Reversible deformation-dependent erythrocyte cation leak

Extreme sensitivity conferred by minimal peroxidation

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ABSTRACT To determine the threshold at which red blood cells (RBC) begin to manifest deformation-dependent leakiness to monovalent cations, we examined net passive potassium leak during elliptical deformation. Normal RBC did not begin to leak appreciable amounts of potassium until shear stress reached 204 dyn/cm^2, at which point they had attained > 96% of their maximal deformation. In striking contrast, RBC that had undergone minimal, physiologically relevant degrees of peroxidative damage induced by *t*-butylhydroperoxide began to leak potassium at only 59 dyn/cm^2 when they had reached only 63% of their maximal deformation. The cation leak identified in this manner is not prelytic, and it is fully reversible. Therefore, these data may be relevant to abnormal cation leaks that develop in sickle red cells that have membranes damaged by autoxidative stress and that manifest an exuberant but reversible leakiness to monovalent cation during sickling-induced deformation of the cell membrane.

INTRODUCTION

The red blood cell (RBC) membrane is remarkable for its ability to provide a permeability barrier even while remaining tolerant of deformation demanded by RBC circulation. There are limits, however, to the membrane's ability to withstand such stresses. For example, there is a shear stress (~1,500 dyn/cm^2) beyond which RBC hemolysis is a predictable consequence (1). Even below this hemolytic threshold, some previous studies performed at rather high shear stresses and/or harsh conditions have identified leaks that are either frankly prelytic or permanent (2, 3). Of greater interest, there are leaks that develop under conditions more clearly relevant to pathophysiology. For example, the membrane deformation accompanying deoxygenation-induced RBC sickling causes a marked but reversible leakiness to cations (4). Notably, the sickle membrane has been damaged by autoxidative stress so that both proteins and lipids reveal oxidative modifications (4, 5). Ney et al. postulated that this might confer an abnormal susceptibility to the potential hazards of cell deformation and documented that RBC subjected to minimal peroxidative stress do develop a unique leak pathway for monovalent cations that is deformation dependent and fully reversible (6). Those experiments, conducted at a single shear stress (220 dyn/cm^2), incidentally documented a small deformation-dependent leak from normal control RBC. The present study was done to precisely identify the threshold at which normal RBC and subtly peroxidized RBC begin to develop reversible leakiness to monovalent cation in response to elliptical deformation.

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METHODS

RBC Preparation

Blood was collected from volunteer donors, and RBC were washed three times with removal of buffy coat using 10-mM Hepes, 10-mM glucose, 4-mM KCl, NaCl to 290 mOsmol/liter, pH 7.4. To induce mild peroxidative stress (6), we treated normal RBC at hematocrit 10% for 30 min at 37°C with this buffer containing 0.8 mM *t*-butylhydroperoxide (tBuOOH). Peroxidation was then halted by addition of butylated hydroxytoluene (0.1 mM final concentration) in ethanol. Control RBC were handled identically except for exposure to tBuOOH. RBC treated with tBuOOH retained their normal biconcave discoid shape.

RBC were then washed three times with 10-mM Hepes, 10-mM glucose, 2-mM $CaCl_2$, 2-mM $MgCl_2$, 0.1-mM ouabain, 1-mM furosemide, NaCl to 290 mOsmol/liter, pH 7.4. Osmolarity was determined by vapor pressure osmometer (6). The ouabain and furosemide were included to inhibit Na^+K^+ATPase and Na^+K^+ cotransport, respectively, so that eventual measurement of K leak would reflect net passive K leak (here referred to as "K leak"). Based upon measured microhematocrit of packed cells, RBC were then suspended to hematocrit 10% in the latter buffer to which 20% dextran (average molecular weight 40 kD; Sigma Chemical Co., St. Louis, MO) had been added before the addition of NaCl for final adjustment of osmolarity. The viscosity of this medium at 37°C is 0.10 poise. The benign nature of this procedure and suspending medium has been amply documented previously (6).

