J. Membrane Biol. 142, 349–362 (1994)

The Journal of
Membrane
Biology

Rate of Activation and Deactivation of K:Cl Cotransport by Changes in Cell Volume in Hemoglobin SS, CC and AA Red Cells

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Received: 18 May 1994/Revised: 24 August 1994

Abstract. Red blood cells (RBC) of subjects homozygous for hemoglobin A (AA), C (CC) and S (SS) exhibit different cell volumes which might be related to differences in cell volume regulation. We have investigated how rapidly K:Cl cotransport is activated and deactivated to regulate the cell volume in these cells. We measured the time course of net K^+ efflux after step changes in cell volume and determined two delay times: one for activation by cell swelling and a second for deactivation by cell shrinkage. Cell swelling induced by 220 mOsm media activated K^+ efflux to high values (10–20 mmol/liter cell × hr) in CC and SS; normal AA had a threefold lower activity. The delay time for activation was very short in blood with a high percentage of reticulocytes (retics): (SS, 10% retics, 1.7 ± 0.3 min delay, $n = 8$; AA, 10% retics, 4 ± 1.5 min, $n = 3$; CC, 11.6% retics, 4 ± 0.3, $n = 3$) and long in cells with a smaller percentage of reticulocytes: (AA, 1.5% retics, 10 ± 1.4 min, $n = 8$; CC whole blood 6% retics, 10 ± 2.0 min, $n = 10$, $P < 0.02$ *vs.* SS). The delay times for deactivation by cell shrinking were very short in SS (3.6 ± 0.4 min, $n = 8$, $P < 0.02$) and AA cells with high retics (2.7 ± 1 min, $n = 3$) and normal retics (2.8 ± 1 min, $n = 3$), but 8–15-fold longer in CC cells (29 ± 2.8 min, $n = 9$).

Density fractionation of CC cells ($n = 3$) resulted in coenrichment of the top fraction in reticulocytes and in swelling-activated cotransport (fourfold) with short delay time for activation (4 ± 0.3 min) and long delay for deactivation (14 ± 4 min). The delay time for activation, but not for deactivation, increased markedly with increasing cell density. These findings indicate that all CC cells do not promptly shut off cotransport with cell shrinkage and high rates of cellular K^+ loss persist after return to isotonic conditions.

In summary, (i) K:Cl cotransport is not only very active in young cells but it is also very rapidly activated and deactivated in young AA and SS cells by changes in cell volume. (ii) Delay times for cotransport activation markedly increased with RBC age and in mature cells with low cotransport rates, long delay times for activation were observed. (iii) The long delay time for deactivation exhibited even by young CC cells induces a persistent loss of K^+ after cell shrinkage which may contribute in vivo to the uniformly low cell volume, low K^+ and water content of CC cells.

Key words: Hemoglobin S — Hemoglobin C — Red blood cells — KCl cotransport — Sickle cell anemia — Cell volume regulation

Introduction

Upon maturation of human and some mammalian reticulocytes, cell volume, K^+ and water content decrease markedly and mean corpuscular hemoglobin concentration (MCHC) increases with cell age [31, 40]. An important characteristic of young human red blood cells (RBC) is their elevated activity of volume-stimulated K:Cl cotransport, a powerful regulator of cell volume that responds to cell swelling or reduced pH by activating a net loss of K^+, Cl^-, and water to re-establish normal cell volume [8, 9, 13, 16, 23, 25]. The physiological

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stimulus for activation of K:Cl cotransport may be the reduced pH characteristic of certain organs such as the kidney or conditions such as low pO_2 or stress.

Several studies independently demonstrated that in RBCs of subjects homozygous for hemoglobin A (AA) and homozygous for hemoglobin S (SS) [9, 13], the high cotransport activity of young red cells decreases with cell age and increased MCHC [*for reviews, see* 6, 12, 29]. In mature RBCs of AA subjects, cotransport activity is very low but it has been extensively characterized for its anion dependence, effect of inhibitors and K^+ kinetic properties [12, 20, 33]. In SS and CC patients, swelling-stimulated K^+ efflux is very active [7, 8] because the red cell lifespan is shortened to approximately 10–14 and 40 days, respectively [34]. As expected from the differences in hemolytic intensity between the two entities, CC whole blood has a mildly elevated reticulocyte count (average 7%) which is less than that of SS whole blood (average 16%) [22, 46]. In spite of the smaller reticulocyte count in CC blood, swelling-stimulated cotransport is very active and nearly equal to that of SS cells [3, 8, 16]. CC cells have a uniformly higher MCHC, a smaller mean corpuscular volume (MCV), lower cell water content and higher density than SS or AA cells because of their low K^+ content [22, 36, 46]. In contrast, SS cells do not exhibit a uniform increase in MCHC and the majority of young red cells in SS blood have a normal MCHC and MCV although there is a variable population of dense red cells [7, 12, 13, 16, 23]. In SS cells, generation of dense and irreversibly sickled cells (ISCs) is strongly associated with enhanced Ca^{2+} influx, elevation of cytosolic Ca^{2+} levels and opening of the Ca-activated K^+ channels which are inhibited by charybdotoxin [*for reviews, see* 6, 12, 29]. Thus, even though cotransport is very high in CC cells and its activation can lead to volume reduction after cell swelling [8], this property is not sufficient to account for the homogenous elevation of MCHC of CC whole blood. The mechanism of the uniformly increased MCHC of CC cells has remained unresolved.

For a physiologically effective regulation of cell volume, cell swelling must rapidly signal the activation of a net efflux of K^+ and Cl^- to reduce MCHC; and, likewise, cell shrinking must signal a rapid deactivation after the initial cell volume is re-established. The hypothesis that we propose to account for the higher density and uniformly reduced volume of CC cells is that changes in cell volume abnormally regulate the K:Cl cotransporter. One mechanism that can contribute to K^+ loss and dehydration is an extended delay in turning off cotransport once the volume has returned to normal values. As a first step to define the role of cotransport regulation in determining the final red cell volume, we have investigated how K:Cl cotransport responds to step changes in cell volume of SS, CC and AA cells. To this end, we have followed the time course of K^+ efflux from these three cell types to determine the delay times for activation by cell swelling and deactivation by cell shrinking. We found that in young AA, SS and CC cells cotransport has a short delay for activation which appeared to increase with cell age. Cotransport deactivation by cell shrinkage was independent of cell age and had a very short delay time in SS and in AA cells. In contrast, in CC cells cotransport responds very slowly to a reduction in cell volume, an abnormality which can account for an enhanced cell K^+ loss and may contribute to their dehydration.

