties rather than a change in cell structure or cell dehydration. A small population of abnormally rigid cells does not significantly alter $P_0$, though it would lead to an increase in the long time slope, $M$, due to filter plugging. Thus, we feel the mean of the cell population has lowered deformability after subhemolytic mechanical trauma.

For normal cells filtered under similar conditions, flow resistance has been attributed primarily to membrane viscosity [24]. Our studies suggest that low level calcium loading may significantly increase erythrocyte membrane viscosity. Much of the previous work on red cell deformability has concentrated on the role of phosphorylation and calcium content, but, logically, the proteinaceous sublayer has more nucleophilic sites for intermolecular bridges or links with calcium ions than electrophilic sites for a covalently bound phos-

phate anion. The recent findings that band 4.1 causes spectrin-actin gels to become thixotropic and that spectrin plus band 4.1 crosslink actin are of interest, since both of these phenomena are regulated by calcium in the micromolar range [25,26]. In addition, calcium appears to initiate aggregation of erythrocyte membrane proteins [27].

Calcium may enter the red cell undergoing shear through membrane holes whereas the disparate magnitudes of the sodium and potassium fluxes indicate that a hyperpolarized membrane may be responsible for calcium uptake. Gardos proposed the latter mode for erythrocytes treated with propranolol [28]. The actual mechanism for calcium accumulation in the shear stressed erythrocytes is likely a combination of these effects, passive diffusion enhanced by electrostatic attraction. The results presented above compliment the recently published data of Larsen et al. [29] which indicate increased $Ca^{2+}$ permeability of human erythrocytes due to lower shear stress application in ATP depleted erythrocytes.

Our in vitro data with the shear-induced deformability defect suggested similar studies of red cell deformability and calcium content of patient groups with certain chronic hemolytic anemias. Of particular interest were patients with prosthetic heart valves and those with chronic renal failure receiving hemodialysis. In both cases, levels of shear stress on erythrocytes higher than in our model studies are known or suspected to occur. However, exposure times are much shorter than in our in vitro viscometer experiments. An individual erythrocyte may see many short, intermittant exposures of high shear stress during the life of the cell. Nevertheless, we have found decreased filterability of red cells and increased intracellular calcium in cardiac patients with prosthetic valves and in hemodialyzed renal failure patients [30,31]. Successful comparisons such as these support the use of in vitro shear stressed normal erythrocytes as a model for studying the deleterious effects of nonphysiologic flow on erythrocytes and for investigating the biochemical control mechanisms of red cell membrane mechanical properties.

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