Biochimica et Biophysica Acta, 691 (1982) 211–219
Elsevier Biomedical Press

BBA 71342

DEFORMABILITY OF ISOLATED RED BLOOD CELL MEMBRANES

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(Received March 9th, 1982)

Key words: Erythrocyte membrane; Deformability; Ca^{2+} effect; Laser diffraction; Ektacytometry

We have used a laser diffraction method (ektacytometry) to directly measure the membrane component of red cell deformability, without contributions from either cell geometry or internal viscosity. This technique was validated by subjecting resealed erythrocyte ghosts to manipulations previously shown to increase the membrane shear modulus. Heating above 45°C, pH greater than 9.0 and less than 5.0, and micromolar concentrations of the cross-linking agents, glutaraldehyde and diamide, all reduced the deformability of resealed erythrocyte ghosts. We have applied this assay to the study of reduced cellular deformability of calcium-loaded red cells, and have shown that, for physiological concentrations of calcium, the effect of calcium on the physical properties of the membrane may be negligible when compared to its effect of promoting cell dehydration and subsequent increased cytoplasmic viscosity.

Introduction

The ability of circulating red blood cells to deform is determined by the internal viscosity of the cell, its shape/volume relationship, and by physical properties of the red cell membrane [1]. Several experimental approaches have been used to study these factors. These include filtration [2], viscometry [3], micropipette aspiration [4] and ektacytometry [5]. The latter technique employs analysis of laser diffraction images of a population of deforming erythrocytes in a defined fluid shear field to obtain measurements of cellular deformability. However, ektacytometric measurements of intact red cells, in common with several other methods, is less sensitive to the effects of membrane physical properties than to internal viscosity or surface area-to-volume ratio effects. To better study the specific influence of the red cell membrane on cellular deformability, we have devised a method for ektacytometric measurement of isolated erythrocyte membranes. This approach allows us to directly measure membrane deformability in a large population of cells in the absence of major internal viscosity effects. Although unequivocal measurements of membrane physical properties can be obtained using 1 μm diameter pipettes [4], these are derived from individual cells and not an entire population. An additional advantage of the ektacytometric method is that it permits study of the deformability of erythrocyte ghosts whose membrane skeleton organization has been modified under controlled conditions.

The red cell membrane skeleton, which underlies, and is in close apposition to, the inner lipid monolayer of the membrane, is thought to be responsible for the regulation of cell shape and may have some role in cell deformability. Perturbation of any or all of the membrane skeletal proteins might be reflected in altered membrane physical properties. (For a review of the red cell membrane skeleton, see Ref. 6.)

In this paper we describe experiments designed to assess the suitability of the ektacytometer for

studies of membrane deformability. Membranes were subjected to heat, extremes of pH and chemical cross-linking agents, treatments known to increase membrane shear modulus [7–9]. As an example of the utility of this method, we describe its use to study the effect of calcium on the red cell membrane. Recent studies have indicated that the major determinant of reduced whole cell deformability of calcium-loaded red cells is increased cytoplasmic viscosity (due to calcium-stimulated loss of cell potassium and water), and not membrane stiffening [10,11]. Ektacytometry of calcium-treated membranes enabled us to assess the extent of calcium-induced membrane stiffening uncomplicated by changes in intracellular viscosity.

Experimental procedures

Materials

Materials used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories. Microcrystalline cellulose, α-cellulose, diamide, and dithiothreitol were from Sigma Chemical Co.; diisopropyl fluorophosphate from Aldrich Chemical Co., Milwaukee, U.S.A.; glutaraldehyde from Polysciences Ltd.; Stractan from St. Regis Paper Company, Tacoma, WA, U.S.A.; Protosol and Liquiscint from National Diagnostics; [^{45}Ca]-calcium chloride from New England Nuclear and ionophore A23187 from Calbiochem. All standard chemicals were reagent grade.

