Exaggerated Cation Leak From Oxygenated Sickle Red Blood Cells During Deformation: Evidence for a Unique Leak Pathway

By Takashi Sugihara and Robert P. Hebbel

An abnormal susceptibility of the sickle red blood cell (RBC) membrane to deformation could compromise its permeability barrier function and contribute to the exuberant cation leakiness occurring during the sickling phenomenon. We examined this hypothesis by subjecting RBCs at ambient oxygen tension to elliptical deformation, applying shear stress in a visco medium under physiologic conditions. Compared with normal and high-reticulocyte control RBCs, sickle RBCs manifest an exaggerated K leak response to deformation. This leak is fully reversible, is both Cl and Ca independent, and at pH 7.4 is fully balanced so that K\text{leak} equals Na\text{leak}. This abnormal susceptibility is also evident in that the K leak in response to deformation occurs at an applied shear stress of only 141 dyne/cm² for sickle RBCs, as compared to 204 dyne/cm² for normal RBCs. Fresh sickle RBC membranes contain elevated amounts of lipid hydroperoxide, the presence of which is believed to provide the biochemical basis for enhanced deformation susceptibility. When examined at pH 6.8, oxygenated sickle RBCs acquire an additional, unbalanced (K\text{leak} > Na\text{leak}) component to the K leak increment specifically ascribable to deformation. Studies with inhibitors suggest that this additional component is not caused by a known leak pathway (eg, either K/Cl cotransport or the Gardos channel). This abnormal susceptibility of the sickle membrane to development of cation leakiness during deformation probably contributes to the exuberant cation leak taking place during RBC sickling.

THE TENDENCY of sickle erythrocytes (red blood cells [RBCs]) to become dehydrated is one of the most important cellular abnormalities in sickle disease pathophysiology, but the underlying abnormalities of cation homeostasis have been elucidated only partially. Oxygenated sickle RBCs are abnormally permeable to monovalent cations, and stimulation of their K/Cl cotransport pathway by cell swelling or acid conditions induces an additional K leak. Even more striking, however, is the exuberant but reversible leak of monovalent cation that develops during deoxygenation-induced sickling. This clearly requires cell deformation by proximate development of hemoglobin polymer, but the physical-mechanical details of this event are not understood. There is some evidence that sickling activates the Gardos channel, but there may well be activation of multiple leak mechanisms.

Rather than promoting exaggerated ion flux through a normal leak pathway, it is possible that sickling induces leakage through an abnormal pathway. In this regard, we earlier hypothesized that the pathologic oxidation characteristic of the sickle RBCs might make its membrane more susceptible to the potentially adverse effects of deformation, such as compromise of permeability barrier function. We confirmed the possible validity of this hypothesis in a model system whereby a subtle degree of RBC membrane peroxidation (induced with t-butylhydroperoxide [tBuOOH]) was shown to induce a novel leak pathway that is reversibly activated by elliptical deformation. In the present studies, we have examined sickle RBCs at ambient oxygen tension for evidence of this unique leak pathway.

MATERIALS AND METHODS

RBC preparation. RBCs were freshly obtained in heparinized blood from volunteer normal or sickle (homozygous) donors. RBCs were washed three times in buffer (10 mmol/L HEPES, 10 mmol/L glucose, 4 mmol/L KCl, NaCl to 290 mmol/L, pH 7.4) with removal of buffy coat. For some experiments, sickle RBCs were fractionated into density subpopulations (top, middle, and bottom thirds) using a discontinuous arabinogalactan (Consulting Associates, Tacoma WA) gradient. High-reticulocyte control RBCs were obtained from patients with paroxysmal nocturnal hemoglobinuria, immunologic hemolytic anemia, and recovery from severe blood loss.

Deformation-dependent K leak. Measurement of net passive K efflux ("K leak") was conducted as previously described. RBCs were first washed three times in 10 mmol/L HEPES, 10 mmol/L glucose, 2 mmol/L CaCl₂, 2 mmol/L MgCl₂, 0.1 mmol/L ouabain (to inhibit Na⁺/K⁺ATPase), 1 mmol/L furosemide (to inhibit Na⁺/K⁺/Cl⁻ cotransport), and NaCl to 290 mmol/L (pH 7.4). They were then suspended to hematocrit 10% in a viscous medium, the same buffer containing 20% dextran (average molecular weight 40,000), Sigma Chemical Co, St Louis, MO), and the RBCs were subjected to elliptical deformation at 37°C by application of shear stress (220 dyne/cm²) in a concentric cylinder viscometer. The resulting K leak from RBCs undergoing deformation or parallel static control incubation was measured by flame photometric (Radiometer model FLM3, Louisville, KY) analysis of triplicate supernatants obtained at zero time and after 2 hours of deformation. The sensitivity of this technique allowed reliable detection of K leak amounting to 0.02 mEq K/L RBCs/h.