Deformation-induced K leak

RBC suspensions were placed in a Couette viscometer having a rotating plexiglass outer cylinder and a stationary metal inner cylinder that was perfused to maintain suspension temperature at 37°C. In

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parallel, an aliquoit of RBC suspension was incubated at 37°C under static conditions. At zero time and after 2 h, triplicate aliquots of each suspension were evaluated for supernatant K content using flame photometry, exactly as previously described (6). Based on hematocrit results were converted to mEq K leak per liter RBC, and in every experiment K leak was corrected for hemolysis (which was always <0.5%), as previously described. The sensitivity of this technique allows reliable detection of K leaks on the order of 0.02 mEq K/liter RBC/h. For some of the reported experiments, single RBC samples (either normal or tBuOOH treated) were split and placed in each of two identical Couette devices run at slightly different speeds so that RBC could be evaluated simultaneously but at somewhat different shear stresses.

Measurement of RBC Deformation

We used ektacytometry (laser diffractometry) to compare the relative deformabilities of normal and tBuOOH-treated RBC. By this method, deformation is expressed as a "deformation index" which simply reflects the change in axial ratio as a function of applied shear stress. This assessment was performed as previously described (7) except that the present measurements were made at 37°C and using 20% dextran so that they would be directly relevant to the leak studies described herein. We also determined the maximal possible RBC deformation, defined as that observed in 35% dextran at room temperature (viscosity = 0.95 poise).

RESULTS

The response of RBC to elliptical deformation is depicted for both normal RBC (n = 70) and tBuOOH-treated RBC (n = 71) in Fig. 1. For clarity, results are expressed specifically as the deformation-induced increment in K leak (i.e., leak during deformation minus leak detected during parallel static incubation) as a function of applied shear stress. As noted in Methods, all data are fully corrected for the small degree of hemolysis occurring in this system.

The threshold shear stress at which normal and tBuOOH-treated RBC begin to develop leakiness was determined by calculating slopes for the leak data using those normal RBC samples analyzed at >200 dyn/cm^2 (n = 27 data points) and those tBuOOH RBC samples analyzed at >60 dyn/cm^2 (n = 64). As shown in Fig. 1, the resulting slopes intercept the abscissa at 204 dyn/cm^2 for normal RBC and at 59 dyn/cm^2 for tBuOOH-treated RBC, suggesting these as appropriate threshold values. Confirmatory data are obtained by examining the subset of experiments from Fig. 1 in which paired runs were performed by splitting a single RBC sample and simultaneously examining it at two slightly different shear rates. This allows us to identify the threshold shear stress above which 100% of paired runs showed the higher leak at the higher shear stress (i.e., the point at which random variability in leak rates due to experimental error gives way to the real enhanced effect exerted by a higher shear stress). The threshold points identified by this second, less precise analysis are ~205 dyn/cm^2 for normal RBC (derived from 26 experiments) and ~70 dyn/cm^2 for tBuOOH-treated RBC (from 23 experiments). Some of the experiments illustrating this point are depicted in Fig. 2.

When they are examined in the viscous medium (viscosity = 0.10 poise) used for these experiments, tBuOOH-treated RBC have somewhat diminished de-

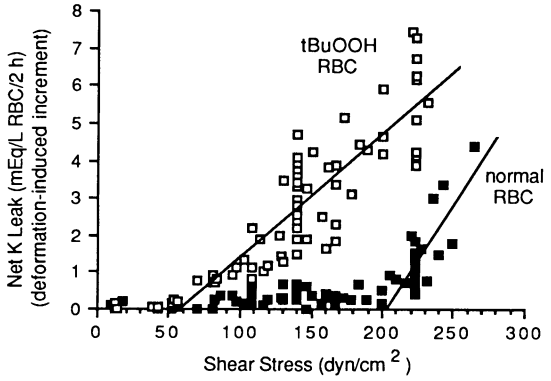

FIGURE 1 Potassium leak from RBC during elliptical deformation. Normal and tBuOOH-treated RBC were subjected to shear stress in 20% dextran at 37°C (viscosity, 0.1 poise). Resulting net passive potassium leak is depicted as the deformation-specific increment in K leak over a 2-h period. Regression lines are shown for the 27 normal RBC data points at >200 dyn/cm^2 and for the 64 tBuOOH RBC data points at >60 dyn/cm^2. For the normal cells, $y = -12.1836 + 0.0598x$ and $r = 0.758$. For the tBuOOH cells, $y = -1.97722 + 0.0336x$ and $r = 0.837$.

