Membrane Stress Increases Cation Permeability in Red Cells

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ABSTRACT The human red cell is known to increase its cation permeability when deformed by mechanical forces. Light-scattering measurements were used to quantitate the cell deformation, as ellipticity under shear. Permeability to sodium and potassium was not proportional to the cell deformation. An ellipticity of 0.75 was required to increase the permeability of the membrane to cations, and flux thereafter increased rapidly as the limits of cell extension were reached. Induction of membrane curvature by chemical agents also did not increase cation permeability. These results indicate that membrane deformation per se does not increase permeability, and that membrane tension is the effector for increased cation permeability. This may be relevant to some cation permeabilities observed by patch clamping.

INTRODUCTION

In recent years, a significant increase in our understanding of the effects of physical forces on mammalian cells has been achieved. This progress has been motivated in part by an interest in biotechnological applications of stirred flow reactors for cultured mammalian cells (Tran-Son-Tay, 1993), as well as studies of the mechanosensitive ion channels detected in many cell types by patch clamping (Morris, 1990; Davies and Dull, 1993). Because of the complexity of eucaryotic cells, it is often useful to examine physiological responses to mechanical forces in simpler situations where both the applied force and the response can be well defined. One such case is the augmented permeability to cations seen in human red cells subjected to various types of deformation. Normal erythrocytes deformed by shear have an increased permeability to monovalent (Johnson and Gannon, 1990; Ney et al., 1990) and divalent (Johnson and Tang, 1992) cations. Similarly, the erythrocytes of sickle cell anemia acquire distinctive elongated spicules upon deoxygenation, and at the same time their permeability to both monovalent and divalent cations increases (Tosteson et al., 1952; Rhoda et al., 1985; Mohandas et al., 1986; Clark and Rossi, 1990; Joiner, 1990, 1993; Joiner et al., 1993). In both cases, the increase in permeability is reversed when the normal shape of the cell is restored. Because of the simplicity of the structure of the erythrocyte, this physically induced response is particularly amenable to detailed study, and may afford insights into the responses of more complex mammalian cells. The permeabilities induced in red cells have been called shape dependent or deformation dependent, but under the circumstances where cation permeability is increased, the cell membrane was also subjected to a shear stress. We here present arguments that shape change per se does not increase cation permeability, but rather that membrane stress is the inducing factor.

MATERIALS AND METHODS

Red cells were obtained in heparin from normal volunteers with informed consent, 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. They were adjusted to a hematocrit of 48 in PBS for subsequent manipulations.

Shear conditions

To facilitate comparisons between the degree of deformation and the magnitude of the increased permeability, cation fluxes and cell deformation were both determined in an ektacytometer (Technicon Instruments, Tarrytown, NY). The ektacytometer was originally designed to quantitate the deformation of a population of red cells subjected to a defined laminar shear stress in a Couette viscometer (Bessis and Mohandas, 1975). It was therefore convenient to measure the cation permeabilities in the ektacytometer, where erythrocyte deformation could be determined in the same conditions of applied shear as those used to induce the cation fluxes. The Couette geometry has the additional advantage that all the cells are subjected to the same shear stress (Tran-Son-Tay, 1993). The viscometer had a radius R of 5 cm and a cylinder gap ϵ of 0.5 mm. The shear rate (s^{-1}) in the cylinder gap is $r = (R/\epsilon) \times (2\pi/60) \times$ rpm, where R = cylinder radius (cm) and ϵ = cylinder gap (cm). The shear stress (dynes/cm^2) is $r \times \nu$, where ν = viscosity of the suspending medium in poise (Groner et al., 1980). These are nominal stresses, because the red cell modifies the flow conditions in its vicinity.

Measurement of cation flux rates

All cation determinations were made by measuring the internal cation content of pelleted erythrocytes rather than by measuring the cations released into the medium, to avoid possible complications from cell hemolysis. Both Na and K were determined by flame photometry (model 343, Instrumentation Laboratory, Lexington, MA).

Cation flux rates in sheared cells

These fluxes were measured in PVP buffer, which was 10 mM sodium phosphate and 1 mM EDTA, with polyvinylpyrrolidone (PVP K-90, average molecular weight 300,000, GAF Chemicals, Wayne, NJ) added to increase

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the solution viscosity. The pH was adjusted to 7.4, 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.

