Retention of Water and Potassium by Erythrocytes Prevents Calcium-Induced Membrane Rigidity

Kenneth L. Dreher, BS, John W. Eaton, PhD, John F. Kuettner, MD, Katia P. Breslawec, BS, Perry L. Blackshear, Jr., PhD, and James G. White, MD

Modest increases in intracellular calcium concentrations, in association with ATP depletion, cause the appearance of pathologic changes in erythrocyte shape and deformability. The loss of erythrocyte ATP and simultaneous increase in cellular calcium have previously been considered the sole requisites for the appearance of erythrocyte membrane rigidity. We report that red cells suspended in high-potassium buffers may be simultaneously loaded with calcium (through exposure to the divalent cation ionophore A23187) and depleted of ATP without incurring drastic changes in shape or in membrane stiffness. Incubation of erythrocytes under these conditions effectively blocks both water and potassium loss normally caused by calcium accumulation. However, the high external potassium has no influence on either the ionophore-induced accumulation of calcium or on the concomitant hydrolysis of cellular ATP. These results suggest the involvement of at least one further parameter, ie, changes in cell water and cation content, in the development of calcium-induced erythrocyte rigidity. (Am J Pathol 92:215–226, 1978)

FLEXIBILITY is an important property of the erythrocyte membrane, being essential for passage of the cell through the microcirculation.[1] Diminished membrane flexibility may contribute to the circulatory incompetence and premature destruction of erythrocytes in a number of congenital and acquired hemolytic diseases.[2,3] One possible cause of decreased membrane flexibility is an increase in red cell calcium content associated with abnormal rigidity of the cell membrane.[4] Calcium-induded rigidity may, for example, underlie the permanent membrane stiffening and loss of deformability characteristic of irreversibly sickled cells which occur in patients with sickle cell anemia.[5-7]

To develop fundamental information on the role of calcium in erythrocyte deformability and pathologic behavior, a series of studies employing the ionophore A23187 was initiated.[6-9] The ionophore selectively transports divalent cations across biologic or artificial membranes until equilibrium is reached.[10,11] Its value for evaluating the role of calcium in a variety

From the Departments of Mechanical Engineering, Medicine, and Pediatrics, University of Minnesota Health Sciences Center, Minneapolis, Minnesota.

Supported by Grants HL-11880, AM-06317, HL-06314, Ca-12607, CA-08832, CA-11996, GM-AM-22167, HL-16833, HL-20695, and AM-15317 from the Public Health Service and by a grant from the Leukemia Task Force.

Accepted for publication March 14, 1978.

Address reprint requests to James G. White, MD, Department of Pediatrics, School of Medicine, University of Minnesota, Box 479 Mayo Memorial Building, Minneapolis, MN 55455.

0002-9440/78/0710-0215$01.00

of cellular processes has been established by investigations in many cell systems.[12-14]

A23187, in the presence of extracellular calcium ions, causes erythrocytes to accumulate calcium, lose substantial amounts of potassium and water, hydrolyze adenosine triphosphate (ATP), convert from biconcave disks into echinocytes and spheroechinocytes, and become strikingly rigid.[6-9,11,15-17] Ionophore-calcium treated erythrocytes also develop increased resistance to osmotic lysis, fail to pass polycarbonate filters which permit the passage of untreated red cells, and become undeformable in the focusing tube of the Coulter sizing system.[6,7,16,18] The diminished flexibility of calcium-loaded, ionophore-treated erythrocytes has been further substantiated by recent studies on the plastic behavior of the stiffened cells, employing the micropipette aspiration technique. Increased resistance to aspiration and decreased ability to recoil after expulsion from the pipette suggest that A23187-induced calcium uptake markedly alters membrane fluidity.[16,18]

The mechanisms through which increased intracellular calcium alters the properties of erythrocyte membranes is not known. However, in the present investigation we have found that prevention of calcium-induced potassium loss may modify a great number of the cellular responses to calcium influx. Erythrocytes suspended in high-potassium buffers showed no evidence of the water and potassium loss normally caused by A23187, although these cells did accumulate calcium and hydrolyze ATP. The high-potassium environment also limited the degree of conversion of the cells to echinocytes and spheroechinocytes and, most importantly, preserved membrane fluidity despite increased cellular calcium levels.