In every experiment, our K leak measurement obtained in this manner was fully corrected for any hemolysis. As previously described, we derived the percent hemolysis from the concentrations of hemoglobin (Hb) in the sample supernatant and in a lysate of the RBC suspension, both prepared using 0.1% Triton X-100. Based on the intracellular K content (at zero time), the percent lysing was converted to the corresponding mEq of K and subtracted from supernatant K contents to yield the K leaks reported here in mEq of K per liter of RBCs. The Hb concentrations were determined spectrophotometrically at 412 nm, the λmax for both lysate and supernatant Hb.

As in our previous studies, RBC lysis during the present experiments was minimal, typically no more than 0.5% over 2 hours of deformation for sickle RBCs. To illustrate, the percent lysis for
the sickle RBCs used for Fig 1 was 0.38% ± 0.13% (mean ± SD) with only 2 of the 17 values being greater than 0.5%; corresponding values for sickle RBCs in Fig 3 were 0.33% ± 0.09% at pH 7.4 and 0.35% ± 0.12% at pH 6.8, with no lysis greater than 0.5%. When applied shear stress was variable (as for Fig 2), there was no correlation between shear stress and percent lysis (r = 0.343, P = NS). Finally, the ratio of supernatant K to Hb at the end of 2 hours of deformation was 13.3 ± 6.8 for sickle RBCs used for Fig 1, 13.8 ± 4.8 for sickle RBCs at pH 7.4 used for Fig 2, and 25.5 ± 13.7 for sickle RBCs at pH 6.8 used for Fig 2. This large excess of K leak over Hb leak confirms that the results are not influenced significantly by lysis.

For some experiments, we altered this basic protocol by adjusting the viscous medium to pH 6.8 rather than 7.4, and for others we included okadaic acid (300 nmol/L) or purified charybdotoxin (50 nmol/L) in the medium. Sometimes the medium was formulated with 1 mmol/L MgEGTA substituted for calcium or with bromide salts substituted for all chloride salts. Finally, we tested the reversibility of the leak pathway by examining K leak over 2 hours of deformation and then for 2 continued hours of static incubation; the resulting K leak rate was compared with that of RBCs undergoing the static incubation only.5

The net K\textsubscript{efflux}/Na\textsubscript{influx} balance was determined by monitoring RBC cation contents.5 Intracellular K and Na were determined by washing RBCs three times in ice-cold 10 mmol/L Tris-buffered MgCl\textsubscript{2} (98 mmol/L; pH 7.4). After 0.2 mL of packed RBCs was lysed by addition to 3.8 mL of diluent (5.3% trichloracetic acid and 5.2 mmol/L LiCl), supernatant K and Na concentrations were determined by flame photometry. We are specifically interested here in the K\textsubscript{efflux}/Na\textsubscript{influx} balance (ie, net ΔK + Na) of the incremental leak caused by deformation per se. We calculated this from RBC [K] and [Na] contents measured at zero time (“zero”) and after 2 hours of deformation (“def”) or parallel static control incubation (“static”) as follows: Net ΔK + Na = ΔK + ΔNa, where ΔK = [K]\textsubscript{def} - [K]\textsubscript{zero} - ([K]\textsubscript{static} - [K]\textsubscript{zero}); and ΔNa is calculated in the same fashion using measurements of RBC [Na].

Shear stress threshold for K leak. Sickle RBC samples from two patients were analyzed for K leak at multiple shear stresses.5 To then estimate the K leak threshold, we calculated the slope for all data points acquired at shear stress greater than 140 dyne/cm\textsuperscript{2}, the point at which K leakiness started to increase above baseline, as judged by visual inspection of the data.

