Deformation of Swollen Erythrocytes Provides a Model of Sickling-Induced Leak Pathways, Including a Novel Bromide-Sensitive Component

By Takashi Sugihara, Yoshihito Yawata, and Robert P. Hebbel

Deoxygenation-induced red blood cell (RBC) sickling probably activates multiple cation leak pathways. In an attempt to model this, we examined the net passive K efflux ("K leak") from normal and sickle RBCs undergoing elliptical deformation in hypotonic media (200 mOsmol/L). This hypotonic deformation activates two deformation-dependent K leak pathways that are not detectable during the balanced leak (K_{\text{efflux}}^{\text{K}} = N_{\text{influx}}^{\text{K}}) resulting from deformation of RBCs in isotonic medium. These are (1) a calcium-dependent leak component and (2) a novel leak pathway that is inhibited by substitution of bromide (but not sulfamate) for chloride, which converts the unbalanced K leak (K_{\text{efflux}}^{\text{K}} > N_{\text{influx}}^{\text{K}}) of hypotonic deformation to a residual balanced leak. This dramatic effect of hypotonic deformation is reversible, is detected in both normal and sickle RBCs, and is inhibited significantly by 4,4'-diethyliothiocyanato-2,2'-stilbene disulfonate.

Remarkably, bromide also inhibits by 55% the K leak resulting from authentic deoxygenation-induced RBC sickling and, thereby, blunts the imbalance of accompanying monovalent cation leaks. The unique effect of bromide is not readily explainable on the basis of known behaviors of known ion leak/transport pathways. The mechanical threshold for triggering K leak during hypotonic deformation is at applied shear stress of 164 dyne/cm², a value similar to the abnormal susceptibility we previously found for oxygenated sickle RBCs during isotonic deformation. These data suggest that membrane stretch accompanying hypotonic deformation activates the same multiple leak pathways that contribute to net K leak during authentic RBC sickling, including a previously unknown bromide-sensitive leak.

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MATERIALS AND METHODS

RBC preparation. We obtained fresh heparinized blood from volunteer normal or sickle (homozygous) donors, and RBCs were prepared for deformation experiments using a three-step protocol.
First, RBCs were washed 3 times in Buffer A (10 mmol/L HEPES, 10 mmol/L glucose, 4 mmol/L KCl, NaCl to 290 mOsmol/L, pH 7.4) with removal ofuffy coat. Second, RBCs were washed 3 times in Buffer B (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 mOsmol/L, pH 7.4). Third, RBCs were suspended to hematocrit 10% in Buffer C, a viscous medium comprised of Buffer B containing 20% dextran (average M, = 40,000; Sigma Chemical Co, St Louis, MO) and having a measured osmolality of 290 mOsmol/L. These cells were then ready for deformation experiments (see below).

For various experiments, we altered this basic preparative protocol by changing the formulation of Buffer C, in which case the corresponding alterations also were made in the proximate wash Buffer B. To expose RBCs to conditions of hypertonic swelling (for hypotonic deformation experiments), the cells were suspended in viscous medium formulated to 200 mOsmol/L. In some experiments, we included okadaic acid (OA; 300 mmol/L) or purified charybdotoxin (CTX; 50 mmol/L) or 4,4′-disothiocyano-2,2′-stilbene disulfonate (DIDS; 10 mmol/L) in the medium. Sometimes the medium was formulated with 1 mmol/L MgEGTA substituted for calcium. For some experiments, it was prepared using bromide salts substituted for all chloride salts. To substitute sulfamate (SMF) for chloride in the medium, we used sodium SFM made by neutralizing methane sulfonic acid (Aldrich Chemical Co, Milwaukee, WI) with NaOH, and we replaced MgCl₂ and CaCl₂ with MgSO₄ and CaSO₄. (In preliminary experiments, we verified that inclusion of 4 mmol/L SO₄ was not effective on exchanger K leak rates.)

