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Characteristics of the Volume- and Chloride-Dependent K Transport in Human Erythrocytes Homozygous for Hemoglobin C

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Summary. In human red cells homozygous for hemoglobin C (CC), cell swelling and acid pH increase K efflux and net K loss in the presence of ouabain (0.1 mM) and bumetanide. We report herein, that K influx is also dependent on cell volume in CC cells: cell swelling induces a marked increase in the maximal rate (from 6 to 18 mmol/liter cell × hr) and in the affinity for external K (from 77 ± 16 mM to 28 ± 3 mM) of K influx. When the external K concentration is varied from 0 to 140 mM, K efflux from CC and normal control cells is unaffected. Thus, K/K exchange is not a major component of this K movement. K transport through the pathway of CC cells is dependent on the presence of chloride or bromide; substitution with nitrate, acetate or thiocyanate inhibits the volume- and pH-dependent K efflux. When CC cells are separated according to density, a sizable volume-dependent component of K efflux can be identified in all the fractions and is the most active in the least dense fraction. N-ethylmaleimide (NEM) markedly stimulates K efflux from CC cells in chloride but not in nitrate media, and this effect is present in all the fractions of CC cells separated according to density. The persistence of this transport system in denser CC cells suggests that not only cell age, but also the presence of the positively charged C hemoglobin is an important determinant of the activity of this system. These data also indicate that the K transport pathway of CC cells is not an electrodiffusional process and is coupled to chloride.

Key Words erythrocyte · K transport · C hemoglobin · KCl cotransport · volume regulation · membrane transport

Introduction

Cell volume is determined by the water content of the cells, which is in turn determined by the nonpermeant and permeant cell solutes (Tosteson, 1967). Volume regulation generally takes place through systems that regulate the cellular concentration of Na and K utilizing the concentration gradients generated by the Na-K pump. As a general model, when cells are swollen, they turn on a ouabain-resistant "K leak," which produces K, Cl and water loss, and when they are shrunken, they turn on a ouabain-resistant "Na leak," which produces Na, Cl (and occasionally K) and water gain.

Volume regulatory processes have been characterized in duck red cells (Schmidt & McManus, 1977*a,b,c*; Kregenow, 1981; Haas, Schmidt & McManus, 1982; Haas & McManus, 1985), amphiuma red cells (Cala, 1980, 1983, 1985; Siebens & Kregenow, 1985), human lymphocytes (Grinstein et al., 1982*a*; Grinstein, Dupre & Rothstein, 1982*b*; Grinstein, Clark & Rothstein, 1983; Sarkadi, Mack & Rothstein, 1984*a,b*), dog red cells (Parker, 1983) and sheep red cells (Dunham & Ellory, 1981).

Human erythrocytes have long been known for the lack of mechanisms able to compensate for acute changes in cation and water content. Na-K pump (Glynn & Karlish, 1975), Na-K-Cl cotransport (Wiley & Cooper, 1974; Brugnara et al., 1986*b*), Ca-activated K channel (Gardos, 1958) and not involved in restoring cell volume after hypotonic swelling or hypertonic shrinkage in human red cells.

We have shown that erythrocytes of patients with homozygous hemoglobin C disease (CC) exhibit a volume regulatory decrease when swollen either isosmotically or hyposmotically (Brugnara et al., 1985). This volume regulatory decrease takes place even when the Na-K pump and the Na-K-Cl cotransport are inhibited by ouabain (0.1 mM) and bumetanide (0.01 mM), respectively, and involves a K loss. A feature of this K movement is the bell-shaped dependence on pH, with maximal activation at pH 6.7–7.0, and inhibition at more alkaline or acid pH. Our studies in CC cells have excluded K/H exchange, Ca-dependent K permeability, and K/Na exchange as possible transport mechanisms (Brugnara et al., 1985). A similar system is also present in SS erythrocytes (Brugnara, Bunn & Tosteson, 1986*a*; Canessa, Spalvins & Nagel, 1986*b*, 1987) and in the least dense, reticulocyte-

rich fraction of normal control (AA) erythrocytes (Brugnara & Tosteson, 1987*a*; Hall & Ellory, 1986*a,b*). Studies by Berkowitz and Orringer (1985, 1987) have indicated that this K transport in CC cells is partially inhibited by high concentrations of bumetanide and furosemide, does not depend on the presence of external Na and is chloride dependent.

Previous studies on K transport in CC cells have not adequately characterized some important properties of this system. In particular, there are no published data on the kinetic properties of K efflux and influx, on the anion selectivity and the dependence of K movement on cell volume. Detailed studies on the properties of K transport in density-separated CC cells have also not been carried out, even though they could provide important information on the role of cell age and C hemoglobin in determining the activity of this system. It is also not clear whether K transport in CC cells is simply due to electrodiffusion or to a specific transport system (e.g., KCl cotransport). In this study, we were able to determine that the swelling-induced K movement in CC cells does not take place through simple electrodiffusion and requires the presence of chloride or bromide. These and other features of this transport system suggest a close similarity with the KCl cotransport system described in LK sheep red cells (Dunham & Ellory, 1981; Lauf, 1985*b*). We also found that this system is present in all the density fractions of CC cells, whereas it is absent in the denser fractions of AA cells. This would indicate that, besides cell age, the presence of the positively charged hemoglobin C is an important determinant of the activity of this pathway in CC cells.

Materials and Methods

Chemicals

NaCl and KCl were purchased from Mallinckrodt, St. Louis, MO. $NaNO_3$, KNO_3, NaBr, NaSCN, KSCN, tri(hydroxymethyl)aminomethane (Tris), albumin (bovine, fraction V), 3(N-morpholino) propanesulfonic acid (MOPS), 2(N-morpholino) ethanesulfonic acid (MES), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), ethyleneglycol-*bis*-(β-aminoethylether) N,N′-tetra-acetic acid (EGTA), N-ethylmaleimide (NEM), dithiotreitol (DTT), ouabain, furosemide, sucrose and nystatin were purchased from Sigma Chemical, St. Louis, MO. Choline chloride and A23187 were purchased from Calbiochem-Behring, La Jolla, CA. Na acetate, $MgCl_2$, and $Mg(NO_3)_2$ were purchased from Fisher Scientific, Fair Lawn, NJ. Radioisotopes (^{22}Na and ^{86}Rb) were purchased from Amersham, Arlington Heights, IL. Bumetanide was a gift from Leo Pharmaceutical Products, Ballerup, Denmark. All chemicals were reagent grade and all solutions were prepared using double-distilled water.

