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Research Article

Membrane rigidity has been widely accepted as the dominant cause of reduced deformability both of ATP-depleted erythrocytes and erythrocytes containing excess calcium (Ca). However, recent studies have shown normal membrane deformability in ATP-depleted erythrocytes. In addition, Ca accumulation causes massive ion and water loss, and it has been shown that extensive dehydration causes an increase in intracellular viscosity with attendant loss of whole cell deformability. To obtain a detailed understanding of the processes accompanying ATP depletion and/or Ca accumulation that limit cell deformability, we have used a viscodiffractometric method to identify the cellular factors contributing to reduced whole cell deformability.

Analysis of the influence of the suspending medium osmolality on deformability showed the presence of two independent processes. One was a Ca-independent reduction in cell surface area/volume ratio, resulting from the spheroechinocyte formation that follows total ATP consumption. The other was a Ca-dependent increase in intracellular viscosity resulting from a Ca-induced loss of intracellular potassium and water. This deformability loss due to increased intracellular viscosity was found for cells depleted of ATP in the presence of Ca and in cells treated with Ca and A23187 without prior depletion. Ionophore-treated cells at high Ca concentration (>500 μM) formed spheroechinocytes with reduced surface area and a further loss of whole cell deformability. The rate of deformability loss associated with Ca-induced spheroechinocytosis was […]

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ABSTRACT Membrane rigidity has been widely accepted as the dominant cause of reduced deformability both of ATP-depleted erythrocytes and erythrocytes containing excess calcium (Ca). However, recent studies have shown normal membrane deformability in ATP-depleted erythrocytes. In addition, Ca accumulation causes massive ion and water loss, and it has been shown that extensive dehydration causes an increase in intracellular viscosity with attendant loss of whole cell deformability. To obtain a detailed understanding of the processes accompanying ATP depletion and/or Ca accumulation that limit cell deformability, we have used a viscodiffractometric method to identify the cellular factors contributing to reduced whole cell deformability.

Analysis of the influence of the suspending medium osmolality on deformability showed the presence of two independent processes. One was a Ca-independent reduction in cell surface area/volume ratio, resulting from the spheroechinocyte formation that follows total ATP consumption. The other was a Ca-dependent increase in intracellular viscosity resulting from a Ca-induced loss of intracellular potassium and water. This deformability loss due to increased intracellular viscosity was found for cells depleted of ATP in the presence of Ca and in cells treated with Ca and A23187 without prior depletion. Ionophore-treated cells at high Ca concentration (>500 μM) formed spheroechinocytes with reduced surface area and a further loss of whole cell deformability. The rate of deformability loss associated with Ca-induced spheroechinocytosis was much more rapid than that associated with ATP-depletion-induced spheroechinocytosis, suggesting different mechanisms for the morphologic changes. No major effects of altered membrane elasticity on the reduced deformability of either ATP-depleted or Ca-loaded cells were observed.

INTRODUCTION

Maintenance of normal erythrocyte deformability appears to be crucial to normal erythrocyte function and survival. ATP-depletion and calcium uptake result in a loss of cell deformability and are generally agreed to be mechanisms of physiologically important cell destruction. Weed et al. (1) proposed that a depletion of cellular ATP impaired the ability of cells to maintain their normally low Ca content and that the intracellular accumulation of Ca caused decreased deformability by inducing a sol-to-gel transformation in the cell membrane. This single mechanism of Ca-induced membrane stiffening has been thought to underlie the reduced deformability of both ATP-depleted cells and cells loaded with Ca without prior depletion. The reduced deformability of irreversibly sickled cells (2, 3) and erythrocytes stored in plasma for extended periods (4) has also been explained on this basis. Recently it has been suggested that in addition to its effect in fueling Ca extrusion, ATP may also have a direct effect on membrane flexibility mediated through membrane-associated "structural" proteins (5–7).

These conclusions about the effects of ATP depletion and Ca uptake on erythrocyte deformability were based on micropipette studies that employed micropipettes with a diameter (~3 μm) not much smaller than the cell diameter (7–8 μm). It has now become apparent that measurements of cell deformability using micropipettes of >1 μm Diam are influenced by cell surface area/volume ratio and intracellular viscosity as well as by membrane

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flexibility (8, 9). Recent evaluations of the deformability of ATP-depleted cells, using >1 μm-Diam micropipettes (10) and a viscometric method (11) have shown normal membrane deformability. This suggests that other effects of ATP depletion, such as this loss of cell surface area accompanying echinocytogenesis (4), should be considered. Similarly, the effect of Ca in causing potassium (K) and water loss from erythrocytes (Gardos effect) (12), with a consequent massive increase in cytoplasmic viscosity (13), was not taken into account in explaining these original observations.