Deformation-dependent K leak. Measurement of net passive potassium efflux ("K leak") was performed exactly as previously described. RBCs in Buffer C were subjected to elliptical deformation at 37°C by application of shear stress (220 dyne/cm²) in a concentric cylinder viscometer. In parallel, control RBCs in the same medium were incubated under static conditions, except for occasional gentle mixing to ensure that they remained in suspension. The resulting K leak from RBCs undergoing deformation or static incubation was measured using flame photometry (model FLM3; Radiometer, Louisville, KY) analysis of triplicate supernatants obtained after 2 hours. In every experiment, our K leak measurement obtained in this manner was fully corrected for the percentage of hemolysis, which we derived from the concentration of hemoglobin in the sample supernatant and in a lysate of the RBC suspension (both prepared using 0.1% Triton X-100). The hemoglobin concentrations were determined spectrophotometrically at 412 nm, the λ₅₅₀ for both lysate and supernatant hemoglobin. Based on the intracellular K content (at zero-time), the percentage of lysis was converted to the corresponding milliequivalent (mEq) K and subtracted from supernatant K contents to yield K leaks in mEq K per liter RBCs. Subtraction of the static control K leak from the total deformation K leak yielded the specific deformation-induced K leak increment, the value of interest for most experiments.

We determined the net Kₑₑₑₑₑₑ／Naₑₑₑₑₑₑ balance by monitoring RBC intracellular K and Na contents. RBCs were washed 3 times in ice-cold 10 mmol/L Tris-buffered MgCl₂ (98 mmol/L; pH 7.4). After 0.2 mL packed RBCs were lysed by addition to 3.8 mL of a lysis buffer (5.3% trichloracetic acid and 3.2 mmol/L LiCl), supernatant K and Na concentrations were determined by flame photometry. Here, we were specifically interested in the Kₑₑₑₑₑₑ／Naₑₑₑₑₑₑ 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 (and after 2 hours of deformation (def) or parallel static incubation (static); as follows: net ΔK + Na = ΔK + ΔNa, where ΔK = ([K]ₑₑₑₑₑₑ - [K]ₑₑₑₑₑₑ) - ([K]ₑₑₑₑₑₑ - [K]ₑₑₑₑₑₑ); ΔNa is calculated in the same fashion using measurements of RBC [Na]. It will be noted that this method of calculation (ie, based on measurements of cation per volume of RBCs) theoretically could serve to somewhat blunt the apparent magnitude of a K leak that is truly unbalanced (Kₑₑₑₑₑₑ ＞ Naₑₑₑₑₑₑ) because of the reciprocal Na-concentrating effect of water loss. Thus, the reported unbalanced leaks could be somewhat understated in magnitude in our results. On the other hand, we have established that this potential effect does not cause leaks that are truly unbalanced to artificially appear as being balanced. For example, during isotonic deformation of both sickle and normal RBCs, true balance of the observed K leak was confirmed earlier by an accompanying lack of change in RBC mean cell hemoglobin concentration and density profile assessed using phalthead esters. In the present context, we documented that hypotonic deformation of normal RBCs in bromide-substituted medium results in a phalthead density profile unchanged from that of cells subjected to static control incubation (data not shown), supporting the actual balance of K and Na net fluxes reported below for this experimental condition.

K leak threshold. We established the thresholds for K leak onset during hypotonic deformation by varying magnitude of applied shear stress from 97 to 246 dyne/cm² (at constant medium osmolality of 200 mOsmol/L) and by varying medium salt concentration to achieve tonicities from 200 to 290 mOsmol/L with chloride salts. Then, constant-volume aliquots of this RBC suspension were added to much larger volumes of Buffer B formulated at 200 mOsmol/L, with substitution of bromide or sulfamate for chloride (or for control to Buffer B with chloride at 290 mOsmol/L). The transferred RBCs were centrifuged and resuspended in a known and uniform total volume of these media, and microhematocrit was measured (five experiments), the change of which directly reflects magnitude of cell volume response to the differing media.

Sickling-induced K leak. Sickle RBCs were washed and suspended to hematocrit 10% in a flask containing the same isotonic bromide-salt or chloride-salt buffers used for deformation experiments. The headspace was flushed with humidified air or nitrogen, and the RBCs were incubated at 37°C with very gentle swirling. As described in the sample range using Buffer B formulated at 290 mOsmol/L with chloride salts. Then, constant-volume aliquots of this RBC suspension were added to much larger volumes of Buffer B formulated at 200 mOsmol/L, with substitution of bromide or sulfamate for chloride (or for control to Buffer B with chloride at 290 mOsmol/L). The transferred RBCs were centrifuged and resuspended in a known and uniform total volume of these media, and microhematocrit was measured (five experiments), the change of which directly reflects magnitude of cell volume response to the differing media.