Materials and Methods

Patient Material

Blood was drawn from six SS and four CC patients followed in the Heredity Clinic of the Bronx Comprehensive Sickle Cell Center after informed consent. We also studied six normal AA subjects with normal reticulocyte counts and three with high reticulocyte counts due to nutritional anemia. The hemoglobin of all patients was characterized by a combination of cellulose acetate (pH 8.6) and citrate agar (pH 6.4) electrophoresis and a solubility test for hemoglobin S [17].

Measurements of Reticulocytes

Reticulocytes were determined by suspending aliquots of cells in centrifuged plasma at hematocrit 50%. Equal volumes of blood and new methylene blue reticulocyte stain were mixed and the samples were allowed to incubate at room temperature for at least 10 min after which smears were made for counting.

Measurements of K:Cl Cotransport Activity

RBC Preparations

RBCs used in these experiments were washed four times with cold preservation solution containing (mM): 135 KCl, 15 NaCl, 10 Tris-MOPS, pH 7.4 at 4°C and shipped overnight on ice to Boston [16]. Transport experiments were performed the next day in Boston, within 24 hr after the blood was drawn. RBCs were washed with cold washing solution (CWS) containing (mM): glucamine-NO_3 149, $MgNO_3$ 1, Tris MOPS 10, pH 7.4, 4°C. A 50% cell suspension was made in CWS to measure hematocrit, hemoglobin, K^+ (1/500 dilution), and Na^+ (1/50 dilution) as previously described [13, 23]. Control experiments to study the effect of 24 hr preservation were done at the Albert Einstein College of Medicine, Bronx, New York.

K:Cl Cotransport Activation by Cell Swelling

We have chosen to use measurements of net K^+ efflux into Na^+ media instead of Rb^+ influx to study the time course of cotransport activation and deactivation. Previous studies in rabbit and LK sheep RBCs [18, 27] have measured Rb^+ influx to avoid interference by cell lysis. As previously reported by us and others [7, 8, 13], SS and CC cells have a very high activity of K:Cl cotransport that is 3–10 times higher than that of rabbit and LK sheep cells which minimizes errors of net K^+ efflux measurements. Net K^+ efflux was determined as previously described [13, 16] incubating cells in K-free (reagent contamination was determined to be 5 μM) Na^+ media of varying osmolarity, pH and Cl^-

concentration. All flux media contained (mM): 0.1 ouabain, 1.0 $MgCl_2$, 0.1 bumetanide, 10 glucose, 2% (v/v) hematocrit, and Tris-MOPS at the pH indicated at 37°C. When red cells are incubated in isotonic media (140 mM NaCl, 300 mOsm, pH 7.4 or 140 $NaNO_3$, pH 7.4), the resting K^+ efflux is low, largely Cl independent and has similar values in SS and CC cells. To activate cotransport, cell swelling was induced by incubation in hypotonic media (100 mM NaCl, pH 7.4). After a step change in the media osmolarity, the change to a new steady-state volume is complete in less than a minute as shown for human RBCs [35] and K^+ efflux into Cl^- media is markedly enhanced with similar values in SS and CC cells. In hypotonic nitrate media (100 mM $NaNO_3$, pH 7.4), cells are swollen but cotransport is not activated. The volume increase in hypotonic nitrate media at 220 mOsm was found to be 18% for SS cells and 15% for CC cells by triplicate measurements of hematocrit and hemoglobin content. Cotransport activity can be determined from the Cl^--dependent component of K^+ efflux which was estimated from the differences in flux in NaCl and $NaNO_3$ media. K:Cl cotransport was also induced by incubation in isotonic media at pH 7.0 (140 mM NaCl, pH 7 and 140 mM $NaNO_3$).

To study the time course of K^+ efflux, the transport reaction was initiated by adding an aliquot of the RBCs to media of different composition prewarmed to 37°C. Duplicate samples were removed at multiple time points between 2 and 60 min, placed in chilled Eppendorf tubes containing 0.4 ml of butyl phthalate oil and centrifuged for 6 sec in an Eppendorf microcentrifuge. The flux medium was removed with plastic Pasteur pipettes at the end of the experiment. The K^+ concentration of the media was determined by atomic absorption spectrophotometry using K^+ standards in Na^+ media as described [13, 16]. In all experiments, the total K^+ content of the flux media at different time periods was corrected for the initial cell volume and K^+ efflux expressed in millimoles of K^+ per liter of the original cells. Because activation of cotransport results in a net efflux of KCl, K^+ efflux was measured for 20 min for SS cells and 30 min for CC cells to minimize changes in the cell volume induced by continuous cellular K^+ loss.

K:Cl Cotransport Deactivation by Cell Shrinkage

To deactivate cotransport by cell shrinking, RBCs were first swollen by incubation for 19 min in hypotonic media or isotonic acid media at 37°C and subsequently cotransport was deactivated by addition to the media of an appropriate volume of 1.5 M NaCl to make the media isotonic or of an appropriate volume of 1 M Tris to restore the pH to 7.4. Duplicate samples were drawn at multiple time points between 20–60 min and processed as described. This time was chosen because by 50–60 min, unidirectional K^+ efflux into hypotonic media also decreases. However, we also performed some experiments following the deactivation up to 90 min to confirm the validity of our measurements.

Kinetic Analysis of the Time Course of K^+ Efflux

The time course of total K^+ accumulation in NaCl medium was analyzed using the equation derived by Jennings and Al-Rohil [27] for a two-state model:

$$R \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} A \tag{1}$$

using R for the relaxed or inactive phosphorylated form of the transporter, A for the active or dephosphorylated form, and the rate constants k_{12} and k_{21} for activation and deactivation, respectively. The equation describing K^+ efflux for this model was generalized from Eq. (7) in reference [27]:

$$K(t) = J_1 t + \tau (J_1 - j_o) [\exp(-t/\tau) - 1] \tag{2}$$

where $K(t)$ is the K^+ content of the media at time t expressed in mmol/liter of the original volume of cells; J_1 is the final steady-state K^+ efflux; J_o is the initial steady-state efflux prior to volume change; and τ is the delay time for relaxation from the first steady-state to the second. In the Jennings two-state model, the relaxation to any new steady-state, after sudden swelling or shrinking of the cells, proceeds at a rate given by the inverse of the sum of the forward and reverse rate constants, τ.