Preparation of Erythrocytes

Blood was collected in heparinized Vacutainer® tubes (Becton, Dickinson & Co., Rutherford, NJ) from three donors homozygous for hemoglobin C (CC cells) and three normal controls (AA cells). Leucocytes were removed with cotton filtration (Diepenhorst, Sprokholt & Prins, 1972). The blood was centrifuged in a Sorvall refrigerated centrifuge (RC-5B, Du Pont Instruments, Sorvall Biomedical Div., Newtown, CT) at 5°C for 10 min at 3,000 × *g* and the cells were washed five times with a washing solution containing 152 mM choline chloride, 1 mM $MgCl_2$, 10 mM Tris-MOPS, 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 determinations 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 contents was determined in a Perkin-Elmer atomic absorption spectrophotometer (model 5000, Perkin-Elmer, Norwalk, CT) using standards in double-distilled water.

When the experiments were not performed on the same day of collection, the erythrocytes were stored at 4°C (20% Hct) in a preservation solution containing 140 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 2.5 mM NaH_2PO_4 buffer, pH 7.4 and 10 mM glucose. All experiments were performed within two days of sampling.

Nystatin Loading Procedure

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. The sucrose concentration in the loading solution varied from 20 to 100 mM according to the desired cell volume (Brugnara et al., 1985).

Choline-washed cells (2 ml) were added to 20 ml of cold loading solution containing 40 μg/ml of nystatin. The nystatin was dissolved fresh every day in dimethylsulfoxide (DMSO, 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 (30 ml) of cold loading solution without nystatin. This procedure allows complete equilibration of the cell cation content with the loading solution. 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.

Measurement of K Efflux

Cell suspension (0.2 ml) in choline washing solution (Hct 30–40%) was added to 7 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 (or 15) min in a shaking water bath at 37°C. This time course meets conditions of 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. From the total volume of medium (*Vm*), the volume (*Vcs*) and hematocrit (Hct) of the cell suspension added, the ratio of milliliters of medium to milliliters of cells was calculated:

$$f = \frac{[Vm + (Vcs \times 100\text{-Hct}/100)]}{(Vcs \times \text{Hct}/100)}. \quad (1)$$

To obtain the efflux values in mmol/liter cell, this factor was used to multiply the K concentration measured in the supernatant.

When pH_o was varied, the medium contained 140 mM NaCl, 1 mM $MgCl_2$, 10 mM glucose, 0.1 mM ouabain, 0.01 mM bumetanide and 10 mM Tris-MES (pH 6.0–6.5 at 37°C) or Tris-MOPS (pH 6.75–8.0 at 37°C). When the osmolarity was varied, the media contained 100 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris-MOPS, pH 7.40 at 37°C, 10 mM glucose, 0.1 mM ouabain, 0.01 mM bumetanide and 0–100 mM choline chloride, with osmolarities ranging from 215 to 405 mosM).

Measurement of Unidirectional Radioactive Influx

Medium (7 ml) was chilled and mixed with 10 μCi of ^{22}Na or 30 μCi of ^{86}Rb. The radioactivity in four aliquots of 20 μl of medium was measured to determine the initial specific activity. Packed cells (0.3 ml) 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 (or 15) min of incubation at 37°C was discarded and the cells were washed three times with 4 ml of cold choline washing solution. Afterwards, the cell pellet was lysed with 1 ml of 0.02% Acationox®. 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, Lynn, MA). The influx in mmol/liter cell · hr was calculated from:

$$\frac{\text{cpm/liter cell } (25 - 5 \text{ min}) \times 60}{\text{ISA (cpm/mmol) } 20} \quad (2)$$

where cpm/liter cells is the counts per minute in the lysate × $1/Vl \times 100/\text{Hct}$. *Vl* is the volume (liters) of lysate counted and Hct is the amount of cells (%) in the lysate. The amount of cells in the lysate was calculated using the Hct and concentration of hemoglobin of the initial cell suspension and the concentration of hemoglobin determined in the lysate. ISA is the initial specific activity of the medium in counts per minute per millimole. The standard error of the slope of the regression of time *vs.* intracellular Na or K content was used to determine the standard error of the influx.

Measurement of Unidirectional Radioactive Efflux

After a first incubation in the desired loading solution in the presence of nystatin, the cells were incubated a second time with 1.5 ml of cold loading solution (50% Hct) containing 5 μCi ^{22}Na or 50 μCi ^{86}Rb. ^{86}Rb was used as a tracer for K fluxes. In preliminary experiments, K efflux was measured in media containing 140 mM NaCl or 140 mM choline chloride using ^{86}Rb and measuring at the same time K efflux by atomic absorption spectroscopy. In both media, similar results of ouabain- and bumetanide (OB) resistant K efflux were obtained using either ^{86}Rb or atomic absorption spectroscopy. After 15 min incubation, the cells were washed four times with a warm (37°C) solution as described above in the nystatin loading technique. Afterwards, the cells were washed five times at 4°C with choline washing solution and used for efflux measurement. Cell suspension (0.3 ml) was added to 7 ml of flux medium. The flux suspension was then processed as previously described for the nonradioactive efflux. At the end of the incubation (5 and 25 or 15 min), the tubes were transferred to an ice bath and, after 2 min, they were spun down at 3,000 × *g* for 5 min. Supernatant (0.8 ml) was removed and counted in a gamma counter. Four aliquots of 1 ml of 1:50 dilution of the initial cells suspension in choline washing solution were counted for determination of the cell initial specific activity.

The efflux in mmol/liter cell · h was calculated from:

$$\frac{1}{\text{cell SA}} \times \frac{1}{Vs} \times f \frac{\text{cpm}}{\text{hr}} \quad (3)$$

where cell SA is counts per minute per millimole, *f* is milliliters flux medium per milliliter of red cells, *Vs* is the volume (liters) of supernatant counted, and cpm/hr is obtained by multiplying by 60 the slope of the values of counts per minute in the supernatant taken at any given time *vs.* time in min. The standard error of the slope of counts per minute *vs.* time was used to calculate the standard error of the fluxes.

Separation of CC and AA Cells with Density Gradients

Erythrocytes were suspended at 30% Hct in a solution containing 10 mM NaCl, 140 mM KCl, 1 mM $MgCl_2$, 10 mM Tris-MOPS, pH 7.4 at 4°C, and 10 mM glucose. The cell suspension was then carefully layered on top of discontinuous gradients of Stractan, prepared according to the specification of Clark, Morrison and Shohet (1978). The densities used for CC cells were 1.092–1.096–1.101–1.110–1.115 g/ml with a cushion of 1.146 g/ml and for AA cells were 1.083–1.092–1.096–1.101 g/ml with a cushion of 1.146 g/ml (values at 25°C). Gradients were centrifuged in a Beckman SW 28 rotor at 4°C for 45 min at 25,000 × *g*. After centrifugation, the various layers of cells were carefully removed with a Pasteur pipette and the cells were then washed four times with choline washing solution. An aliquot of cells was used for determination of MCHC, cell Na and K content.

