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Research Article

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Regulation of Cation Content and Cell Volume in Hemoglobin Erythrocytes from Patients with Homozygous Hemoglobin C Disease

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Abstract

Erythrocytes from patients with homozygous hemoglobin C disease (CC cells) contain less K, Na, and water than do erythrocytes from normal subjects that contain only hemoglobin A (AA cells). In this paper, we provide evidence that the reduced K content and volume of CC cells are due to the activity in these but not in AA cells of a K transport system that is: (a) insensitive to ouabain and bumetanide, and (b) stimulated by increased cell volume, and dependent on internal pH (pH_i). When the cation and water content of CC cells was increased (by making the membrane temporarily permeable to cations with nystatin) and the cells were then incubated in an isotonic medium containing 140 mM NaCl and 4 mM KCl, they lost K and shrunk back toward the original volume. This regulatory K and volume decrease was not inhibited by ouabain or bumetanide. When CC cells were incubated in a hypotonic medium, with ouabain and bumetanide, they also lost K and shrunk toward the original volume. This behavior was not observed in control AA cells. The ouabain- and bumetanide-resistant K efflux from CC cells was volume and pH dependent: K efflux from CC cells rose from 5–6 to 20–25 mmol/liter of cells \times h, when cell volume was increased by increasing cell solute content (nystatin method) or by exposure to hypotonic media. In CC cells, the dependence of K efflux on pH_o had a bell shape, with a maximal flux (20–25 mmol/liter of cells \times h) at pH_o 6.8–7.0. In contrast, the K efflux from control cells was minimal at pH 7.4 (1.2 mmol/liter of cells \times h) and was slightly stimulated by both acid and alkaline pH. In order to study the effect of pH_i and pH_o on K efflux, CC cells were incubated with 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (150 μ M) and acetazolamide (1 mM) at different pH_i (6.7, 7.3, and 7.8), and resuspended in media with different pH_o (6.75, 7.4, and 8): K efflux was stimulated by reducing pH_i but was independent of pH_o . The ouabain- and bumetanide-resistant K efflux from CC cells was not inhibited by some inhibitors of the Ca^{2+} -activated K permeability. It seems likely that the genetically determined change in the primary structure of hemoglobin C directly or indirectly causes this modification in K transport. One possible mechanism could involve an electrostatic interaction between C hemoglobin and components of the erythrocyte membrane.

An abstract of this work was presented at the 97th Meeting of the Association of American Physicians in 1984 and at the 38th Annual Meeting of the Society of General Physiologists in 1984.

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Introduction

The difference between hemoglobin C and hemoglobin A involves a single base substitution in the $\beta 6$ -codon, leading to a replacement of glutamate by lysine (1). Because, at a given pH, the net negative charge on hemoglobin is reduced in CC erythrocytes (cells from patients with homozygous hemoglobin C disease) as compared to AA erythrocytes (normal subject cells containing only hemoglobin A), one would expect the chloride ratio ($[Cl_i]/[Cl_o]$) to be higher, if the cation content of CC and AA cells were the same. However, Murphy (2, 3) reported that the cation and water content of CC cells is reduced, but did not investigate how cation transport in CC cells produces this reduced cation and water content. The experiments described in this paper were designed to answer that question.

We recently found (4) that the cell isoelectric point is higher (7.15 vs. 6.95), and the absolute charge of the impermeant erythrocyte solutes is lower (-10 vs. -50 meq/kg of dry cell solids) in CC than in AA cells. This difference of 40 meq/kg of dry cell solids in the charge of impermeant cell solutes accounts for +3 change in the charge of each hemoglobin molecule compared to the +4 expected. We attributed this difference to changes in other nonpermeant solutes, such as 2,3-diphosphoglyceric acid (2,3-DPG).¹ A greater reduction was found in the total cation content (212 vs. 265 mmol/kg of dry cell solids). In contrast to previous reports (2, 3), we found that the Cl^- distribution ratio (and therefore membrane potential and internal pH) is the same in CC and AA erythrocytes.

In this paper we examine the transport pathways for Na and K in CC erythrocytes. We show that CC cells regulate their volume through a ouabain- and bumetanide-insensitive pathway for K. This pathway seems to be responsible for the reduction in K and water content observed in CC cells. The pathway is dependent on cell volume and internal pH, and does not share any of the properties of the Ca-activated K transport system first described by Gardos (for a review see reference 5).

A preliminary report of this work has previously appeared in another publication (6).

Methods

Preparation of erythrocytes. Blood was collected in heparinized Vacutainer tubes (Becton, Dickinson & Co., Rutherford, NJ) from two

1. *Abbreviations used in this paper:* AA cells, normal erythrocytes that contain only hemoglobin A; CC cells, erythrocytes from patients with homozygous hemoglobin C disease; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; 2,3-DPG, 2,3-diphosphoglyceric acid; MCHC, mean corpuscular hemoglobin concentration; MOPS, 3-(*N*-morpholino)propanesulfonic acid; OB, ouabain and bumetanide.

donors homozygous for hemoglobin C (CC cells) and three normal controls (AA cells). The blood was centrifuged in a Sorvall centrifuge (RB 5B, DuPont Instruments, Sorvall Biomedical Div., Newtown, CT) at 5°C for 10 min at 3,000 g. Plasma and buffy coat were carefully removed and the cells were washed four times with a washing solution containing 152 mM choline chloride, 1 mM MgCl₂, 10 mM Tris-MOPS [tris(hydroxymethyl)aminomethane-3-(*N*-morpholino)propane sulfonic acid], pH 7.4, at 4°C. An aliquot of cells was then suspended in an approximately equal volume of choline washing solution, and from this cell suspension determination of hematocrit (Hct), cell Na (1:50 dilution in 0.02% Acationox, American Scientific Products, McGaw Park, IL), cell K (1:500 dilution), hemoglobin (optical density at 540 nm in Drabkin's solution), and mean corpuscular hemoglobin concentration (MCHC) were carried out. The erythrocyte Na and K content was determined in a Perkin-Elmer atomic absorption spectrophotometer (model 5000, Perkin-Elmer Corp., Norwalk, CT) using standards in double-distilled water. Dry weight and ³⁶Cl distribution ratio were measured in whole blood and washed cells according to Freedman and Hoffman (7).

When the experiments were not performed on the same day of collection, the erythrocytes were stored at 4°C (20% hematocrit) in a preservation solution containing 140 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 2.5 mM NaH₂PO₄ buffer, pH 7.4, and 10 mM glucose. All experiments were performed within 2 d of sampling.