Preparation of a homogeneous population of resealed erythrocyte ghosts

Fresh blood was washed in 5 mM Tris, 140 mM NaCl (pH 7.4) and passed through a microcrystalline/α-cellulose column to remove the white cells [12]. Ghosts were prepared by lysing red cells in either 20 vol. or 80 vol. of ice-cold 5 mM Tris, 7 mM NaCl (pH 7.4) to yield 'pink' or 'white' ghosts, respectively. The former contained 2–3% of their original haemoglobin and the latter less than 0.5% of their original haemoglobin. To prevent premature resealing, all manipulations of ghosts were performed at 4°C. After centrifugation and removal of the supernatant haemoglobin, the ghost pellet was resuspended in 10 vol. of isotonic Tris-NaCl pre-warmed to 37°C. The suspension was then incubated at 37°C for one hour to promote resealing [13]. The ghost suspension was subsequently centrifuged at 15000 rpm for 5 min to produce a concentrated ghost suspension for deformability measurements. The concentrations of ghosts were determined using a Coulter counter. Initially, flotation ultracentrifugation using Stractan gradients (density 1.032 g/ml) was used to obtain a homogeneous population of resealed ghosts. This step was later omitted since very few ghosts failed to reseal during the incubation.

Deformability measurements

Ghost deformability measurements were obtained using the ektacytometer, a viscometric instrument originally developed to measure whole cell deformability. The generation of a deformability signal and its analysis is described in detail elsewhere [5].

30 μl of packed, resealed ghosts (approx. $250 \cdot 10^6$ ghosts) were suspended in 3.0 ml Stractan (22 cp viscosity, 290 mosM, pH 7.4) for routine measurements. (Stractan was chosen as the suspending medium for ghost deformability measurements since it has a high refractive index compared to the internal milieu of ghosts, and a refractive index difference between particle and medium of at least 0.03 is required to produce a well-defined laser diffraction pattern in the ektacytometer.) Deformability curves recording the value of the deformability index (DI) generated as a function of the applied shear stress, were obtained. Numerical values of the maximum deformability index reached, defined as DI_{max}, were also recorded for each sample. All measurements of ghost deformability were carried out at 25°C.

Temperature and pH-mediated changes in membrane deformability

Resealed ghosts were suspended in 10 vol. of isotonic Tris-NaCl buffer maintained at temperatures ranging from 4 to 50°C for 10 min. After heating, membranes were rapidly cooled to room temperature, and concentrated by centrifugation prior to resuspension in Stractan for deformability measurements.

The influence of pH on membrane deformability was examined by performing ghost deformabil-

ity measurements in Stractan of various pH values (pH 3.5–12.0). Resealed ghosts were mixed with Stractan and deformability measurements carried out within 1 min.

Membrane protein modification with cross-linking agents

Diamide and glutaraldehyde were used to induce membrane protein cross-linking in resealed erythrocyte ghosts [14,15]. Resealed ghosts were incubated at 37°C for 30 min in isotonic medium (90 mM KCl, 45 mM NaCl, 10 mM Na_2HPO_4, 4 mM sucrose, pH 8.0 containing diamide at concentrations of 1–25 μmol/$1.5 \cdot 10^{10}$ ghosts. Resealed ghosts were also incubated in medium (5 mM KCl, 135 mM NaCl, 10 mM Na_2HPO_4, 10 mM glucose, pH 7.4) containing 1–50 μmol glutaraldehyde per $1.5 \cdot 10^{10}$ ghosts). After incubation in the presence of cross-linking agents, the ghosts were washed and deformability measurements performed. In one series of experiments, diamide-induced cross-links were reversed by treatment with dithiothreitol. Ghosts, pre-treated with 10 μM diamide, were washed and the pellet resuspended in 20 vol. of medium containing 10 mM dithiothreitol. After incubation at 37°C for 1 h, the resealed ghosts were washed and deformability measurements carried out.

Calcium-mediated changes in membrane deformability

Two methods were used to prepare calcium-treated membranes for deformability studies. Method 1 involved the preparation of resealed membranes from red cells previously incubated with ionophore A23187 and calcium (25 μM–10 mM), and method 2 involved resealing untreated membranes in isotonic buffers containing calcium (1–20 mM).

Method 1. Freshly drawn erythrocytes were washed in isotonic sodium phosphate media (pH 7.4) containing either low potassium (5 mM KCl) or high potassium (140 mM KCl) and resuspended to 20% hematocrit. An aliquot of the red cell suspension was added to an equal volume of the appropriate medium containing 15 μM of the calcium-specific ionophore A23187 (1 mg/ml in DMSO). Immediately after mixing, calcium was added (from 100-fold stock solutions) to give final calcium concentrations of 25 μM–10 mM. The red cell suspension was incubated for 2 h at 37°C. After incubation, the A23187 was washed from the cells using 0.1% bovine serum albumin in either high or low K^+ medium and resealed ghosts prepared as described.