Materials and Methods

The procedures used in this laboratory to obtain blood from normal adult donors; to mix the samples with 3.8% trisodium citrate or citrate–citric acid, pH 6.5, in a ratio of 9 parts blood to one part anticoagulant; to separate platelets and leukocytes from red cells by differential centrifugation at room temperature; and to multiply wash and resuspend the erythrocytes in buffered salt solutions were described in detail in previous communications.[5-9,15,17] In the present study the cells were washed three times and resuspended in either sodium- or potassium-rich Hanks' balanced salt solution (HBSS), free of both calcium and magnesium but containing 0.1% bovine serum albumin, pH 7.4. In potassium-rich HBSS the concentrations of potassium salts and sodium salts were reversed, yielding a final ratio of 130 mEq of potassium to 10 mEq of sodium. This ratio of potassium to sodium is very close to that found within the cell water of normal human erythrocytes. The washed erythrocytes in different buffers were incubated with and without the ionophore A23187, with or without calcium, and in buffer alone or buffer with the ionophore diluent dimethylsulfoxide (DMSO) under conditions identical to those described in earlier reports.[6-9,15,17,18] The ionophore A23187 was dissolved in DMSO to a concentration of 10^{-2}

M. Stock solution of A23187 was further diluted in distilled water to a concentration of 5×10^{-4} M. The addition of 0.1 ml of this dilution to red blood cell suspensions yielded an ionophore concentration of 5×10^{-6} M. Control samples containing the same final DMSO concentration without ionophore were included in each experiment. In many experiments calcium chloride was added to a final concentration of 5×10^{-3} M. The concentrations of ionophore and calcium chloride were varied in each set of experiments and, when critical to interpretation, the precise concentrations of the two are provided in results, texts, and legends. Samples of erythrocytes exposed to the test conditions were fixed, dehydrated, critical-point dried, and evaluated in the scanning electron microscope by the methods described in detail previously.[9,15] The micropipette aspiration procedure established in our laboratory was the subject of complete description in an earlier publication.[18] In the present study we employed pipettes of different internal diameters, but in all other respects the procedure was employed in the manner reported. Erythrocyte calcium content was measured by atomic absorption spectroscopy on extracts of red cells washed rapidly in saline, as published earlier.[6] Erythrocyte sodium and potassium concentrations were determined by flame photometry on lysates prepared from cells washed three times in 110 mM $MgCl_2$. Mean corpuscular hemoglobin concentration was calculated from hemoglobin (measured as cyanmethemoglobin) and microhematocrit determination. Analysis of ATP was carried out by the method of Beutler.[19] All of the results obtained in the study are based on a minimum of four separate experiments. In all of the replicate biochemical analyses the values obtained varied less than 10%. Therefore, for the sake of simplicity, the results presented in the text-figures represent values from single experiments.

Results

Control Samples

Erythrocytes washed and resuspended in either sodium- or potassium-rich HBSS with or without DMSO and A23187, but in the absence of added calcium, retained their biconcave discoid form (Figures 1 and 2). Mean corpuscular hemoglobin concentrations (MCHC), sodium, potassium, calcium, and ATP levels remained normal during periods of incubation up to 1 hour (Text-figures 1 through 5). In addition, the erythrocytes exposed to either buffer with and without ionophore and DMSO exhibited identical behavior when aspirated into or discharged from a 1.5-μ diameter pipette (Table 1). After 30 minutes of incubation at 37 C, erythrocytes in both buffer solutions fragmented within 1 second after aspiration at a negative pressure of -120 cm of water and, at a pressure of -50 cm of water, produced 10- to 12-μ tongues which recoiled abruptly when the cells were extruded back into the medium. The discharged cells converted immediately into crenated forms. Aspiration into a slightly larger pipette (2-μ internal diameter) also demonstrated the similarity of erythrocytes suspended in either the sodium- or potassium-rich buffers. At negative pressures of -150 to 200 cm of water the cells in either medium extended 11- to 13-μ tongues on aspiration and subsequently lysed.