Measurement of K efflux from fresh cells. K efflux from CC cells and controls was measured into Na or choline medium, as a function of external pH (pH_o), at constant osmolarity (295–300 mosM), and as a function of osmolarity, at constant pH_o (7.4). When the pH_o was varied, the medium contained 140 mM NaCl, (or 140 mM choline chloride), 1 mM MgCl₂, 10 mM glucose, 0.1 mM ouabain, 0.01 mM bumetanide, and 10 mM Tris-MOPS, pH 6.2–8.0, at 37°C. When the osmolarity was varied, the medium contained 100 mM NaCl (or 100 mM choline chloride), 1 mM MgCl₂, 10 mM Tris-MOPS, pH 7.4, at 37°C, 10 mM glucose, 0.1 mM ouabain, and 0.01 mM bumetanide. The osmolarity was varied from 220 to 400 mosM by adding choline chloride. For the flux assay, 0.4 ml of cell suspension in choline washing solution (hematocrit ~ 30%) was added to 9 ml of flux medium, and this flux suspension was then distributed into six previously chilled 5-ml tubes. After capping, three tubes were incubated for 5 min and three for 25 min in a shaking water bath at 37°C. The time course of K efflux into hypotonic, isotonic, and hypertonic media was determined in preliminary experiments. A 25-min incubation was chosen in order to meet conditions of linear initial rate in the flux measurement. At the end of the incubation, the tubes were transferred to an ice bath and, after 2 min, they were spun at 3,000 g for 5 min. The supernatant was removed and the K concentration was measured by atomic absorption using standards for K with the same composition of the flux medium.

Measurement of unidirectional radioactive influx in fresh cells. 9 ml of medium was chilled and mixed with 10 μCi of ²²Na, or 30 μCi of ⁸⁶Rb (⁸⁶Rb was used as a tracer for K fluxes). The radioactivity in five aliquots of 20 μl of medium was measured for determination of initial specific activity. 0.5 ml of packed red cells was added to the medium. The flux suspension was then distributed into six previously chilled 5-ml tubes, and after capping the tubes were incubated in a water bath at 37°C, under shaking. The supernatant of the samples, collected after 5 and 25 min of incubation at 37°C, was discarded and the cells were washed three times with 4 ml of cold Na washing solution containing 150 mM NaCl, 1 mM MgCl₂, and 10 mM Tris-MOPS, pH 7.4, at 4°C. Afterwards, the cell pellet was lysed with 1 ml of double-distilled water containing 0.02% Acationox (American Scientific Products). The tubes were vortexed and spun for 20 min at 3,000 g. Aliquots of 50 μl were diluted 50 times with an automatic dilutor, and the concentration of hemoglobin was determined at 540 nm. Aliquots of 0.8 ml of the lysate were counted in a gamma counter (Auto-Gamma 500, Packard Instrument Co., Lynn, MA).

The influx in millimoles/liter of cells × hour was calculated from influx = counts per minute/liter of erythrocytes (25 min – 5 min of

incubation)/initial specific activity (counts per minute/millimole) · 60 min/20 min, where counts per minute/liter of erythrocytes = counts per minute in the lysate × 1/VI × 100/Hct, where VI is the volume (liters) of lysate counted and hematocrit is the amount of cells (%) in the lysate. The quantity of erythrocytes present in the lysate was calculated using the hematocrit and concentration of hemoglobin of the initial cell suspension, and the concentration of hemoglobin determined in the lysate.

Nystatin-loading procedure. The procedure developed by Canessa et al. (8) was used. The loading solution contained 140 mM cations and 20–100 mM sucrose. The Na and K concentrations in the loading solution were reciprocally changed according to the desired cell Na and K content (e.g., 10 mM NaCl and 130 mM KCl, to yield 7 mmol/liter of cell Na and 90 mmol/liter of cell K). The sucrose concentration in the loading solution varied from 20 to 100 mM according to the desired cell volume. In the presence of nystatin, cell volume is set by the concentration of nonpermeant solutes in the external solution (7). Erythrocyte water contents similar to those measured in plasma were obtained with 70 mM sucrose (CC cells) and 55–60 mM sucrose (AA cells). Sucrose concentrations below or above these values, resulted in swollen or shrunken cells as desired. 1 ml of choline-washed cells was added to 5 ml of cold loading solution, containing 40 μg/ml of nystatin. The nystatin was dissolved fresh every day in dimethyl sulfoxide (5 mg in 0.25 ml). The cell suspension was incubated at 4°C for 20 min and vortexed every 5 min. The cold suspension was then centrifuged for 5 min at 2,000 g and the supernatant removed. The cells were incubated again in a larger volume (20–30 ml) of cold loading solution without nystatin. This procedure allows complete equilibration of the cell cation content with the loading solution. When measurements of unidirectional radioactive Na or K efflux were performed, the cells were incubated a final time with 1 ml of cold loading solution containing 5 μCi ²²Na or 50 μCi ⁸⁶Rb. The cells were then washed four times with a warm (37°C) solution having the same composition as the loading solution, with the addition of 1 mM Na or K phosphate buffer, pH 7.4, 10 mM glucose, and 0.1% albumin. Afterwards, the cells were washed five times at 4°C with choline washing solution and used for efflux or influx measurement. An aliquot of cells was used to measure hematocrit, cell Na and K, hemoglobin and MCHC, and dry weight.

Measurement of maximal rates of Na-K pump, Na-K cotransport, and Na-Li countertransport. To assay the maximal rate of Na-K pump and Na-K cotransport, the red cells were made to contain equal amounts of Na and K (50 mmol/liter of cells each). This procedure allows saturation of the internal sites for both transport systems. The nystatin-loading solution contained 70 mM NaCl, 70 mM KCl, and 65 mM sucrose for CC cells or 55 mM sucrose for AA cells. The Na-K pump was estimated as the ouabain-sensitive fraction of the Na efflux into a medium containing 130 mM choline chloride and 10 mM KCl. The incubation times were 5 and 25 min at 37°C, with triplicate samples. The hematocrit of the efflux cell suspension was 1%. The Na-K cotransport was estimated as the furosemide or bumetanide sensitive fraction of the Na and K efflux into a medium containing 140 mM choline chloride and 0.1 mM ouabain. The efflux times were 5 and 25 min, with triplicate samples. The hematocrit of the efflux cell suspension was 2%. To assay the maximal rate of the Na-Li countertransport, the nystatin-loading solution contained 10 mM LiCl, 130 mM KCl, and sucrose (65 mM for CC cells and 55 mM for AA cells). This procedure yields cells with 7 mmol Li/liter of cells and 1.5 mmol Na/liter of cells, with near saturation of the internal sites with Li and little competitive inhibition by internal Na. The Li efflux was measured into media containing either 140 mM choline chloride or 140 mM NaCl, in the presence of both ouabain and bumetanide. The incubation times were 5 and 65 min, with triplicate samples. The hematocrit of the efflux cell suspension was 3%. The Na-Li countertransport was estimated as the difference between the Li efflux into Na and Na-free media (8). MgCl₂ was kept constant in all media and washing solutions at 1 mM. All media contained 10 mM Tris-MOPS, pH 7.4, at 37°C, and 10 mM glucose.

Concentrations of the inhibitors were 0.1 mM for ouabain, 0.01 mM for bumetanide, and 1 mM for furosemide.