Method 2. Erythrocyte ghosts were prepared by hypotonic lysis in Tris-NaCl and resealed in isotonic Tris-NaCl buffers containing 1–20 mM calcium. Resealed ghosts were washed with isotonic Tris-NaCl and centrifuged at 15000 rpm in a Sorvall RC5-B for 5 min.

Samples of ghosts treated by all of the methods described above were taken for deformability measurements and membrane protein and morphological analyses.

^{45}Ca uptake measurements

[^{45}Ca]Calcium chloride was used to measure calcium uptake and accumulation in A23187-treated red blood cells. Tracer amounts of the label were added to 300 μl aliquots of red cell suspensions containing ionophore and calcium. After 2 h incubation at 37°C, the suspension was centrifuged in a Beckman Microfuge (Beckman Instrument Co., Palo Alto, CA) and the counts in the packed cell volume were compared to the counts in the cell suspension. Cell samples were solubilized with Protosol, decolourized with hydrogen peroxide and counted in Liquiscint. Similarly, [^{45}Ca]calcium chloride was used to quantify intracellular calcium accumulation in ghosts resealed around calcium-containing medium.

Analysis of membrane proteins

Erythrocyte ghosts were solubilized in sodium dodecyl sulphate and membrane proteins analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis using either the Laemmli system [16] or the method of Fairbanks et al. [17]. Cleavable disulphide cross-links in membrane proteins and cross-linked protein complexes were analyzed by comparative polyacrylamide gel electrophoresis in the presence and absence of a reducing agent, dithiothreitol.

Morphological analysis

Resealed ghosts were suspended in isotonic Stractan to permit their easy visualization by con-

ventional phase microscopy. The large difference in the refractive indices between ghosts and Stractan enhanced the contrast and produced an image similar to that of intact cells in buffer. Erythrocytes were fixed in 1% phosphate-buffered glutaraldehyde prior to examination by phase microscopy.

Results

Ghost deformability measurements

When resealed erythrocyte ghosts were subjected to a linearly increasing shear field in the ektacytometer, a well-defined diffraction pattern was produced which changed from a circle to a sharp ellipse as the ghosts deformed. A signal proportional to the ellipticity of the diffraction pattern and designated here as the deformability index (or DI) was recorded as a function of the applied shear stress producing the characteristic ektacytometer deformability curve. The deformability curves for resealed ghosts and intact erythrocytes were virtually identical (Fig. 1). The amount of residual haemoglobin in the ghosts (less than 0.5–5% original haemoglobin) had no effect on the deformation curve.

We studied the effect of varying ghost concentration, suspending medium viscosity and osmolality on the characteristics of the ghost deformability curve. At concentrations between $75 \cdot 10^6$ and $200 \cdot 10^6$ ghosts per ml Stractan, variations in ghost number had no effect on either the shape of the deformability curve or the maximum value for the DI (DI_{max}). At ghost concentrations below $7.5 \cdot 10^7$/ml, the diffraction pattern generated was of low intensity, resulting in a low signal-to-noise ratio. At concentrations above $2 \cdot 10^8$ ghosts/ml, where the ghosts no longer formed a single, non-overlapping layer of cells, the diffraction image was diffuse. Suspending medium viscosity had a profound effect on the deformability curve (Fig. 1). Although ghosts deformed at low viscosities, maximum deformation was only observed when the viscosity of Stractan exceeded 15 cP. At viscosities in excess of 50 cP, the ghosts underwent fragmentation. An intermediate viscosity of 22 cP produced a curve identical to that obtained for intact red cells at the same viscosity, and this value was used for subsequent ghost deformability measurements.

The effects of suspending medium osmolality

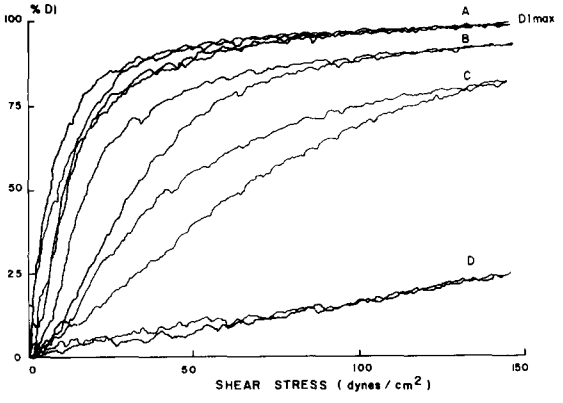

Fig. 1. Dependence of membrane deformability on the viscosity of the suspending medium. Each deformability curve records the value of the deformability index (DI) as a function of the applied shear stress. DI_{max} represents the maximum value for the deformability index. (A) Resealed ghosts and intact cells in 22 cP medium; (B) resealed ghosts in 13 cP medium; (C) resealed ghosts in 9 cP medium; (D) resealed ghosts in 4 cP medium.