$$\tau = [k_{12} + k_{21}]^{-1} \tag{3}$$

The relation between the variable (t) and the constants (τ, J_1, and J_o) in Jennings Eq. (7) [27] is the same as in Eq. (2) in the three-state model described by Dunham, Klimczak and Logue [19] for LK sheep red cells:

$$A \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} B \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} C \tag{4}$$

where swelling-induced activation of $A \rightarrow B$ is the rate limiting step and the steps $B \rightarrow C$ and $B \rightarrow A$ are fast. The three states represent three different levels of activity ranging from low (A) to the highest (C) and the transitions are determined by the cell volume or the intracellular Mg^{2+} concentration. In the three-state model [19] described by Dunham et al., the form of Eq. (2) remains the same but the relation of the constant τ to the rate constants in Eqs. (1) and (4) (this manuscript) is different. The justification for applying the Jennings equation to a three-state model was that the second conversion in the linear three-state models was claimed to be very fast compared to the slow, rate limiting conversion. For the two-state or three-state models, the value of τ is equivalent to the lag that can be estimated visually from the time at which the perturbation occurs to the point of intersection of lines representing the initial and steady-state fluxes and thus is equal to what one would intuitively call the delay time.

Nonlinear regression analysis of the data provides a quantitative estimation of the delay time, final rate, and error analysis of these parameters. A 486 computer and Statgraphics Plus software program (Statistical Graphics, Rockville, MD) were used to fit the experimental data to Eq. (2). This program uses a modified Marquart algorithm to perform an iterative nonlinear least squares procedure to fit the experimental data to J_1 and τ in Eq. (2). Statgraphics Plus also gave r^2, which is a measure of the percent of the variance the model accounts for or how well the model fits the data and standard errors (SE) for both J_1 and τ, which are estimated from the residual sum of squares and the partial derivatives of J_1 and τ. The ratio of the estimated value to the standard error (SE) was greater than 8 for all J_1 and greater than 3 for all τ and the analysis of variance for the full regression as given by r^2 was usually >0.96.

For swelling-stimulated experiments, the total initial potassium efflux into isotonic media (J_o) was measured in a separate experiment. The total activated rate of K^+ efflux (J_1) induced by swelling the cells and τ were calculated from measurements of the time course of K^+ appearance into hypotonic media. To determine the steady-state value of the deactivated rate of K^+ efflux induced by cell shrinking and the τ associated with it, the steady-state activated flux was used as the J_o. J_1 was relatively insensitive to moderately large variations (±20%) in J_o, but τ was more sensitive. All values of J_o and J_1 refer to total potassium fluxes. The chloride-sensitive component (K:Cl cotransport) was determined by measuring the potassium efflux in the corresponding medium in which nitrate was substituted for chloride and subtracting the two values.

The very long delay time for deactivation of cotransport in CC

cells resulted in larger standard errors for J_1 and τ and lower r^2 values than for SS cells. This occurs because K^+ efflux in CC cells has not reached its steady-state value even after 40 min incubation in isotonic media. We selected this period of observation because in some experiments at time periods longer than 50–60 min, cell K^+ and K^+ efflux decrease. The validity of the estimated parameters for CC cells was tested by following the deactivation process for a longer period of time (90 min) after induction of cell shrinking. In this experiment when time points up to 60 min were used, the activated flux was 21 ± 0.6 mmol/liter cell × hr and the delay time for deactivation was 11.5 ± 7 min (r^2 = 0.971 ± 0.006); when time points up to 90 min in isotonic media were used, the delay time was 9.3 ± 3 (r^2 = 0.991 ± 0.002). The calculated steady-state J_1 deactivated flux at 90 min was 9.3 ± 3.1 mmol/liter cell × hr and 10.6 ± 4 at 60 min; these fluxes are close to the measured isotonic K^+ efflux (8.8 ± 0.4 mmol/liter cell × hr). This implies that the 20–60 min interval is a sufficiently long observation period to determine the rate and delay time for deactivation.

The delay times for activation and deactivation can also be estimated from the point of intersection of the linear regression lines of the initial and final steady-state [15]. This procedure yields values similar to the nonlinear regression analysis in SS cells where the on and off delay times are between 2 and 5 min; but in CC cells, the delay time for deactivation is long and linear regression fitting underestimates the delay time and the final steady-state J_1 efflux. Hence nonlinear least squares analysis was used to fit all data.

The kinetic parameters for K:Cl cotransport in SS, CC and AA red cells were compared using an unpaired *t*-test for comparison between cell types and a paired *t*-test for comparison within a cell type, i.e., delay for deactivation *vs.* delay for activation (Statgraphics Plus). The null hypothesis was rejected at $P < 0.05$.

Density Gradient Fractionation of CC Red Cells

All density separations of CC blood were performed on the same day as the blood was drawn and the fractions were shipped to Boston for transport measurements. Density-defined cell fractions were isolated from CC cells by density gradient separation as we previously described for SS cells [23]. Cells from CC blood were separated into three density fractions at 4°C: Top, density < 1.095 and MCHC < 38 which has the highest percent reticulocytes; middle, 1.095 < density < 1.105 with 38 < MCHC < 40; bottom, density > 1.105 and MCHC > 40.

Results

Density Characteristics of SS, CC and AA Cells

The mean cellular hemoglobin concentration (MCHC) is proportional to red cell density and cell water content. SS blood is characterized by two distinct populations of red cells with different densities (Fig. 1) whose proportions vary from patient to patient and with the health of individual patients [21]: one population is similar in density to that of normal AA individuals and the other population consists of very dense red cells some of which are irreversibly sickled cells. In contrast, CC blood is characterized by a uniformly elevated density and MCHC. Most CC red cells are denser than the densest AA red cells. Note that AA cells, which have the smallest percent reticulocytes, are also the least dense.

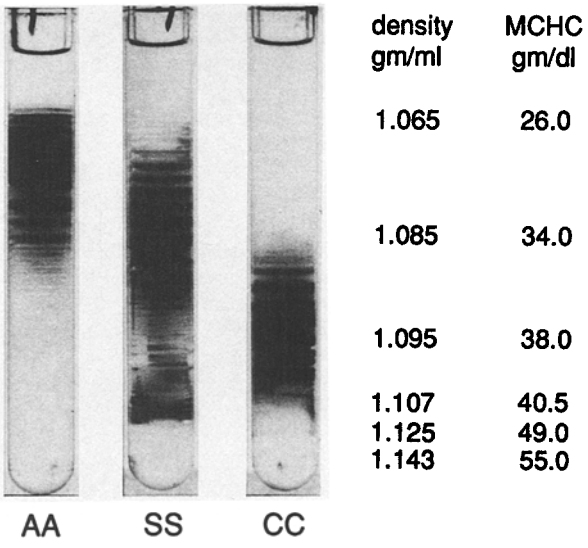

Fig. 1. Red cell distribution as a function of density for AA, SS, and CC whole blood. Red cell distribution as a function of density on Percoll-Larex continuous density gradients run at 37°C, pH 7.4, 280 mOsm. Note that many SS red cells have densities comparable to those of AA red cells and that there is little overlap of CC and AA red cells.