Recently, we have shown that measurement of whole cell deformability with the ektacytometer at various osmotic strengths can be used to distinguish the separate contributions of internal viscosity, surface area/volume ratio, and membrane shear modulus to the behavior of abnormally deformable cells (14). We have used these methods to study cells during prolonged and acute depletion and after rapid Ca loading using the ionophore A23187. The salient conclusion drawn from these studies is that the deformability loss in ATP-depleted and Ca-loaded cells proceeds by two distinct mechanisms. First, the gradual reduction in cell deformability that occurs after ATP depletion in the absence of Ca is associated with the Ca-independent generation of spheroechinocytes and loss of membrane surface area. Second, in the presence of added Ca, an independent, further reduction in deformability occurs, caused by an increase in intracellular viscosity mediated by the Gardos effect. This intracellular viscosity increase and additional loss of cell deformability can be duplicated by rapid Ca loading at low Ca concentrations without prior ATP depletion. At higher Ca concentrations, spheroechinocyte formation and membrane fragmentation also occur, at a rate much faster than that observed during metabolic depletion. Little or no contribution to the deformability loss from increased membrane shear modulus (increased rigidity) was found in either ATP-depleted or Ca-loaded cells.

METHODS

Deformability measurements. Whole cell deformability was measured by a laser diffraction method employing the ektacytometer (15) with the new image analysis system as described (14). In this instrument, deformation of the laser diffraction pattern of suspended cells provides a measure of cell deformation. The most recent version of the ektacytometer employs an automatic image analysis system that records a deformability index (DI)[1] as a continuous function of applied shear stress. We have previously shown that the DI and the ellipticity respond identically to applied shear stress, and thus the DI curve provides an accurate measure of cell deformation (14). From the DI curve, two pieces of information that characterize cell deformability can be obtained. First, the DI_{max} represents the cell defromation at maximum shear stress, and varies with suspending medium osmolality. Measurement of DI_{max} under varying osmotic conditions permits the identification of erythrocyte surface area/volume ratio and increased internal viscosity as factors responsible for reduced whole cell deformability (14). Second, the initial slope of the DI curve, which contains all the information about membrane resistance to cell deformation, was shown to permit identification of increased membrane shear modulus in those cases where a normal DI_{max} could be obtained (14).

Metabolic depletion. Freshly drawn erythrocytes were washed three times in isotonic Tris-buffered saline solution (10 mM Tris, pH 7.4) and then resuspended to 10% hematocrit in the same medium with the addition of penicillin-streptomycin. The cell suspensions were then incubated at 37°C for period up to 48 h. At various times, samples were withdrawn for measurement of deformability and for other properties as described below. To determine the influence of Ca, paired samples were incubated in the presence of 1 mM EDTA or Ca at various concentrations up to 100 μM. In some experiments, the rapid depletion of ATP was induced by the addition of 1 mM sodium iodoacetate (buffered to pH 7.4 with Tris) and 5 mM glucose.

ATP measurement. ATP was measured by the firefly bioluminescence method of Aledort et al. (16). Intracellular ATP was expressed as micromoles per milliliter of erythrocytes as derived from the calibration curve and after correction for hematocrit and dilution. We also verified that iodoacetate does not interfere with the ATP assay.

Calcium loading. Freshly drawn erythrocytes were washed three times and then resuspended to 20% hematocrit in isotonic solutions of NaCl or KCl buffered with 10 mM Na or K phosphate, or 10 mM Tris-HCl, pH 7.4. The suspended cells were added to an equal volume of the same buffered medium to which we had previously added the Ca ionophore A23187 (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) as a stock solution in dimethyl sulfoxide (1 mg/ml). The final concentrations of A23187 were varied from 2 to 20 μM. Immediately after adding the cells to the A23187, a 100-fold concentrated solution of $CaCl_2$ was added to give final Ca concentrations of 5 μM–2 mM. At intervals after the addition of Ca, changes in cell volume were determined by measuring the microhematocrit of the cell suspension. Additional samples were removed for correlative deformability measurements. Samples were also fixed in 1% glutaraldehyde (prepared in Isoton, Coulter Electronics Inc., Hialeah, Fla.) for subsequent morphologic evaluation. Deformability was measured in isotonic dextran and various hypotonic dextran solutions during the course of each experiment. In some experiments the dextran medium also contained Ca at the same concentration used for loading to assure that the restoration of deformability in hypotonic medium was not caused by loss of Ca from the cells during the deformability measurements. Hemoglobin concentrations of cell suspensions were determined from absorbance of the cyanomethemoglobin complex. Mean corpuscular hemoglobin concentration (MCHC) was calculated from the hemoglobin and hematocrit values. All operations were conducted at ambient temperature (23°C).