Statistical treatments. Data were evaluated for statistical significance using the Students t-test, either paired or unpaired as appropriate.

RESULTS

For these studies, we subjected normal and sickle RBCs to elliptical deformation (at 220 dyne/cm²) for 2 hours (at 37°C and pH 7.4) under either isotonic (290 mOsmol/L) or hypotonic (200 mOsmol/L) conditions. The resulting net passive Kₑₑₑₑₑₑ ('"K leak"') values observed during deformation and parallel static incubation are depicted in Fig 1, and the corresponding specific deformation-induced K leak increments are calculated in Table 1.

From these initial data, three observations are apparent. First, during isotonic deformation the K leak increment is significantly greater for sickle RBCs than for normal RBCs (Table 1); these results are identical to those we recently described. Second, during static incubation under hypotonic...
conditions, the K leak from sickle RBCs that have a high level of K-Cl cotransport is markedly increased compared with that of the normal RBCs, as expected (Fig 1). Most importantly, for both RBC types the specific deformation-induced K leak increment is significantly greater during hypotonic deformation than during isotonic deformation (Table 1). The magnitude of this change is greater for normal RBCs, which are more deformable than sickle RBCs.

As for all our previous studies of K leak during RBC deformation, all results are reported here only after full correction for any contribution from RBC lysis, which was trivial in these experiments. For example, sickle RBCs after 2 hours of hypotonic deformation typically showed only 0.5% lysis. Further studies showed that for both normal RBCs (Fig 2) and sickle RBCs (data not shown) this exaggerated leak during hypotonic deformation is fully reversible so that it stops when deforming stress ceases.

Although loop diuretics are reported to potentiate K-Cl cotransport stimulation under some experimental conditions, performance of control experiments with and without furosemide (which is included in our standard media) shows no influence whatsoever of this agent on magnitude of K leak during hypotonic deformation (data not shown).

Threshold for K leak during hypotonic deformation. By varying the magnitude of applied shear stress or medium salt concentration, we established the thresholds for onset of exaggerated K leak during hypotonic deformation. At constant shear stress of 220 dyne/cm², increasing the medium osmolarity from 200 mOsmol/L results in a decreasing magnitude of the deformation-induced K leak increment (Fig 3, left). Remarkably, however, this experiment shows maintenance of increased leak rates at osmolarities only slightly below normal, and there appears to be an inflection point in the data at 245 mOsmol/L. In the experiment performed at constant 200 mOsmol/L, increasing the applied shear stress results in precipitous onset of deformation-induced K leak at 164 dyne/cm², with a linear increase in leak rate as shear stress increases further (Fig 3, right). Considering experimental error, we estimate that this derived shear stress threshold value is within ±10 dyne/cm² of the true threshold value.

Bromide inhibition. As in all our previous studies, substitution of bromide for all chloride salts has no influence on magnitude of the specific deformation-induced K leak increment for RBCs undergoing deformation under isotonic conditions. However, in striking contrast, bromide substitu-
DEFORMATION OF SWOLLEN RBCs

Fig 3. Thresholds for onset of K leak during hypotonic deformation. RBCs were examined for K leak over 2 hours of hypotonic deformation, and the specific deformation-induced K leak increments are shown. (A) RBCs from three normal donors were examined at shear stress of 220 dyne/cm², with medium salt concentration varied to achieve different tonicities. (B) Normal RBCs were examined at 200 mOsmol/L, with variable applied shear stress. Linear regression analysis of the illustrated, pooled data from three donors shows an x-intercept at 164 dyne/cm².

The inhibiting effect of bromide on K leak during hypotonic deformation is not explained by an absence of chloride per se, because substitution of SFM for chloride provides no inhibition of the hypotonic deformation-induced K leak (Fig 5). The magnitude of RBC volume increase achieved by suspension in these different media (shown at the bottom of Fig 5) provides evidence that the bromide effect is not explained simply by chaotrope-induced cell shrinkage.