The reticulocyte count of the CC patients was found to be elevated (6.5 ± 1.1%, range 5–8%) but to a lesser extent than in SS patients (10.5 ± 1.9%, range 9–14%) (Table 1).

Activation and Deactivation Kinetics of K:Cl Cotransport in SS Red Cells in Response to Step Changes in Osmotic Pressure

In the following results, all fluxes are given as total potassium efflux (either J_o for the preperturbation flux or J_1 for the postperturbation flux). The chloride-dependent flux (due to K:Cl cotransport) is calculated by subtracting the flux in the corresponding NO_3^- media. The τ values refer to the delay for activation or deactivation calculated from the time of perturbation.

Figure 2 depicts a representative experiment in one SS patient of the time course of K^+ accumulation in media at different osmolarities. In isotonic media (Fig. 2*A*), K:Cl cotransport is in the inactive state and linear regression analysis of the data yielded low values of total K^+ efflux which is mainly Cl independent (J_o = 4.0 mmol/liter cell × hr) as can be seen by comparing the flux into isotonic NO_3^- media. Figure 2*B* shows the time course of K^+ accumulation in the media after exposure at time zero to the 220 mOsm media (isotonic → hypotonic transition) to maximally activate cotransport [11]. As shown by electron spin resonance measurements [35], volume increases in less than 1 min. Measurements done in our laboratory have found an increase in cell volume

Table 1. Properties of CC, SS, and AA red blood cells

	AA	SS	CC
Reticulocytes (%)	1.5 ± 1	10.5 ± 1.9	6.5 ± 1.1
Red cell life span (days)[a]	120	10–14	40
MCHC (g/dl)	33 ±2	35 ±3	38 ±2

Number of subjects: AA = 8; SS = 8; CC = 4. ([a]) [Ref. 34].

averaging 18% under these conditions. Cotransport activation consisted of two phases: a time lag and a phase of increased K^+ efflux. Nonlinear regression analysis of the experimental points fit the theoretical predictions of Eq. (2) with a r^2 of 0.993. In this experiment, there was a delay time of 3.0 ± 1.1 min before total K^+ efflux was activated to a new steady-state flux J_1 of 19.6 ± 0.1 mmol/liter cell × hr, which is fivefold higher than the J_o flux into isotonic NaCl media. SS cells incubated in hypotonic $NaNO_3$ media (Fig. 2*B*) also swell but K^+ efflux is only 4.5 mmol/liter cell × hr, indicating that K:Cl cotransport is not activated in the absence of Cl^-. This experiment also demonstrates that errors in K^+ concentration of the media due to lysis in hypotonic media are small. In this SS patient, the Cl-dependent component of the swelling-activated K^+ efflux (K:Cl cotransport) was 15 mmol/liter cell × hr.

The deactivation of K:Cl cotransport was studied by incubating the cells for 19 min in hypotonic media to induce cell swelling and then restoring the osmolarity of the hypotonic media to isotonicity by a pulse of NaCl. The preincubation period in hypotonic media gives sufficient time to activate the K^+ efflux to 98% of the steady-state value. As shown in Fig. 2*C*, deactivation consisted of two phases: a delay followed by a phase of reduced K^+ efflux. In this SS patient, the delay for deactivation, τ, was 4.5 ± 1.7 min. After this short delay, K^+ efflux was deactivated to a new steady-state flux of 5.2 ± 1.2 mmol/liter cell × hr.

Table 2 summarizes results from eight experiments performed on whole blood from six SS patients where the delay time for activation was 1.7 ± 1.0 min and varied between 0 and 3.7 min. The total steady-state, swelling-activated K^+ efflux (300 → 220 mOsm transition) varied in these patients between 15.2 and 26.9 mmol/liter cell × hr with an average value of 19.9 ± 1.2 mmol/liter cell × hr. The steady-state deactivated K^+ efflux was 4.8 mmol/liter cell × hr which represents a 71% inhibition of the activated flux and is of similar magnitude to the flux measured in isotonic media. In SS whole blood, which has a large percentage of young cells, the delay time for deactivation of K:Cl cotransport was 3.6 ± 0.45 min, longer than the delay time for activation ($P < 0.005$). Corresponding measurements in nitrate media allow these results to be interpreted in terms of activation and deactivation of K:Cl cotransport.

Activation and Deactivation Kinetics of K:Cl Cotransport in CC Red Cells in Response to Step Changes in Osmotic Pressure

Figure 3 shows a representative experiment of the time course of K^+ efflux from CC cells into isotonic and hypotonic media. Figure 3*A* shows that CC cells incubated in isotonic NaCl media have a slow rate of K^+ efflux. In a separate flask, CC cells were incubated in 220 mOsm media to induce cell swelling and maximally activate cotransport [8]. As previously shown by Brugnara et al. [8], incubation of CC cells in this hypotonic media increases cell water content from 60 to 65% wt/wt. Measurements done in our laboratory have found an increase in cell volume averaging 15% under these conditions. The experimental data were analyzed by nonlinear regression analysis using Eq. (2). This analysis indicated that there was a delay time of 10.6 ± 0.6 min before total K^+ efflux was activated to a new steady-state flux of 21.8 ± 0.6 mmol/liter cell × hr, a value 4.4-fold higher than that in isotonic NaCl media (4.9 mmol/liter cell × hr). The fit to Eq. (2) had a r^2 of 0.985 which implies that the model accounts for nearly all of the variance in extracellular K^+. In addition, the SE for the estimated τ and J_1 were very low. Because reduction of osmotic pressure increases cell volume in seconds [18], the long delay time of CC cells can be accounted for by differences in the regulation of cotransport. CC cells incubated in hypotonic $NaNO_3$ media (Fig. 4*B*) also swell and cell volume increases but K:Cl cotransport is inhibited by the lack of Cl^-.