Membrane peroxidation byproducts. Freshly obtained RBCs were washed as above and evaluated for content of thiobarbituric acid reactive substances (TBARS) after the addition of butylated hydroxytoluene to 0.1 mmol/L and using two different wavelengths to correct for non-TBARS chromogen.5 Membrane lipid hydroperoxide (LOOH) content was determined iodometrically.5 Control experiments showed that membrane LOOH content in this analysis was unchanged if the extensively washed RBCs were further subjected to cellulose filtration in an attempt to even more thoroughly remove white blood cells (WBCs) (data not shown).

Statistical treatments. Data were evaluated for statistical significance using the Student’s t-test, either paired or unpaired as appropriate.

RESULTS

Deformation-induced K leak. Oxygenated RBCs were examined for net passive K efflux (“K leak”) during 2 hours of elliptical deformation and parallel static control incubation. The resulting K leaks are shown in Fig 1 and the corresponding deformation-induced increments are calculated in Table 1. Normal RBCs manifested the same small deformation-induced increment in K leak previously observed.5 Compared with normal RBCs, high-reticulocyte control RBCs (average reticulocyte count, 12%; range, 5% to 25%) showed somewhat greater static and deformation-induced incremental K leaks. Sickle RBCs (average reticulocyte count, 8%; range, 3% to 15%) manifested an even higher K leak during static incubation as expected and an incremental response to deformation that was significantly exaggerated compared with that of either normal or high-reticulocyte control RBCs. There was no correlation evident between reticulocyte count and leak response to deformation for either high-reticulocyte control or sickle RBCs.

Shear stress threshold for K leak. Multiple experiments performed using two sickle donors yielded an estimate of 141 dyne/cm\textsuperscript{2} as the shear stress threshold at which deformation-induced K leak begins to appear (Fig 2).

Membrane LOOH content. Compared with normal and high-reticulocyte control RBCs, membranes of fresh sickle RBCs had increased TBARS and LOOH. An experiment

<table>
<thead>
<tr>
<th>RBC Type</th>
<th>n</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>K Leak Increment (mEq/L RBCs/2 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>21</td>
<td>0.84 ± 0.19</td>
<td>0.44-1.18</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>Sickle</td>
<td>17</td>
<td>1.75 ± 0.98</td>
<td>0.94-4.04</td>
<td>P = .035</td>
</tr>
<tr>
<td>High-reticulocyte</td>
<td>12</td>
<td>1.08 ± 0.39</td>
<td>0.54-1.58</td>
<td>P = .02</td>
</tr>
</tbody>
</table>

Data for all the experiments in Fig 1 are shown here in terms of the specific deformation-induced K leak increment (K leak during 2 hours of deformation minus K leak during 2 hours of static incubation).
Fig 2. Sickle RBCs are abnormally susceptible to deformation. Sickle RBCs were examined for K leak in response to deformation at various applied shear stresses. A series of experiments on RBCs from a single sickle donor is shown at the left for RBCs under static incubation conditions (□) and during deformation (●). On the right, pooled data (shown as the deformation-induced K leak increment) from multiple experiments on two sickle donors are shown for all data points greater than 140 dyne/cm². Using the best-fit line for these data \( y = 0.03076x - 4.33521 \) to calculate the x-intercept, we estimate that the leak threshold is 141 dyne/cm².

Performed on density-separated sickle RBCs indicated that the accumulation of peroxidation byproduct was greatest for most-dense sickle RBCs, although the least-dense, reticulocyte-rich fraction was clearly abnormal as well (Table 2).

**Deformation at low pH.** We also examined RBCs at pH 6.8 to determine whether a stimulated KCl cotransport pathway might be further activated by deformation. For this set of experiments, K leaks are shown in Fig 3 and deformation-induced increments are calculated in Table 3. Low pH had the expected effect of increasing the leak from sickle and high-reticulocyte control RBCs under static conditions. The incremental K leak ascribable to deformation was seen to be diminished for normal RBCs at acid pH, which is their typical response to pH in this system. This pattern also was seen for six of the seven high-reticulocyte control RBC samples deformed at the lower pH. In clear contrast, the deformation-induced incremental K leak for sickle RBCs actually increased at pH 6.8 compared with pH 7.4.