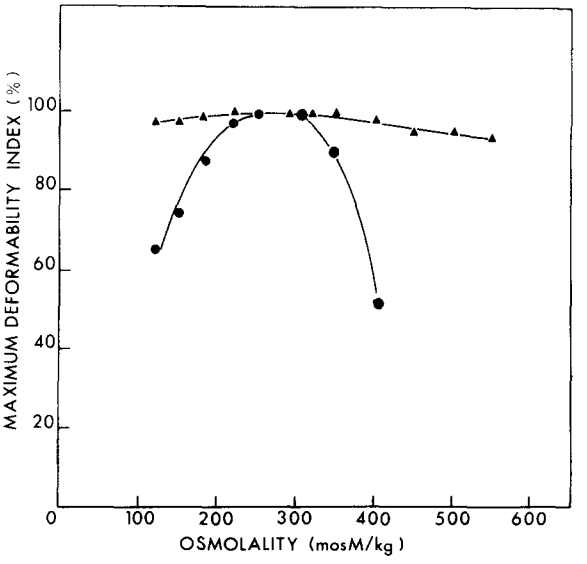

Fig. 2. Effect of suspending medium osmolality on whole cell and membrane deformability. Resealed ghosts and intact cells were suspended in hypotonic and hypertonic media to alter the surface area-to-volume ratio. Whole cell deformability (●) is profoundly influenced by changes in medium osmolality, whereas ghost deformability (▲) is largely unaffected by variations in medium osmolality.

on the deformability behavior of resealed ghosts was also examined (Fig. 2). Unlike intact red blood cells, the deformability of ghosts resealed in 290 mosM buffers remained largely unchanged over the entire range of osmolalities studied (120–550 mosM). In hypertonic suspending media, intact erythrocytes became progressively undeformable as they lost water and the concentration and viscosity of haemoglobin increased. In contrast, ghosts, which contain little or no haemoglobin, did not undergo large changes in intracellular viscosity when placed in hypertonic media. In hypotonic media, intact erythrocytes took up water, became swollen and less deformable. Ghosts resealed in 290 mosM buffer had approximately half the volume of intact red cells and were therefore able to take up correspondingly greater volumes of water without approaching spherical geometry. Thus resealed ghosts continued to deform maximally down to osmolalities of 120 mosM. Ghosts resealed in hypertonic buffer and subsequently suspended in 290 mosM Stractan had the same volume as intact cells. These ghosts showed the same dependence of DI_{max} on decreasing osmolality as intact cells, confirming that ghost deformability in hypotonic media was simply a function of initial ghost volume. In addition, this observation confirmed that the ghosts were relatively impermeable to cations and behaved as osmometers.

The effect of temperature and pH on membrane deformability

Fig. 3 shows the effect of mild heating on membrane deformability. When resealed ghosts were incubated at temperatures above 40°C for 10 min, and then subjected to fluid shear stress at room temperature, they showed a progressive loss of deformability with increasing incubation temperature. After heating above 45°C, membranes were markedly undeformable. These ghosts were spherical and showed evidence of spontaneous fragmentation. High molecular weight protein complexes, which did not penetrate polyacrylamide gels, were present in heated membranes.

The effect of pH on membrane deformability is shown in Fig. 4. There was little or no change in ghost deformability over the range pH 5.5 to pH 9.0. On either side of this range however, there was an abrupt and significant loss in membrane deformability, which correlated with increased sphering and fragmentation. In addition, the pH extremes produced extensive membrane protein cross-linking, detected as high molecular weight material remaining at the top of polyacrylamide gels.