Deactivation of swelling-stimulated K:Cl cotransport of CC cells was induced by re-establishing isotonic conditions (Fig. 3*C*). In this CC patient, deactivation also consisted of two phases: a time lag followed by a phase of reduced K^+ efflux. Notably, the delay time for deactivation, τ, was 21.8 ± 10 min, and much longer than that observed in SS cells. After this long delay, total potassium efflux was deactivated to a J_1 flux of 4.4 mmol/liter cell × hr. The experimental data fit Eq. (2) with an r^2 of 0.97, but the standard errors of τ and J_1 were larger than those for activation.

Table 3 summarizes results obtained in ten experiments performed on whole blood from four CC patients, (most CC patients were studied two or three times). The swelling-activated K^+ efflux, (J_1) for CC cells was very similar to that observed for SS cells. The mean value for the delay time for activation of K:Cl cotransport in CC cells was significantly shorter than that for deactivation ($P < 0.02$). CC cells have delay time for K:Cl cotransport activation (10.6 min) and deactivation (29 min) 6–8-fold higher than in SS cells ($P < 0.02$).

Whole blood from three CC patients was also examined immediately after their blood was drawn and again 24 hr later after storage in preservation solution. We found that neither the delay time for deactivation, nor

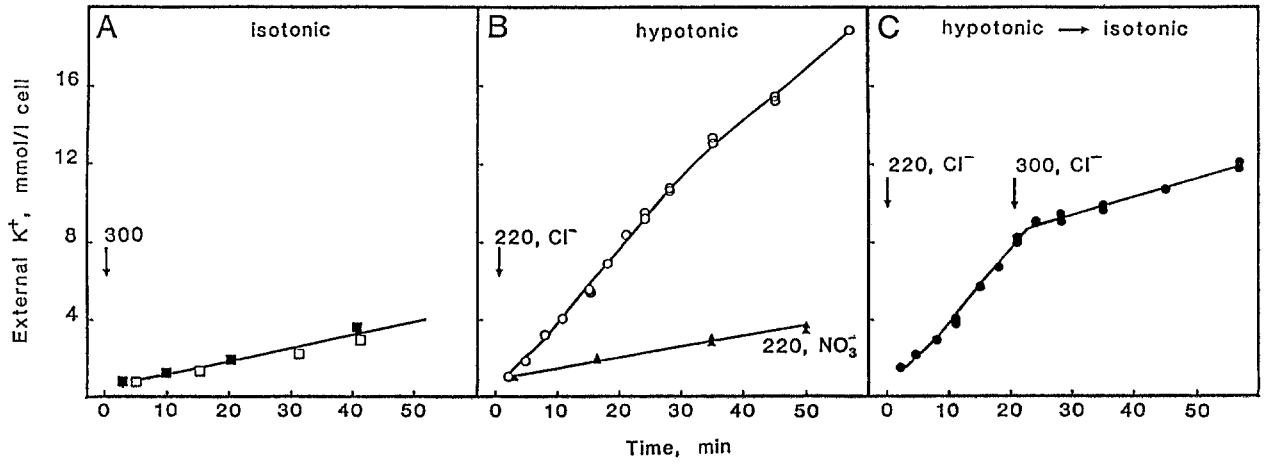

Fig. 2. Activation kinetics of K^+ efflux by cell swelling and deactivation by cell shrinking in whole blood of one SS patient. (*A*) SS cells were incubated in 300 mOsm Cl (-□-□-□-) or NO_3 (-■-■-■-) media and K^+ efflux was measured for 40 min. Linear regression analysis of the data yielded low values of K^+ efflux (J_o = 4.0 mmol/liter cell × hr). Comparison of values in Cl and NO_3 indicate that the isotonic potassium efflux is mainly Cl independent. (*B*) SS cells were transferred into hypotonic 220 mOsm Cl (-○-○-○-) or NO_3 (-▲-▲-▲) media at 0 min (arrow) and potassium efflux was observed for 55 min. The line is drawn connecting the experimental values. The nonlinear regression analysis of these experimental points fit very well the theoretical predictions of Eq. (2) with a r^2 of 0.993 and low SE. There was a delay time of 3 ± 1 min for activation of K^+ efflux to 19.6 ± 0.1 mmol/liter cell × hr. In flask (-▲-▲-▲-), K^+ efflux into hypotonic NO_3 was 4.3 ± 0.2 mmol/liter cell × hr. (*C*) SS cells were incubated in hypotonic 220 mOsm Cl (-●-●-●-), for 19 min starting at 0 min (left arrow) and then at 19 min (right arrow), 450 μl of 1.5 M NaCl was added to establish isotonicity and a similar volume of hypotonic media was added to the control flask. The nonlinear regression analysis of the experimental points fit very well the predictions of Eq. (2) with a r^2 of 0.96. After a lag (τ) of 4.4 ± 1.3 min from the time of addition of NaCl (at 19 min), K^+ efflux was deactivated to 5.2 ± 1.2 mmol/liter cell × hr which is a value similar to that obtained under isotonic conditions and represents a 100% inhibition by cell shrinking of the volume-stimulated K^+ efflux.

Table 2. Activation and deactivation kinetics of K:Cl cotransport by step changes in osmolarity in SS red blood cells

Osmotic changes Initial cell volume	Steady-state K^+ efflux mmol/liter cell × hr	τ Delay times minutes	Model fitting r^2
Isotonic, normal volume (300 → 300 mOsm)	4.1 ± 0.96		
Swelling activated (300 → 220 mOsm)	19.9 ± 1.166	1.7 ± 0.35	0.99 ± 0.003
Shrinking deactivated (220 → 300 mOsm)	4.8 ± 0.81	3.6 ± 0.45	0.96 ± 0.001

Mean ± SE. Eight experiments were performed in seven SS patients. The parameters were calculated using a nonlinear regression analysis for model fitting to Eq. (2).

the total activated K^+ efflux changed from day one to day two. In two patients, the delay time for activation increased from 2 to 10 min on day two and in the third patient it did not change. Thus, neither the delay time for deactivation nor the activated flux were sensitive to storage.

Density Gradient Distribution of Reticulocytes, Cotransport Activity and Regulation in CC RBC

RBCs of three CC patients with an average reticulocyte count of 7% were density fractionated by density gradient centrifugation into three equal fractions: top, middle, and bottom. Each fraction was studied for the time course of K^+ efflux during the swelling → shrinking protocol to estimate J_1 and the delay times for activation and deactivation as previously done in whole blood. Figure 4*A* shows that the least dense fraction (top) has the highest reticulocyte count (12%). The denser middle and bottom fractions contained 4 and 2.7% reticulocytes, respectively. Thus, CC blood at 4°C also contained some very dense reticulocytes. Figure 4*B* shows that volume-stimulated K^+ efflux had the highest values in the top and middle fractions; the denser bottom fraction had a very low activity of volume-stimulated K^+ efflux. The percent reticulocytes was strongly correlated with J_1, the total activated K^+ efflux, measured in all fractions (r^2 = 0.90, $P < 0.01$). Measurements of cotransport activity had very small errors in the top and middle fractions. In the bottom fraction, cotransport activity calculated by Eq. (2) had a large SE because the delay times were very long.