CC cells suspended in a medium with the osmolarity of plasma are shrunken compared to AA cells. As a result, 1 liter of CC cells contains more cells and more total membrane area than 1 liter of AA cells. To enable comparison of fluxes per unit of membrane area (independent of cell volume), we calculated the surface area of CC and AA erythrocytes, using Murphy's data (2) and Ponder's equation. An important assumption in this calculation is that all water is osmotically active in both CC and AA erythrocytes, as we previously demonstrated (4). This results in a lytic volume of $165 \mu\text{m}^3$ and a cell membrane area of $145 \mu\text{m}^2$, in both CC and AA cells. From the mean corpuscular volume data, 1 liter of cells contains 1.11×10^{13} cells in AA cells and 1.43×10^{13} cells in CC cells. Therefore, the total membrane area is $1.6 \times 10^7 \text{ cm}^2$ in 1 liter of AA cells and $2.07 \times 10^7 \text{ cm}^2$ in CC. Making use of these estimates, the reader can convert the unit of the fluxes to unit of membrane area, as desired.

Chemicals. KCl, NaCl, and MgCl_2 were purchased from Mallinckrodt, Inc., St. Louis, MO. Tris, albumin (bovine, fraction V), MOPS, EGTA, ouabain, trifluoperazine, chlorpromazine, acetazolamide, quinine, and 3,4,5-trimethoxybenzoic acid 8-(diethylamino)-octyl ester (TMB-8) were purchased from Sigma Chemical Co., St. Louis, MO. *N*-methyl D-glucamine was purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. Choline chloride, A23187, and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) were purchased from Calbiochem-Behring Corp., La Jolla, CA. Radioisotopes (^{22}Na and ^{86}Rb) were purchased from New England Nuclear, Boston, MA. Furosemide was a gift of Hoechst-Russel Pharmaceuticals, Inc., Somerville, NJ. Nystatin was from E. R. Squibb & Sons, Inc., Princeton, NJ. Bumetanide was a gift from Laboratoire Leo, Vernouillet, France. 3,3'-diethylthiadicarbocyanine iodide was from Koch-Light Laboratories Ltd., Colnbrook Books, England. Amiloride was a gift of Merck Sharp & Dohme, Rahway, NJ.

All solutions were prepared using double-distilled water.

Results

Electrolyte composition and water content

CC cells contain fewer cations and less water than AA cells, as shown in Table I. Despite a measured reduction of 40 meq/kg of dry cell solids in the concentration of impermeant negative anions, CC cells have normal Cl^- (and OH^-) ratio (4). This occurs because of the reduction in cation content. The reduction of cation content is due mainly to a reduction of K content (-45 mmol/kg of dry cell solids); the Na content is also somewhat reduced (-10 mmol/kg of dry cell solids). CC cells have also an increased 2,3-DPG concentration (9 vs. 6.8 mmol/liter of cell water). MCHC was 37 g/dl in CC cells and 33 g/dl in AA cells, whereas the reticulocyte count was 2.8% in CC as compared with 1% in AA cells.

Regulation of cell K content and volume

The K and water content of CC cells was increased by the nystatin procedure (without changing cell Na). The cells were then incubated in a solution containing 140 mM NaCl, 4 mM KCl, 1 mM phosphate, 10 mM Tris-MOPS, pH 7.4, at 37°C , and 10 mM glucose. As shown in Fig. 1, swollen CC cells (initial water content, wt/wt, 66.8%) shrank back toward their original volume within an 8-h period (final water content, wt/wt, 61%). This process was not inhibited by ouabain or bumetanide. As shown in Table II, this process took place through a reduction of the total cation content (from 272 to 200 mmol/kg of dry cell solids), mainly due to a loss of K (from 250 to 190 mmol K/kg of dry cell solids). Swollen AA

Table I. Electrolyte Composition of CC and AA Erythrocytes

	CC	AA
Water content		
(% weight/weight)	59.5 ± 0.5	62.7 ± 0.8
(% weight/volume)	65.5	69
(kg/kg of dry cell solids)	1.47	1.68
Cation content		
	mmol/liter of cells	
Na	5.1 ± 0.5	9.4 ± 1.4
K	89.7 ± 2.8	97.9 ± 2.2
Total	94.8 ± 2.7	107.2 ± 2.4
	mmol/liter of cell water	
Na	8	14
K	137	142
Total	145	156
	mmol/kg of dry cell solids	
Na	11	23
K	201	238
Total	212	261
Chloride ratio		
(Cl_i/Cl_o)	0.71 ± 0.05	0.70 ± 0.025
2,3-DPG		
(mmol/liter of cell water)	9.0 ± 0.3	6.8 ± 0.35

The data are the mean \pm standard deviation of at least three measurements in each of the two CC patients and of five normal controls (AA).

cells showed no evidence of volume regulation, during the 8-h incubation (Fig. 1; Table II). The reduction of water and cation content of swollen CC cells was inhibited when the external medium contained 140 mM KCl.

When AA or CC cells are incubated in hypotonic medium

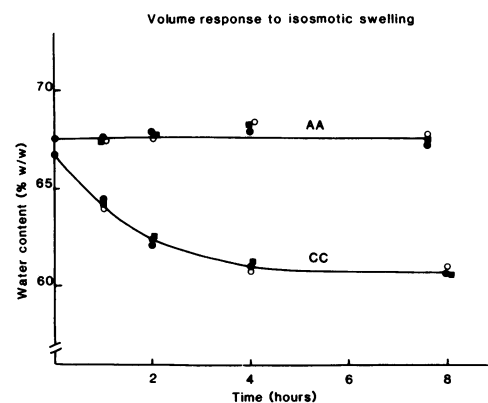


Figure 1. Cell water content (% weight/weight) as a function of time in CC and AA cells. The cation and water content were increased by the nystatin-loading procedure. The nystatin-loading solution contained 10 mM NaCl, 130 mM KCl, and 20 mM sucrose. The cells were then incubated in a medium containing 140 mM NaCl, 4 mM KCl, 1 mM phosphate, 10 mM glucose, and 10 mM Tris-MOPS, pH 7.4 at 37°C , without (●) and with 0.1 mM ouabain (■) and with 0.1 mM ouabain and 0.01 mM bumetanide (○). Dry weight was measured in duplicate at each time interval. The values for CC cells are from two different experiments in the same subject.

Table II. Regulation of Cation and Water Content in CC and AA Erythrocytes

Time (h) + inhibitor	Content		Cation		
	Water		Na	K	Na + K
	wt/wt	kg/kg of dry cell solids	mmol/kg of dry cell solids		
CC					
0	66.8	2.01	22	250	272
8	61.0	1.56	12	190	202
8 + ouabain	60.8	1.55	39	158	197
8 + ouabain + bumetanide	60.9	1.56	39	158	197
AA					
0	67.4	2.07	30	287	317
8	67.2	2.05	19	286	305
8 + ouabain	67.5	2.07	50	259	309
8 + ouabain + bumetanide	67.7	2.09	51	260	311

The cells were swollen by increasing cation content isosmotically. Data from the experiments reported in Fig. 1.