Fig 4. K leak during hypotonic deformation is partially inhibited by bromide substitution. RBCs were examined for K leak during 2 hours of deformation under isotonic and hypotonic conditions (290 and 200, respectively). The resulting specific deformation-induced K leak increments are shown (mean ± SD) for normal (n = 4) and sickle (n = 3) RBCs. Substitution of bromide (Br) for chloride (Cl) had no effect on K leak increment under isotonic conditions, but it inhibited K leak increments during hypotonic deformation.

The inhibiting effect of bromide on K leak during hypotonic deformation is not chloride dependent. RBCs were examined for K leak during 2 hours of deformation under hypotonic conditions, and the specific deformation-induced K leak increment is depicted. Compared with K leak in standard chloride-containing medium, substitution of bromide is inhibitory, whereas substitution of SFM is not. The bottom of this figure shows the percentage of increase in RBC volume (n = 5 experiments) resulting from suspension in these hypotonic media before any deformation experiments (calculated versus the volume of reference normal RBCs in medium formulated with chloride and having 290 mOsmol/L).

Fig 5. K leak during hypotonic deformation is not chloride dependent. RBCs were examined for K leak during 2 hours of deformation under hypotonic conditions, and the specific deformation-induced K leak increment is depicted. Compared with K leak in standard chloride-containing medium, substitution of bromide is inhibitory, whereas substitution of SFM is not. The bottom of this figure shows the percentage of increase in RBC volume (n = 5 experiments) resulting from suspension in these hypotonic media before any deformation experiments (calculated versus the volume of reference normal RBCs in medium formulated with chloride and having 290 mOsmol/L).
undergoing 2 hours of deformation were examined to determine if the specific deformation-induced K leak increment was balanced as the deformation-induced change in RBC monovalent cation content (net $\Delta K + Na$) and calculated as described in Materials and Methods). Studies were performed with normal and sickle RBCs ($n = 3$ each), under both isotonic (290) and hypotonic (200) conditions, and with media containing either chloride or bromide salts. See text for interpretation of results.

Balance of $K^{\text{efflux}}$ and $Na^{\text{influx}}$. We examined the balance between $K^{\text{efflux}}$ and $Na^{\text{influx}}$ for the specific deformation-induced K leak increment itself by calculating the net change in RBC $K + Na$ contents. For RBCs of all types deformed under isotonic conditions with chloride salts, this deformation-induced K leak increment is again balanced (Fig 6), as was the case in our previous studies of normal and sickle RBC.7 Also as predicted, the K leak from both RBC types during hypotonic static incubation is unbalanced ($K^{\text{efflux}} > Na^{\text{influx}}$) because of activation of K-Cl cotransport (data not shown). However, it is of note that, during hypotonic deformation with chloride salts the specific deformation-induced K leak increment also becomes unbalanced ($K^{\text{efflux}} > Na^{\text{influx}}$) for both RBC types (Fig 6), indicating the additional appearance of a new type of leak pathway in response to hypotonic deformation.

Remarkably, substitution of bromide for chloride salts eliminates this unbalanced component (Fig 6), even though bromide only partially inhibits the overall deformation-dependent K leak (Fig 4) and does not limit the K-Cl cotransport activation in these hypotonically swollen cells (data not shown). This balancing effect of bromide substitution is accounted for mainly (77%) by a decrease in the $K^{\text{efflux}}$ stimulated by deformation and only partially (23%) by an enhancement of $Na^{\text{influx}}$ (data not shown).

Inhibitors. Under hypotonic conditions, addition of OA, a K-Cl cotransport inhibitor,13 appropriately diminishes the magnitude of the static K leak rate (data not shown) but does not affect the magnitude of the deformation-induced K leak increment at all (Fig 7). CTX, a blocker of RBC calcium-activated K channels14 and EGTA substitution also inhibit somewhat (Fig 7). Addition of DIDS markedly inhibits the K leak increment. Because of the magnitude of the K leaks being examined, the percentage of inhibition of leak from normal RBCs is a more reliable measurement, but comparable data are obtained from the sickle RBC samples exposed to these inhibitors (Fig 7). None of these inhibitors that exert the illustrated influence during hypotonic deformation had any influence on K leak during isotonic deformation (data not shown) or previous7 studies.