^{45}Ca uptake. To determine the quantity of Ca accumulated by the cells during ATP depletion or Ca loading with A23187, we conducted several experiments with a ^{45}Ca isotopic label (2.5 μCi/ml). At the same times that cell

[1] *Abbreviations used in this paper*: DI, deformability index: DI_{max}, deformability at maximum shear stress; MCHC, mean corpuscular hemoglobin concentration.

deformability was measured, the cells were spun down in microhematocrit tubes, and radioactivity in the packed cells was compared to that for an equal volume of supernate. The cell samples were decolorized with hydrogen peroxide and were counted in Aquasol (New England Nuclear, Boston, Mass.) or scintillation fluid (619 ml toluene, 293 ml Triton X-100, 88 ml H_2O, containing 3.53 g PPO and 44 mg POPOP).

Preparation of cells with specific deformability defects. In some experiments normal cells were treated with diamide and in others with gentle heating, to provide a model in which the viscoelastic modulus of the membrane is known to be increased, as described (14). It has also been shown that such cells have minimal alterations in surface/volume ratio or internal viscosity (17, 18). As described (14), cells were also treated with lysolecithin (19) to provide a model in which the surface area/volume ratio was reduced but neither internal viscosity nor membrane shear modulus was increased (14). For comparison with the cells loaded with Ca in high K medium, we chose a lysolecithin concentration that gave approximately the same maximum DI value.

Cell morphology. At the same time that deformability measurements were performed, cell samples were fixed in glutaraldehyde (1% in isotonic phosphate-buffered saline) for subsequent morphologic examination by phase contrast and scanning electron microscopy. Samples were prepared for scanning electron microscopy by the method described by Bessisand Weed (20). Echinocytes were classified according to the Bessis nomenclature (21).

Documentation of changes in cell water and membrane area. Changes in cell water content and membrane surface area were assessed on the basis of both osmotic fragility and cell density distribution. Osmotic fragility was measured by the method originally described by Parpart (22). In the absence of changes in MCHC, this technique provides a measure of surface area/volume ratio. The density distribution of the various cell samples was determined by centrifugation on discontinuous gradients of Stractan II (St. Regis Paper Co., Tacoma, Wash.) (23, 24). 12 layers of Stractan were used, ranging in density from 1.079 to 1.124 g/ml. The equilibrium position of the cells on the gradients provided an indication of the range of alteration in cation and water content throughout each cell sample, since erythrocyte density is primarily determined by the mean cell hemoglobin concentration. Changes in cell density provided information about changes in cell water content, unaffected by changes in surface area/volume ratio.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using the Laemmli system (25) and membranes prepared (5) after various periods of incubation.

RESULTS

ATP-depleted cells. When cells were incubated in the absence of metabolic substrate and with added EDTA, the first detectable decrease in cell deformability occurred ~18 h after the beginning of the incubation. At this stage the DI_{max} in isotonic medium was normal, but in hypotonic medium it was noticeably reduced (Fig. 1). Continued incubation resulted in a subsequent marked loss of deformability at 200 mosmol/kg and a gradual decrease in deformability at 290 mosmol/kg. When Ca was added to the incubation medium (1, 10, and 100 μM concentrations), there was no difference from the EDTA-containing suspensions up to 18 h. However, after this point an abrupt reduction in isotonic deformability was observed (Fig. 2). The rate of this decline in cell deformability was more rapid with increasing Ca concentration (Fig. 2). In contrast to cells in EDTA, which showed a monotonic decrease in deformability with decreasing osmolality, the Ca-treated cells began to show a substantial increase in DI_{max} in hypotonic medium beginning after 30 h incubation (Fig. 3). This increase in deformability with decreasing osmolality was observed only down to 150 mosmol/kg. Below this value, cell deformability declined. During this period of declining cell deformability, a distinct difference was noted in the appearance of the laser diffraction patterns produced by cells in EDTA vs. those in Ca solutions. The superposition of a circular and an elliptical diffraction pattern from the Ca treated cells indicated the presence of a double population of undeformable and deformable cells. With time, the relative intensity of the circular pattern from the undeformable subpopulation increased. EDTA-treated cells produced a single pattern of gradually diminishing ellipticity.