N-Ethylmaleimide (NEM) Treatment

CC and AA cells were exposed to 1 mM NEM as specified by Lauf (1983*a*). The red cells were washed twice with Na nitrate washing solution, and then incubated at 15% Hct for 15 min at 37°C in a medium containing 140 mM $NaNO_3$, 1 mM $Mg(NO_3)_2$, 10 mM Tris-Mops, pH 7.10 at 37°C, 10 mM glucose, 0.1 mM ouabain, 0.01 mM bumetanide and 1 mM NEM. Afterwards, DTT was added to a final concentration of 1 mM and the cells were then washed twice with Na nitrate washing solution and used for K efflux measurement.

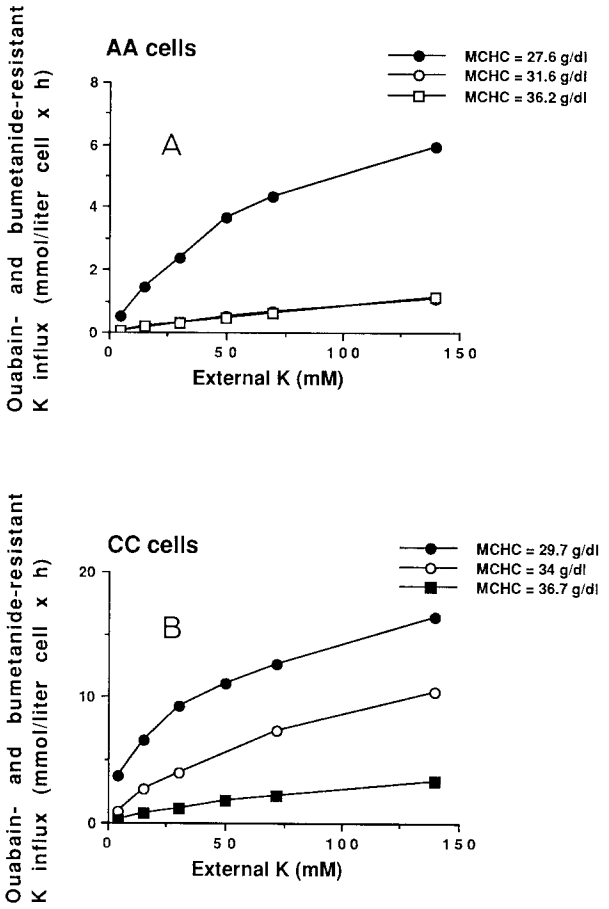

Fig. 1. K influx in AA and CC cells as a function of external K at different cell volumes. Cell volume was varied using the nystatin technique, and increasing cell K content at constant cell Na content (10 mmol/liter cell). The red cells were incubated in media containing from 5 to 140 mM KCl and from 135 to 0 mM choline chloride, respectively. All the media contained 1 mM $MgCl_2$, 10 mM Tris-MOPS, pH 7.4 at 37°C, 10 mM glucose, 0.1 mM ouabain and 0.01 mM bumetanide. The incubation times at 37°C were 5 and 25 min (5 and 15 min for swollen CC cells). ^{86}Rb was used as tracer for K movement. (*A*) The data points for AA cells at MCHC = 27.6 and 36.2 g/dl are the mean ± SD of two different determinations in the same subject. (*B*) The data points for CC cells are the mean ± SE of the fluxes of single experiments in the same CC subject. All the fluxes are expressed per liter of original cells (MCHC of 35 g/dl for CC and 32 g/dl for AA). Similar results were obtained in another CC patient

Results

Dependence of K Influx on External K

In control AA cells incubated in an isosmotic medium containing ouabain (0.1 mM) and bumetanide (0.01 mM) at pH 7.40, K influx from a Na-free medium (choline substitution) linearly increases when external K is increased (Fig. 1*A*). When AA cells are isosmotically swollen (nystatin procedure, MCHC from 34 to 27.5 g/dl), K influx is stimulated, and becomes a saturable function of the external K concentration (values of $K_{0.5}$, calculated from the Eadie plot, were 87 ± 6 mM and 120 ± 29 mM in two separate experiments).

Figure 1B shows the dependence on external K of K influx in CC cells at different cell volumes. In CC cells, the dependence on external K varies according to the cell volume. When CC cells are shrunken compared to their volume in vivo, the maximal rate of K influx is reduced (5.9 ± 1 mmol/liter cell × hr), and the $K_{0.5}$ for external K is 83 ± 13 mM and 71 ± 16 mM (both parameters were calculated from the Eadie plot, using data of two separate experiments in CC cells with MCHC of 37 g/dl). When the volume of CC cells is close to the volume they display in vivo (MCHC = 35 g/dl), the maximal rate of K influx and its affinity for external K are increased (13.0 ± 1.5 mmol/liter cell × hr and 54 ± 11, mM respectively; Eadie plot). These changes are more marked in swollen CC cells, where the maximal rate and affinity for external K of the OB-resistant K influx are further increased (18.1 ± 1 mmol/liter cell × hr, and 28 ± 3 mM, respectively; Eadie plot). These findings indicate that cell swelling has two effects on the properties of K influx in CC cells: (*i*) increase of the maximal rate and, (*ii*) increase of the affinity for external K. In AA cells, isosmotic cell swelling induces a smaller increase of OB-resistant K influx and a smaller change in the dependence on external K.

Dependence of K Efflux on External K

The dependence of K efflux on external K was studied in AA and CC cells, incubated in choline chloride media, at constant pH (7.40) and different cell volumes (isosmotic swelling), in the presence of ouabain and bumetanide. As can be seen in Fig. 2*A*, K efflux from AA cells is stimulated by isosmotic swelling, but is unaffected by external K (0 to 140 mM). A much larger stimulation by isosmotic swelling is seen in CC cells (Fig. 2*B*), and, as in AA cells, K efflux is practically unaffected when the external K concentration is varied from 0 to 140 mM. Thus, the lack of stimulation by external K of K efflux in AA and CC cells suggests that K_i/K_o exchange is not a sizable component of this K movement. Similar conclusions were recently reported by Berkowitz and Orringer (1987) in CC cells, but not by Kaji (1986) in normal cells.