Table 1—Micropipette Responses of Erthrocytes in Either Potassium-Rich or Sodium-Rich Buffer With and Without A23187 and Calcium

	Initial morphology	Tongue extension (μ) (2.0-μ pipette, 150 cm H_2O pressure)	Tongue extension (μ) (1.5-μ pipette, 50 cm H_2O pressure)	Tongue relaxation time (seconds) (50 cm H_2O pressure)	Cell type on recovery	Fragmentation time (seconds) (1.5-μ pipette, 150 cm H_2O pressure)	Fragmentation time (seconds) (1.5-μ pipette, 50 cm H_2O pressure)
Control cells							
Na^+ HBSS	Discocytes	11-13	10-12	$<< 1$	Crenated	1	40-80
K^+ HBSS	Discocytes	11-13	10-12	$<< 1$	Crenated	1	40-80
Treated cells							
HBSS K^+ A23187, 10^{-6} Ca^{++}, 0.5×10^{-6}	Macro-echinocytes	10-12	8-10	< 1	Macro-echinocytes	1	40-80
HBSS Na^+	Discocytes	12-14	10-12	1-3	Crenated	1	8-10
A23187, 10^{-6}	E_1	17-19	15-17	5-12	E_1	1, lyse	3-5
Ca^{++}, 0.5×10^{-6}	E_2	17-19	15-17	65-85	E_2	1, lyse	3-5
	Sphero-echinocytes	6-8	6-8	25-35	Sphero-echinocytes	3, lyse	—

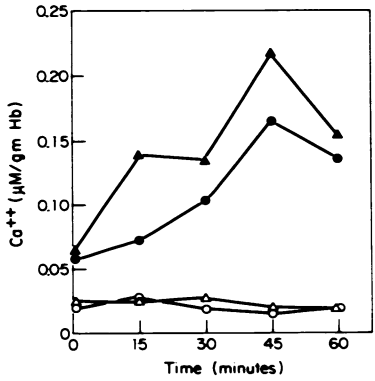
The micropipette aspiration technique was used to evaluate the responses of erythrocytes in sodium- or potassium-rich HBSS with red blood cells in the same buffer system after incubation with A23187 and calcium. Negative pressures required to aspirate the cells into 1.5- and 2.0-μ pipettes were established in preliminary experiments, and tongue extension lengths, fragmentation and relaxation times, and cell recovery types were determined after exposure to the fixed negative pressures. Cells in sodium- or potassium-rich HBSS alone or in either buffer with A23187 alone, calcium alone, or DMSO alone behaved identically in the micropipette. Erythrocytes suspended in potassium HBSS after exposure to calcium and A23187 also responded in the same manner as control cells to micropipette aspiration, despite their high content of calcium and low level of ATP. Ionophore-calcium-treated erythrocytes in sodium-rich HBSS demonstrated the altered morphology and prolonged relaxation times after extrusion from the micropipette observed earlier with pipettes of large diameter (2.4).[18]

Sodium-Rich Buffer, A23187, and Calcium

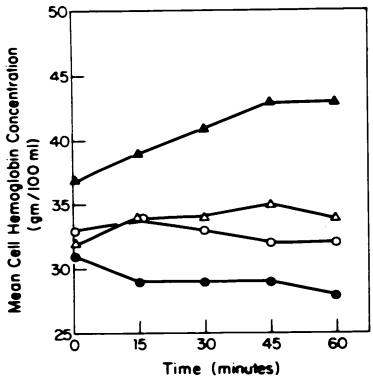
Erythrocytes incubated for 30 minutes in sodium-rich HBSS were converted from typical biconcave forms into echinocytes and spheroechinocytes [9,15] (Figure 3). Exposure to the ionophore and calcium ions in high-sodium buffer resulted in uptake of calcium and in losses of ATP, potassium, and cell volume (Text-figures 1 through 5). On aspiration into the micropipette, the cells did not fragment at high negative pressures that regularly fragmented control erythrocytes, and they exhibited prolonged tongue relaxation times after extrusion from the pipette (Figures 5 and 6; Table 1). Cells incubated for 60 minutes were predominantly spheroechinocytes which required higher negative pressures for deformation and produced shorter tongues. In the 2.0-μ pipette, residual discocytes extended 12- to 14-μ tongues and passed through the pipette at negative pressures of -100 to 150 cm of water. Echinocytes 1 (E_1) and echinocytes 2 (E_2) extended 20- to 22-μ tongues on aspiration into the pipette and passed through it at -50 cm of water pressure. In the 1.5-μ pipette, ionophore-calcium-treated discocytes extended 10- to 12-μ tongues and fragmented at a negative pressure of -50 cm of H_2O. E_1 and E_2 cells extended 15- to 17-μ tongues and lysed or fragmented at the same negative pressure. In both the larger and smaller diameter pipettes, aspirated and ejected discocytes incubated with calcium and A23187 in sodium-rich HBSS had a relaxation time of 1 to 3 seconds; E_1, 5 to 12 seconds; E_2, 65 to 85 seconds; and spheroechinocytes, 25 to 35 seconds (Table 1).

