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DIDS inhibition of deformation-induced cation flux in human erythrocytes

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The permeability of human erythrocytes to sodium, potassium and calcium increases when the cells are deformed by shear. We now report that the anion-exchange inhibitor DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid) inhibited 55–60% of the deformation-induced flux with an apparent $K_{1/2}$ of 1 μM. Covalently bound DIDS was also effective. In cells partially derivatized at 0°C (pH 7.4), anion exchange and the deformation flux were inhibited in parallel, implying that lysine a is the site of inhibition for both fluxes. Ektacytometry showed that DIDS does not inhibit by lowering the cell's ability to deform. Crosslinking of lysines in Band 3 was not required for inhibition of the stress flux, as demonstrated by electrophoretic analysis of chymotrypsin-cleaved Band 3 after DIDS treatment. Chymotrypsin cleavage itself did not affect the cation flux rates. DNDS, an anion exchange inhibitor that binds to the chloride site on Band 3 but is unable to derivatize lysine a, is an ineffective inhibitor of the deformation flux. Other high-affinity inhibitors of anion exchange were also relatively ineffective against the deformation flux, and anion exchange itself was unchanged by shear. These results suggest that 55–60% of the deformation-induced cation movement traverses a route that includes Band 3, but is distinct from the pathway utilized by anion exchange. Chloride-dependent cation pathways do not participate in the stress induced cation flux, since complete exchange of intracellular chloride for sulfate had no effect on the rates. Deformation of erythrocytes by laminar shear appears to increase the non-specific cation permeability.

Introduction

Deformation of human red cells can increase Na and K fluxes. This has been observed in normal erythrocytes subjected to shear in a viscometer [1–5], and in the extremely distorted forms of deoxygenated sickle cells [6–10]. An increase in ion permeability in response to shear is not limited to red cells, however. Mechanosensitive ion channels have been found in a variety of cell types [11], usually by patch clamping techniques. It is possible that some of these channels are artifacts of the large membrane tensions produced by the patch clamp [12]. DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid), a potent inhibitor of anion exchange, also inhibits the monovalent cation permeabilities induced by sickling [9], which suggested that DIDS might inhibit the shear-induced cation fluxes. To test this, we have determined the effect of anion exchange inhibitors on deformation fluxes in normal erythrocytes. The results imply that part of the stress-induced cation fluxes are mediated by Band 3, but that cations do not traverse the same pathway used by anion exchange.

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Materials and Methods

The general procedures used were described earlier [1]. Red cells were obtained in heparin from normal volunteers, and were washed three times in phosphate-buffered saline (PBS): 9 mM sodium phosphate (pH 7.4), 1 mM EDTA, 5 mM glucose, 150 mM NaCl.

Buffers

The effect of mechanical deformation on both cation and anion fluxes was determined. For the experiments measuring cation fluxes, a phosphate buffer was prepared (P_i-PVP), which was 10 mM sodium phosphate and 1 mM EDTA, with about 9% polyvinylpyrrolidone (PVP K-90, average molecular weight 300000, GAF Corporation) added to increase the solution viscosity. The pH was adjusted to 7.45 and sufficient NaCl was added to raise the osmolality to 290 mosM, as determined by a vapor pressure osmometer (Wescor, Logan,

UT). On the day of use, 5 mM glucose was added. Usually, 0.1 mM ouabain (Sigma, St. Louis, MO) and 0.01 mM bumetanide were present to inhibit the Na^+/K^+-ATPase and the $(Na^+,K^+,2Cl^-)$-cotransport, respectively. Anion fluxes under stress were determined in SST-PVP: 5 mM Na_2SO_4, 20 mM Tris-HCl (pH 7.45), 9% PVP, with sufficient NaCl to raise the osmolality to 290 mOsm.