Net K Extrusion and Equilibrium Point of K Fluxes

We studied the equilibrium point (external and internal K concentrations at which efflux = influx) of the unidirectional K fluxes in CC and AA cells. This

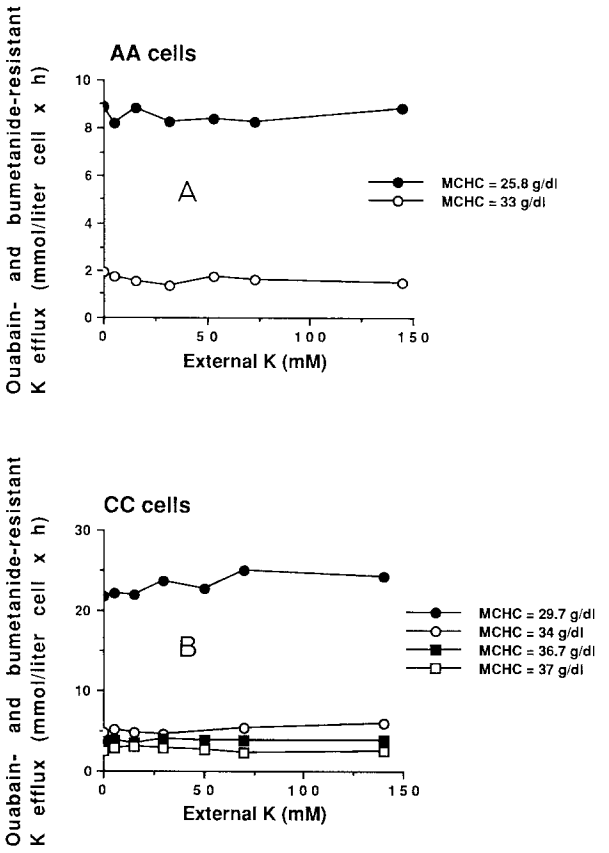

Fig. 2. K Efflux in AA and CC cells as a function of external K at different cell volumes. Experimental conditions were the same as in Fig. 1. ^{86}Rb was used as tracer for K. (*A*) The data points for AA cells are the mean ± SE of the fluxes of single determinations in the same subject. Similar results were obtained in another control. (*B*) The data points for CC cells are the mean ± SE of the fluxes of single experiments in the same CC subject. Similar results were obtained in another CC patient. All the fluxes are expressed per liter of original cells

kind of analysis is useful in determining whether or not this K movement takes place through electrodiffusion. When CC cells were shrunken compared to their volume in vivo, K fluxes reached equilibrium (efflux = influx) at 80–100 mM external K (*data not shown*). These values are not significantly different from those of normal AA control cells (Fig. 3). However, as shown in Fig. 3, in CC cells with volume similar to that of in vivo conditions, the K fluxes reached equilibrium at external K = 45–50 mM. According to Ussing's flux ratio analysis for independent electrodiffusional processes (Ussing, 1978)

$$^{i}k/^{o}k = [Cl]_o/[Cl]_i$$

that is, at the equilibrium point of the K fluxes, the ratio of the rate constants of influx to efflux ($^{i}k/^{o}k$) is equal to the chloride ratio (Cl_o/Cl_i). In AA cells and in shrunken CC cells, the equilibrium point of the K

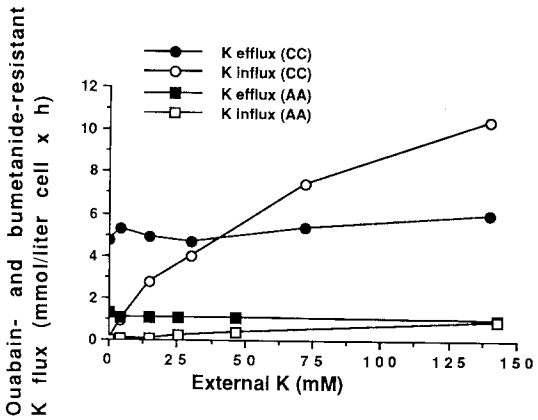

Fig. 3. Simultaneous measurement of K efflux and influx in CC and AA cells at different external K concentrations. Experimental conditions were the same as in Fig. 1. MCHC values were those of fresh CC and AA cells (34.5 and 32.5 g/dl, respectively)

fluxes is reached around 100 mM K_o (Fig. 3) and the ratio of the rate constants (1.5) fits with simple electrodiffusion. In fresh CC cells, the ratio of the rate constants for K movement at the equilibrium point is 2.5 instead of 1.5. This would suggest that the swelling-induced K transport of CC cells is not due to simple electrodiffusion, and some other kind of transport mechanism is involved.

Dependence of K Efflux on Internal K

Previous reports have shown that K influx in CC cells does not depend on external Na (Brugnara et al., 1985; Berkowitz & Orringer, 1987), thus excluding inward Na-K-Cl cotransport as a possible transport mechanism. However, the role of internal K or Na on K efflux was not examined in those studies. The dependence of K efflux on internal K and Na was studied in nystatin-treated CC cells, with MCHC of 35.8 g/dl, varying internal K from 2 to 100 mmol/liter cell (Na substitution). It can be noticed (Fig. 4), that the dependence of K efflux on internal K in the range 30 to 100 mmol K/liter cells markedly differs in CC and AA cells. K efflux from AA cells is constant when cell K is increased from 30 to 100 mmol/liter cells (Fig. 4 and Canessa et al., 1986*a*), whereas K efflux from CC cells increases monotonically with the internal K concentration. Figure 4 also shows that K efflux is not affected when internal Na is increased from 0 to 20 mmol Na/liter cells, thus excluding outward Na-K-Cl cotransport as a possible transport mechanism.

Dependence of K Efflux on Cell Volume (MCHC)

Figure 5 shows the effect of isosmotic swelling on the pH dependence of K efflux from CC cells. It is worth noting that isosmotic cell swelling not only

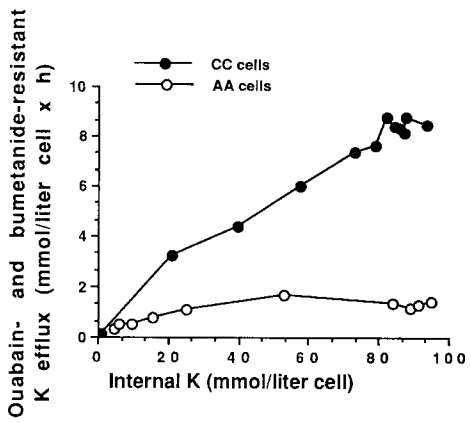

Fig. 4. K Efflux from CC and AA cells into choline medium at different internal K concentrations. K content was varied with the nystatin technique (Na substitution). MCHC was 35.8 g/dl in CC and 32.5 g/dl in AA. K efflux was measured into a medium containing 145 mM choline chloride, 1 mM $MgCl_2$, 10 mM Tris-MOPS, pH 7.4 at 37°C, 10 mM glucose, 0.1 mM ouabain and 0.01 mM bumetanide. Incubation times were 5 and 25 min

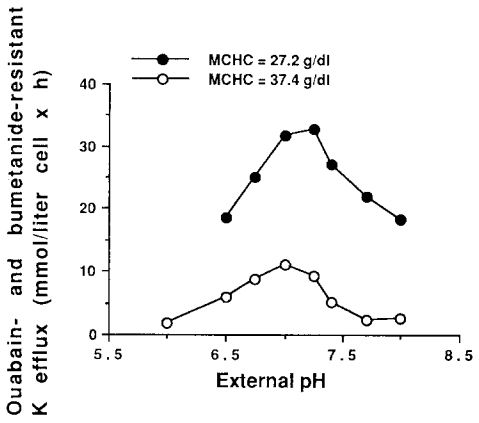

Fig. 5. pH dependence of K efflux from CC cells at different cell volumes. Cell volume was varied with the nystatin technique. K efflux was measured into a medium containing 145 mM sodium chloride, 1 mM $MgCl_2$, 10 mM Tris-MOPS, 10 mM glucose, 0.1 mM ouabain and 0.01 mM bumetanide. Incubation times were 5 and 25 min

markedly increases K efflux but also changes the pattern of its pH dependence. In fact, in swollen CC cells, the inhibitory effect of alkaline pH is markedly reduced. These results differ from our previous findings on the combined effect of pH and volume under conditions of hyposmotic swelling (Brugnara et al., 1985), where we showed that alkaline pH still maintained its inhibitory effect on K efflux from CC cells. This would suggest that cell swelling at constant osmolality can override the inhibitory effect of alkaline pH.