Potassium-Rich Buffer, A23187, and Calcium

Most erythrocytes incubated in potassium-rich HBSS with A23187 and calcium lost their biconcave discoid form and became oval or lenticular

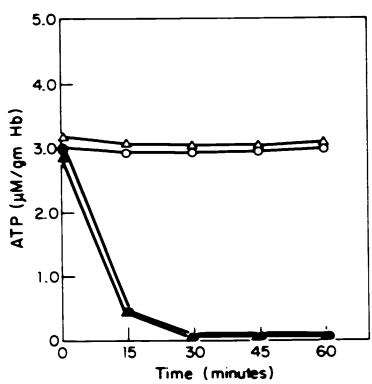


TEXT-FIGURE 1—Changes in intracellular calcium in erythrocytes incubated with (*solid symbols*) and without (*open symbols*) A23187. Samples suspended in potassium-rich Hanks' balanced salt solution are indicated by *circles*, and those in sodium-rich Hanks' balanced salt solution are indicated by *triangles*. Calcium concentration of the suspending medium (5×10^{-5} M) was the same in all samples.

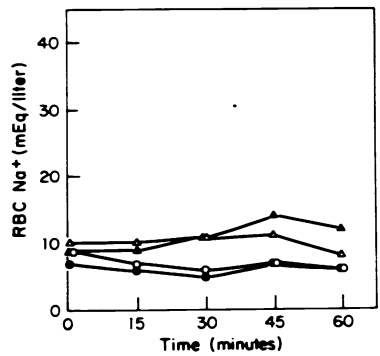


TEXT-FIGURE 2—Changes in mean cell hemoglobin concentration (MCHC) in red cells incubated with (*solid symbols*) and without (*open symbols*) A23187. Whereas erythrocytes incubated in sodium-rich Hanks' balanced salt solution with A23187 (*solid triangles*) showed large increases in MCHC, similarly treated red cells in potassium-rich Hanks' balanced salt solution (*solid circles*) did not. Erythrocytes incubated without A23187 in either sodium-rich (*open triangles*) or potassium-rich (*open circles*) Hanks' balanced salt solution underwent no changes in MCHC. Calcium concentration (5×10^{-4} M) in the medium outside the cells was the same in all samples.

(Figure 4). A few spike-like pseudopods were evident on many cells, but their altered morphology did not approach the changes observed in red cells exposed to calcium and A23187 in sodium-rich HBSS. Levels of ATP were sharply reduced in ionophore-calcium–treated erythrocytes suspended in potassium-rich HBSS, and the calcium concentration was increased to the same level found in red cells suspended in sodium solutions. However, the MCHC of ionophore-calcium–treated erythrocytes in potassium HBSS was increased slightly and there were no changes in the concentrations of intracellular sodium and potassium compared with control cells (Text-figures 1 through 5). The membrane plasticity and elasticity of the calcium-loaded cells in high-potassium buffer remained very similar to normal untreated erythrocytes (Table 1). Thus, on aspiration into a 1.5-μ pipette, the cells formed 8- to 10-μ tongues and fragmented at negative pressures greater than -50 cm of water. When aspirated at negative pressures up to -50 cm of water and released into the medium, the cells immediately returned to their original form (Figures 7 and 8). The A23187-calcium–treated red cells in potassium HBSS lysed when



TEXT-FIGURE 3—Erythrocyte ATP concentrations during incubations with (*closed symbols*) and without (*open symbols*) A23187. Note that the ATP within red cells exposed to A23187 in either sodium-rich (*closed triangles*) or potassium-rich (*closed circles*) Hanks' balanced salt solution (HBSS) underwent rapid hydrolysis, whereas the ATP content of erythrocytes incubated without A23187 in either sodium-rich (*open triangles*) or potassium-rich (*open circles*) Hanks' balanced salt solution was unchanged. Calcium concentration (5×10^{-4} M) was the same for all samples.