Washed red cells (hematocrit = 48) and PVP buffer, both prewarmed to 37°, were mixed at a 1:5 ratio to make the final hematocrit equal to 8 and the final PVP concentration approximately 7%. The suspension was placed in the Couette viscometer of an ektacytometer, whose cylinders were wrapped in heating coils to maintain the internal temperature at 37°, 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. Triplicate 0.2-ml volumes of cell suspension were removed from the side port and placed in chilled 1.5-ml centrifuge tubes containing ice-cold Tris-Mg solution (10 mM Tris-Cl, 107 mM $MgCl_2$, pH 7.4). The cells were immediately pelleted by a 90-s spin in a chilled Eppendorf microcentrifuge at 4° (Brinkmann Instruments, Westbury, NY). The supernatant was completely removed and an aliquot saved for hemoglobin determination with Drabkin's reagent to estimate hemolysis. As noted previously (Groner et al., 1980; Johnson and Gannon, 1990), flow in the viscometer is laminar under the conditions of the experiment, and little hemolysis was observed (<3%) even at the highest stresses used. This is consistent with the finding of Leverett et al. (1972) that red cells are not hemolyzed by shear forces below 1500 dynes/cm^2 in Couette viscometers, even in turbulent flow. After a second wash in ice-cold Tris-Mg solution, which was sufficient to reduce external Na and K to negligible levels, the cells were lysed in 1.0 ml of distilled water. Hemoglobin was determined with Drabkin's reagent, and Na and K were determined.

Unstressed controls were obtained by repeating the entire experiment with the same cell suspension in the same buffer, but with a cylinder rotation speed of 5 rpm.

As reported (Johnson and Gannon, 1990), 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 one or two additional times in the linear region. The values for flux under stress were corrected by subtracting the flux observed in unstressed cells (5 rpm). Rates are reported as mmol K released or Na taken up/kg hemoglobin/min. Applied shear forces were calculated as described above from the rotation rate and the actual viscosity of the PVP-red cell suspension, determined in a thermostated Cannon viscometer.

Shape estimation during stress

Under laminar shear in the Couette viscometer of the ektacytometer, the membrane processes around the circumference of the cell, in the motion termed tank-treading (Fischer et al., 1978). This motion does not disrupt the normal intramolecular associations of the membrane (Weaver et al., 1990). At the same time, red cells elongate and orient themselves with their long axis normal to the rotational axis (Bessis and Mohandas, 1975; Groner et al., 1980). Because of its high hemoglobin concentration, the interior of the red cell has a refractive index greater than that of the medium, and scatters light. The Couette viscometer of the ektacytometer is constructed of clear plastic, and information about cell shape is derived from the diffraction pattern produced by a laser beam directed normal to the axis of rotation. The diffraction pattern is circular when the cells are at rest, but becomes elliptical as they are stressed and elongate. For the measurement of cell deformation, the length L and width W of the first diffraction ring are measured, and the ellipticity index $EI = (L - W/L + W)$ is calculated by the automated image analyzer, which is part of the Technicon instrument. It can be shown (Groner et al., 1980; Mohandas et al., 1980) that this image analyzer produces a quantitatively accurate measure of the red cell ellipticity. For the measurement, a suspension of cells in isotonic PVP buffer (20 μl whole blood in 6 ml isotonic diluent) were introduced into the nonrotating viscometer. Shear stress was varied by gradually increasing the rotation speed at a rate of 1 rpm/s, while EI was measured continuously. The analog output of the ektacytometer was digitized with a digitizing tablet (Summagraphics MM 1812).

Effect of shape-altering compounds

To determine the effect of various shape-altering compounds on the cation permeability of red cells, washed erythrocytes were brought to 37° and mixed with five volumes of PBS containing the compounds, also at 37°. A zero-time sample was taken immediately, and additional samples were taken at intervals over the next 30 min. Cation fluxes were measured as described.

To determine whether they affected the shear-induced flux, the compounds were made up as 100× concentrated solutions in methanol and added to the red cell-PVP suspensions, which were then incubated for 30 min at 37° before measuring the stress-induced permeabilities as described above. The compounds were also present in the PVP solutions used in the ektacytometer during the stress run.