Stress conditions

The red cell suspension (hematocrit 48) and the flux buffer, both pre-warmed to 37°C, were mixed at a 1:5 ratio to make the final hematocrit equal to 8 and the final PVP concentration between 7.0 and 7.5%, yielding viscosities between 70–85 cP at 37°C. The red cell-PVP suspension was loaded into a cylindrical Couette viscometer, originally part of an ektacytometer (Technicon Corporation, Tarrytown, NY), with a radius of 5 cm and a gap between the cylinders of 0.5 mm. The cylinders were wrapped in heating coils to maintain the internal temperature at 37°C, monitored with a thermocouple inserted through the outer cylinder wall. Rotation of the inner cylinder was begun at zero time, and samples were removed at appropriate intervals through a sampling port drilled in the cylinder wall midway between the top and bottom of the cylinder. Care was taken to obtain samples only from regions of undisturbed flow and uniform shear rates. Red cell-PVP suspensions were examined at a constant shear rate of 1300/s (250 rpm). Since shear stress equals shear rate × viscosity in poise [13], the applied stresses were between 1000 and 1250 dyne/cm^2. Unstressed controls were obtained by repeating the entire experiment with the same cell suspension in the same buffer, at a cylinder rotation speed of 5 rpm.

Calculation of cation flux rates

All cation flux measurements were made by determining the intracellular cation content of the erythrocytes. As reported previously [1], hemolysis was minimal (1–3%) at all shear stresses. Triplicate 0.2 ml samples from the viscometer side port were placed in a chilled 1.5 ml centrifuge tube containing 1.0 ml ice-cold Tris-Mg solution (10 mM Tris-HCl, 107 mM $MgCl_2$, pH 7.4). The cells were immediately pelleted by a 90 s spin in a chilled Eppendorf microcentrifuge at 4°C. The supernatant was completely removed and an aliquot was saved for hemoglobin determination as an estimate of hemolysis. The red cells were washed a second time in Tris-Mg solution, which was sufficient to reduce external Na^+ and K^+ to negligible levels. The pelleted cells were lysed in 1.0 ml of distilled water. Hemoglobin was determined with Drabkin's reagent [14], and Na^+ and K^+ were determined by flame photometry (Instrumentation Laboratories model 343).

Cation release and uptake was linear for 15–20 min. Rates were calculated from least squares fits of triplicate data points at zero time and two additional times in the linear region (usually 8 and 15 min), and are reported as micromoles of K^+ released or Na^+ taken up per g Hb per min. The stressed values were corrected for the flux in unstressed cells (5 rpm), which was 0.05–0.15 μmol/g Hb per min for both Na^+ and K^+.

Inhibition of stress flux by DIDS

To determine the deformation-induced cation flux rates in the presence of DIDS, appropriate volumes of a fresh stock solution of 5 mM DIDS in PBS was added to the red cell suspension in P_i-PVP immediately before the stress runs were performed at 37°C. The total volume of PBS added was equal in all samples, to maintain viscosity constant. These experiments were done at 250 rpm in a medium viscosity of 80 cP, so that the applied shear stress was 1050 dyne/cm^2.

Covalent labeling with DIDS

This was performed under various conditions, described in Results. In all cases, DIDS was added from a 5 mM stock solution in the appropriate buffer made immediately before the experiment, and the cells with DIDS were incubated in the dark. To remove unbound DIDS, the cells were washed twice with buffer containing 0.5% bovine serum albumin [15], and then twice with buffer only.

Red cell deformability

It was important to ascertain that the various drug treatments did not alter cation flux rates by diminishing cell flexibility, which would limit the extent of membrane deformation induced by stress. Erythrocyte deformability was determined before and after the stress runs using osmotic scan ektacytometry [16]. The ektacytometer (Technicon Instruments, Tarrytown, NY), quantitates the deformation of red cells subjected to a defined laminar shear stress in a Couette viscometer [13,16,17]. For the osmotic scan [16], 0.4 ml of the red cell suspension in stress flux buffer was mixed with 2.6 ml of osmoscan buffer (9 mM NaP_i (pH 7.4), 1 mM EDTA, 40 μg/ml sodium azide, 3% PVP K-90), adjusted to 290 mosM with NaCl. The red cells were then added to a continuous gradient of osmoscan buffer in which the osmolality varied from 40 to 700 mosM before entering the ektacytometer.