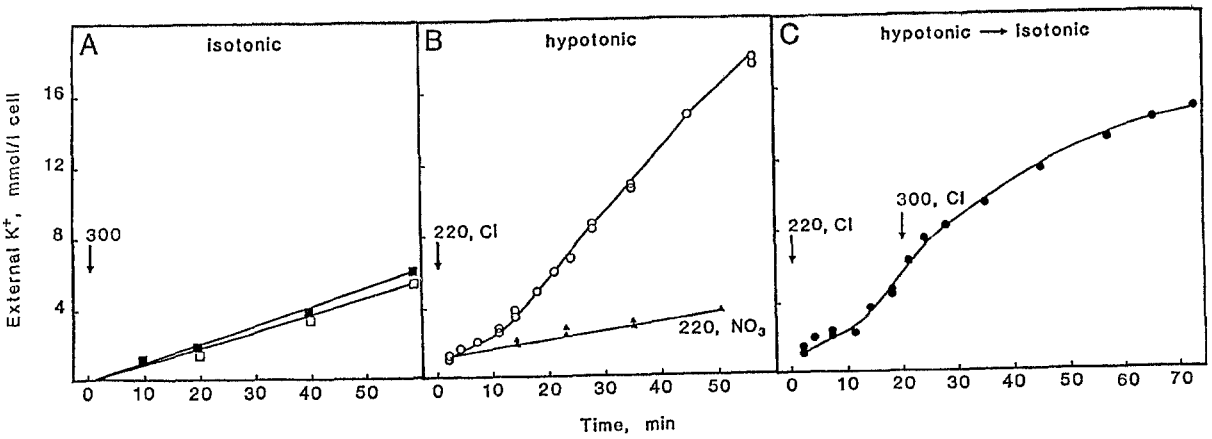

Fig. 3. Activation kinetics of K^+ efflux by cell swelling and deactivation by cell shrinking in whole blood of one CC patient. (*A*) CC cells were incubated in isotonic, 300 mOsm, Cl^- (-□-□-□-) and NO_3 media (-■-■-■-) and K^+ efflux was measured for 60 min. Linear regression analysis of the data yielded low values of K^+ efflux (J_o = 4.0 mmol/liter cell × hr). Comparison of values in Cl and NO_3 indicate that the isotonic potassium efflux is slightly Cl dependent. (*B*) CC cells were transferred into hypotonic, 220 mOsm, Cl (-○-○-○-) or NO_3 (-▲-▲-▲-) media at 0 min (arrow) and potassium efflux was observed for 55 min. The nonlinear regression analysis of the experimental points fit the theoretical predictions of Eq. (2) very well with a r^2 0.997 and low SE. There was a delay time of 10.8 ± 0.5 min for activation of K^+ efflux to 21.7 ± 0.57 mmol/liter cell × hr. In flask (-▲-▲-▲-), K^+ efflux into hypotonic NO_3 was 3.2 ± 0.3 mmol/liter cell × hr. Therefore the Cl-dependent potassium efflux for this patient is 18.5 mmol/liter cell × hr. (*C*) CC cells were incubated in hypotonic, 220 mOsm, Cl^- media (-●-●-●-) and K^+ efflux was measured for 19 min starting at 0 min (left arrow) and then isotonicity was re-established by addition of NaCl at 19 min (right arrow). The nonlinear regression analysis of the experimental points fit well the theoretical predictions of Eq. (2) with a r^2 of 0.97. After a lag (τ) of 21.8 ± 13 min from the time of addition of NaCl (at 19 min), K^+ efflux was deactivated to 4.4 ± 1.2 mmol/liter cell × hr, a value similar to that obtained under isotonic conditions and represents a 100% inhibition by cell shrinking of the volume-stimulated K^+ efflux.

Table 3. Activation and deactivation kinetics of K:Cl cotransport by step changes in osmolarity in CC red blood cells

Osmotic changes Initial cell volume	Steady-state K^+ efflux, J_1 mmol/liter cell × hr	τ Delay times minutes	Model fitting r^2
Isotonic, normal volume (300 → 300 mOsm)	6.1 ±0.91		
Swelling activated (300 → 220 mOsm)	23.42 ± 1.8	10.6 ± 2.0	0.989 ± 0.004
Shrinking deactivated (220 → 300 mOsm)	4.3 ±0.69	29.2 ± 2.8	0.95 ±0.01

Mean ± SE. Ten experiments were performed in four CC patients. The parameters were calculated using a nonlinear regression for model fitting to Eq. (2).

Figure 5 shows the mean values for the delay time for activation of cotransport in the three fractions separated from the three CC patients. In the top fraction, the mean value for the delay time in the three CC patients was 4 min, a value significantly lower than that observed in whole blood (10 min, $P < 0.01$). The delay for activation in the middle fraction increased to 15 min and increased again to 74 ± 12 min in the bottom fraction. Thus, the reticulocyte-enriched top fraction not only has the highest cotransport activity but also a significantly shortened delay time for activation by cell swelling. In addition, the delay time for activation markedly increased with cell density, and the densest CC cells had the longest delay time for activation. These findings indicate that cotransport in the densest cells cannot be turned on in less than 1 hr to regulate the cell volume.

In contrast to the delay time for activation, the delay time for deactivation did not increase significantly with cell density (Fig. 5). In the top fraction, the delay time for deactivation (14 ± 4 min) was longer than the delay time for activation (3.3 ± 1.3 min). In the middle fraction, both delay times were of about equal length and in the bottom fraction the delay time for activation was significantly longer (74 ± 12 min) than the delay time for deactivation (20 ± 8 min). In the whole blood of these three subjects, the mean ± SE for the delay time for deactivation was 23.8 ± 2.1 min (n = 7), a value not significantly different from those for the top, middle and bottom fractions.