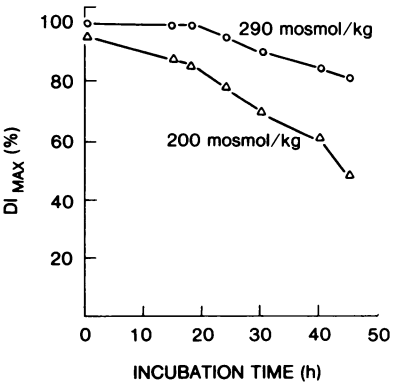

FIGURE 1 Changes in isotonic and hypotonic deformability during metabolic depletion in incubation medium containing 1 mM EDTA. Note the modest decrease in isotonic deformability after 20 h depletion and the more pronounced loss in hypotonic deformability.

Cells incubated in the Tris-buffered medium without added EDTA of Ca showed slightly less impairment in cell deformability than cells with 1 μM Ca added, but were otherwise similar. The addition of glucose to the incubation medium (without added EDTA or Ca) prevented any loss of deformability.

The decrease in intracellular ATP preceded the decrease in isotonic cell deformability by ~6 h, falling gradually from 1.3 μmol/ml cells to undetectable levels from 0 to 18 h. The rate at which ATP levels fell was identical in EDTA- and Ca-containing cell suspensions. To be certain that the loss of observed deformability in these experiments was directly associated with ATP depletion, we depleted cells

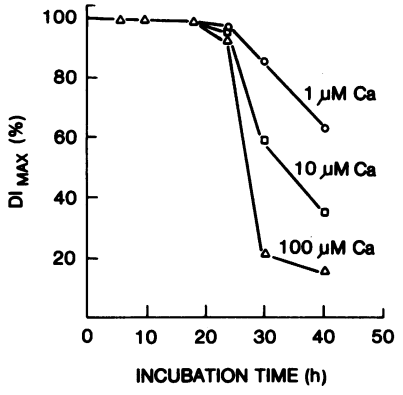

FIGURE 2 Changes in isotonic deformability during metabolic depletion in incubation media containing various concentrations of calcium. A marked loss in isotonic deformability that increases with increasing Ca concentration is seen after 24 h incubation.

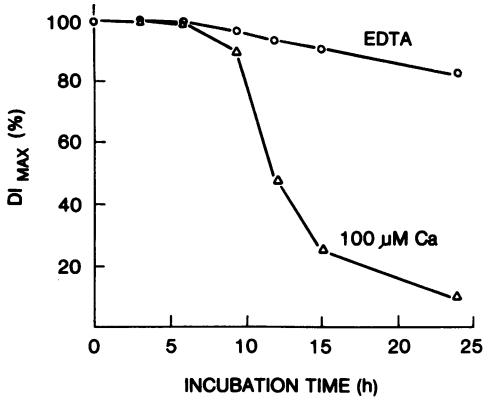

FIGURE 4 Changes in isotonic deformability of erythrocytes rapidly depleted of ATP in medium containing 1 mM iodoacetate and glucose. Note the gradual loss of isotonic deformability for cells incubated in EDTA-containing medium beginning at 9 h of incubation. This is in contrast to the gradual depletion study (Fig. 1) in which a similar change was noted starting at 24 h. A similar 15 h displacement is seen for cells depleted in Ca-containing medium (cf Fig. 2).

rapidly, using iodoacetate and glucose. As before, EDTA or Ca was included in the medium. ATP levels fell to zero within 1 h. A reduction in cell deformability became apparent 6 h after total ATP depletion, as in the gradual depletion study. The changes in isotonic and hypotonic deformability over the course of the experiment were closely similar to those in the experiment using gradual depletion. When isotonic cell deformability was plotted vs. time for both sets of experiments, a 15 h displacement to earlier time was seen for the onset of deformability loss, with little change in the rate of loss (Fig. 4).

These observed deformability changes could be correlated with changes in cell morphology and water content. Cells incubated both with EDTA and Ca showed the same chronological development of spheroechinocyte morphology. Moreover, the onset of rapid deformability loss in hypotonic medium coincided in time with conversion of the entire cell population to spheroechinocytes. This reduction in deformability was the result of the decreased surface area/volume ratio of the spheroechinocytes, as indicated by a progressive increase in the osmotic fragility of EDTA incubated cells.