(100 mM NaCl), in the presence of both ouabain and bumetanide, they instantly swell. The water gain is different between CC and AA cells (wt/wt indices change from 60 to 65.5% for CC and from 62.5 to 69% for AA). This difference in water gain reflects the differences in the cation content of the fresh CC and AA cells. Again, unlike AA cells, CC cells shrunk back toward their original volume over a 4-h time period (Fig. 2). Thus, CC cells can regulate their volume even when incubated in hypotonic medium, in the presence of ouabain and bumetanide.

Properties of the ouabain- and bumetanide-resistant K and Na transport in CC and AA cells

The ouabain and bumetanide (henceforth abbreviated OB)-resistant Na and K fluxes (leak pathways) were studied in CC

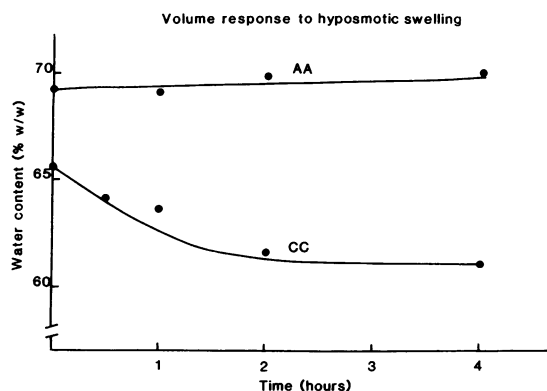


Figure 2. Cell water content (% weight/weight) as a function of time in CC and AA cells. The cells were incubated in a medium containing 100 mM NaCl, 1 mM MgCl₂, 10 mM Tris-MOPS, pH 7.4 at 37°C, 10 mM glucose, 0.1 mM ouabain, and 0.01 mM bumetanide (osmolarity = 220 mosM). Dry weight was measured in duplicate at each time interval. The results shown here are from one experiment in a normal subject and three experiments in two CC patients.

and AA cells. Table III shows measurements of unidirectional ²²Na and ⁸⁶Rb fluxes for CC and AA cells in a medium containing 140 mM NaCl and 4 mM KCl. The intracellular Na content was 4 mmol/liter of cells in both CC and AA cells (nystatin procedure). It can be seen, that CC cells had a 25-fold greater rate constant for K influx and a sixfold greater rate constant for K efflux than was observed in AA cells. The rate constants for Na efflux and influx were greater in CC than in AA cells by a factor of 3.

Volume dependence

Dependence of OB-resistant K efflux on external osmolarity. The osmolarity of the medium was changed by adding varying amounts of choline chloride to 100 mM NaCl. In hypotonic medium, (100 mM NaCl, 220 mosM) the cell water content of CC cells increases to 65.5 (wt/wt), the concentration of negative hemoglobin charges is therefore reduced, Cl⁻ moves into the cell, and the Cl⁻ ratio increases (0.76 at 220 mosM compared to 0.71 at 300 mosM). The increase in the Cl⁻ ratio, at constant external pH, is accompanied by an increase in the internal pH (+0.025 pH U) and also makes the membrane potential more positive by 2.5 mV. All of these variables change in the opposite direction when choline chloride is added and the osmolarity of the medium is increased above normal values. As shown in Fig. 3, the dependence of OB-resistant K efflux on osmolarity is different in CC and AA cells. In hypotonic medium, the K efflux is greatly increased in CC but not in AA cells. At normal osmolarity (300 mosM), OB-resistant K efflux is about five times higher in CC than in control AA cells. It is worth noting that K permeability is the same in CC and AA cells, when the osmolarity of the medium is above 350 mosM.

Dependence of OB-resistant K efflux on cell volume. To distinguish between the roles of osmolarity and cell volume in regulating the K leak, we measured the OB-resistant K efflux in cells with different volumes at constant external pH and osmolarity. Cell volume was varied by varying the cell cation content using the nystatin procedure. The internal Na concentration was kept at 4–5 mmol/liter of cells. As shown in Fig. 4, the K leak is dependent on cell volume (or the concentration of hemoglobin and/or other nonpermeant intracellular solutes) in both normal and CC cells. The stimulation of OB-resistant K efflux produced by cell swelling is significantly greater in CC cells. An increase in outward K leak was also described in swollen AA human red cells by Adragna and Tosteson (9).

pH dependence

Dependence of OB-resistant K efflux and influx on both external and internal pH. The OB-resistant K efflux was measured from CC and AA cells into media with a constant osmolarity and chloride concentrations, but with the pH varying from 6.25 to 8.0. Under these conditions, when the external pH is reduced, internal pH also falls, and the negative charges on hemoglobin are titrated, Cl⁻ enters to preserve electroneutrality, and the Cl⁻ ratio increases. The increase in internal Cl⁻ content increases water content and cell volume and also results in a more positive membrane potential (10). On the other hand, when the external pH is increased, internal pH rises, hemoglobin is titrated to become more negatively charged and Cl⁻ moves out to preserve electroneutrality. As a consequence, the chloride distribution ratio and cell volume are decreased, and the membrane potential becomes more negative.

Table III. OB-resistant Na and K Fluxes (Leak Pathways) in CC and AA Erythrocytes

	CC	AA	CC/AA ratio	Rate constant		CC/AA ratio
				CC	AA	
	mmol/liter of cells × h	mmol/liter of cells × h		h ⁻¹	h ⁻¹	
Na efflux	0.720±0.12	0.200±0.01	3.6	0.155	0.047	3.3
Na influx	1.875±0.09	0.670±0.01	2.8	0.013	0.005	2.6
K efflux	6.060±0.24	1.175±0.07	5.9	0.077	0.013	5.9
K influx	0.900±0.05	0.035±0.01	25.7	0.225	0.009	25.0

The unidirectional ²²Na and ⁸⁶Rb fluxes were measured in CC and AA cells containing 4 mmol Na/liter of cells and 92 mmol K/liter of cells (nystatin-loading procedure). The external medium contained 140 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 10 Tris-MOPS, pH 7.4 at 37°C, 10 mM glucose, 0.1 mM ouabain, and 0.01 mM bumetanide. The data are the mean±standard deviation of two experiments in the same CC patient and in two AA controls.

As shown in Fig. 5, the p*H*_o dependence of OB-resistant K efflux differed in CC and AA cells. The OB-resistant K efflux from CC cells had a bell-shaped dependence on external pH, with maximal activation (20–30 mmol/liter of cells × h) at pH 6.8–7.0. At external pH 7.4, the K leak was about five times higher in CC than in normal cells. The K leaks in CC and AA cells were equal when the external pH was higher than 7.5.