RESULTS

Rate dependence on applied stress

In previous experiments (Johnson and Gannon, 1990; Johnson and Tang, 1992), red cells suspended in 7% PVP K-90 were subjected to stresses ranging up to 910 dynes/cm^2 by varying the rotation speed at a constant viscosity (70 cP). The maximal rotation speed of the Technicon ektacytometer is 250 rpm, and to increase the applied stress and the flux rate, it was necessary to produce higher viscosities by increasing PVP concentrations, up to a maximum of 7.5%. After mixing the red cells and the PVP solutions, the viscosities of the suspensions as determined in a thermostated Cannon viscometer were between 70 and 85 cP at 37°. They were loaded into the Couette viscometer of the ektacytometer and subjected to a constant shear rate of 1300/s (250 rpm). Shear stresses up to 1250 dynes/cm^{2}were obtained. As shown in Fig. 1, the induced cation permeabilities gradually increased until applied shears of 800–1000 dynes/cm^2 were attained. Thereafter, the permeabilities increased in an apparently exponential manner. Earlier work at lower shear stress (Johnson and Gannon, 1990; Ney et al., 1990; Hebbel and Mohandas, 1991) had indicated a linear response.

Deformation response to shear stress

The deformation of sheared red cells in PVP suspensions was monitored by light scattering (Fig. 2). The maximal stress shown in Fig. 2 was 1233 dynes/cm^2, which approaches but does not attain the hemolytic stress of 1500 dynes/cm^2 (Leverett et al., 1972). As reported earlier (Bessis and Mohandas, 1975; Fischer et al., 1978; Mohandas et al., 1980), elongation initially increases rapidly with applied stress, up to an ellipticity of 0.7, but further elongation requires considerably greater stress. The top panel of Fig. 2 shows that the ellipticity continued to increase at the higher stresses, without attaining a plateau value. Similar behavior was reported (Pfafferott et al., 1985) for erythrocytes under direct visual observation in the rheoscope: cell elongation did not plateau, but continued to increase slowly as higher stresses were applied.

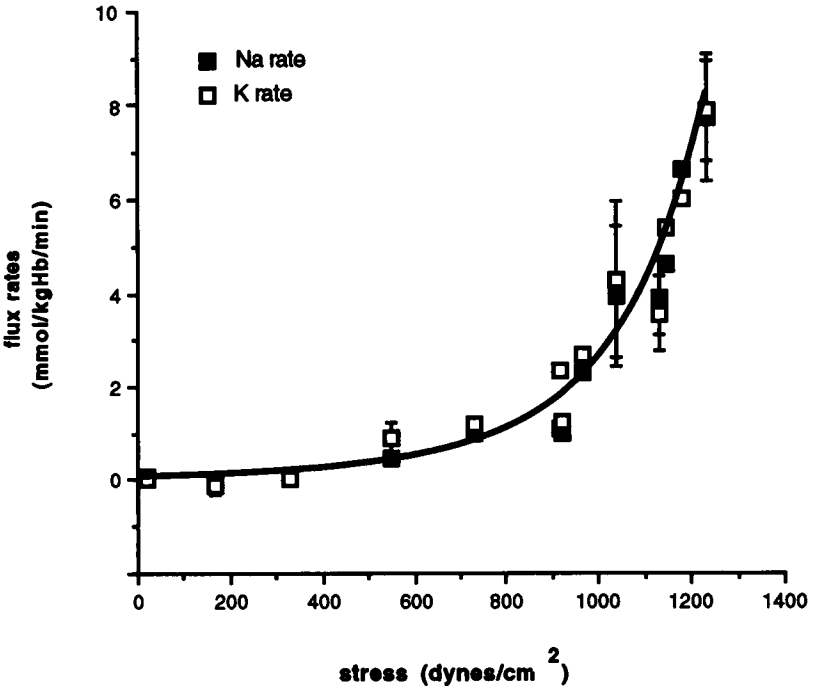

FIGURE 1 Effect of applied shear stress on flux rates. For the lowest four data points, stress was varied by changing the shear rate in 7% PVP. For the rest of the points, shear rate was constant at 1300/s and viscosity was varied. Each point on the figure represents the average rate with a different PVP buffer. Each PVP solution was used for a different number of experiments (1–10), and the error bars show the SE of the flux rates for those viscosities used more than once. Shear stress is calculated in dynes/cm^2 as shear rate × viscosity in poise.

Relation between elongation and cation flux

In Fig. 3, data from light scattering and cation flux measurements are compared. It can be seen that cation flux did not begin until 96% of the maximal cell elongation was attained. Thereafter, the rate of cation flux rose rapidly with small increments of elongation. As the last 1% of the cell extension was attained (EI = 0.770 to EI = 0.780), the rate of cation flux increased fivefold.