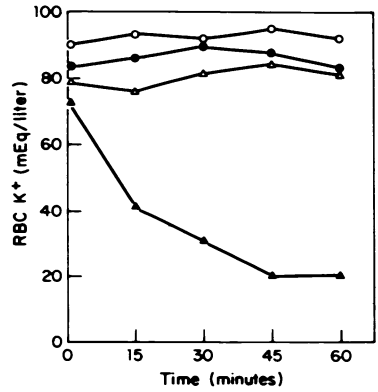


TEXT-FIGURE 4—Sodium content of erythrocytes incubated with (*closed symbols*) and without (*open symbols*) A23187 in either sodium-rich (*triangles*) or potassium-rich (*circles*) Hanks' balanced salt solution. Note the lack of significant changes in erythrocyte sodium content. Calcium concentration (5×10^{-4} M) in the medium outside the cells was constant for all samples.

aspirated into a 2-μ pipette at negative pressures in the range of -150 to 200 cm of water. This behavior in the micropipette was nearly identical to that of control cells.

Discussion

Results of the present study demonstrate that exchanging potassium for sodium as the major monovalent cation in the suspending medium drastically alters erythrocyte response to the calcium influx induced by the ionophore A23187. In sodium-rich buffers, the loading of erythrocytes with calcium was associated with loss of water, potassium, and ATP; a marked decrease in cell volume; conversion of discocytes to echinocytes and spheroechinocytes; development of rigidity as measured by several methods; and significant changes in membrane plasticity as determined by micropipette aspiration.[6-9,11,15-18] Reversing the concentrations of potassium and sodium did not affect all of the responses of the red cells to the ionophore. Exposure to A23187 and calcium in the potassium-rich buffer resulted in the expected uptake of calcium, hydrolysis of ATP, and some



TEXT-FIGURE 5—Changes in the potassium content of erythrocytes incubated with (*solid symbols*) and without (*open symbols*) A23187 in either sodium-rich (*triangles*) or potassium-rich (*circles*) Hanks' balanced salt solution. The potassium content of red cells incubated with A23187 in sodium-rich Hanks' balanced salt solution (*closed triangles*) fell markedly during the incubation, while that of cells in the other treatment groups was unchanged. Calcium concentration (5×10^{-4} M) outside the cells was the same in all samples.

alterations in morphology. However, cells in potassium-rich buffer did not lose potassium or water and developed a slight increase in volume rather than undergoing the marked shrinkage observed in sodium-rich buffers. The morphologic alterations observed in calcium-loaded erythrocytes suspended in potassium- or sodium-rich buffers also differed. In contrast to echinocytes 1 and 2, and spheroechinocytes with multiple spiky pseudopods found when erythrocytes in sodium solutions were exposed to calcium and A23187,[9,15] the cells in potassium solutions for the most part became relatively spherical with only a few short spicules. The changes in morphology were always far less dramatic when potassium was the major monovalent cation in the buffer than when sodium solutions were employed as the suspension medium.

The outstanding difference between calcium-ionophore–treated erythrocytes in either sodium or potassium solutions was demonstrated by the micropipette aspiration technique. Calcium-loaded red cells in potassium-rich medium retained their deformability. When aspirated and then ejected from the pipette, the cells recovered their original form within 1 second. The recovery time was similar to that of control erythrocytes in either potassium- or sodium-rich buffers which had not been exposed to calcium and A23187. However, the cells suspended in potassium with ionophore and calcium did not show an increased number of spikes or loss of volume and subsequent crenation following extrusion from the pipette, a phenomenon which occurred regularly in the control cells with either buffer.

The ability of the A23187-calcium–treated erythrocytes in potassium solution to deform in the same manner as normal cells in the micropipettes and to recover as rapidly after ejection indicates that neither loading of the erythrocyte with calcium nor complete hydrolysis of cellular ATP is sufficient to significantly alter membrane plasticity. Concomitant losses of potassium and water must be crucial factors in the development of erythrocyte rigidity, which occurs regularly in red cells suspended in sodium-rich solution after treatment with calcium and A23187.