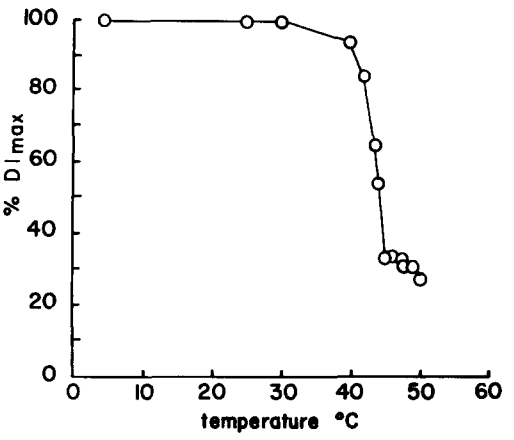

Fig. 3. Red cell membrane deformability is affected by temperature. Experimental points show that resealed ghosts became progressively indeformable as they were heated above 40°C.

The effect of membrane protein cross-linking on membrane deformability

Experiments designed to assess the role of erythrocyte membrane proteins in cellular deformability indicated that cross-linking of the pro-

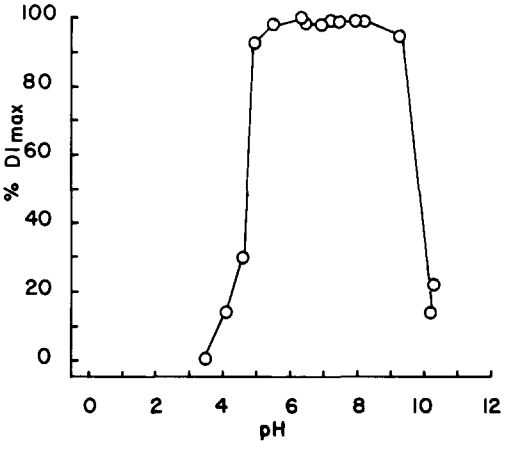

Fig. 4. Effect of pH on deformability of erythrocyte membranes. Resealed ghosts show an abrupt loss in deformability at extremes of pH.

TABLE I

DIAMIDE REDUCES ERYTHROCYTE MEMBRANE DEFORMABILITY

The deformability of ghosts pre-treated with diamide is expressed as the percentage of the maximum deformability index (DI_{max}) for untreated ghost membranes. Values are given ± S.D.

Diamide (μM)	*n*	% DI_{max}
0.0	4	100.0
1.0	3	96.0 ± 0.8
5.0	4	57.3 ± 19.7
10.0	4	30.3 ± 14.8

teins with either diamide, which induces the formation of both intermolecular and intramolecular disulphide bonds, or glutaraldehyde, which generates cross-linking between amino-groups, reduced membrane deformability (Tables I and II). This effect was observed to be time- and concentration-dependent for both cross-linking agents and, in the case of diamide, to be reversible by reduction with dithiothreitol.

Polyacrylamide gel electrophoresis of membrane proteins showed that spectrin and band 3 were the major proteins affected by glutaraldehyde and diamide cross-linking. A high molecular weight protein complex which did not penetrate the gel was formed. At high concentrations of glutaraldehyde (in excess of 25 μM), which caused a marked loss of ghost deformability, all ghost proteins except haemoglobin failed to enter the gel. Incubation of resealed ghosts with glutaraldehyde or diamide had no effect on ghost morphology. The amount of residual haemoglobin did not affect the extent of membrane deformability loss induced by protein cross-linking, suggesting that cross-links within membrane proteins rather than cross-links involving haemoglobin were responsible for the reduced membrane deformability.

TABLE II

GLUTARALDEHYDE REDUCES ERYTHROCYTE MEMBRANE DEFORMABILITY

The maximum deformability index (DI_{max}) of glutaraldehyde-treated membranes is expressed as the percenrage of the DI_{max} ± S.D. of untreated membranes. Details of incubation conditions are given in the text.

Glutaraldehyde (μM)	*n*	% DI_{max}
0.0	4	100.0
1.0	4	98.0 ± 0.0
5.0	5	84.8 ± 4.8
10.0	5	48.6 ± 13.5
20.0	3	10.3 ± 6.5

The effect of calcium on membrane deformability

Incubation of erythrocytes for 2 h at 37°C in a medium containing the divalent cation ionophore A23187 and various concentrations of calcium resulted in the intracellular accumulation of calcium. In addition, cells became echinocytes and spheroechinocytes upon calcium loading, depending on the calcium concentration.