The unidirectional K influx (⁸⁶Rb tracer) was measured in a medium containing 140 mM NaCl, 5 mM KCl, and OB. It showed a p*H*_o dependence similar to that of the K efflux, with maximal stimulation at p*H*_o 7.0, and inhibition at alkaline pH. In hypotonic medium (100 mM NaCl) at p*H*_o 7.4, K influx was stimulated, whereas hypertonic media inhibited K influx. Therefore, the dependence of K influx on pH and volume was similar to that found for the K efflux.

The interpretation of these results is made difficult by the simultaneous variations of internal pH, Cl⁻ ratio, and membrane potential, when the external pH is changed. In order to determine the relative importance of these parameters, we

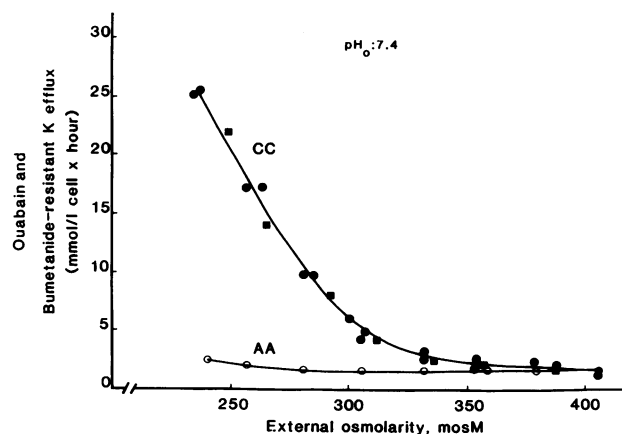


Figure 3. OB-resistant K efflux as a function of osmolarity in CC and AA cells. The medium contained 100 mM NaCl (■) or 100 mM choline chloride (●), 1 mM MgCl₂, 10 mM Tris-MOPS, pH 7.4 at 37°C, 10 mM glucose, 0.1 mM ouabain, and 0.01 mM bumetanide. The osmolarity was increased by adding choline chloride (0–100 mM). The values for AA cells are the mean of two experiments in two subjects. The values for CC cells are from two different experiments in the same patient. Similar results were obtained in a second CC patient.

studied the effect of external pH at constant internal pH, Cl⁻ concentration, and membrane potential.

Dependence of OB-resistant K efflux on external versus internal pH. The dependence of K transport on p*H*_o was studied in cells treated with DIDS (150 μM) and acetazolamide (1 mM). Under these conditions, facilitated Cl⁻ and HCO₃⁻ exchange and carbonic anhydrase are inhibited, the rates of equilibration of chloride and protons are very low, and therefore the external pH can be changed whereas internal pH, chloride concentration and membrane potential remain relatively constant (11). As can be seen in Fig. 6, at any given external pH, K efflux was inhibited when the internal pH became alkaline. At any given internal pH, there was no difference in the K efflux with external pH 6.7 or 7.4. However, external pH 8.0 partially inhibited K efflux in cells with acid internal pH. This effect could be related to some proton equilibration due to the high proton concentration gradient present in the experiment.

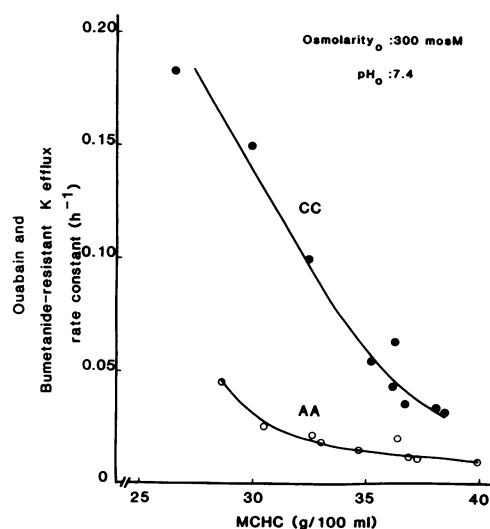


Figure 4. OB-resistant K efflux as a function of cell volume (MCHC) in CC and AA cells. The water content was varied with the nystatin-loading procedure, incubating CC and AA cells in a solution containing 10 mM NaCl, 130 mM KCl, and 20–100 mM sucrose. K efflux was measured in a medium containing 140 mM NaCl, 1 mM MgCl₂, 10 mM Tris-MOPS, pH 7.4 at 37°C, 10 mM glucose, 0.1 mM ouabain, and 0.01 mM bumetanide. The data are from one of two experiments in the same CC patient.

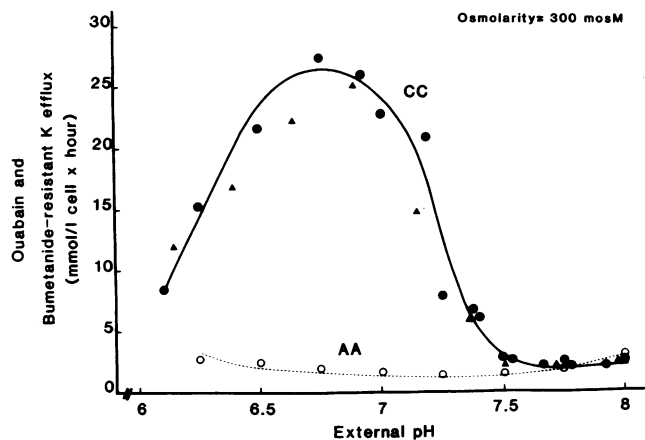


Figure 5. OB-resistant K efflux as a function of external pH in CC and AA cells. The cells were incubated in a medium containing 140 mM NaCl (Δ) or 140 mM choline chloride (\bullet), 1 mM $MgCl_2$, 10 mM Tris-MOPS, pH 6.2–8.0 at 37°C, 10 mM glucose, 0.1 mM ouabain, and 0.01 mM bumetanide. The values for AA are the mean of two experiments in two subjects. The values for CC are from two different experiments in the same patient. Similar results were obtained in a second CC patient.

We conclude that OB-resistant K efflux is not dependent on external pH or on the pH gradient across the membrane. This result makes it unlikely that the K transport pathway involves a K/H exchange. However, the simultaneous changes in internal pH, internal chloride concentration, membrane potential, and cell volume do not allow a clear separation between the effect of these factors on OB-resistant K transport. Clearly, further experiments are necessary to resolve this issue.

Dependence of OB-resistant K efflux on external pH at different osmolarities. To study the relative importance of external (and internal) pH and cell volume, the pH_o dependence

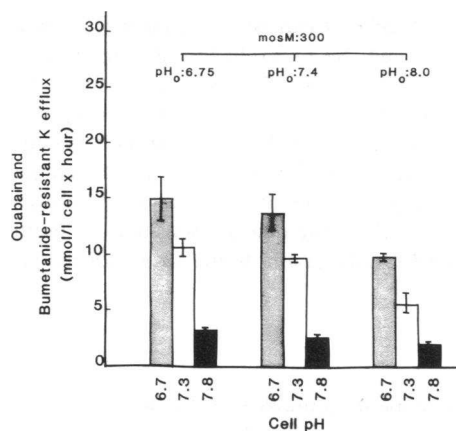


Figure 6. OB-resistant K efflux at three different external pH values from CC cells having three different internal pH values. CC cells were incubated in a medium similar to that described in Fig. 5, pH 6.75, 7.4, and 8.0, with addition of DIDS (150 μ M) and acetazolamide (1 mM). The internal pH was calculated from the external pH and the Cl distribution ratio. K efflux was measured from each of the three sets of cells in media with three different pH values (6.75, 7.4, and 8.0). The results are the mean \pm standard deviation of two experiments in the same CC subject.