Anion exchange

In unstressed cells, Band-3-mediated anion exchange was determined by measuring either exchange sulfate-sulfate or sulfate-chloride exchange. Inhibition of sulfate fluxes by DIDS correlates exactly with inhibition of Cl^-/Cl^- exchange [18,19], but sulfate fluxes are

slower and can be measured at 37°C. Sulfate-sulfate exchange was measured as described by Joiner [9]. Cells were washed four times in SST (140 mM sodium chloride, 5 mM sodium sulfate, 20 mM Tris-HCl, 10 mM glucose, pH 7.45 at 37°C), with removal of the buffy coat, and then incubated at a hematocrit of 5 for 1 h in SST at 37°C. After the various experimental manipulations, including any covalent modification with DIDS, anion exchange was determined by adding 10 μl of SST with 10 μCi $K_2{}^{35}SO_4$ to 1 ml cells at 16 hematocrit. After 5–10 min, the first 200 μl sample was taken as the zero time point, and additional samples were collected over the next hour. Samples were mixed with 1 ml cold Tris-Mg and washed as described for the measurement of stress-induced cation fluxes. The pellet was lysed in 1 ml water and an aliquot was scintillation counted in Safety-Solve (RPI, Mount Prospect, IL).

Anion exchange in PVP media

To determine anion exchange in unstressed cells under the same conditions as the stress flux experiment (i.e., cells are washed in PBS but not equilibrated with SST), sulfate-chloride exchange was measured. Erythrocytes were washed in PBS and mixed with P_i-PVP buffer at 37°C, exactly as for the measurement of cation flux rates. To initiate exchange, appropriate amounts of fresh 5 mM DIDS and 600 mM $K_2{}^{35}SO_4$ (415 mCi/mmol) (final sulfate concentration, 5 mM) were added simultaneously to the red cell-PVP suspension. Samples were removed at zero min and subsequent time points (5, 8, or 10 min) and washed in ice-cold Tris-Mg as above. ^{35}S in the pellet was determined by scintillation counting.

Effect of shear on anion exchange

For efflux measurements, red cells were washed four times in SST and then equilibrated for 1 h, 37°C, with SST containing 0.1–1.0 μCi/ml [^{35}S]sulfate. They were then washed three times at 0°C to remove exogenous ^{35}S and adjusted to an hematocrit of 48. The cells were mixed with SST-PVP to make the final hematocrit equal to 8, PVP 7.2%, and viscosity 83 cP. The application of shear and sampling into Tris-Mg was exactly as described for cation flux measurements. After centrifugation for 90 s, hemoglobin in the supernatant was determined as a measure of hemolysis, and an aliquot was mixed with an equal volume of 10% TCA at 0°C. After a 10 min centrifugation, the clear supernatant was neutralized and scintillation counted in Safety-Solve.

For measurements of exchange rates in the inward direction, cells equilibrated in SST without radioactivity were mixed as above with SST-PVP containing 1 μCi/ml [^{35}S]sulfate. After the suspensions were subjected to shear in the viscometer, 0.2 ml samples were either washed in Tris-Mg as usual or alternatively, layered on 1.0 ml of 20% sucrose in SST and centrifuged. The supernatant and the sucrose layer were removed by aspiration and the pellet was lysed in 1 ml water. Hemoglobin in the pellet was determined and an aliquot of the supernatant was counted.

Chymotrypsin digestion of Band 3

The methods of Passow and coworkers were used [20,21]. Washed red cells in PBS-glucose (hematocrit = 10) were treated with 1 mg/ml chymotrypsin for 1 h at 37°C. They were then washed twice with 0.5% BSA in PBS-glucose and twice in PBS-glucose. These cells were then incubated with 10 μM DIDS at pH 7.4, 37°C, and stress flux determined. The extent of chymotryptic cleavage and DIDS cross-linking between the Band 3 fragments was determined by SDS-gel electrophoresis [20] of washed membranes, using the StrataScan densitometry analysis program (Stratagene, La Jolla, CA).

Effect of intracellular chloride on stress-induced permeability

The method of Katnik and Waugh [22] was used to remove internal chloride, completely replacing it with sulfate. Washed red cells were suspended in 110 mM K_2SO_4, 5 mM K-Hepes (pH 7.5) at a hematocrit of 5 for 2 h, 37°C. The cells were pelleted and resuspended in a fresh volume of the same buffer every 30 min. Katnik and Waugh [22] demonstrated that this method produces essentially complete exchange of chloride for sulfate. Controls were cells incubated in PBS. The cells were chilled to 0°C, quickly washed twice in ice-cold PBS, and adjusted to a hematocrit of 48. Na^+ and K^+ fluxes under stress were immediately measured as described above.

Other methods

Red cell parameters were determined by standard methods using the Coulter counter Zf with a Channelyzer attachment for cell size and number, and Drabkin's reagent [14] to determine hemoglobin. Recrystallized 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS) from ICN was a gift of Dr. C. Joiner. Dipyridamole was a gift of Burroughs-Wellcome. DIDS and all other compounds were obtained from Sigma.