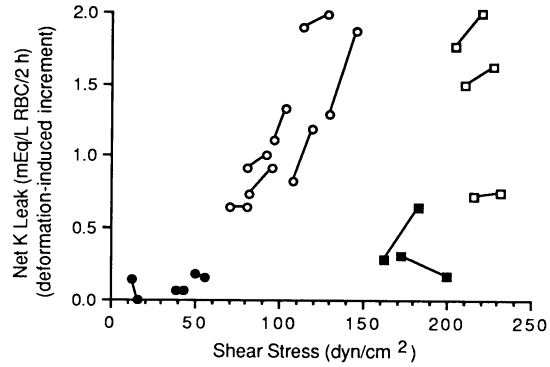

FIGURE 2 Selected paired experiments. A subset of experiments plotted in Fig. 1 consisted of paired runs with split RBC samples examined simultaneously at two shear rates. Only some of these are depicted here for illustrative purposes to avoid interpretive difficulty due to overlapping symbols and lines. Experiments on normal RBC (□, ■) and tBuOOH RBC (○, ●) are shown, with split pairs connected by lines. In each case, open symbols denote paired runs above the apparent leak threshold, whereas closed symbols denote runs below the apparent threshold (as discussed in text).

formability (Fig. 3). Although the ektacytometer reflects various and complex cellular properties (7), this is consistent with earlier observations of membrane stiffness in tBuOOH-treated cells made by others using micropipette techniques (8). Not shown is the fact that we find the deformability index at maximal RBC deformation (defined in medium having viscosity 0.95 poise) to be nearly identical for normal and tBuOOH-treated RBC (0.70 and 0.67, respectively). Thus, the development of leakiness from normal RBC at 204 dyn/cm^2 occurs only after RBC have reached >96% of their maximal deformation. In striking contrast, leakiness from tBuOOH-treated RBC develops at 59 dyn/cm^2 when RBC have reached only 63% of their maximal deformation.

To interpret these results, it is important to note that experimental variability due to apparatus, methods, and technique is minimal in this system. The simultaneous analysis of duplicate split samples yields identical leak rates if examined at identical shear stress (data not shown). A small degree of run-to-run experimental variability is revealed by sequential analysis of the same sample, as previously reported (6). This, plus the degrees of day-to-day and donor-to-donor variability, are all of the same magnitude (data not shown) and are evident in the width of the band described by normal RBC data points between zero and 200 dyn/cm^2 in Fig. 1. In contrast, the greater variability apparent in leak rates from tBuOOH-treated RBC reflects inconstancy of biologic effect of this peroxidant even though the treatments were always done in the same manner. Repeated use of some donors illustrated that this is due to treatment-to-treatment variability in biologic effect rather than differences in individual susceptibility to peroxidant (data not shown). For this reason, statistical treatments of the data sets contributing to the slopes shown in Fig. 1 would not be helpful in estimating the error in our assignments of threshold points. Rather, it is more informative to rely upon simple inspection of Fig. 1 and note that the threshold point for tBuOOH cells is clearly at <70 dyn/cm^2, while that for normal RBC is clearly >200 dyn/cm^2.