Chemically induced shape changes do not affect cation permeability

It has long been known that anionic amphipaths induce echinocytic shape change in red cells (reviewed in Steck, 1989). Similarly, cationic amphipaths induce stomatocytic shape change (Deuticke, 1968). However, neither of these shape changes is associated with an increase in cation flux in unstressed cells (Table 1). The failure to induce any abnormal cation flux as a result of shape change is especially striking in the echinocytic cells (Fig. 4), where the membrane protrusions have very small radii of curvature, estimated to be 250 nm (Ferrell et al., 1985).

The effect of shape-altering compounds on the stress-induced cation flux was also determined (Table 1). Chlorpromazine (20 μM) did not inhibit the stress-induced flux. The echinocytic compound tetracaine appeared to augment slightly the sensitivity of the cell to shear. None of the compounds lowered cell deformability under the conditions of the experiment as determined by osmotic scan ektacytometry (Clark et al., 1983) (data not shown).

DISCUSSION

When erythrocytes are deformed by hemoglobin S polymerization (Joiner, 1993) or by shear (Johnson and Gannon, 1990; Ney et al., 1990), monovalent and divalent cation permeabilities are augmented. This might be a consequence of membrane curvature per se, but evidence presented here argues against this. First, curvature induced by amphipathic compounds did not increase either Na or K flux. Secondly, the results shown here indicate that human erythrocytes subjected to laminar shear achieved 96% of their maximal elongation before significant cation flux was seen. Higher shear stresses then markedly stimulated both Na influx and K efflux with only a marginal increase in cell elongation.

Other more limited studies are consistent with the data on amphipathic compounds. Jennings and Schultz (1990) found no effect of chlorpromazine or echinocytic agents on ^{86}Rb uptake in rabbit erythroyctes, and 10 μM chlorpromazine has no effect on cation transport in human cells (Brugnara et al., 1985; Kaji, 1986). There is therefore no evidence that chemically induced membrane curvature can activate cation permeabilities in the red cell. This is in contrast with *E. coli* membranes, where a well-documented (Sukharev et al., 1993, 1994) mechanosensitive ion channel exists, which is activated by amphipathic compounds (Martinac et al., 1990).

With regard to erythrocyte elongation, Hebbel and Mohandas (1991) have made similar observations for K efflux at low-shear stresses. Na influx was not measured. They also found that pretreating erythrocytes with an oxidizing agent, *t*-butylhydroperoxide (*t*BHP), lowered the threshold for K efflux (Ney et al., 1990; Hebbel and Mohandas, 1991), so that an increased K efflux could be detected when cells were