These results indicate that defective regulation of cotransport by cell shrinking is already present in the CC reticulocyte. In addition, we found that the delay time

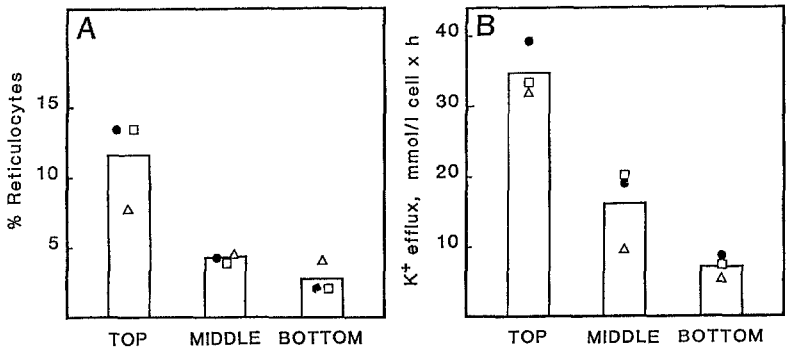

Fig. 4. Density gradient distribution of reticulocytes and swelling-stimulated K^+ efflux in CC red cells. Cells from blood of three CC patients (-△-, -□-, -○-) were separated into three density fractions at 4°C: Top, density < 1.095 and MCHC < 38; middle, 1.095 < density < 1.105 with 38 < MCHC < 40 and bottom, density > 1.105 and MCHC > 40. (*A*) Percent reticulocyte of the top, middle and bottom fractions. The least dense (top) fraction had the highest reticulocyte count. (*B*) Swelling-stimulated K^+ efflux induced by hypotonic-Cl media, (J_1) had the highest activity in the reticulocyte-enriched top fraction and decreased markedly with cell density.

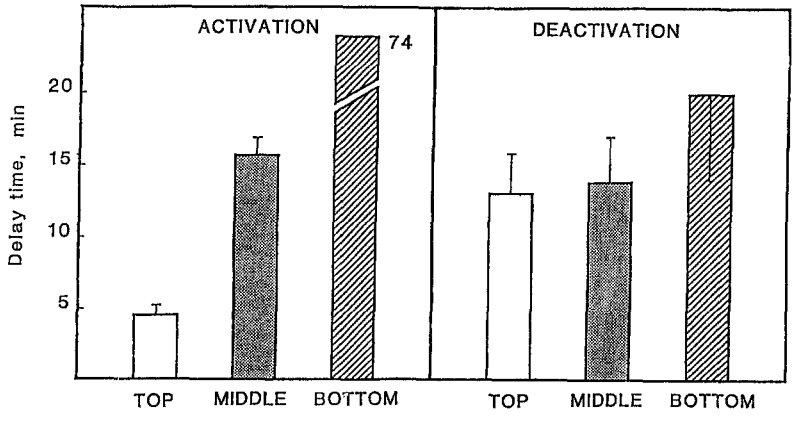

Fig. 5. Delay time for activation and deactivation of K:Cl cotransport in CC cells fractionated by cell density. Cells from CC blood from the same three patients shown in Fig. 4*A* and *B* were separated into three density fractions at 4°C: Top, density < 1.095 and MCHC < 38 and middle, 1.095 < density < 1.105 with 38 < MCHC < 40. The delay time ± SE for cotransport activation of the top fraction was significantly shorter than that for the middle and bottom (74 ± 12 min) fractions. The mean values ± SE for the delay time for cotransport deactivation by cell shrinking in the three fractions were not significantly different.

for cotransport activation was significantly shorter in the reticulocyte-enriched top fraction than in the mixed cell population present in whole blood.

Activation and Deactivation Kinetics of K:Cl Cotransport in AA Red Cells in Response to Step Changes in Osmotic Pressure

Normal AA cells exhibit a low activity of swelling-stimulated K:Cl cotransport which may reflect the small number of young cells present [9, 12, 13]. Figure 6 shows a representative experiment on RBCs from a subject with normal AA cells. When AA cells were incubated in hypotonic NaCl media (Fig. 6), there was a delay time of 16.3 ± 3.6 min before K^+ efflux was activated to a new steady-state flux $J_1 = 7.1 \pm 0.6$ mmol/liter cell × hr. The experimental data analyzed by nonlinear regression analysis fit very well the predictions of Eq. (2) ($r^2 = 0.986$). The deactivation of swelling-induced K:Cl cotransport in AA cells after re-establishing isotonic conditions to induce cell shrinking (Fig. 6) also consisted of two phases: a time lag followed by a phase of reduced K^+ efflux. The time lag for deactivation, τ, was 3 ± 1 min. After this delay, total potassium efflux was deactivated to a J_1 value of 2.6 ± 0.8 mmol/liter cell × hr. The experimental data fit Eq. (2) model with an $r^2 = 0.958$.

Table 4 summarizes results obtained in several experiments performed on whole blood from eight normal AA subjects. The swelling-activated K^+ efflux, J_1, was about threefold smaller for AA cells than for CC or SS cells. We selected subjects with volume-stimulated K^+ efflux larger than 2.0 mmol/liter cell × hr to reduce the error in the estimate of the delay time. The mean value of the delay time for activation in AA cells was 10.2 ± 1.4 min, a value sixfold longer than in SS but similar to that of CC cells. There was large interindividual variability in the delay time for activation (range 6.0 to 16 min). However, the mean value for delay time for deactivation was 2.8 min and significantly shorter than in CC cells but similar to that of SS cells.

To investigate the effect that a younger red cell population might have on the K:Cl cotransport, similar kinetic experiments were performed in whole blood of three AA subjects with a 11.5% reticulocyte count (Table 4, section II). The mean value for the delay time for activation in these AA subjects was 4.0 ± 1.5 min, a value significantly ($P < 0.05$) shorter than whole blood of normal AA subjects. In contrast, the delay time deactivation was very short (2.7 min) and not significantly different than that in normal AA cells. These results support the contention that cotransport in young human cells is characterized by a short delay time for activation, while the deactivation appears to be cell-age independent.