Cells incubated for 2 h in low potassium medium containing ionophore and calcium showed a concentration-dependent loss of whole cell deformability. Treated cells containing 100 μM calcium became completely undeformable. These cells were spherical. The membrane deformability of the cells was also reduced, though to a lesser degree. The DI_{max} of resealed ghosts prepared from these cells decreased by 30%. The ghosts were echinocytic. When calcium-stimulated loss of cell potassium and water was prevented by incubating cells in high potassium media, the DI_{max} for the whole cells decreased by 10% for 100 μM calcium. The majority of the cells were early echinocytes. Even at 15 mM calcium, whole cell deformability loss was only 30–35%. Resealed ghosts prepared from cells loaded with calcium in high potassium media showed a loss of deformability which depended on the concentration of calcium (Fig. 5).

Gel electrophoretic analysis of membrane proteins from these cells showed that calcium loading at extracellular concentrations greater than 1 mM produced a high molecular weight complex which failed to penetrate the gel and could not be reduced by dithiothreitol treatment. The formation of this complex was accompanied by a reduction in the staining intensity of band 3 and spectrin, and the total loss of band 4.1 (data not shown). This observation did not differ significantly from those reported by others [18,19]. The formation of new cross-links between the proteins of the membrane skeleton was probably due to the activity of a calcium-activated transglutaminase [20].

To further study the direct effects of calcium on

membrane deformability, we prepared resealed ghosts which had been exposed to calcium at the time of resealing. These ghosts, the majority of

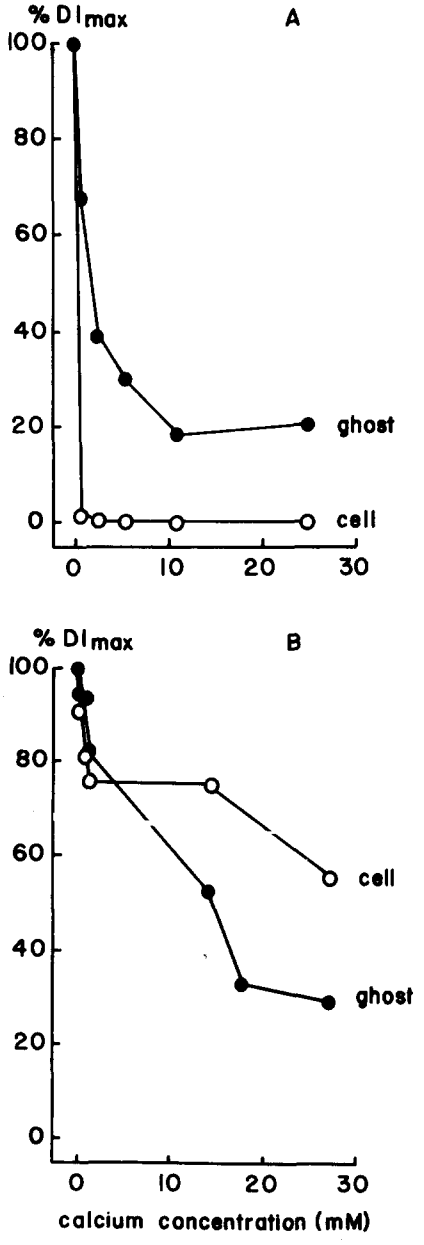

Fig. 5. Effect of calcium accumulation on cellular and membrane deformation. Whole red cell and membrane deformability are affected by calcium loading. Cellular dehydration appears to be the major determinant of whole cell deformability loss since incubation in K^+-rich medium reduces calcium-mediated whole cell deformability loss. (A) The effect of incubating cells with A23187 and calcium in K^+-poor media; (B) the effect of incubation in K^+-rich media.

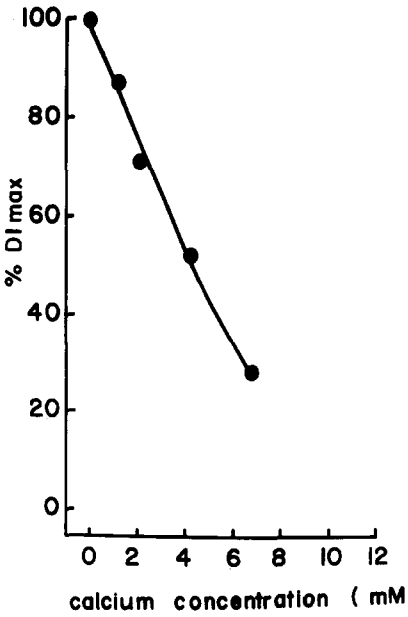

Fig. 6. Effect of calcium on erythrocyte membrane deformability. Resealing ghosts in the presence of calcium causes a calcium concentration-dependent loss of membrane deformability.