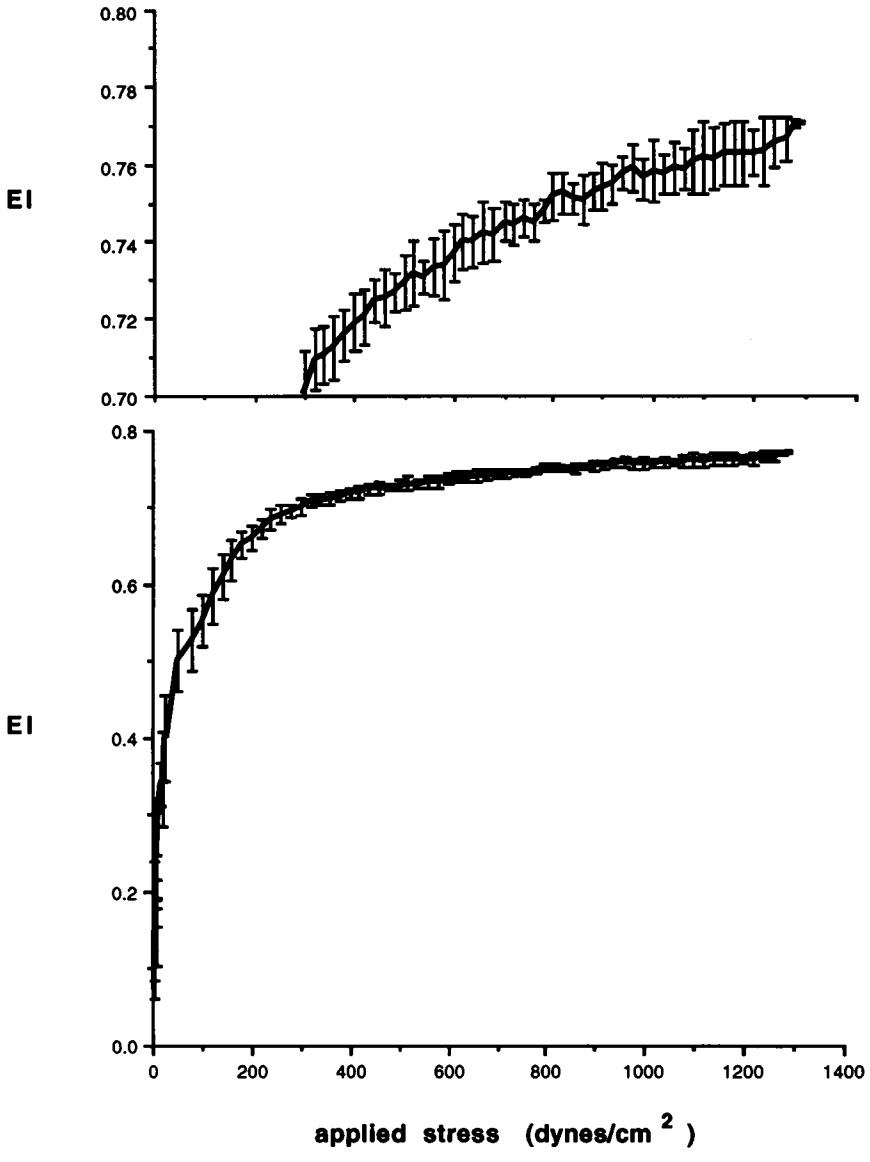

FIGURE 2 The deformability index under shear stress. Red cells suspended in 7.5% PVP in phosphate buffer were subjected to an increasing shear stress in the Couette viscometer of the ektacytometer. The analog traces of ellipticity (*EI*) *vs.* applied shear stress from the ektacytometer were digitized at intervals of 20 dynes/cm^2. Six traces were averaged, and the mean and SE are shown.

deformed to only 63% of their maximal possible elongation. The interpretation of these results is difficult, as *t*BHP has multiple effects on the erythrocyte membrane. It is known, for example, that even less vigorous treatment with *t*BHP than that used by Hebbel and Mohandas (1991) inhibits the transport ATPases (Moore et al., 1989), and that comparable concentrations can significantly increase membrane malondialdehyde levels (Chen et al., 1991; Sugihara et al., 1991), convert 20% of the hemoglobin of the cell to the met form (Chen et al., 1991), and lower membrane mechanical stability (Chen et al., 1991). Slightly higher concentrations augmented K efflux even in unstressed cells (Van der Zee et al., 1989). Nevertheless, a specific role for oxidized lipid in activating the stress-induced flux was suggested by the observation that lipid peroxides inserted with the phospholipid exchange protein increased the flux rates (Sugihara et al., 1991). In sum, these results can be interpreted as evidence that oxidation increases the sensitivity of the cell to membrane tension. Anion exchange is not affected by mechanical shear (Johnson and Tang, 1993).

It is difficult to estimate the membrane tension required to initiate cation flux. Tran-Son-Tay et al. (1987) have provided an algorithm to calculate the membrane tension in a tank-treading erythrocyte, which indicates that the tension is not uniform throughout the membrane, being greatest on the flattest part of the central streamline and decreasing toward the the more curved regions. This calculation requires values of the semiaxes of the ellipsoidal red cell and its angle of inclination, which are not known for cells at high shear rates.

Deformation or stress-induced permeabilities have been observed in a number of physiological contexts. The maximal stress experienced by erythrocytes in the circulation is estimated to be 400 dynes/cm^2 in the capillaries (Chien, 1975), which is within the range that will increase the permeability of the membrane to cations. The stress experienced by the membrane of sickle cells during sickling is likely to