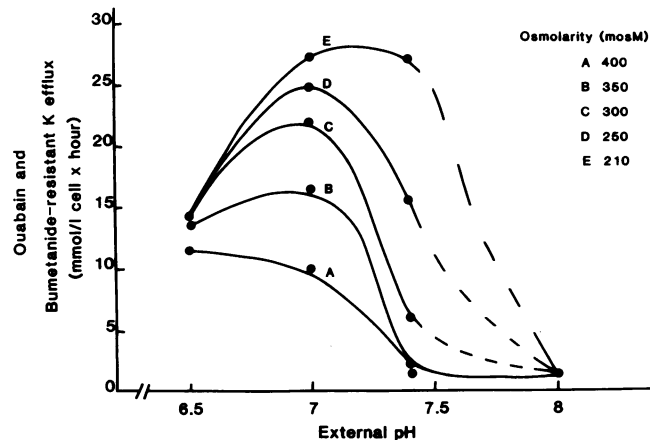


Figure 7. OB-resistant K efflux as a function of external pH, at different osmolarities in CC cells. The medium contained 100 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris-MOPS, pH 6.5–8.0, at 37°C, 10 mM glucose, 0.1 mM ouabain, and 0.01 mM bumetanide. The osmolarity was increased by adding choline chloride (0–100 mM). The data are from one of two experiments in the same CC patient.

of OB-resistant K efflux was measured at different osmolarities. As can be seen in Fig. 7, alkaline (8.0) and acid (6.5) pH, inhibited OB-resistant K efflux in hypotonic, isotonic, and hypertonic media. In contrast, when K efflux was activated by pH_o 7.0, cell swelling further increased and cell shrinkage inhibited the OB-resistant K efflux in CC cells.

Effect of different inhibitors on the OB-resistant K efflux and its relationship with the Ca-activated K permeability

In order to understand better the properties of the OB-resistant K leak in CC cells, the effect of different inhibitors was tested. When the inhibitor of Na transport amiloride (12) (1 mM) was added to the incubation medium, it inhibited 20% of the K efflux, under both basal conditions, and when the K efflux was stimulated by pH 7.0 or hypotonicity. It is possible that this effect is related to an alkaline shift in internal pH produced by the entry of the weak base amiloride (12), rather than a specific effect of the drug.

To investigate the role of Ca in the K permeability of CC cells, the concentration of internal free Ca was reduced by adding the ionophore A23187 (40 μ mol/liter of red cells; hematocrit = 1%) and EGTA (0.1–1.0 mM) (13). This treatment did not significantly affect the K efflux from CC cells, even when it was stimulated by hypotonicity or pH_o 7.0 (Table IV). The effect of various inhibitors of the Ca-activated K channel was also tested (5). As can be seen in Table V, none of the inhibitors of the Gardos pathway that we tested significantly reduced the volume- or pH-dependent K efflux from CC cells. We conclude that the increased K leak in CC cells is not a Ca-dependent process.

Because divalent cations inhibit Na and K fluxes in normal human red cells (14), we also tested the effect of external Mg. At low concentrations (0–8 mM) of external Mg, only a slight inhibition of the OB-resistant K efflux was found in CC cells, compared to the 50% inhibition found in AA cells. High Mg concentrations (16–20 mM) slightly stimulated K efflux from CC cells, but inhibited K efflux from AA cells. Therefore, another distinguishing characteristic of the OB-resistant K

Table IV. Effect of Internal Ca Removal on OB-resistant K Efflux from CC Cells

pH _o	Osmolarity <i>mosM</i>	Ouabain and bumetanide-resistant K efflux	
		EGTA	A23187 and EGTA
		<i>mmol/liter of cells × h</i>	
7.4	300	8.6±1.5	7.4±0.9
6.75	300	26.9±2.4	24.4±1.7
7.4	210	36.0±2.6	35.1±2.1

The efflux was measured in media containing 100 or 142 mM choline chloride, 0.15 mM MgCl₂, 10 mM Tris-MOPS, 10 mM glucose, 0.1 mM ouabain, and 0.01 mM bumetanide. The A 23187 concentration was 40 μmol/liter of cells for a hematocrit of the flux medium of 1%. The EGTA concentration was 0.1–1.0 mM. The results are the mean±standard deviation of three different experiments in the same CC patient.

efflux in CC cells seems to be the lack of inhibition by divalent cations.

Effect of external Na and external K on the OB-resistant K efflux

To demonstrate that the increased K leak in CC cells does not take place through a K_i/Na_o exchange mechanism, the OB-resistant K efflux was measured in Na media and in Na-free media (choline substitution), at constant external MgCl₂ (1 mM), as a function of pH_o and osmolarity. As can be seen in Figs. 3 and 5, there was no effect of removing external Na. K efflux has the same magnitude and the same pH and volume dependence in Na-free medium as in Na medium. Therefore, the increased K permeability of CC cells does not take place through a K_i/Na_o exchange mechanism.

To determine the effect of external K on the OB-resistant K efflux from CC cells, K efflux was measured into choline media, varying external K from 0 to 140 mM, at constant pH_o (7.4) and osmolarity (300 mosM). Because external K did not stimulate K efflux from CC cells, it is reasonable to infer

Table V. Effect of Inhibitors of the Ca-activated K Permeability on the OB-resistant K Efflux from CC Cells

pH _o Osmolarity (<i>mosM</i>)	OB-resistant K efflux	
	7.0	7.40
		<i>mmol/liter of cells × h</i>
Control	28.1±3.3	30.6±5.1
Quinine (75 μM)	29.5±3.2	33.6±5.1
Carbocyanine (10 μM)	31.2±1.6	33.0±3.9
Trifluoperazine (10 μM)	33.5±2.6	37.1±4.1
Chlorpromazine (10 μM)	34.6±1.9	37.5±3.3
TMB-8 (20 μM)	27.4±1.9	33.2±6.6

The medium contained 100 mM NaCl, with or without 50 mM choline chloride, 10 mM Tris-MOPS, 1 mM MgCl₂, 10 mM glucose, 0.1 mM ouabain, and 0.01 mM bumetanide. The results are the mean±standard deviation of two experiments in the same CC patient. The hematocrit of the flux media was 1%. TMB-8, trimethoxybenzoic acid 8(diethylamino)-octyl ester.

that K efflux does not take place through a K_i/K_o exchange mechanism. On the other hand, OB-resistant K influx was stimulated by external K. Although more work is needed on the effect of external K on the K efflux and influx pathways at different cell volumes, these properties suggest that the OB-resistant K movement in CC cells is driven primarily by the electrochemical gradient for K.