Dependence of K Efflux on Chloride

To study the effect of different anions on the swelling-induced K flux, chloride was substituted by other permeant anions in the flux media and K efflux from CC cells was measured in conditions of hyposmotic swelling and isosmotic swelling. As shown in Table 1, the swelling-induced K efflux requires the presence of chloride and/or bromide, and is not activated in the presence of nitrate, thiocyanate and acetate. A similar experiment was performed in AA control cells (Table 2). In this case, the effect of different anions was studied in control conditions, and in cells hyposmotically and isosmotically swollen. As previously reported (Adragna & Tosteson, 1984; Brugnara et al., 1985) isosmotic swelling of normal cells induces a much larger increase of K efflux than hyposmotic swelling. Both effects are increased by substitution with bromide and inhibited by nitrate. The anion sequence preference $Br > Cl > SCN > acetate > NO_3$ is similar to that reported by Lauf (1988*b*) and Dunham and Ellory (1981) in LK sheep erythrocytes and differs from that reported by Funder and Wieth (1967) in human red cells. However, in Funder and Wieth's study (1967), ouabain-resistant K fluxes were measured in cells with normal volume and in the absence of inhibitors of the Na-K-Cl cotransport system. In other studies, we examined the chloride dependence of K efflux at different pH and osmolarity values. Figure 6 shows that when external and internal chloride are both substituted with nitrate, the pH- and volume-dependent K efflux of CC cells is completely abolished and K efflux from CC cells becomes similar to that of AA cells. The same is true when acetate was used as a chloride substitute in the study of the pH and volume dependence of K efflux (*data not shown*). However, we found that acetate induces a large increase in the OB-resistant K efflux from normal AA cells at pH higher than 7.4, and we did not use this anion at higher pH. As can be seen in Fig. 6, when nitrate is substituted for chloride in the flux medium, K efflux in AA control cells is increased. Increase of K efflux with nitrate substitution was also reported by Funder and Wieth (1967), but not by Dunham, Stewart and Ellory (1980).

To study the dependence of the swelling-induced K movement of CC cells on chloride concentration, CC cells were incubated in hypotonic media (100 mM Na, 215 mosM) and external (and internal) chloride was varied from 0 to 100 mM. Three different anions (nitrate, thiocyanate and acetate) were used. As can be seen in Fig. 7, K efflux was progressively stimulated when the external Cl concentration was increased.

Table 1. Effect of anion substitution on K efflux from CC cells[a]

Medium	K efflux (mmol/liter cell · hr)		
	Isosmotic control	Hyposmotic swelling	Isosmotic swelling
NaCl	5.94 ± 0.15	26.03 ± 0.31	25.41 ± 0.52
NaBr	5.77 ± 0.20	21.89 ± 0.43	25.97 ± 0.55
NaSCN	3.12 ± 0.27	5.39 ± 0.40	8.05 ± 1.05
$NaNO_3$	4.19 ± 0.38	5.30 ± 0.41	7.53 ± 1.29
NaAcetate	2.21 ± 0.25	2.77 ± 0.3	4.46 ± 0.54
NaCl (hypertonic)	2.52 ± 0.35	—	—

[a] In the control conditions, K efflux was measured from fresh cells into media containing 140 mM Na and the specified anion (osmolarity 295–300 mosM). In the hyposmotic swelling conditions, K efflux was measured from fresh cells into media containing 100 mM Na and the specified anion (osmolarity 215–220 mosM). In the isosmotic swelling conditions, the cells were swollen by using the nystatin technique (MCHC from 37.4 to 27.2 g/dl) and K efflux was then measured in the same media as control conditions. All the media contained 1 mM Mg, 10 mM Tris-MOPS, PH 7.4 at 37°C, 10 mM glucose, 0.1 mM ouabain and 0.01 mM bumetanide. The hematocrit of the flux suspension was 1% and the incubation times were 5 and 25 min.

Table 2. Effect of anion substitution on K efflux from AA cells[a]

Medium	K efflux (mmol/liter cell · hr)		
	Control	Hyposmotic swelling	Isosmotic swelling
NaCl	1.78 ± 0.19	3.32 ± 0.1	5.81 ± 0.2
NaBr	2.18 ± 0.43	4.72 ± 0.16	8.14 ± 0.47
$NaNO_3$	2.89 ± 0.11	2.15 ± 0.1	2.26 ± 0.54
NaAcetate	3.52 ± 0.11	2.43 ± 0.11	4.09 ± 0.44
NaSCN	3.51 ± 0.08	3.08 ± 0.02	5.22 ± 0.24
NaCl (hypertonic)	1.69 ± 0.14	—	2.45 ± 0.23

[a] Experimental conditions were as detailed in Table 1. For the isosmotic swelling conditions, MCHC was varied from 33 to 28 g/dl. The hematocrit of the flux suspension was 1.5% and the incubation times were 5 and 35 min.

Cell Density and Swelling-Induced K Efflux From CC Cells

We have recently reported (Brugnara & Tosteson, 1987*a*), that a system similar to the one we described in CC cells is also present in the least dense (reticulocyte-rich) fraction of normal AA cells. Some other investigators have suggested that the reason for the high magnitude of this system in CC cells is just the presence of young cells (Canessa et al., 1986*b*, 1987). If this were the case, one would expect the system to be inactive in the denser fractions of CC cells, as it is in AA cells. As can be seen in Fig. 8, when CC cells are separated according to density (Clark et al., 1978), and K efflux is measured in hyposmotic media, there is a large K flux in the least dense fractions (5% of the total cells) and all the denser fractions have K efflux values similar to those of whole CC blood and substantially larger than AA control cells. In AA cells, the volume-dependent K flux is present in only 5–10% of the cells and is lost when the cells become denser (*see* Fig. 8 and Brugnara & Tosteson, 1987*a*). Thus, in CC cells the volume-dependent component of K movement is present in all the density fractions and cannot be due just to the presence of young cells. We attribute the presence of an active volume-dependent K flux in dense CC cells but not in dense AA cells, to the presence of the positively charged C hemoglobin.