Cells depleted in the presence of Ca also showed increased osmotic fragility at 24 h, but over the next 20 h, the osmotic fragility curves showed both broadening and a shift to decreased osmotic fragility. This suggestion of cellular dehydration in the Ca-treated cells was confirmed by analysis of the cell density distribution on Stractan density gradients. As shown in Fig. 5, very dense dehydrated cells began to appear at about 30 h. Thereafter the proportion of these dense cells increased steadily. Cells incubated with EDTA or in Tris-buffered medium with glucose alone maintained normal density for the entire 40-h period.

To investigate possible changes in membrane shear modulus, we focused attention on the low shear stress region of the DI curves. We have previously shown that a reduction in the initial slope of the DI vs. shear stress curve provides an indication of reduced membrane deformability. It should be noted, however, that this criterion can be applied only when the DI_{max} plateau is close to that for normal cells. The cells depleted in the presence of EDTA showed no reduction in this initial slope, indicating the absence of detectable changes in membrane shear modulus. Curves for cells depleted in the presence of Ca could

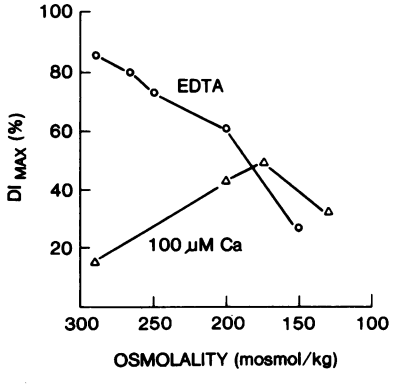

FIGURE 3 Maximum deformation vs. suspending medium osmolality for cells depleted for 40 h in incubation medium containing either 1 mM EDTA or 100 μM Ca. Cells depleted in EDTA-containing medium showed a monotonic decrease in deformability with decreasing osmolality. In contrast, cells depleted in Ca-containing medium showed an increase in deformability with a subsequent decrease as the osmolality was progressively reduced.

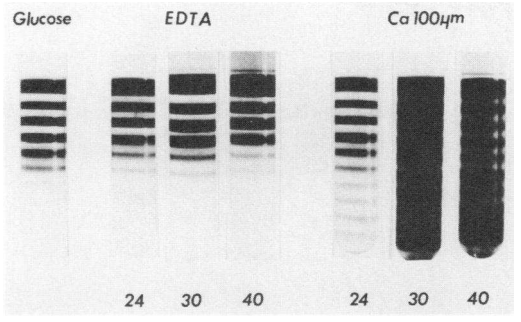

FIGURE 5 Analysis of the density distribution of erythrocytes metabolically depleted in media containing 1 mM EDTA and 100 μM calcium. Stractan density increased from 1.070–1.124 in 12 equal increments. Note minimal changes in cell density after 24 h incubation. At 30 and 40 h, cells depleted in EDTA medium showed no changes in cell density distribution, whereas cells depleted in calcium-containing medium became progressively more dehydrated as indicated by the increased percentages of high density cells.

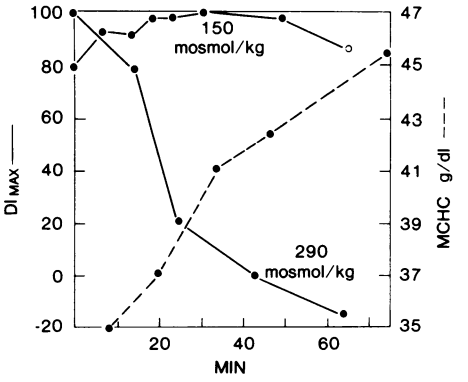

FIGURE 6 Changes in erythrocyte deformability and MCHC subsequent to Ca loading in low K medium. Cells were incubated for the indicated periods after the addition of 6 μM A23187 and 100 μM Ca. Deformability, shown as DI_{max} at 290 and 150 mosmol/kg, is plotted as the percentage of the DI_{max} for untreated control cells. Solid line, deformability; dashed line, MCHC. Note that the isotonic deformability curve has a shape complementary and opposite to that of the MCHC curve. Also, deformability in hypotonic medium shows only slight variation after Ca loading.

not be evaluated because of failure of attain near-normal DI_{max} values.

To obtain an estimate of the quantity of Ca taken into the cells during the long incubation, parallel experiments were conducted with the addition of ^{45}Ca isotope. No appreciable accumulation of isotope was evident during the first 20 h. A Ca concentration dependent linear increase in intracellular isotope content was found for the following 20 h. Cells incubated with EDTA and glucose showed no accumulation of the isotope.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of glucose-free samples showed small percentages of dithiothreitol-reducible high molecular weight aggregates at each time point, regardless of whether the incubation medium included EDTA or Ca. The absence of nonreducible aggregates indicates the absence of transamidative cross-linking, as anticipated from the ATP and Ca requirements for activation of the transglutaminase enzyme (26).