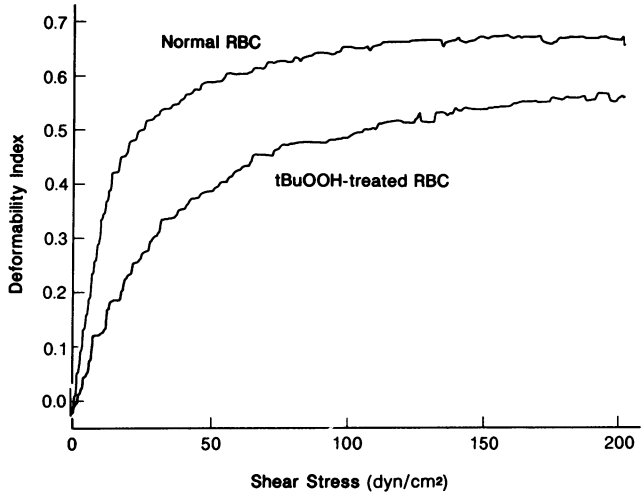

FIGURE 3 RBC deformability as assessed by ektacytometry. Normal and tBuOOH-treated RBC were evaluated in 20% dextran at 37°C (viscosity, 0.1 poise). Deformability is measured as a "deformation index." In separate experiments (not shown) performed at room temperature and with 35% dextran (viscosity, 0.95 poise), the maximal achievable deformability index was found to be 0.7 for normal RBC and 0.67 for tBuOOH-treated RBC.

DISCUSSION

These data precisely identify the threshold at which RBC develop leakiness to monovalent cation as a consequence of the elliptical (tank treading) deformation that they undergo when acted upon by fluid shear stress. For normal RBC this threshold is at 204 dyn/cm^2, a value that is consistent with the small deformation-dependent leak we observed earlier in separate experiments using a single shear stress, 220 dyn/cm^2 (6). It is somewhat lower than that identified by Johnson and Gannon (9), undoubtedly because our use of a longer observation period allows reliable detection of lower leak rates. We also have precisely defined the extreme susceptibility of tBuOOH-treated RBC to deformation, with the remarkably low threshold of 59 dyn/cm^2 identified for leak from such cells. As presented in Results, these threshold assignments are probably accurate within 5 dyn/cm^2, certainly within 10 dyn/cm^2.

Previous studies clearly documented that this leak is not explained by development of prelytic pores, RBC fragmentation, wall effects, or tank-treading frequency (6). Ney et al. referred to this unique leak pathway as a "synergistic oxidation-plus-deformation leak pathway" (6). The leak so described is clearly distinguishable from the so-called "chemo-mechanical leak" examined by Thelan et al., a leak observed in RBC that were sheared after exposure to a crosslinking agent to the extent that RBC deformability was completely abolished and a prelytic pore was established (10). Likewise, the leak pathway we describe clearly is different from the prelytic pore induced by enormously greater tBuOOH effects (11). Indeed, a remarkable feature of our studies is that they have utilized physiologically relevant degrees of physical and biochemical stress. For normal RBC, abnormal cation leak develops only at >96% of maximal RBC deformation, but for tBuOOH-treated RBC it develops when RBC are at only 63% of maximal deformation. During deoxygenation of sickle RBC the degree of stress

on the membrane probably far exceeds that achieved in our model which employs simple elliptical deformation. Even the degree of peroxidation induced by tBuOOH is appropriate since it is nearly identical to that found in membranes of unmanipulated sickle RBC (unpublished data). Thus, the extreme susceptibility of tBuOOH-treated cells observed in these studies is likely to be relevant to sickle RBC.

The actual molecular identity of the leak pathway revealed by these studies is not known. Our ongoing studies suggest that the responsible biochemical component of peroxidative stress induced by tBuOOH is the development of lipid hydroperoxides (12). Beyond this, this leak pathway is definable only by its phenomenology. It is fully reversible so that cells return to their normal state upon cessation of deformation (6). The leak is balanced so that induced Na influx equals K efflux, it is lower at low pH, and it is not chloride dependent (6, 9). Previous studies, performed under somewhat different conditions, suggested that application of shear stress can cause leakiness to calcium (13). The leak pathway we have studied is not calcium dependent (6, 9), indicating that if calcium does gain entry during deformation it does not reach levels able to stimulate calcium-activated K channels. Whether calcium in lesser amounts is entering RBC during our studies is not determined yet. Notably, this phenomenologic description applies to the deformation-induced leak pathway of both normal and tBuOOH-treated RBC evaluated at 220 dyn/cm^2 (6).

Thus, these observations suggest that the same leak pathway is present in the two cell types, so the lower threshold for tBuOOH-treated cells may reflect altered susceptibility to deformation rather than something truly unique about the peroxidized cells. At present we can only speculate regarding the biophysical nature of this phenomenon, but preliminary data suggest that it is related to perturbing effects of lipid hydroperoxides exerted at the lipid/protein interface (12).

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