Effect of N-Ethylmaleimide (NEM) on K Efflux From CC Cells

As shown by Lauf in low K sheep red cells (1983*a*,*b*) and in human erythrocytes (Lauf, Adragna & Garay, 1984; Kaji & Kahn, 1985; Canessa et al., 1986*b*, 1987), NEM promotes chloride-dependent K transport. We and others have

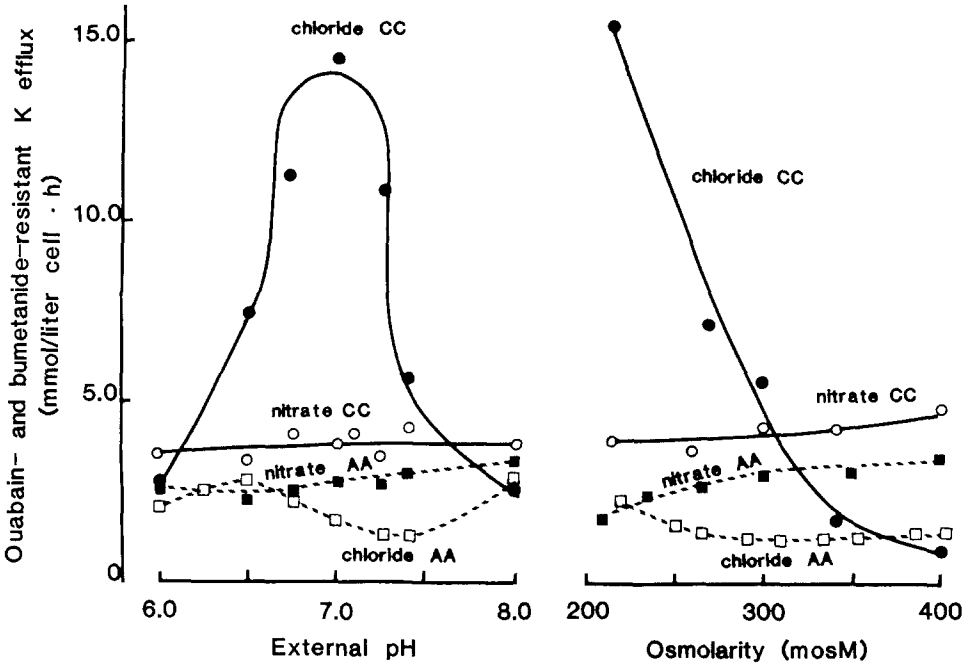

Fig. 6. Dependence of K efflux on external pH and osmolarity in CC and AA cells and the effect of chloride substitution by nitrate. To study the pH dependence, the cells were incubated in a medium containing 140 mM Na, 1 mM Mg (with the specified anion), 10 mM Tris-MES (pH 6–6.5) or Tris-MOPS (pH 6.75–8). To study the dependence on osmolarity, the cells were incubated in media containing 100–200 mM Na and 1 mM Mg (with the specified anion), 10 mM Tris-MOPS, pH 7.4 at 37°C. All the media contained 10 mM glucose, 0.1 mM ouabain and 0.01 mM bumetanide. Incubation times were 5 and 25 min. Similar results were obtained in another CC patient

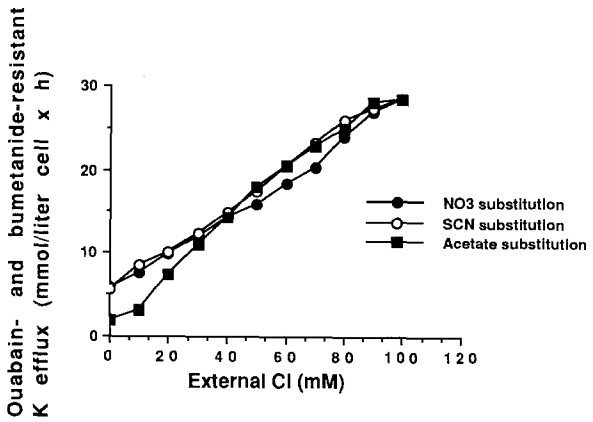

Fig. 7. K efflux from CC cells as a function of the external chloride concentration. Fresh CC cells were incubated in hyposmotic media containing 100 mM Na. Chloride concentrations was varied substituting with three different anions (nitrate, thiocyanate and acetate). Experimental conditions were as in Fig. 7. Similar results were obtained in another CC patient

shown that the effect of NEM in AA cells is limited to cells of the least dense fraction possessing the pH-, volume- and chloride-dependent system for K transport (Berkowitz, Walstad & Orringer, 1987; Brugnara & Tosteson, 1987*a*). As shown in Fig. 9, K efflux from fresh CC cells was increased by treatment with NEM (1 mM). The dependence on pH was also affected by NEM treatment, since there was no inactivation by alkaline pH (8.0). There was a small dependence on cell volume in NEM-treated CC cells, with 20% inhibition by hypertonic media (400 mosM) as compared to hypotonic media (215 mosM). In control cells, there was a sizable stimulation of OB-resistant K efflux by NEM, but to values much lower than those of CC cells. The effect of NEM was completely abolished by replacement of chloride by nitrate, and K efflux into nitrate media in both AA and CC cells was not affected by NEM treatment (*data not shown*). We also studied the effect of NEM on K efflux into isosmotic medium at pH 7.40 from density-separated CC cells. As shown in Fig. 10, all the density fractions of CC cells studied exhibited a sizable stimulation of K efflux by NEM. The presence of this stimulation in both young and denser (and presumably older) CC erythrocytes suggests that cell youth is not the only determinant of the activity of this pathway in CC cells.

Discussion

This paper provides a further characterization of K transport in CC erythrocytes. We show here that

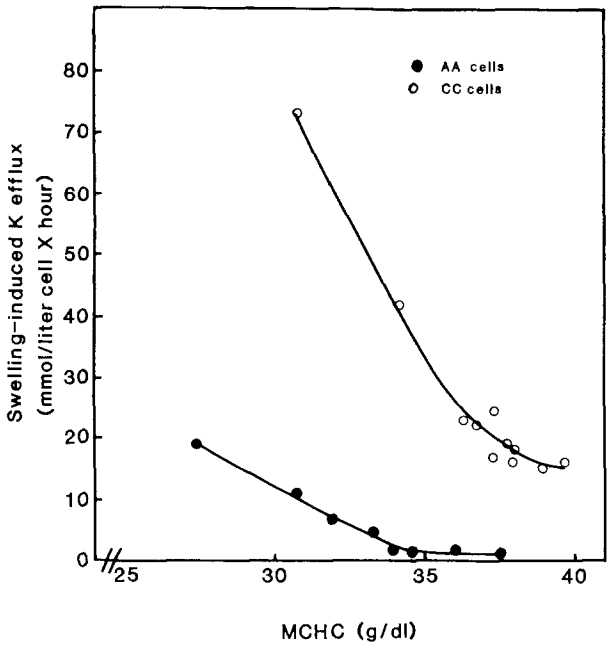

Fig. 8. Effect of hyposmotic swelling on the K efflux from CC cells separated according to density. CC cells were separated according to density by using Stractan II gradients. K efflux was measured in isotonic (140 mM NaCl) and hypotonic (100 mM NaCl) media. All the media contained 1 mM $MgCl_2$, 10 mM Tris-MOPS, pH 7.4 at 37°C, 10 mM glucose, 0.1 mM ouabain and 0.01 mM bumetanide. Incubation times were 5 and 15 min