Ca-loaded fresh cells. As observed by others (27–29), the addition of Ca and A23187 to fresh erythrocytes in K-poor medium caused a marked reduction in hematocrit and a marked increase in MCHC. A concomitant decrease in deformability in isotonic medium, measured as DI_{max}, mirrored this change in MCHC (Fig. 6). At low Ca/A23187 concentrations, this loss of deformability was completely reversed by suspension of the treated cells in hypotonic medium (Fig. 6).

To confirm that the observed deformability changes depended only on changes in cell water and internal viscosity, we carried out Ca loading in high-K medium. The presence of high concentrations of external K is known to block water and K loss from Ca loaded cells. In our experiments, it also eliminated the deformability loss (Fig. 7). Normal deformability, both as DI_{max} and initial slope of the DI curve, was observed over a range of Ca-loading concentrations from 10 to 100 μM (Table I), despite increasingly abnormal cell morphology. Only a few slightly crenated cells were observed at 10 μM Ca, but at 100 μM Ca, up to 90% of the cells were echinocytes of which approximately half were stage III echinocytes. At higher Ca concentrations, from 500 μM up to 2 mM, the hypotonic deformability of the cells loaded with Ca in high-K medium was abnormally decreased, although isotonic deformability was unchanged (Table I). This loss of hypotonic deformability was associated with conversion of the entire cell population to spheroechinocytes and a marked increase in

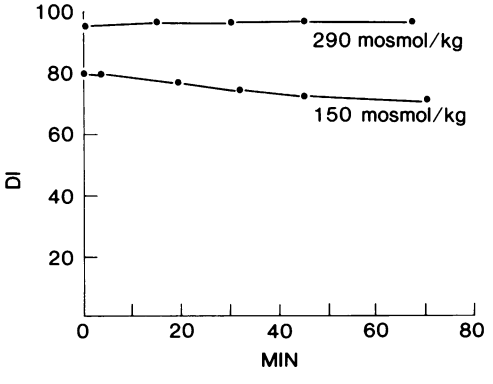

FIGURE 7 Absence of appreciable deformability changes after Ca loading of erythrocytes in high K medium. Isotonic and hypotonic deformability are expressed as in Fig. 6.

TABLE I
Maximum Deformation of Cells Loaded with Ca in High-K Medium

Ca concentration	DI_{max} at various osmolalities		
mosmol/kg	290	200	150
	% of DI_{max} for normal cells at 290 mosmol		
0	100	98	78
10 μM	101	97	83
100 μM	99	93	77
500 μM	100	88	62
2 mM	97	76	hemolysis

The plateau value of the DI vs. shear stress curve is given as the percentage of the value found for normal cells in isotonic medium. The A23187 concentration used for Ca loading was 5 μM.

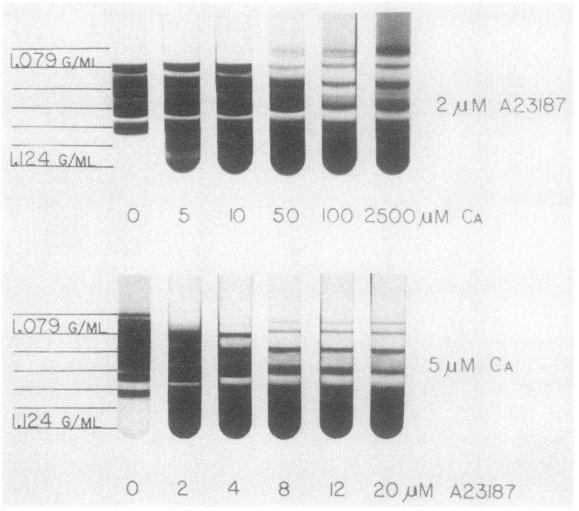

FIGURE 8 Analysis of density distribution of Ca-loaded cells. Erythrocytes were treated with the indicated concentrations of Ca and A23187. After incubation for 2 h at room temperature, they were centrifuged on discontinuous gradients of Stractan over the indicated density range. Increasing concentrations of either Ca or A23187 led to increased proportions of very dense cells.

osmotic fragility (data not shown). In contrast to the depletion experiments, in which spheroechinocytes were seen only 4–5 h after total depletion, Ca loading caused a rapid morphologic response. Sphereoechinocytes were apparent within 30 min after Ca and ionophore addition. The observed deformability behavior was independent of the buffer used during Ca loading. Tris and phosphate-buffered medium gave essentially the same results, even at 2 mM Ca concentrations.