Results

DIDS inhibits stress-induced cation fluxes

Red cells subjected to mechanical stress have increased permeability to Na^+ and K^+ [1–5]. When red cells were stressed in the presence of DIDS, however, the induced K^+ efflux and Na^+ influx were both inhibited. Fig. 1 shows the averages for four experiments in which a wide range of DIDS concentrations

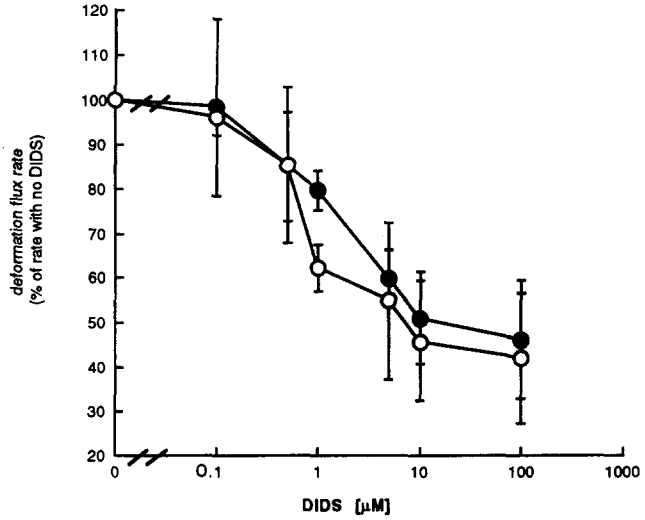

Fig. 1. Effect of DIDS on stress-induced cation fluxes. Appropriate volumes of a fresh stock solution of 5 mM DIDS in PBS was added to the red cell suspension in P_i-PVP immediately before the stress runs were performed at 37°C. The total volume of PBS added was equal in all samples, to maintain viscosity constant. All experiments were done at 250 rpm in a medium viscosity of 80 cP, so that the applied shear stress was 1050 dyne/cm^2. The maximal cation flux rates in stressed cells were 1.4 ± 0.23 μmol/g Hb per min. The cation flux rates in unstressed cells were 0.12 ± 0.04 (Na^+) and 0.20 ± 0.06 (K^+) μmol/g Hb per min. These values were subtracted from the stressed rates before plotting the data. Means and standard deviations for four complete titrations are given. (○), K^+ efflux rate; (●), Na^+ influx rate.

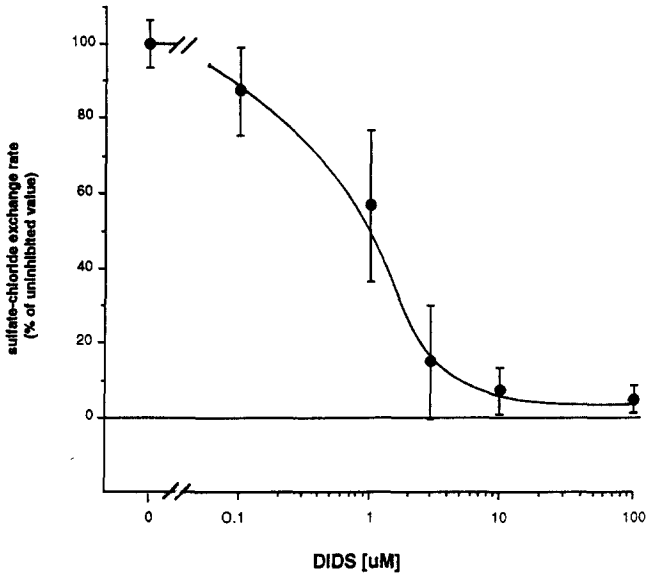

Fig. 2. DIDS inhibition of anion exchange in stress flux media. Red cells were washed in PBS and mixed with P_i-PVP buffer at 37°C, exactly as described for the measurement of flux rates. To initiate the exchange, appropriate volumes of a fresh stock solution of DIDS in PBS and $K_2{}^{35}SO_4$ (415 mCi/mol, 5 mM final) were added simultaneously to the suspension. Duplicate 0.2 ml samples were removed and washed twice with cold Tris-Mg containing 0.1% BSA at zero and 10 min. The cell pellet was lysed in distilled water and ^{35}S determined by scintillation counting. The data are the means of six experiments.