For one experiment, we had sufficient cells available to further compare the inhibitory effects of CTX and bromide alone and in combination (Table 2). For the deformation-induced K leak increment of both normal and sickle RBCs, the degree of inhibition exerted by the combination is less than expected if the two effects were fully additive (which would be the expected consequence of inhibiting completely separate pathways) but more than expected if there were no additive effect at all.

**Sickling-induced K leak.** The ability of bromide to inhibit sickling-induced K leak apparently has never been tested. We find that bromide significantly inhibits the K leak induced by deoxygenation of sickle RBCs (Fig 8), and the degree of this effect (on average 55%) is comparable with that exerted by bromide during hypotonic deformation (as shown in Fig 4). During one of these experiments, we had sufficient sample to simultaneously monitor RBC monovalent cation content (total $K + Na$) and found the following changes: $-3.5$ and $-1.0$ mEq/L RBCs/h during sickling in chloride and bromide containing media, respectively (with corresponding values of $-0.3$ and $-0.1$ from RBCs incubated in parallel at ambient oxygen concentration). Direct microscopic observation showed no detectable influence of bromide as compared with chloride on sickling morphology (data not shown).

**DISCUSSION**

We have examined RBCs subjected to elliptical deformation under hypotonic conditions (hypotonic deformation) as
Deformation of Swollen RBCs

Table 2. Combined Inhibiting Effect of CTX and Bromide

<table>
<thead>
<tr>
<th>RBC Type</th>
<th>Deformation-Induced K Leak Increment (mEq/L RBC/h)</th>
<th>Percentage of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CI +CTX</td>
<td>+Br</td>
</tr>
<tr>
<td>Normal</td>
<td>8.67</td>
<td>5.57</td>
</tr>
<tr>
<td>Sickle</td>
<td>2.84</td>
<td>1.68</td>
</tr>
</tbody>
</table>

RBCs were subjected to 2 hours of hypotonic deformation with and without various additions/substitutions in the composition of the viscous medium. Actual deformation-induced K leak increments are shown to the left, and the percentage of inhibition is calculated on the right. See text for interpretation. =, Defined as.

Fig 8. Bromide inhibits sickling-induced K leak. Sickle RBCs from five donors were incubated under ambient oxygen tension or nitrogen, and net K leak occurring over 60 minutes was measured. Substitution of bromide for chloride salts significantly inhibits sickling-induced K leak to about the same extent that it inhibits the K leak response to hypotonic deformation (as shown in Fig 4). Data are shown as mean ± SD (n = 5 each). Three of the experiments were performed with RBCs in dextran-containing viscous medium, and two of the studies were performed with RBCs in dextran-free buffer; because results were no different under the two conditions, the five experiments have been grouped together here.

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Because we are interested in the effects of deforming stress, here, we emphasize the analysis of the specific deformation-induced K leak increment, which is to be distinguished from the any effect of experimental manipulation on the simple rate of static K leak (eg, as results from activation of K-Cl cotransport). It also must be noted that observation of K leak in this experimental system absolutely requires that erythrocytes be responsive to deforming stress (ie, that they be deformable). Presumably, this explains why the leak from normal cells is greater than that from sickle cells which have markedly deficient deformability. For this reason, our data are more developed for normal RBCs, but is apparent that the same leak pathways also are evident during hypotonic deformation (at ambient oxygen tension) of sickle cells. We find that hypotonic deformation of erythrocytes induces reversible K leak because of activation of multiple leak pathways, very probably the same pathways active during authentic polymerization-induced membrane deformation during the sickling process.

Comparison of isotonic and hypotonic deformation versus sickling. The present results must be contrasted with those derived from isotonic deformation, during which the deformation-induced K leak from normal, minimally peroxidized, hydroperoxide-loaded, and oxygenated sickle erythrocytes has the following characteristics: fully reversible (leak ceases when deforming stress is stopped); balanced (K\textsubscript{influx} = Na\textsubscript{outflux}); independent of external calcium (not inhibited by EGTA); not dependent on chloride (not inhibited by nitrate or bromide substitution); and leak rate is lower at lower pH, 6.7-7.15.