OB-resistant Na influx and efflux in CC and AA cells: effect of pH_o and osmolarity

The OB-resistant ²²Na influx was measured as a function of pH_o, at constant osmolarity (140 mM NaCl), and as a function of osmolarity (100 mM NaCl + choline) at constant pH_o (7.4). Under these conditions, we found that Na influx is independent of both pH and osmolarity (Table VI). In addition, there is no inhibition of this pathway by amiloride (1 mM). In Table VI, measurements of OB-resistant K influx under similar conditions (5 mM KCl) are included. From a comparison of the two rate constants for Na and K influx, it can be seen that this pathway prefers K to Na by at least a factor of 10.

To further characterize the cation selectivity of the volume- and pH-dependent increase in K permeability in CC cells, they were made to contain 86 mmol/liter of cells Na and 7 mmol/liter of cells K (nystatin procedure). The OB-resistant Na efflux was then measured in media containing choline chloride, as a function of pH_o and osmolarity (Table VI). Under these conditions, the Na efflux was not dependent on osmolarity, but was stimulated by alkaline pH. In the same table, we included measurement of OB-resistant K efflux from fresh cells into choline medium. Again, from a comparison of the two rate constants for Na and K efflux, it can be concluded that the OB-resistant, volume- and pH-dependent pathway for cation movement in CC cells prefers K to Na by at least a factor of 10. More accurate definition of the cation selectivity of this pathway must await further experiments.

Maximal rates of Na-K pump, Na-K cotransport, and Na-Li countertransport in CC and AA cells

Table VII shows the maximal rates for the three transport systems in CC and AA cells. It can be seen that the maximal rates of Na-K pump and Na-Li exchange are significantly higher in CC than in AA cells. The bumetanide-sensitive Na-K cotransport is lower in CC cells than in control cells. The differences in the maximal rates of the three transport systems remains significant even when the fluxes are calculated per number of cells or per unit of membrane area (see Methods). The affinity for internal Na of the ouabain-sensitive Na pump was measured in one CC subject and found to be normal (12 mmol/liter of cells).

Discussion

The erythrocytes of patients homozygous for hemoglobin C contain fewer impermeant negative charged groups than do erythrocytes from normal individuals homozygous for hemoglobin A (4). The cation content (and volume) of CC cells are also reduced to such an extent that the internal Cl⁻ and pH are the same as in AA cells. This reduction in cation content is the result of differences in the cation transport systems in CC as compared with AA cells.

The reduction in cation content observed in CC cells in vivo can be duplicated in vitro. When the cation content of

Table VI. OB-resistant Na and K Fluxes in CC Cells: Dependence on External pH and Osmolarity

	External pH (300 mosM)		Osmolarity (pH 7.4)		pH 7.4 (300 mosM)	
	7.0	8.0	220 mosM	400 mosM		
	CC	CC	CC	CC	CC	AA
A						
Na influx (mmol/ liter of cells × h)	1.81±0.01	2.27±0.06	1.28±0.07	1.51±0.03	1.87±0.1	0.67±0.02
Rate constant (h ⁻¹)	0.013	0.016	0.013	0.015	0.013	0.005
K influx (mmol/ liter of cells × h)	1.79±0.03	0.10±0.001	2.58±0.04	0.10±0.01	1.05±0.05	0.04±0.005
Rate constant (h ⁻¹)	0.358	0.018	0.516	0.020	0.21	0.009
K/Na influx Rate constant ratio	27	1.1	39.7	1.3	16.2	1.8
B						
Na efflux (mmol/ liter of cells × h)	8.70±0.1	13.10±0.15	9.38±0.2	11.68±0.2	11.76±0.48	5.32±0.2
Rate constant (h ⁻¹)	0.101	0.152	0.109	0.135	0.136	0.060
K efflux (mmol/ liter of cells × h)	24.24±1.2	2.39±0.25	25.34±0.2	1.09±0.1	6.06±0.25	1.43±0.08
Rate constant (h ⁻¹)	0.269	0.026	0.281	0.012	0.077	0.015
K/Na efflux Rate constant ratio	2.7	0.17	2.6	0.09	0.57	0.25

(A) Na influx into fresh cells was measured from media containing 140 mM NaCl (different pH) and 100 mM NaCl (different osmolarities). K influx into fresh cells was measured from media containing 140 mM NaCl and 5 mM KCl (different pH) and 100 mM NaCl and 5 mM KCl (different osmolarities). (B) Na efflux was measured into 140 mM choline chloride medium from CC cells containing 86.2 and 7.2 mmol/liter of cells of Na and K, respectively. K efflux was measured into 140 mM choline chloride medium from fresh CC cells (5 and 90 mmol/liter of cells of Na and K, respectively). Control experiments in AA cells were performed under similar conditions. The results are the mean±SD of two different determinations in the same CC patient and in two AA controls.

Table VII. Maximal Rates of Na-K Pump, Na-K Cotransport, and Na-Li Countertransport in CC and AA Erythrocytes

Cation	Cellular content		Flux	
	CC	AA	CC	AA
	mmol/liter of cells		mmol/liter of cells × h	
Na	44.4±4	45±3		
K	50±8	53±7		
Na-K pump				
Ouabain-sensitive Na efflux			12.1±3.2	4.9±0.9
Na-K cotransport				
Bumetanide-sensitive Na efflux			0.1±0.1	1.1±0.35
K efflux			0.1±0.1	1.2±0.35
Na	2±1	2.5±2.0		
Li	9.5±1.7	7.5±1.0		
K	78±2	88±5.5		
Na-Li countertransport			0.56±0.1	0.27±0.13

The intracellular Na and K content were reciprocally varied with the nystatin-loading procedure. Na-K pump was measured as ouabain-sensitive Na efflux into 130 mM choline and 10 mM KCl medium. Na-K cotransport was measured as bumetanide-sensitive Na and K efflux, in the presence of ouabain, into 140 mM choline medium. Na-Li countertransport was measured as Li efflux, in the presence of ouabain and bumetanide, into 140 mM choline and 140 mM NaCl media. All the media contained 1 mM MgCl₂, 10 mM Tris-MOPS, pH 7.4, at 37°C, and 10 mM glucose. The concentrations of the inhibitors were 0.1 mM for ouabain and 0.01 mM for bumetanide. The results are the mean±standard deviation of three determinations in two CC subjects and in three AA controls.