were used. Three other experiments have been done with a restricted number of DIDS concentrations. The apparent inhibition constant $K_{1/2}$ for DIDS was 1–2 μM. Inhibition was never complete, averaging $58 \pm 14\%$ (mean ± S.E.) for K^+ efflux and $54 \pm 13\%$ for Na^+ influx, even at the highest DIDS concentrations tested (100 μM). We found no DIDS effect on cation flux rates in unstressed human cells, in common with most [9,15,18,23,24], but not all [2] previous studies. DIDS inhibited anion exchange in PVP buffers, with an apparent $K_{1/2}$ of 1 ± 0.5 μM (Fig. 2). The deformation-induced flux is unlikely to change membrane potential very much, since the inward Na^+ flux is nearly equal to the outward K^+ flux [1,2]. The permeability constant for K^+ and Na^+ in maximally DIDS inhibited stressed cells can then be estimated from the observed flux and the concentration gradient, and was approx. 0.01–0.02 μmol/g Hb per min per mM.

Cell deformability with DIDS

DIDS did not inhibit the stress-induced flux by limiting the extent of deformation. Ektacytometric osmotic scans (Fig. 3) showed no loss of deformability after shearing in the presence of DIDS. Higher concentrations (> 1 μM) caused a small expansion of cell volume [25] which could be detected in the osmotic scans [16] by a shift in the position of the low osmolality minimum from 150 to 160 mosM.

Inhibition of stress-induced fluxes by covalent DIDS binding

The reaction between Band 3 and DIDS is complex (reviewed in Ref. 19). Non-covalent binding occurs within 1 s, completely inhibiting anion exchange. Covalent bonding then follows, primarily with a specific lysine, Lys a, but also with a second site, Lys b. Lys a

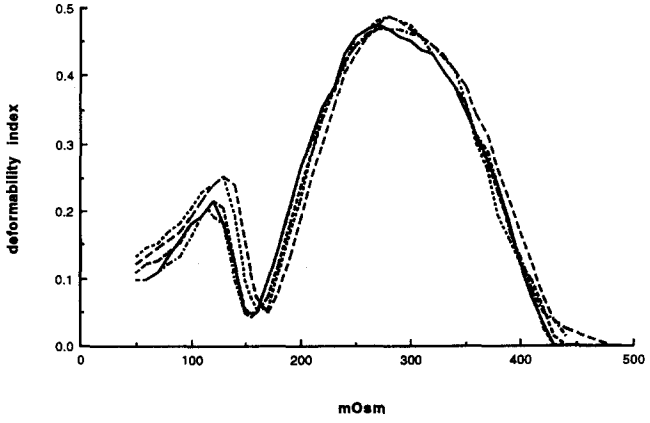

Fig. 3. Osmotic scan ektacytometry of DIDS treated cells after stress. ———, 0 μM DIDS; - - -, 10 μM DIDS; –·–, 0.1 μM DIDS; – – –, 100 μM DIDS; – – –, 1 μM DIDS.

reacts three times more quickly than Lys b at pH 7.4, presumably because of an abnormally low pK. If the reaction is allowed to proceed, Lys a and Lys b are cross-linked by DIDS. It is well-established that at 37°C, 15–20% of the Band 3 molecules immediately form covalent bonds with DIDS, and that the reaction with Lys a is complete in 20 min [18,24]. It is therefore inevitable that when stress flux in the presence of DIDS was measured (Fig. 1), both covalent and non-covalent binding must have occurred.

To determine the effects of covalent binding alone, cells were treated with DIDS and washed with BSA to remove unreacted DIDS before determining rates. In initial experiments with high concentrations of DIDS (100 μM) at 37°C, the deformation-induced cation flux was inhibited, but to variable extents. Inhibition was more consistent at lower DIDS levels, but often less than that observed when DIDS was present throughout the run. For example, when cells were incubated with 10 μM DIDS at 37°C for 30 min, Na^+ and K^+ stress-induced fluxes were reduced by $39 \pm 5\%$ ($n = 4$). The reason for inconsistent results at 37°C is unclear. DIDS reacts with other membrane proteins besides Band 3, especially at concentrations above 10 μM [18], and these sites may have an independent effect on the the membrane's response to shear. These problems could be circumvented, however, by treating the erythrocytes with DIDS at 0°C, which gave reproducible inhibition of the deformation-induced flux.