**Leak pathway characteristics.** As previously described, the deformation-induced K leak pathway for both normal RBCs and minimally peroxidized RBCs is Cl-independent and Ca-independent and balanced so that \( K^\text{out} = Na^\text{in} \). In the present studies of sickle RBCs at pH 7.4, we also found this to be true so that net AK + Na = −0.1 ± 0.5 mEq/L RBCs over 2 hours of deformation (n = 4 experiments), which is not a significant change. Other measurements documented that the deformation-dependent leak from sickle RBCs was not inhibited by the substitution of Br

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**Table 2. Peroxidation Byproducts in RBC Membranes**

<table>
<thead>
<tr>
<th>RBC Type</th>
<th>TBARS (nmol/mL RBCs)</th>
<th>Lipid Hydroperoxide (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal RBCs</td>
<td>0 ND</td>
<td></td>
</tr>
<tr>
<td>Peroxidized RBCs*</td>
<td>3.29 ± 1.01 0.08 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>High-reticulocyte RBCs</td>
<td>0 ± 0 ND</td>
<td></td>
</tr>
<tr>
<td>Sickle RBCs</td>
<td>8.01 ± 2.61 0.17 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>Sickle density fractions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Top one-third</td>
<td>6.55 0.10</td>
<td></td>
</tr>
<tr>
<td>Middle one-third</td>
<td>7.77 0.09</td>
<td></td>
</tr>
<tr>
<td>Bottom one-third</td>
<td>11.89 0.23</td>
<td></td>
</tr>
</tbody>
</table>

Peroxidation byproducts were measured in freshly obtained RBCs as described in Materials and Methods. Data are shown (mean ± SD) for normal (n = 5), minimally peroxidized (n = 3), high-reticulocyte control (n = 4), and sickle RBCs (n = 4). A fifth sickle patient contributed RBCs for the density separation experiment.

Abbreviation: ND, not detectable (detection threshold is 0.02% of phospholipid).

*RBCs treated with 0.8 mmol/L t-butylhydroperoxide for 30 minutes in the presence of glucose, after which butylated hydroxytoluene was added.®

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**Fig 3.** Deformation-induced K leak at low pH. The magnitude of the deformation-induced K leak was determined as described for Fig 1 except that RBCs were examined in parallel in media having pH 6.8 versus pH 7.4. Data are shown as mean ± SD for normal (n = 5), sickle (n = 6), and high-reticulocyte control (n = 7) RBCs. The statistical significance of differences in K leak rates during static incubation is shown. Deformation-induced K leak increments for this set of experiments, and their statistical treatment, are shown in Table 3.
Table 3. Deformation-Induced K Leak Increments for Figure 3 Data

<table>
<thead>
<tr>
<th>RBC Type</th>
<th>pH</th>
<th>n</th>
<th>K Leak Increment (mEq/L RBCs/2 h) (mean ± SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>7.4</td>
<td>3</td>
<td>0.89 ± 0.06</td>
<td>.001</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>3</td>
<td>0.55 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Sickle</td>
<td>7.4</td>
<td>6</td>
<td>1.24 ± 0.27</td>
<td>.013</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>6</td>
<td>1.60 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>High-reticulocyte</td>
<td>7.4</td>
<td>7</td>
<td>1.21 ± 0.34</td>
<td>.005</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>7</td>
<td>0.99 ± 0.26</td>
<td>.022</td>
</tr>
</tbody>
</table>

The specific deformation-induced K leak increment for each of the experiments in Fig 3 is calculated here as described in Table 1.

Abbreviation: NS, not significant.

for Cl or by the substitution of EGTA for calcium (data not shown). Moreover, it was fully reversible as evidenced by the fact that, upon cessation of deforming stress, the rate of K leak promptly returned to that of cells undergoing static incubation (Fig 4), exactly as previously shown for normal and minimally peroxidized RBCs.5

In contrast, however, during deformation of sickle RBCs at pH 6.8, K outflux was greater than Na influx so that the specific deformation-induced AK + Na = -1.6 ± 0.9 mEq/L RBCs (n = 3; P = .037). Despite the fact that the deformation-dependent K leak shifted from being balanced (at pH 7.4) to being unbalanced (at pH 6.8), the addition of the K:Cl cotransport inhibitor okadaic acid10 during deformation at pH 6.8 had no inhibiting effect whatsoever on the magnitude of the incremental K leak due to deformation (Fig 5).