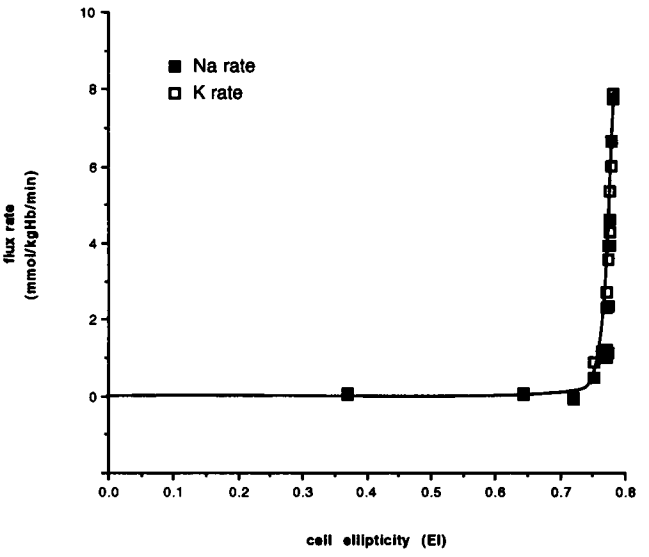

FIGURE 3 Relation between cell ellipticity and cation permeability.

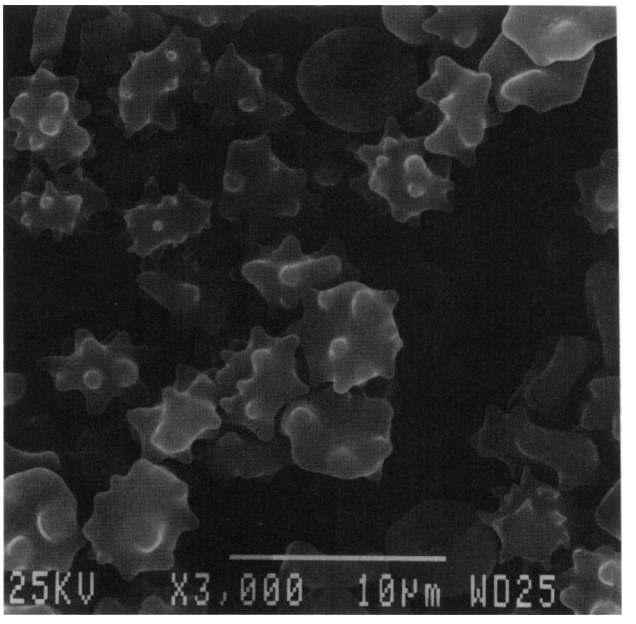

FIGURE 4 Scanning electron micrographs of human erythrocytes in 500 μM tetracaine.

TABLE 1 Effect of shape-altering compounds on cation flux rates

	5 rpm		250 rpm	
Compound	Na influx	K efflux	Na influx	Kefflux
Control	0.14 ± 0.13	0.15 ± 0.21	3.54 ± 0.70	3.56 ± 0.15
CPZ (20 μM)	0.14 ± 0.14	0.21 ± 0.16	3.78 ± 0.88	3.31 ± 0.16
Tetracaine (500 μM)	0.22 ± 0.24	0.28 ± 0.11	4.31 ± 1.35	4.03 ± 0.59

These flux rates were measured as described in Materials and Methods. Mean ± SD. $N = 3$.

be much greater, and higher shear stresses examined in this study are probably a better model than low-shear exposures. This idea is supported by the observation that the high stress flux' is inhibited by 4,4'-Diisothiocyanatostilbene-2,2'disulfonic acid (DIDS) (Johnson and Tang, 1993), as is the sickling-induced flux (Joiner, 1990), whereas DIDS had no effect at low applied stresses (Ney et al., 1990; Sugihara et al., 1991). Our observations may also be relevant to studies of the mechanosensitive channels of eucaryotic cells (Morris, 1990; Davies and Dull, 1993). Most of these channels have been detected by patch-clamping techniques, which applies large pressures to a limited area of the membrane. Morris has pointed out (Morris, 1990, 1992) that these large pressures may well induce nonspecific effects on permeability. In support of this hypothesis, Morris and Horn (1991) found that macroscopic mechanosensitive currents could not be elicited in *Lymnaea* neurons by applying various mechanical stimuli, even though patch clamping had indicated that mechanosensitive channels were present in these cells, leading them to suggest that some examples of single-channel mechanosensitivity were artifacts of patch recording. In our work, large membrane tensions comparable to those produced by patch-clamping were shown to induce a cation permeability in erythrocytes, which are not likely to have true mechanosensitive channels. We propose that an increase in cation permeability in response to high membrane tension may represent a general physical property of cell membranes.

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