the kinetic properties of K influx change according to red cell volume; when CC cells are isosmotically swollen, there is a large increase in the affinity for external K and in the maximal rate of K influx (Fig. 1*B*). This behavior differs from that of AA cells. In fact, although cell swelling stimulates K influx in AA cells, this stimulation is much lower than in CC cells, and takes place without a similar increase in the affinity for external K (Fig. 1*A*). In both CC and AA cells, increasing external K from 0 to 140 mM does not stimulate K efflux (Fig. 2*A* and *B*), thus, K_i/K_o exchange is not an important component of this K movement. From the simultaneous measurement of unidirectional K fluxes in CC cells at different external K concentrations, it was shown that the equilibrium position of the K fluxes (external K concentration at which K efflux = K influx) in shrunken CC cells is not significantly different from that of control AA cells (100 mM K_o, flux ratio = 1.5) and fits with simple electrodiffusion. Moreover, when the net flux from shrunken CC cells is plotted against the electrochemical potential ($E_m - E_K$), zero net flux is obtained when the electrochemical potential is also zero (*data not shown*). When CC cells have a volume similar to the one in vivo, the equilibrium position is shifted to lower external K concentrations, so that the flux ratio is 2.5 instead of 1.5. This suggests that in CC cells, the swelling-induced K transport takes place through a mecha-

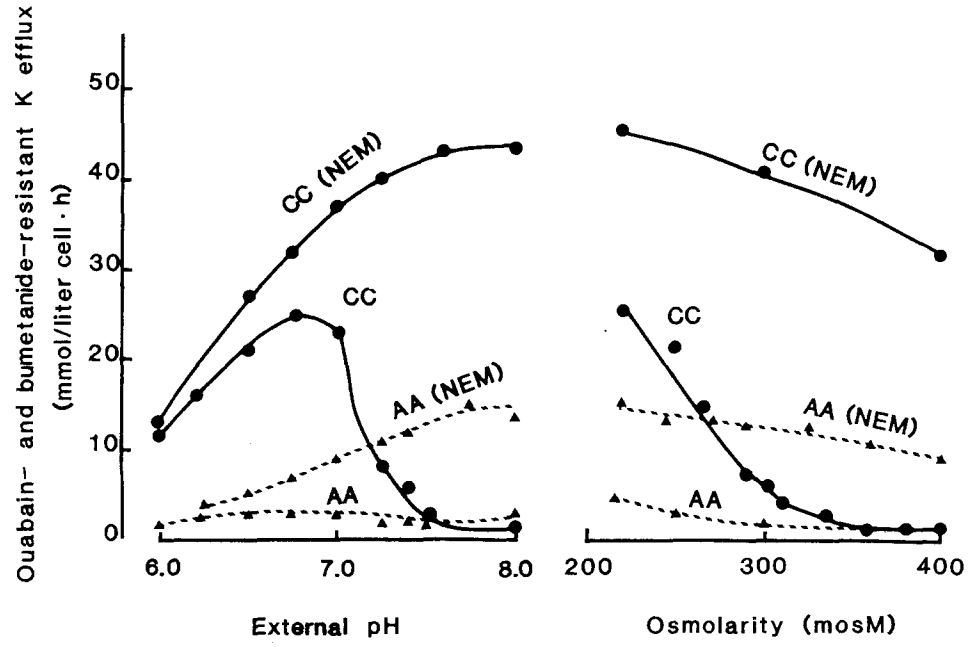

Fig. 9. Effect of NEM treatment on the pH- and volume-dependence of OB-resistant K efflux from fresh CC and AA cells. NEM treatment was performed according to the protocol developed by Lauf (1983*a*). The cells were then washed and K efflux was measured in media not containing NEM. Incubation times at 37°C were 5 and 15 min

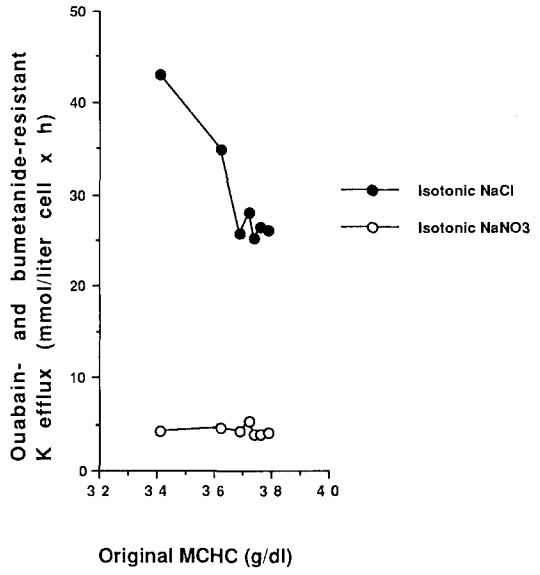

Fig. 10. Effect on NEM treatment on the K efflux from CC cells separated according to density. CC cells were separated according to density by using Stractan II gradients. K efflux was measured in isotonic media containing either 140 mM NaCl or 140 mM $NaNO_3$. All the media contained 1 mM $MgCl_2$ [or $(NO_3)_2$], 10 mM Tris-MOPS, pH 7.4 at 37°C, 10 mM glucose, 0.1 mM ouabain and 0.01 mM bumetanide. Incubation times were 5 and 15 min

nism different from simple electrodiffusion. Using a similar approach, Lauf (1988*a*) reported that the ratio of K fluxes was close to 2 in diamide-treated LK sheep erythrocytes, thus indicating a nondiffusional transport process. Since K movement in CC cells is chloride dependent (Figs. 6 and 7, and Table 1), it is likely that the swelling-induced K transport of CC cells is mediated by a K-Cl cotransport mechanism. Recent studies in AA and SS erythrocytes and in LK sheep and rabbit red cells suggest that K and Cl fluxes are coupled through a volume-dependent KCl cotransport mechanism, since in these cells it is possible to drive K uphill against an electrochemical gradient with a Cl gradient at constant membrane potential (Brugnara, Van Ha & Tosteson, 1989*b*). Berkowitz and Orringer (1987) had suggested that the chloride-dependent pathway for K transport of CC cells might not be responsible for the reduced volume of CC cells, since there was no inhibition by nitrate of K efflux from CC cells at original volume. Table 1 shows that there is substantial inhibition of K efflux from fresh CC cells when SCN and acetate are substituted for chloride, thus suggesting that this pathway is active in fresh CC cells.