At the lower range of Ca and ionophore concentrations, the extent of overall increase in MCHC increased continuously with increasing Ca and A23187 concentrations. The ektacytometer diffraction patterns revealed the presence of mixed populations of deformable and undeformable cells. As the Ca and ionophore concentrations were increased, the proportion of undeformable cells increased. This heterogeneity was caused by differences in cell water content, as revealed by analysis of the cell density distribution on Stractan gradients (Fig. 8). In these experiments, an increase of Ca or A23187 concentration caused the transfer of an increasing proportion of cells into the high density region of the gradient. In addition, the majority of cells appeared at either normal or very high density, with very few cells at a position of intermediate density.

Cells treated with low concentrations of diamide or heated to 47°C for 10 min have been shown to undergo a loss of deformability caused principally by an increase in the shear modulus of the membrane (17, 18). Although the membrane effects of diamide treatment or heating are undoubtedly different from those induced by Ca, these treatments are the only examples so far in which measurements with small diameter micropipettes have provided unequivocal evidence for an increase in membrane shear modulus. In ektacyometric measurements, the membrane deformability loss in such cells is expressed as a more gradual approach of the DI to its plateau value with increasing shear stress, and with only a slight effect on DI_{max} (14). The reduced initial slope of the DI curve represents the effect of membrane resistance in limiting cell deformation in the low shear stress range (17). Combined effects of increased internal viscosity and decreased surface area/volume ratio in cells loaded with high levels of Ca in low-K medium obscure the effect of alterations in membrane properties (14). Therefore, an increase in membrane shear modulus would be detectable only in cells loaded in high-K medium, where dehydration does not occur. When we examined the initial slopes of the DI curves in these high K experiments, we found that in about half the experiments, a slight reduction in the initial slope could be detected at Ca concentrations from 500 μM to 2 mM. In the other experiments, no change in slope was found.

From a comparison of the DI curves for the diamide-treated cells with those of heated cells for which direct micropipette measurements of the membrane shear modulus had been obtained (18), we estimated the upper limit of the membrane shear modulus in these most severely affected Ca-loaded cells to be 175% of the normal value. This estimate did not include any consideration of the slight decrease in slope from effects of reduced membrane surface, such as that shown by lysolecithicin-treated cells (14). Because it has been suggested

recently that a Ca-mediated transamidative cross-linking of proteins associated with the membrane might cause a reduction in membrane flexibility at Ca concentrations in the millimolar range, we looked for effects of histamine, a transglutaminase inhibitor, on the Ca-loaded cells. There was no effect of histamine (50 mM) on the initial slope of the DI curve. That is, in experiments in which a reduction in the initial slope was observed, this reduction was equal in cells loaded with Ca in the presence and absence of histamine.

Finally, to obtain a measure of the amount of Ca that entered the cells during the loading period, we measured the uptake of ^{45}Ca isotope included in the medium in parallel experiments. These measurements indicated that the concentration of Ca in the cells, expressed as quantity per volume of cells, was always at least twice that in the original extracellular medium. The absence of appreciable ^{45}Ca isotope in the packed cell samples without A23187 confirmed that ^{45}Ca was not artifactually precipitated by the phosphate buffer.

DISCUSSION

Analysis of the osmotic dependence of the deformability of metabolically depleted cells has shown the presence of two independent and simultaneous processes causing reduced whole cell deformability: First, a Ca-independent transformation of discoid cells to spheroechinocytes, accompanied by loss of that membrane surface area necessary to accommodate cell deformation; second, a Ca-dependent loss of intracellular ions and water, accompanied by a massive increase in intracellular viscosity. The Ca-independent process of membrane loss was identified on the basis of increased osmotic fragility and a reduction in deformability that was most pronounced in hypotonic medium. The Ca-dependent process of cell dehydration was identified on the basis of an increase in cell density, a progressive reduction in osmotic fragility, and a reduction in isotonic deformability that was partially reversed in hypotonic medium. This water loss from cells depleted in the presence of Ca appeared to be the result of the Gardos effect (12), in which Ca accumulation leads to a specific loss of potassium salts and associated water.

The experiments employing A23187 to introduce Ca into cells without prior depletion confirm this dual process for deformability loss in cells metabolically depleted in Ca-containing medium.