The development of rigidity in erythrocytes loaded with calcium has previously been attributed to calcium-induced cross-linking or precipitation of extrinsic membrane proteins. Lorand et al [20] suggested that the increased levels of intracellular calcium activate the enzyme transglutaminase, which, through the formation of γ-glutamyl:ϵ-lysine bridges, cross-links membrane proteins. Although the present study does not rule out prior explanations for the development of calcium-induced erythrocyte stiffness, our results suggest that the combination of increased levels of cellular calcium and loss of ATP is not sufficient to cause the loss

of deformability. The reductions in potassium, water, and volume, which occur rapidly in sodium buffer but are prevented in potassium solutions, appear to be critical events for the calcium-induced changes in erythrocyte plasticity.

In conclusion, results of this study indicate that simultaneous losses of water, potassium, and ATP are essential for the calcium-induced development of erythrocyte rigidity. Suspension of erythrocytes in potassium-rich buffers before exposure to A23187 and calcium prevents the losses of water, potassium, volume, and membrane fluidity which occur in ionophore-calcium–treated red cells in sodium-rich solutions. Suspension in potassium-rich solutions does not block uptake of calcium caused by the ionophore nor does it modify the rapid hydrolysis of ATP or conversion of cells to mildly spiculated, spherocytic forms. The retention of volume, potassium, and water appears to be a major factor modifying the influence of calcium on extrinsic membrane proteins. The results emphasize the complex nature of the responses of cells and cell membranes to metabolic and ionic perturbations and suggest further experimental approaches to understanding the mechanisms of calcium-induced erythrocyte rigidity and membrane damage. Such experiments are in progress.

References

1. Schmid-Schönbein H: Erythrocyte rheology and the optimization of mass transport in the microcirculation. Blood Cells 1:285–306, 1975
2. La Celle PL: Pathologic erythrocytes in the capillary microcirculation. Blood Cells 1:269–284, 1975
3. Lessin LS, Kurantsin-Mills J, Weems HB: Deformability of normal and sickle erythrocytes in a pressure-flow filtration system. Blood Cells 3:241–262, 1977
4. Weed RI, LaCelle PL, Merrill EW: Metabolic dependence of red cell deformability. J Clin Invest 48:795–809, 1969
5. Eaton JW, Skelton TD, Swofford HS, Kolpin CE, Jacob HS: Elevated erythrocyte calcium in sickle cell disease. Nature (Lond) 246:105–106, 1973
6. Eaton JW, Berger E, White JG, Jacob HS: Effects of calcium on hemoglobin SS erythrocytes: Evidence that calcium accumulation underlies the formation of irreversibly sickled red cells. Proceedings of the Symposium on Molecular and Cellular Aspects of Sickle Cell Disease. Edited by JI Hercules, GI Cottam, MR Waterman, AN Schechter. US Department of Health, Education and Welfare Publication No. (NIH) 76-1007, 1976, pp 327–342
7. Eaton JW, Berger E, White JG, Jacob HS: Metabolic and morphologic effects of intraerythrocytic calcium: Implications for the pathogenesis of sickle cell disease. Zinc Metabolism: Current Aspects in Health and Disease. Edited by GJ Brewer, AS Prasad. New York, Alan R. Liss, Inc., 1977, pp 275–293
8. White JG, Rao GHR, Mundschenk DD: Effects of a calcium ionophore, A23187, on the surface morphology of normal erythrocytes. Blood 44:909, 1974
9. White JG: Scanning electron microscopy of erythrocyte deformation: The influence of a calcium ionophore, A23187. Semin Hematol 13:121–132, 1976
10. Reed PW, Lardy HA: Antibiotic A23187 as a probe for the study of calcium and magnesium function in biological systems. The Role of Membranes in Metabolic Regulation. Proceedings of the Symposium, The University of Nebraska Medical