In contrast, something strikingly different happens during deformation of the swollen erythrocyte. Although still completely reversible, the expressed net leak now is unbalanced (K\textsubscript{influx} > Na\textsubscript{outflux}), and use of inhibitors clearly shows that it consists of multiple components. At least three are definable: (1) a component inhibitable by EGTA or CTX; (2) an unbalanced component that is inhibitable by substitution of bromide for chloride salts; and (3) a residual balanced component similar to that previously observed during hypotonic deformation. The novel bromide-inhibitable leak component was unexpected and has not been observed previously in physiologic studies of RBCs.

Remarkably, in studies of authentic deoxygenation-induced membrane deformation in sickle RBCs, we also find that this unique bromide-sensitive leak pathway accounts for a significant portion (55%) of sickling-induced K loss. That bromide only partially brings the sickling-induced K and Na net fluxes into balance requires further investigation, but this might reflect a stronger contribution of calcium to K loss during sickling compared with hypotonic deformation. Calcium dependence is characteristic of at least one sickling component, as are a sensitivity to DIDS and a probable presence of an underlying balanced leak component. Thus, our model of hypotonic deformation has successfully reproduced the character of sickling-induced leak, including the participation of multiple leak pathways. Because these leaks are still definable only by their phenomenology, further proof of identity must await definitive leak pathway identifications.

Mechanism of hypotonic deformation-induced leakiness. The induction of different leak pathways during deformation...
of swollen RBCs clearly is not dependent on stimulation of K-Cl cotransport, per se, because its stimulation by acid pH did not have the same effect and because OA does not inhibit the incremental leak induced by hypotonic deformation. Thus, the present unique response to deforming stress must be explained either by something inherent in the increased membrane stretch of swollen erythrocytes or by some other biochemical attendant of hypotonic swelling.

The literature predicts that conditions of 200 mOsmol/L would promote an RBC volume increase of about 15% to 20%, which is far less than the limiting threshold for cell lysis. In our media that additionally contain a high concentration of dextran, the actual volume increase is slightly less than this. Nonetheless, even this degree of volume change does induce a detectable resistance to extensional deformation during ektacytometry, an effect predicted to be somewhat augmented given the greater external/internal viscosity gradient under our experimental conditions. Our measurement of K leak rate as a function of variable medium tonicity shows an inflection point in the data, perhaps suggesting that something different develops at tonicity of about 245 mOsmol/L. A full explanation for this surprising sensitivity to hypotonicity will require additional investigation.

The imposition of elliptical deformation would generate increased membrane tension and dilation stress that are likely to develop in a nonuniform manner across the cell surface (Sutera, personal communication, December 1993). In addition, tank-treading deformation causes an increase in cytoplasmic pressure that increases rapidly as the deformed cell reaches its limiting geometry. The threshold for these effects would be reached more quickly if RBCs are swollen. Thus, during hypotonic deformation, the shear stress threshold at which exaggerated K leak is triggered is identified here to be 164 dyn/cm². This is remarkably similar to the value of 141 dyn/cm² found for oxygenated sickle cells and reflects a marked increase in deformation susceptibility compared with that for normal cells (threshold of 204 dyn/cm²). Our previous studies showed that leak is not simply proportional to tank-treading frequency; therefore, the fact that leakiness increases in parallel with shear stress (after the threshold is reached) indicates that consequences of membrane tension are the responsible agent for leakiness during deformation. Unfortunately, the extent to which the mechanical stress of tank-treading deformation actually simulates that of actual sickling is not known. It is of interest that physiologic mechanosensitive channels are known to be gated by tension. Whether the membrane structure responsible for erythrocyte leak responsiveness to hypotonic deformation (or sickling) is mechanosensitive by design or by accident remains to be discerned.

**Calcium leakiness.** In our earlier studies we were unable to detect any entry of radiocalcium during isotonic deformation of normal RBCs (Hebbel, unpublished data). The present induction of a component of calcium-stimulated K leak during hypotonic deformation is reminiscent of the calcium leakiness that develops in erythrocyte membranes as a prelytic event during greater degrees of hypotonic swelling and that is reported during elliptical deformation at much higher shear stress (approximately 1000 dyn/cm²) than we use in our model. Calcium entry has also been observed during deformation of adenosine triphosphate-depleted erythrocytes. Interestingly, it has been predicted that the erythrocyte’s voltage-activated, increased permeability (to calcium and monovalent cations) would be activated by hypotonic swelling. Thus, calcium entry seems to accompany excess membrane tension, for which there is apparently some threshold.