Correlation of anion exchange inhibition and stress flux inhibition

In addition to greater reproducibility, modification with DIDS at 0°C had a second advantage, in that the covalent reaction is much slower, and cells with partially inhibited anion exchange can be isolated [18,21,24,26,27]. A correlation between the degree of anion-exchange inhibition and of stress flux inhibition would argue that the same covalent modification is involved.

Erythrocytes were first equilibrated with SST (pH 7.4, 37°C) for 1 h, to allow subsequent measurements of sulfate-sulfate exchange [9]. In separate experiments (not shown), it was demonstrated that SST equilibration itself had no effect on the stress induced flux rates or on cell deformability. The cells were then washed three times with SST, whose pH was adjusted to 7.4 at 0°C, and suspended at a hematocrit of 15, 0°C. Freshly dissolved 5 mM DIDS in SST (pH 7.4 at 0°C) was added to a final concentration of 10 μM. Samples were removed at intervals, washed twice with 0.5% BSA in SST (pH 7.4 at 37°C) to remove unbound DIDS, and twice with SST. Two aliquots of these modified cells were taken, one for measurement of sulfate exchange, and the second for measurement of the stress-induced cation flux. A linear relationship was found between the two inhibitions (Fig. 4).

Lysine crosslinking is not required for inhibition of stress flux

Chymotrypsin cleaves Band 3 at a site between Lys a and Lys b, yielding two fragments of 60 kDa and 35 kDa on SDS gel electrophoresis. Jennings and Passow showed that these lysines can be crosslinked by H_2DIDS, and the electrophoretic mobility of the crosslinked fragments is that of uncleaved Band 3 [19–21], offering a simple assay for the degree of crosslinking. Jennings and Passow used H_2DIDS rather than DIDS in their original work. We found that covalent DIDS was also able to crosslink the fragments of Band 3, although somewhat less efficiently than H_2DIDS. When the DIDS modified cells were exposed to pH 9, which accelerates reaction at Lys b [20], 40% of the cleaved Band 3 was crosslinked, as shown by SDS-gel electrophoresis. Under the same conditions, covalent H_2DIDS crosslinked about 75% of the fragments.

To show that cross-linking did not occur in cells with an inhibited deformation-induced flux, we first verified that chymotrypsin treatment itself did not affect the stress-induced cation flux (Table I). Cells in which all Band 3 molecules were cleaved by chymotrypsin were

TABLE I

Effect of chymotrypsin digestion and DIDS crosslinking of Band 3 on stress-induced cation fluxes

	Cation flux rate (mean and S.E.)				% of band 3 migrating at 95 K
	Unstressed		Stressed		
	Na^+ influx	K^+ efflux	Na^+ influx	K^+ efflux	
Undigested cells	0.12 ± 0.42	0.05 ± 1.30	2.83 ± 0.37	3.00 ± 1.58	100
CHT	0.12 ± 0.49	0.04 ± 0.35	3.04 ± 0.86	2.89 ± 0.46	0
CHT + DIDS	0.00 ± 0.67	0.24 ± 0.59	1.86 ± 0.68	1.91 ± 0.32	0

CHT = erythrocytes were digested with 1 mg/ml chymotrypsin, 1 h, 37°C.
DIDS = incubated with 10 μM DIDS (pH 7.4), 30 min.
Shear stress = 1075 dyne/cm^2
Cation flux rates in μmol/g Hb per min. $n = 3$.