School, Omaha, Nebraska, May 1972. Edited by MA Mehlman, RW Hanson. New York, Academic Press, Inc., 1972, pp 11–131
11. Reed PW: Calcium-dependent potassium efflux from rat erythrocytes incubated with the antibiotic A23187. Fed Proc 32:635, 1973 (Abst)
12. White JG, Rao GHR, Gerrard JM: Effects of the ionophore A23187 on blood platelets. I. Influence on aggregation and secretion. Am J Pathol 77:135–150, 1974
13. Foreman JC, Mongar JL, Gomperts BD: Calcium ionophores and movement of calcium ions following the physiological stimulus to a secretory process. Nature (Lond) 245:249–251, 1973
14. Lukasen JR, White JG, Kersey JH: Mitogenic properties of a calcium ionophore, A23187. Proc Natl Acad Sci USA 71:5088–5090, 1974
15. White JG: Effects of an ionophore, A23187, on the surface morphology of normal erythrocytes. Am J Pathol 77:507–518, 1974
16. Kirkpatrick FH, Hillman DG, LaCelle PL: A23187 and red cells: Changes in deformability, K^+, Mg^{++}, Ca^{++} and ATP. Experientia 31:653–64, 1975.
17. Eaton JW, Berger E, White JG, Jacob HS: Calcium-induced damage of haemoglobin SS and normal erythrocytes. Br J Haematol 38:57–62, 1978
18. Kuettner JF, Dreher KL, Rao GHR, Eaton JW, Blackshear PL Jr, White JG: Influence of the ionophore A23187 on the plastic behavior of normal erythrocytes. Am J Pathol 88:81–94, 1977
19. Beutler E: Red Cell Metabolism: A Manual of Biochemical Methods, Second edition. New York, Grune & Stratton, 1975
20. Lorand L, Weissmann LB, Epel DL, Bruner-Lorand J: Role of the intrinsic transglutaminase in the Ca^{++}-mediated crosslinking of erythrocyte proteins. Proc Natl Acad Sci USA 73:4479–4481, 1976

Legends for Figures

Figure 1—Scanning electron micrograph of erythrocytes washed and resuspended in sodium-rich Hanks' balanced salt solution (HBSS) prior to fixation. Samples of red cells in the same buffer incubated with A23187 alone, with dimethylsulfoxide (DMSO) alone, and with added calcium (5×10^{-5} M) alone presented an identical appearance. (× 3600)

Figure 2—Erythrocytes washed and resuspended in potassium-rich HBSS prior to fixation. The cells have retained their biconcave discoid configuration. Other samples of red blood cells incubated in the same buffer with A23187 alone, DMSO alone, or calcium (5×10^{-5} M) alone exhibited an identical appearance. (× 3600)

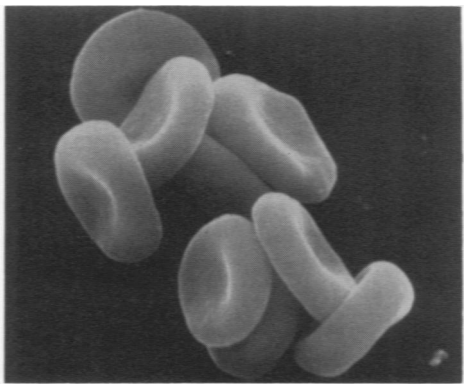
Figure 3—Sample of erythrocytes in sodium-rich HBSS fixed after exposure to both A23187 and calcium for 30 minutes. Most of the cells have converted from discocytes (*1*) to echinocytes (*2*) and spheroechinocytes (*3*). (× 3800)

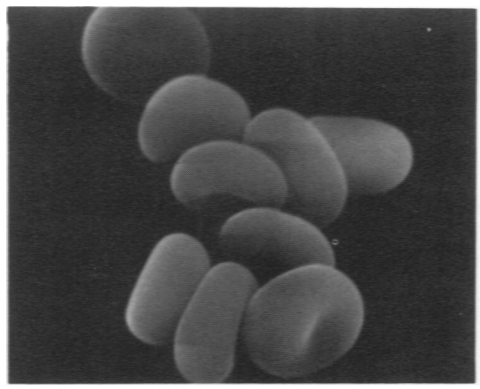
Figure 4—Sample of red blood cells in potassium-rich HBSS fixed after incubation with A23187 and calcium for 30 minutes. The cells appear slightly swollen (*1*) and some have a few short spicules on their surfaces (*2*). (× 3600)

Figures 5 and 6—Micropipette aspiration of erythrocytes in sodium-rich HBSS after incubation for 30 minutes with A23187 and calcium. The cell shown in Figure 5 has been drawn into and extruded from a 2-μ pipette. In Figure 6 the cell has been aspirated into and discharged from a 1.5-μ pipette. In both examples the erythrocyte retains the deformity imposed by the pipette (*arrows*). Eventually the tongues recoil completely and the cells recover the form they had prior to aspiration. (The photographs were taken through the phase contrast microscope under direct observation. Since the cells were in suspension, a slight distortion of the image was inevitable. However, the specific form of each cell was clearly evident in the microscope and was recorded to match the photograph. Final magnifications of the printed illustrations vary from 1100 to 1600 ×.)