It is now clear that Ca accumulation exerts its primary effect on erythrocyte deformability by increasing membrane permeability to K. This specific K leak promotes overall ion and water loss in low-K medium, with an attendant increase in MCHC and intracellular viscosity. The inhibition of deformability changes by high-K and their reversal in hypotonic medium show that Ca does not rigidify erythrocyte membranes sufficiently to limit whole cell deformability. Although at higher Ca concentrations (100 μM) an additional decrease in deformability that could not be attributed to cell dehydration was observed, this further change was associated with the formation of spheroechinocytes. These spherical cells had less excess surface area to accommodate large deformations, especially at reduced osmolality (14). It should be noted, however, that in low-K medium such as plasma, the preceding cell water loss would compensate for the loss in membrane surface area, causing minimal net reduction in surface/volume ratio. On this basis, Ca-mediated membrane loss is expected to contribute little to a deformability loss under physiologic conditions.

The mechanism of spheroechinocyte formation at higher Ca concentrations is not known. The fact that Ca loading in the presence of A23187 causes rapid depletion of ATP (30) raises the possibility that the morphologic change could be the result of ATP depletion. However, in the ionophore experiments, spheroechinocytes were formed much more rapidly than in the depletion experiments. This difference suggests that Ca may exert a direct, ATP-independent effect on membrane components to produce spheroechinocytes.

Previous micropipette studies of metabolically depleted and Ca-containing erythrocytes apparently did not give sufficient consideration to the factors other than membrane elasticity that influence cell deformability. In seeking a mechanism for the impaired ability of ATP-depleted cells to be drawn into micropipettes, Weed et al. (1) concluded that increased membrane rigidity was the dominant factor. Recent analyses of the micropipet method have shown that micropipettes larger than 1 μM in Diam cannot distinguish the effects of altered membrane elasticity from those of altered cell geometry and internal viscosity (8, 9). In addition, reexamination of the deformability of ATP-depleted cells with small micropipettes has shown that ATP depletion has minimal effects on membrane shear modulus (10). Our experiments, showing virtually normal deformability at low shear stress for cells depleted in the absence of Ca, are in accord with these recent observations. Because of the opposing effects of cell sphering and dehydration in cells depleted in the presence of Ca, we were unable to obtain information on membrane properties in the experiments using Ca. However, Heusinkveld's Ph.D. thesis (31) showed that cells depleted of ATP for 40 hr in the presence of Ca and ghosts treated with Ca had a normal shear modulus. The present studies of Ca-loaded cells further confirm the absence of a major contribution to deformability loss by membrane stiffening.

A previous micropipette study by Dreher and coworkers (32) also showed that high K medium blocked the loss of deformability otherwise induced by Ca loading. These authors concluded that the high external K blocked a Ca-mediated membrane stiffening process. However, our observation that normal deformability was restored in hypotonic medium to cells loaded in low-K medium supports the simpler explanation that the primary effect of Ca is exerted through cell ion and water loss. This mechanism is amply supported by three experimental observations: (*a*) the Gardos effect of Ca, causing K and water loss (12); (*b*) the asymptotic increase in hemoglobin viscosity with increasing concentration in the range 35–45 g/dl (13); and (*c*) the profound effect of increased cytoplasmic viscosity in reducing whole cell deformability (14).

The implication of these findings for studies of pathologic cells deserves some consideration. Irreversibly sickled cells, in particular, have been described by many authors as cells with compromised deformability caused by Ca effects on the elasticity of the membrane (2, 3). The present observations on Ca loaded normal cells reinforce our previous conclusion that, for irreversibly sickled cells, high internal viscosity is far more important in limiting the deformability of these cells (33). Even if Ca does cause protein cross-linking (34) in addition to its influence on membrane permeability, it would seem that such cross-linking does not contribute appreciably to reduced cell deformability.

The present experiments on ATP-depleted and Ca-loaded normal cells re-emphasize that normal MCHC and water content, as well as normal cell geometry, are crucial to the preservation of normal cell deformability. Furthermore, they suggest that the widespread belief that elevated Ca in various pathologic cells causes premature erythrocyte destruction through membrane rigidification should be abandoned. Consideration of the influence of Ca on membrane permeability and cell water content should provide a more useful understanding of the role of Ca in reducing cell survival. In addition to the indirect effect of ATP depletion, which permits Ca accumulation and consequent increase of internal viscosity, a direct effect of depletion appears to be a loss of membrane stability. This leads to progressive cell fragmentation and a reduction in surface area/volume ratio that limits deformability. The molecular mechanism of this process and its relationship to the spheroechinocyte formation induced by Ca remain to be determined.

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