**Novel inhibition by bromide.** The most intriguing question relates to how bromide exerts its inhibiting influence. That K leak remains unimpaired despite substitution of sulfamate for chloride rules out a strictly chloride-dependent leak pathway. Bromide is such a mild chaotrope that it does not alter RBC volume and, similarly, is unlikely to directly affect some protein responsible for this deformation-dependent leakiness. Thus, we believe that the mechanism of action of bromide is unique. It might be postulated that bromide somehow affects the calcium-dependent component of this leak. However, the calcium leak response to hypotonic swelling (under static conditions) is not inhibited by chaotropes, indicating that they inhibit neither the erythrocyte’s response to calcium (Gardos channel activation) nor the proximate calcium entry itself. However, it remains conceivable that the situation during deformation of swollen cells is different; therefore, we cannot yet exclude the possibility that calcium entry, per se, is inhibited by bromide during hypotonic deformation (and during sickling). Indeed, the overlapping inhibition effect of bromide and CTX suggests that bromide is at least partially inhibitory for the calcium-dependent part of our observed response. This could occur, for example, if bromide exerts its effect indirectly by altering rate of anion leak that necessarily accompanies a cation leak (for reasons of charge conservation).

It is of note that DIDS has such a nonspecific inhibitory influence on the Gardos leak. Its significant influence in the present studies might be exerted in the same manner, although more indirect actions of DIDS can be hypothesized. We previously found DIDS to cause a slight decrease in the isotonic deformation-induced K leak increment for minimally-peroxidized erythrocytes, but that was wholly accounted for by an increase in rate of static K leak, with no effect on magnitude of K leak during deformation. Therefore, we were hesitant to accept that effect as one of DIDS-mediated inhibition, but, in view of the present results, it may have been a real, albeit minimal, inhibition effect. DIDS does inhibit the leakiness that accompanies the greater membrane stress during isotonic deformation at very high shear rates.

How might an indirect bromide/DIDS effect be occurring during hypotonic deformation? If we assume that bromide limits the anion leak that accompanies cation leak, we may be implicating the anion channel protein, Band 3. It is known that, for anion exchange, flux shows Br⁻ < Cl⁻ and is completely inhibited by DIDS. However, by its very nature, exchange itself should not account for an unbalanced leak. Net (conductive) leak is much slower than exchange and is...
about 60% inhibitable by DIDS, but flux rates show Br$^-$ > Cl$^-$, making this route incompatible with our results. Thus, elimination of an unbalanced component by bromide is intriguing and remains unexplained by the reported/observed behavior of known ion transport pathways. Our data presumably imply that bromide acts by inhibiting a unique pathway which has high conductive permeability during deformation (relative to its state, if any, absent deformation) but has only a low copy number.

Interestingly, Joiner et al. found that the DIDS-inhibitable component of sickling-induced cation leak has a Ki that is an order of magnitude higher than that of DIDS-inhibited anion transport, which argues that another protein (or an unknown aspect of Band 3) is responsible. Our observation of partial inhibition (approximately 50%) of K leak during hypotonic deformation using 10 μmol/L DIDS is consistent with this type of effect rather than with inhibition of the anion channel per se.

Conclusions. We have shown that hypotonic deformation of RBCs activates multiple K leak pathways, each of which probably participates in sickling-induced K leak from deoxygenated sickle erythrocytes. Therefore, the present experimental model appears to reproduce the membrane changes that accompany sickling-induced mechanical deformation. Nonetheless, our results raise a number of new questions that, we hope, can be answered by further experimentation. In particular, the mechanism underlying the unexpected, novel ability of bromide to significantly inhibit the K leak that accompanies both hypotonic deformation and authentic sickling requires elucidation.

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REFERENCES


Deformation of swollen erythrocytes provides a model of sickling-induced leak pathways, including a novel bromide-sensitive component

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