Figures 7 and 8—Micropipette aspiration of red blood cells suspended in potassium-rich HBSS and incubated with A23187 and calcium for 30 minutes. Figures 7 and 8 are sequences of the same cell during aspiration and after extrusion from the pipette (1.5 μ). In Figure 7 the extent of deformation produced during aspiration is apparent (*arrow*). After extrusion (Figure 8) the cell assumes the configuration it had prior to aspiration and is similar to other cells in the medium (evident in Figure 7). (× 1500)

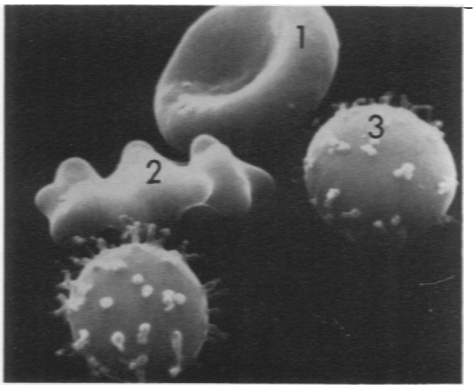
1

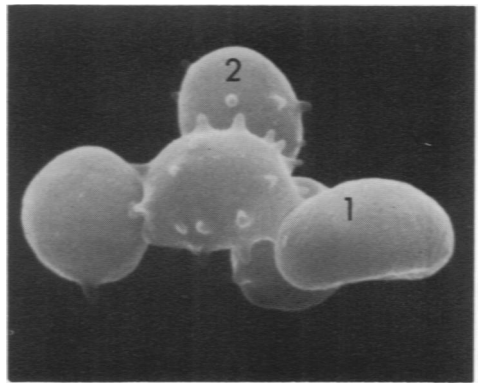




2

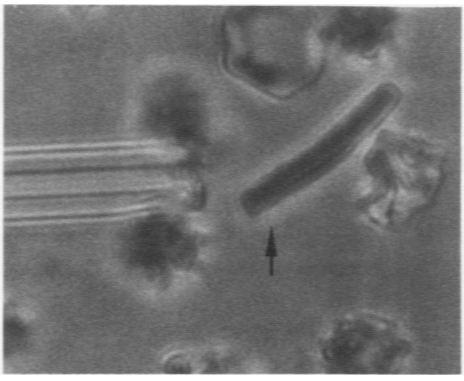
3

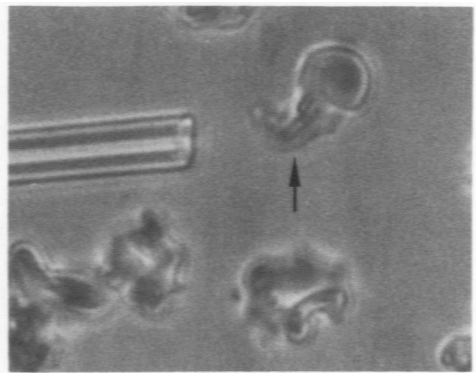




4

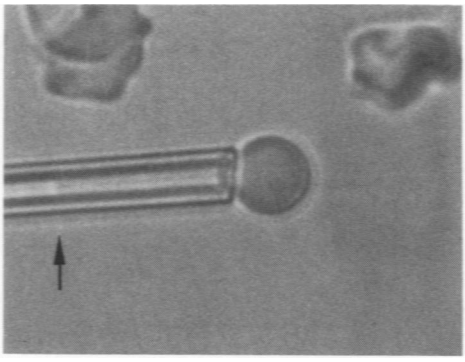
5

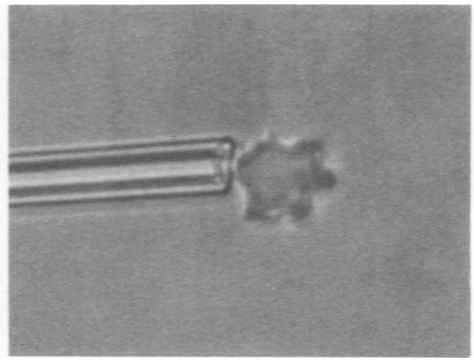




6

7





8