One of the distinguishing features of K transport of CC cells, namely the inhibition by alkaline pH (>7.5) is almost completely abolished when CC cells are isosmotically swollen (Fig. 5), exposed to NEM (Fig. 9) or depleted of their Mg content (*unpublished data;* for SS cells *see* Brugnara & Tosteson, 1987*b*). Since we have previously shown that alkaline pH inhibits K efflux when CC cells are hyposmotically swollen (Brugnara et al., 1985), the first finding suggests that the inhibition by alkaline pH can be overridden by a reduction of nonpermeant cell solutes at constant osmolality. A similar effect can be obtained by removing internal Mg in SS cells (Brugnara & Tosteson, 1987*b*). This would suggest that the presence of internal Mg is required for the inhibitory action of alkaline pH. K efflux from swollen or Mg-depleted CC cells is inhibited by hypertonic shrinkage, thus, the inhibitory effects of hypertonicity and alkaline pH can be dissociated in these two conditions. NEM treatment (Fig. 9) abolishes the inhibitory effect of alkaline pH and most of the inhibition by hypertonicity. A similar change in pH dependence was reported by Zade-Oppen and Lauf (1987) in NEM-treated LK sheep red cells.

Recent reports have indicated that cell swelling activates a Cl-dependent K permeability in normal human red cells (Kaji, 1985, 1986; O'Neill, 1987). In this paper, we have compared some of the properties of this swelling-induced K permeability with the pH- and volume-dependent K transport of CC cells. However, we have found that, in normal AA blood, a small fraction of cells (least dense or reticulocyte-rich fraction) exhibits a pH- and volume-dependent movement for K similar to that of whole CC blood (Brugnara & Tosteson, 1987*a*). Hall and Ellory (1986*a*,*b*) have also reported the presence of a volume-sensitive and chloride-dependent KCl transport in young red cells. The presence of a pH- and volume-dependent K transport in the least dense (reticulocyte-rich) fraction of normal AA cells and its complete disappearance in the denser fractions suggests that this system is probably inactivated at an early stage in the red cell life span. The heterogeneous transport properties of whole AA blood pose some difficulties in the interpretation of the control experiments. The stimulation of K fluxes in AA cells by cell swelling (Kaji, 1985, 1986; O'Neill, 1987), or by NEM (Lauf, Perkins & Adragna, 1985) reflects a large change in the K fluxes of the least dense fraction and a small change in the other denser fractions (Berkowitz et al., 1987; Brugnara & Tosteson, 1987*a*). A similar cell heterogeneity has been recently described for the Ca-activated K permeability of human red cells (Lew & Garcia-Sancho, 1985) and for patch-clamp studies of human red cells (Grygorczyk, Schwartz & Passow, 1984). Clearly, future investigations will have to consider very carefully the problem of AA cell het-

erogeneity, and select the most appropriate fractions as controls.

We also studied the dependence of K transport in CC cells separated according to density. We had previously hypothesized that the presence of the positively charged hemoglobin C was responsible for the high activity of this system in CC cells. A pH-, volume-, and chloride-dependent K movement is present in red cells of patients homozygous for hemoglobin S, a positively charged hemoglobin variant (Brugnara et al., 1986*a*; Canessa et al., 1986*b*). We have also shown that a similar system is present in the least dense fraction of cells containing normal hemoglobin A and is then inactivated when the red cells become denser (Brugnara & Tosteson, 1987*a*), thus suggesting also a role for cell age. If K transport in CC cells were determined only by cell age, one would expect the system to be inactive in the denser fractions of CC cells. However, Fig. 8 shows that the volume-dependent K flux is present in all the fractions of CC cells. This K movement is highest in the 5% least dense fraction of CC cells, but contrary to AA cells, it does not disappear when the cells become denser. C hemoglobin could have two possible effects on the K transport mechanism: (*i*) to increase the number of transport sites per cell (through a reduction of its inactivation), and (*ii*) to change the set point of the system; in fact, in CC cells the system is active at values of MCHC and water content, which inhibit operation of the system in AA cells from the least dense fraction. If cell age were the only factor determining the presence of this K transport pathway, one would expect the system to be inactive in AC cells. However, a sizable volume- and pH-dependent K transport can be demonstrated in AC cells, which have normal life span and no reticulocytosis (Brugnara et al., 1989*a*). Moreover, studies of Ballas et al. (1987) have indicated that the cell dehydration in SC cells compared to AS cells is due to a greater ouabain- and bumetanide-resistant K loss, induced by the presence of C hemoglobin.

From the data presented in this paper and those previously published (Berkowitz & Orringer, 1985, 1987; Brugnara et al., 1985), it seems clear that the pH-, volume-, and chloride-dependent K transport of CC cells differs from the mechanisms described in duck red cells, *Amphiuma* red cells and in lymphocytes. The K transport system described in LK sheep red cells has many properties in common with the K transport of CC cells. The dependence on chloride and bromide and the inhibition by other anions (SCN, nitrate and acetate) on the swelling-induced K flux, the saturable dependence of K influx on external K, and the lack of effect of internal Ca shown in CC cells have also been shown in LK sheep red cells (Dunham & Ellory, 1981; Lauf, 1984). The stimulation by NEM is also present in mature LK but much less in HK sheep red cells (Logue et al., 1983; Lauf, 1985*b*; Fujise & Lauf, 1987). However, the dependence on chloride of the swelling-induced K efflux is linear in CC cells (Fig. 8) and has a sigmoid kinetic in LK cells (Dunham & Ellory, 1981), and moreover, external K inhibits K efflux in LK cells (Lauf, 1983*a*) but not in CC cells (Fig. 2*B*). Another property shared by LK and CC cells is the inhibition of the swelling-induced K flux by divalent cations (Lauf & Mangor-Jensen, 1984) and by high concentrations of furosemide and bumetanide in the presence of external K (Lauf, 1984, 1985*a*). Moreover, the swelling-induced K transport is present in reticulocytes of both LK and HK red cells, and is then inactivated when HK cells become mature red cells (Lauf, 1985*b*); similarly, the swelling-induced K transport is present in the least dense fraction (reticulocyte rich) of normal AA cells, but is not evident in the whole blood or densest fraction of AA cells (Brugnara & Tosteson, 1987*a*).

This study in CC cells, as well as other studies in SS and SC cells (Brugnara et al., 1986*a*; Ballas et al., 1987), show that the presence of positively charged hemoglobins has profound effects on K transport, cell volume, cation and water content of the erythrocyte. The challenge for future studies is to elucidate the mechanisms responsible for these effects.

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