Similarly, neither substitution of EGTA for Ca nor inclusion of the Gardos channel inhibitor charybdotoxin11 had any inhibiting effect (data not shown). Examination of density-separated (one-third fractions) sickle RBCs (n = 3, data not shown) showed that the most-dense RBCs had no detectable incremental leak response to deformation and that the least-dense RBCs (depleted of poorly deformable dense cells) manifested a response that was even greater than that of the unfractionated RBCs (by 46% to 60%).

DISCUSSION

We have examined the hypothesis that the sickle RBC membrane is abnormally susceptible to potential adverse effects of cell deformation. In these experiments, deformation was achieved by application of shear stress to cause elliptical shape change.2 This eliminates any physical/chemical factors directly related to Hb polymerization, although it has the disadvantage of not achieving the extreme degree of membrane derangement actually occurring during spicule-induced membrane deformation per se. However, despite the lower degree of membrane stress, the present model is sufficient to show an abnormal susceptibility whereby sickle RBCs at ambient oxygen tension show exaggerated deformation-dependent leakiness to monovalent cation. Notably, at pH 7.4 the characteristics of this leak are like those previously found for the deformation-dependent leak from the tBuOOH-treated model RBCs: not dependent on Cl or external Ca, fully reversible, and...

Fig 4. Deformation-induced K leak is reversible. Sickle RBCs were examined for K leak over 4 hours of deformation (●). After 2 hours, deforming stress was stopped for one aliquot of the RBCs (■), which promptly resumed the lower leak rate observed for cells from the same donor kept in parallel static control incubation (□). A representative experiment (1 of 2) is shown.

Fig 5. Deformation-induced K leak increment is not inhibited by okadaic acid. Sickle RBCs (n = 3) were examined for K leak (mean ± SD) during deformation and parallel static incubation at pH 7.4 and pH 6.8. At the lower pH, the addition of okadaic acid (+OA) diminished K leak under both static and deformation conditions by a corresponding degree, so that the specific deformation-induced K leak increment was unaffected by OA.
balanced so that $K_\text{efflux}$ equals $Na_\text{influx}$.$^5$ Thus, by its phenomenologic description, this seems to be the same leak pathway.

We had originally hypothesized that deformation might even further activate a stimulated KCI cotransport pathway in sickle RBCs. In fact, there is an interesting relationship between this pathway and oxidative processes because its activation by the thiol perturbant N-ethyl-maleimide is further enhanced by peroxidation.$^12$ In the present studies, we examined its activation by acid pH, the most likely physiologic stimulus. While addition of deforming stress does indeed, have a significantly greater effect on K leak for sickle RBCs at low pH$_c$ compared with pH$_c$ 7.4, this deformation-dependent K leak increment clearly was not inhibited by okadaic acid, an inhibitor of the KCI cotransport pathway.$^10$ It was also not influenced by the substitution of EGTA for calcium or by the addition of a Gardos pathway inhibitor, charybdotoxin.$^11$ Thus, this unique effect of lower pH$_c$ on the deformation response of sickle RBCs (compared with normal or tBuOOH-treated RBCs) apparently is not due to a known leak pathway.

Our previous studies on minimally peroxidized RBCs$^5$ suggested that exaggerated responsiveness to membrane deformation reflects an abnormal susceptibility rather than the existence of a new leak pathway structure.$^6$ Consistent with this, the susceptibility of sickle RBCs is abnormal as evidenced by the low shear stress threshold at which leak develops (141 dyne/cm$^2$) compared with normal RBCs (204 dyne/cm$^2$, as documented in earlier studies$^{6,13}$). Our previous studies also suggested that it is the abnormal presence of LOOH that confers this unusual property upon the RBC membrane.$^7$ In the present work, we do find elevated amounts of peroxidation byproducts in sickle RBCs. Indeed, fresh sickle RBC membranes contain even more LOOH than do the tBuOOH-treated RBCs used in our earlier model studies. These results might be greater accumulation of LOOH in the most-dense sickle RBCs, but the least-dense cells are abnormal as well.

In aggregate, these data are consistent with the notion that this unique property of sickle RBCs is a consequence of oxidative modification of the sickle RBC membrane.$^1$ We suggest that the exaggerated susceptibility to development of monovalent cation leak as shown here contributes to the disordered cation homeostasis resulting from the even greater membrane deformation occurring during the sickling process. Either or both of the deformation-responsive leaks described here (balanced at pH$_c$ 7.4 and unbalanced at pH$_c$ 6.8) could be among the multiple leak pathways that are probably activated during this event.

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REFERENCES

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