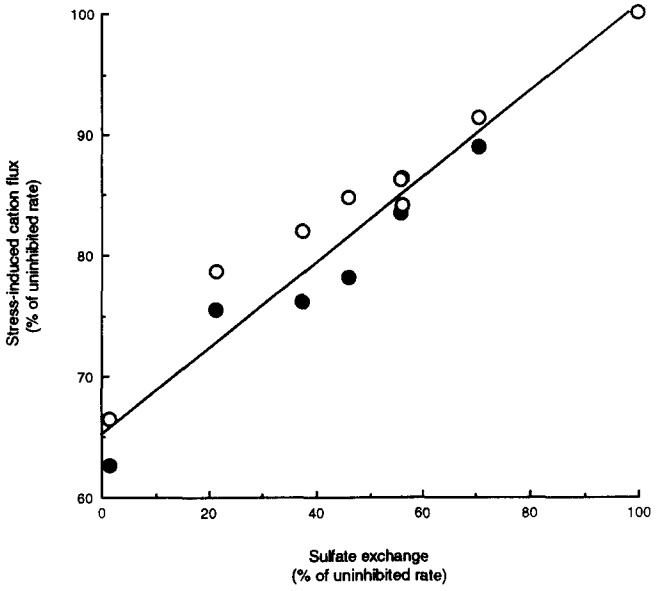

Fig. 4. Comparison of irreversible DIDS inhibition of sulfate exchange and stress flux. Red cells were equilibrated with SST (pH 7.4, 37°C) for 1 h. They were then washed three times with SST, whose pH was adjusted to 7.4 at 0°C, and suspended at a hematocrit of 15, 0°C. Stock 5 mM DIDS was prepared in SST (pH 7.4 at 0°C) and added to a final concentration of 10 μM. Samples were removed at intervals and washed twice with 0.5% BSA in SST (pH 7.4 at 37°C). Sulfate exchange and stress flux were determined on aliquots of these cells. •, Na^+ influx; ○, K^+ influx. Two separate experiments are shown. The line is a least-squares fit of all the points ($R = 0.967$).

then covalently modified with 10 μM DIDS, 30 min, 37°C, and washed with BSA. DIDS was found to inhibit the deformation-induced cation flux in these cells (Table I), and SDS gel analysis showed that no cross-linking had occurred. Neither chymotrypsin digestion nor 10 μM DIDS at 0°C had a significant effect on cell deformability (data not shown).

TABLE II

Effect of anion-exchange inhibitors on stress-induced cation fluxes

For DNDS inhibition, appropriate volumes of a fresh stock solution of 5 mM DNDS in PBS was added to the red cell suspension in P_i-PVP immediately before the stress runs ($n = 3$). Dipyridamole was added directly before and was present throughout the stress run ($n = 1$).

	Concentration (μM)	deformation-induced flux (% of control flux)	
		Na^+	K^+
DNDS	0.1	114.8 ± 0.0	92.0 ± 0.0
	1	104.4 ± 7.56	96.5 ± 6.14
	10	103.3 ± 11.7	84.5 ± 19.3
	100	83.1 ± 23.16	82.1 ± 12.9
	1000	54.3 ± 13.8	57.0 ± 2.0
Dipyridamole	20	100	97
	100	55.0	57

Effect of other anion exchange inhibitors on deformation-induced flux

DNDS, an analog of DIDS which is unable to form covalent linkages, was tested (Table II). As for DIDS, the maximal inhibition of stress-induced flux obtainable with DNDS was 50%, but the K_i was much higher (approx. 100 μM). Dipyridamole did not inhibit stress flux at 20 μM, but reduced the rate by half at 100 μM (Table II). Sulfophenyl isothiocyanate [28] had no effect on stress flux rates (not shown). In order to facilitate comparison with the deoxygenation induced cation exchange of sickle cells, the conditions used here to inhibit the stress-induced flux were the same as those used by Joiner [9] in his study of sickle cells. Similar levels of inhibition were found for both fluxes. None of the compounds affected cell deformability.

Effect of shear on anion exchange

In contrast with cation fluxes, the rate of anion exchange was not significantly altered by shear induced deformation. The observed rates of [^{35}S]sulfate uptake were $5.19 \pm 1.56 \cdot 10^{-3}$ min^{-1} at 5 rpm and $6.02 \pm 0.29 \cdot 10^{-3}$ min^{-1} at 250 rpm ($n = 4$). Efflux rates were also measured with similar results, although hemolysis of cells pre-incubated in SST made the calculation of rates imprecise.

Effect of internal chloride on the stress-induced permeabilities

DIDS is an effective inhibitor of the volume-sensitive K^+,Cl^--cotransport in duck [29] and sheep [30] erythrocytes, but in human cells, either no [31,32] or marginal [9] inhibition has been reported. To show that the stress-induced fluxes are not a manifestation of this cotransport, we removed all internal chloride by exchanging it for sulfate [22]. When these exchanged cells were subjected to shear, the rate of cation movement was the same as control cells that had been incubated for a similar period in PBS (Table III).

TABLE III

Effect on stress induced cation fluxes of exchanging intraerythrocytic chloride for sulfate

Intracellular chloride was exchanged for sulfate as described by Katnik and Waugh [22].