CC cells is increased to the value observed in AA cells (by adding cell K through the nystatin method), the CC cells lose K (and water) until they return to the cation content and volume that they display in vivo (Fig. 1, Table II). This response is not blocked by the simultaneous presence of OB and, therefore, apparently does not involve either the Na-K pump or Na-K cotransport. This response occurs both when the cell Na is low in the absence of ouabain and when it is high in the presence of the glycoside (Table II). In the second case, the return of the cation content of the CC cells to the reduced value that they exhibit in vivo, involves a greater loss of K. The response is not the result of increasing K content per se, in that it can also be initiated by swelling cells in hypotonic media (Fig. 2). Therefore, the response is produced by a direct or indirect effect of cell swelling. Because this response does not occur in AA cells, it apparently involves a OB-resistant K transport pathway that is active in CC but not in AA cells. This pathway does not seem to carry out K/K, K/H (Fig. 6), or K/Na (Figs. 3 and 5) exchange. It occurs even when the cell free Ca concentration is markedly reduced (Table IV) or in the presence of various inhibitors of the Ca-activated K channel of the erythrocyte membrane first described by Gardos (5) (Table V). Therefore, it does not have the same activation and inhibition characteristics as the Ca-activated K channel. In contrast to what has been reported by others (15), our data do not support the hypothesis that the Ca-dependent pathway for K transport is the mechanism responsible for the reduction of K content of CC cells.

This pathway for K movement in CC cells is regulated by cell volume and pH. The interpretation of these effects is not clear because of the interactions between these variables. When internal pH is reduced at constant external pH (see Fig. 6), cell chloride concentration, OH^- , and volume rise and membrane potential becomes more inside-positive. Sorting out the relative importance of these parameters will require further studies. It seems clear that the actions of pH and volume are partly additive, because swelling increases further the flux when pH_i is at the optimum value (Fig. 7). However, the two effects are not completely independent because OB-resistant K efflux does not increase upon swelling of CC cells when the pH_o is 8.0 or 6.5 (Fig. 7).

There have been many previous reports of volume-related K transport pathways in several types of cells. Normal human (AA) erythrocytes have increased OB-resistant K transport when swollen under isosmotic conditions (pH_o 7.4) by the nystatin method (9). However, the magnitude of the increase is substantially less than we have observed in CC cells (compare in Fig. 4), and is apparently insufficient to accomplish volume regulation over the time course required for this process in CC cells (Fig. 1).

Deoxygenation of erythrocytes containing only hemoglobin S (SS cells), has long been known to produce an increase in Na and K permeabilities (16). The increased K transport in de-oxygenated SS cells has more recently been shown to be resistant to ouabain and bumetanide (17). Roth et al. (18) showed that the deoxygenation-dependent K efflux from SS red cells, in the presence of ouabain, had the same pH dependence as in AA cells, and that it was inhibited by incubation in hyposmotic medium. In SC cells, the deoxygenation-induced K efflux is inhibited when the cells are swollen in hyposmotic medium (19). Clearly, hemoglobin polymerization plays a very important role in determining this behavior. It would be interesting to investigate whether oxygenated SS cells have a volume- and pH-dependent OB-resistant K transport pathway similar to that which we have observed in CC cells.

Cala has described a volume-related K transport system in *Amphiuma* erythrocytes (20). This system apparently differs from the pathway that we have described in human CC red cells. The *amphiuma* system carries out K/H exchange, coupled to the $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism, and is activated by increasing the concentration of internal Ca (21). None of these properties are characteristics of the CC system. Volume-related K transport systems have also been reported in duck and other avian erythrocytes (22). These systems are inhibited by bumetanide and appear to involve K/K exchange and a Na-K co-transport, again clearly different from the OB-resistant K transport pathway in CC cells reported in this paper.

Human lymphocytes also have a volume-related K transport pathway, which is resistant to OB (23). The volume regulatory response in lymphocytes also includes the opening of a Cl channel (24). The OB-resistant K channel in lymphocytes differs from the pathway that we describe in CC cells in that it is activated by internal Ca and inhibited by quinine and calmodulin inhibitors (23).

The most interesting question raised by the observations reported in this paper is how a point mutation in the β -chain of hemoglobin can produce a change in cation transport across the erythrocyte membrane. If one makes the parsimonious and plausible assumption that the point mutation in globin is

the only inherited genetic abnormality affecting CC red cells, it is likely that the abnormal globin interacts directly with the cation transport system or its regulators in such a way as to produce the observed transport abnormalities or with a membrane component not normally involved in cation transport. It has been shown that hemoglobin binds to the erythrocyte membrane (25, 26) and that hemoglobin C binds with a higher affinity than hemoglobin A at neutral pH (27, 28). An important site of interaction with the membrane is the N-terminal segment of band 3, which contains a sequence of 23 residues of which 14 are either glutamate or aspartate (29). This sequence contains no amino acids with positively charged side chains and thus constitutes an intensely negatively charged binding site for positively charged residues in hemoglobin and other molecules. The binding of hemoglobin A to the 23,000-mol wt N-terminal segment of band 3 shows a bell-shaped dependence on pH, with optimum binding occurring at pH 5.6–6.0 (29). The binding of hemoglobin C to band 3 persists at higher pH (7.0) (27).

It is plausible to postulate that the binding of hemoglobin C to band 3 activates the volume- and pH-dependent OB-resistant K transport pathway that reduces the K content of CC red cells. The bell-shaped curve of dependence of K transport on pH (Fig. 5) can be interpreted to mean that increasing the intracellular proton concentration has at least two effects on the system. One might be to increase the binding of some activator, e.g., hemoglobin, and the other to titrate, and thus inhibit the transport system itself. Such a model could account for the failure of AA cells to display activation of OB-resistant K transport with decreasing pH. When the pH becomes low enough to produce the net charge on hemoglobin A necessary to promote binding to and activation of the transport system, the transport system may already be inhibited by direct titration.

This hypothesis is consistent with the recent proposal that band 3 is the locus of a K "leak" in human red cells (30), and earlier reports of interactions between monovalent anion and cation transport (31). It may also be related to the bell-shaped dependence of Cl transport through band 3 on pH (32). Certain relevant experimental findings cannot be easily accommodated by this model for K leak via hemoglobin–band 3 interaction. Cation transport is known to occur on band 3 in the form of ion pairs of hydroxyl acids, e.g., NaCO_3^- (33). However, such ion pairs form to an appreciable extent with Li and Na but not with K. Furthermore, such transport of Li and Na as anionic ion pairs on band 3 is inhibited by DIDS. The cation transport system that we have described in CC cells is selective for K and is not inhibited by DIDS.

Secondly, we have found significant differences in the maximal rates of the Na-K pump, Na-K cotransport, and Na-Li exchange between CC and AA cells (Table VII). The elevation in the Na-K pump may be responsible for the reduced Na content of CC red cells. It is not clear to what extent the differences in these transport systems reflect the younger age of circulating CC cells (2–3% reticulocytes) owing to the mild hemolytic anemia encountered in these patients. The abnormalities in these transport systems do not seem to be directly involved in producing the reduced K content of CC cells. Clearly, further research is necessary to elucidate the connection between the point mutation in β -globin and the cation transport characteristics of CC red cells.

Acknowledgments

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