which were discocytic, showed a calcium concentration-dependent decrease in deformability (Fig. 6). This response was specific for calcium as the divalent cation (magnesium having no effect) and was maximal within 15 min of resealing. Inclusion of a potent protease inhibitor (1 mM diisopropyl fluorophosphate) along with 10 mM calcium in the resealing medium did not alter the deformability loss. Polyacrylamide gel electrophoresis of treated membranes showed that the calcium-mediated deformability loss could be correlated with the production of a high molecular weight protein aggregate which was not reducible by dithiothreitol. As in calcium-loaded intact cells, the appearancce of this aggregate was associated with a reduction in the staining intensity of band 3 and spectrin and the disappearance of band 4.1. Protease inhibitor did not substantially alter the appearance of the membrane protein profile of calcium-treated ghosts; band 4.1 was still missing and band 3 and spectrin reduced.

Discussion

Our previous studies using the ektacytometer have demonstrated the importance of cytoplasmic

viscosity and cell surface area-to-volume relationship in determining whole cell deformability. In these studies, the contribution of the cell membrane was frequently obscured by the other two factors. A resealed ghost preparation might be ideally suited for ektacytometric measurements of membrane deformability. Therefore, we describe here the development of such a procedure.

In a series of validation experiments, we have shown that heat, extremes of pH and cross-linking agents cause a loss in membrane deformability, presumably by altering membrane skeletal protein organization. Similar observations have been made by others using alternative methods, including viscometry [14] and micropipette aspiration [21]. The consistency of the deformability losses detected by these various methods validates the use of the ektacytometer for detecting alterations in membrane deformability. Recent correlative ektacytometer and micropipette measurements on diamide-treated resealed ghosts show that the deformability index measures the membrane shear modulus (μ) (Mohandas, N. and Evans, E., unpublished data).

We have used ghost ektacytometry to study the effect of calcium on the red cell membrane. Calcium accumulation by intact red cells has been shown to induce a number of changes, each of which is potentially capable of limiting cellular deformability. One effect of calcium uptake is the loss of cell potassium and water, the Gardos phenomenon [22]. This may lead to greatly increased cytoplasmic viscosity and hence reduced deformability. Additionally, calcium-mediated lipid loss, and subsequent sphering of cells, may also contribute to reduced deformability [23]. Finally, calcium accumulation may also affect the viscoelastic properties of the membrane by inducing changes in membrane skeletal proteins. The relative importance of each of these factors has been a matter for controversy, and recent reports have suggested that the primary determinant of reduced deformability is increased internal viscosity and not membrane rigidity [10,11]. By directly measuring the deformability of calcium-treated membranes, we have been able to demonstrate that calcium does reduce membrane deformability, but only at concentrations greatly exceeding those measured in senescent red cells and in irreversibly sickled cells, which have been reported to have elevated intracellular calcium. At physiological concentrations of calcium any membrane stiffening effect of calcium on whole cell deformability is overwhelmed by the effect of calcium on cell water content.

The studies described here demonstrate the applicability of the ektacytometer for measurements of membrane deformability and illustrate the usefulness of such a technique for separating the relative importance of each of the contributing factors of cellular deformability. By isolating the effects of membrane shear modulus from intracellular viscosity, a direct measurement of the membrane component of cellular deformability may be made. Furthermore, since resealed ghosts used in this procedure deform maximally in hypotonic, as well as in hypertonic media, the effects of variation in the ratio of the surface area to the volume became negligible. Finally, the similarity between the deformability curves of intact red cells and the resealed ghosts strongly suggests that ghost preparation does not significantly alter physical properties of the membrane, and that this preparation may be reliably used for studies of membrane deformability.

Acknowledgements

We wish to thank Dr. Margaret R. Clark for helpful discussions. This work was supported by grants from the National Institutes of Health (AM 23077, AM 26263 and AM 16095). This is publication number 32 from the MacMillian-Cargill Hematology Research Laboratory, University of California, San Francisco, CA 94143 (U.S.A.)

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