		Stress flux rate (μmol/g Hb per min)	
		control	sulfate loaded cells
Stressed cells	Na^+ influx	3.75	3.92
	K^+ efflux	3.97	3.88
Unstressed cells	Na^+ influx	0.49	0.27
	K^+ efflux	0.06	0.04

Discussion

An important motivation for this work [1,5] has been to model the deoxygenation-induced cation exchange of sickle cells. Deoxygenated sickle cells become permeable to monovalent cations [6–10] as a result of the extreme deformation of the membrane [7]. This phenomenon is of clinical interest since it may trigger the pathological dehydration of sickle cells [33,34]. Similarities between the two pathways emerged from independent studies of the stress-induced flux [1,2] and the sickling induced flux [8–10], which showed that both are approximately equimolar exchanges of Na^+ and K^+, have a similar pH-dependence, are not dependent on external chloride, and are reversed when the deformation ceases. This paper adds the observations that inhibition by DNDS, dipyridamole and sulfophenyl isothiocyanate are similar in stressed normal red cells and in deoxygenated sickle cells [9], and the rate of anion exchange is unaltered in stress-deformed erythrocytes (Results, above), as in deoxygenated sickle cells [35]. However, the response of the two pathways to DIDS is quite different. The $K_{1/2}$ for DIDS inhibition in deoxy sickle cells is 10 μM, which is significantly higher than the $K_{1/2}$ for deformation flux (1 μM). Moreover, DIDS failed to inhibit deoxy sickle cell fluxes after incubation at 0°C [9], and the parallel inhibition of deformation flux and anion exchange (Fig. 4) was not seen in the sickle cell [9]. Therefore, it appears that the cation exchange of deoxygenated sickle cells is not the same as that induced in normal red cells by deformation. To some extent, the stress-induced flux resembles the increase in cation permeability induced by low-Cl media, which also has little selectivity for cations [36] and is inhibited by DIDS in parallel with anion exchange [27].

Although DIDS inhibits the deformation-induced cation flux, it is not likely that the anion pathway through Band 3 is the route followed by cations during mechanical stress. DNDS, an effective inhibitor of anion exchange which binds to the chloride site on Band 3 [37], only weakly inhibits the stress-induced cation flux, and other good inhibitors of anion exchange were also relatively ineffective against the deformation flux. Anion exchange itself was not stimulated by mechanical stress. Nevertheless, the parallel inhibition of anion exchange and the deformation flux by DIDS suggests that the same site is involved in both processes, and under the conditions of the experiment in Fig. 4 (neutral pH, 0°C), Lys a of Band 3 is the major modified amino acid in the membrane [18,19,23].

A speculative model for the effect of mechanical shear and DIDS can be suggested. The insertion of transmembrane proteins into lipid bilayers non-specifically increases ion permeability by a large factor [38,39]. This and other evidence [40] suggests that the protein-lipid interface is a major route for passive cation permeation through biological membranes. High membrane tensions are created by mechanical shear in our experimental arrangement [41]. This tension can affect the conformation of integral proteins and their interaction with membrane lipids, thereby increasing non-specific ion permeability. Compounds that alter integral protein conformation will also be expected to exert an influence on non-specific permeability, and DIDS alters Band 3 conformation (review in Ref. 42). In support of this model, it has been observed that the insertion of either Band 3 or glycophorin into liposomes increases their ion permeability and, remarkably, the permeability is reduced by DIDS [43]. Related work has shown that modification of Band 3 sulfhydryls by *p*-chloromercuriphenyl sulfonate increases non-specific membrane cation permeability [44]. Although the normal unstressed red cell is relatively impermeable to cations despite a large amount of Band 3 in the membrane, these findings suggest that chemical modification of Band 3 can increase or decrease non-specific permeabilities.

The DIDS-inhibitable stress-induced cation transport observed in this study (1–2 μmol/g Hb per min) would be accounted for by a flux through Band 3 of about 1 cation/Band 3 per s. This is much lower than the chloride exchange rate (50 000/s), and even lower than the conductive rate of about 5/s [23,26]. This low rate of permeation is consistent with a pathway formed by rare fluctuations in the membrane structure. Finally, it can be noted that only about half of the deformation-induced cation flux is inhibited by DIDS, suggesting the presence of multiple pathways through the membrane.

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