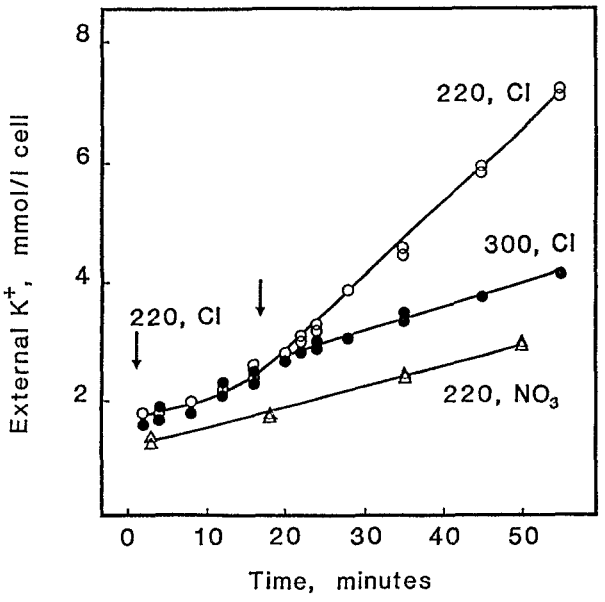

Fig. 6. Activation kinetics of K^+ efflux by cell swelling and deactivation by cell shrinking in whole blood of one Hb AA subject. In flask (-○-○-○-), RBCs were transferred into hypotonic, 220 mOsm, Cl^- media at 0 min (left arrow) and K^+ efflux was measured for 55 min. The nonlinear regression analysis of the experimental points fit very well the theoretical predictions of Eq. (2) with a r^2 of 0.957. There was a delay time of 16.3 ± 0.3 min for activation of K^+ efflux to 7.1 ± 0.6 mmol/liter cell × hr. In flask (△-△-△-△-), K^+ efflux into hypotonic NO_3 was 2.0 ± 0.2 mmol/liter cell × hr and the Cl-dependent, volume-stimulated K^+ efflux (K:Cl cotransport) was 6.7 mmol/liter cell × hr. In flask (-●-●-●-), AA cells were transferred into hypotonic-Cl media at 0 min (left arrow) and then at 19 min (right arrow), 450 μl of 1.5 M NaCl were added to establish isotonicity and a similar volume of hypotonic media was added to the control flask. After a lag (τ) of 3 ± 1 min from the time of addition of the NaCl at 19 min, K^+ efflux into isotonic media decreased to 2.6 ± 0.8 mmol/liter cell × hr, a flux similar to the Cl-independent K^+ efflux.

Activation and Deactivation Kinetics of Cotransport in Response to Step Changes in pH in CC and SS Red Cells

Early experiments reported by Brugnara et al. [8] showed that maximal stimulation of K:Cl cotransport in CC cells can also be achieved by exposure to pH 7.0. Because this maneuver may closely reproduce the conditions likely to trigger cotransport in vivo, K:Cl cotransport was activated by incubation of SS and CC cells in isotonic Cl and NO_3 media with a pH 7.0 (Fig. 7). Under isotonic conditions, an external pH of 7.0 reduces the internal pH and Cl^- enters to counter the increased positive charge of hemoglobin; the increase in intracellular Cl^- increases water content and cell volume. In CC cells exposed to pH 7.0 (Fig. 7), the K^+ efflux was 19.9 ± 0.7 mmol/liter cell × hr with a delay time of 11.6 ± 1.9 min. K^+ efflux into NO_3 media, pH 7.0 was 1.5 ± 0.2 mmol/liter cell × hr. After incubation in pH 7.0-Cl media for 19 min, the external pH was brought to pH 7.4 by addition of Tris-OH. The delay time predicted by the nonlinear regression fitting was longer than 30 min and with a r^2 value of 0.8 K^+ efflux between 19–55 min was 11 ± 0.1 mmol/liter cell × hr which represents a 27% inhibition of the activated flux by cell shrinking.

In SS cells incubated in Cl media at pH 7.0 (Fig. 7) there was a delay of 3 min before cotransport activation to 18.5 ± 0.7 mmol/liter cell × hr. Acid stimulation increased Cl-dependent K^+ efflux from 1.0 to 18.5 mmol/liter cell × hr. After 19 min, the external pH was brought to pH 7.4 by addition of Tris OH to deactivate cotransport. There was a delay time of 6 min for deactivation of K^+ efflux to a 6.5 ± 0.1 mmol/liter cell × hr.

Similar results were obtained in three CC and SS patients (Table 5). The acid-stimulated K^+ efflux was about equal in SS (16.4 ± 1.2 mmol/liter cell × hr) when compared to CC cells (14 ± 2.6 mmol/liter cell × hr). Thus, as previously observed for activation of K:Cl cotransport by hypotonic media, CC cells had a significantly longer delay time for activation than SS cells (23 *vs.* 5 min) and deactivation (15 *vs.* 7 min). No effort was made to exclude CO_2 or HCO_3^- in these experiments and therefore low levels were certainly present.

Discussion

SS, CC and AA Cells Have Different Delay Times for Activation and Deactivation of K:Cl Cotransport by Changes in Cell Volume

In this study, we measured two separate times for K:Cl cotransport: the delay time for activation following a step change in tonicity from isotonic to hypotonic and the delay time for deactivation following a step change in tonicity from hypotonic to isotonic. If either of these delay times becomes long, cell volume regulation becomes either ineffective or aberrant. Our results indicate that the equation derived from the two-state model is an accurate mathematical description of the time course of K^+ efflux and is therefore useful for describing the data; however, as with all kinetic results, the demonstration of an accurate fit does not imply that the model used to generate the equation is the only description of the physiological events. It should be remarked that the analysis of our experiments in terms of a *minimal* two-state model does not exclude the possibility that activation or deactivation occurs as part of a process with three or more states.

The main experimental result of this study is that the delay times for activation and deactivation of K:Cl cotransport are very different in the three cell types (Fig. 8). SS whole blood showed very short delay times for both activation and deactivation while in CC whole blood, the delay times for activation and deactivation

Table 4. Activation and deactivation kinetics of K:Cl cotransport by step changes in osmolarity in AA red blood cells

Osmotic changes Initial cell volume	Steady-state K^+ efflux, J_1 mmol/liter cell × hr	τ Delay times minutes	Model fitting r^2
I. Normal reticulocyte count			
Isotonic, normal volume (300 → 300 mOsm)	2.4 ± 0.33		
Swelling activated (300 → 220 mOsm)	5.9 ± 0.57	10.2 ± 1.4	0.980 ± 0.004
Shrinking deactivated (220 → 300 mOsm)	2.0 ± 0.24	2.8 ± 0.66	0.950 ± 0.034
II. High reticulocyte count			
Swelling activated (300 → 220 mOsm)	9.8 ± 3.40	4.0 ± 1.5	0.977 ± 0.006
Shrinking deactivated (220 → 300 mOsm)	2.9 ± 0.37	2.7 ± 0.37	0.974 ± 0.040

Mean ± SE. In normal AA blood (1.5% reticulocytes), cotransport activation was studied in eight subjects and deactivation in five subjects. In AA blood with high reticulocyte count (11.5% ± 4), three subjects were studied.

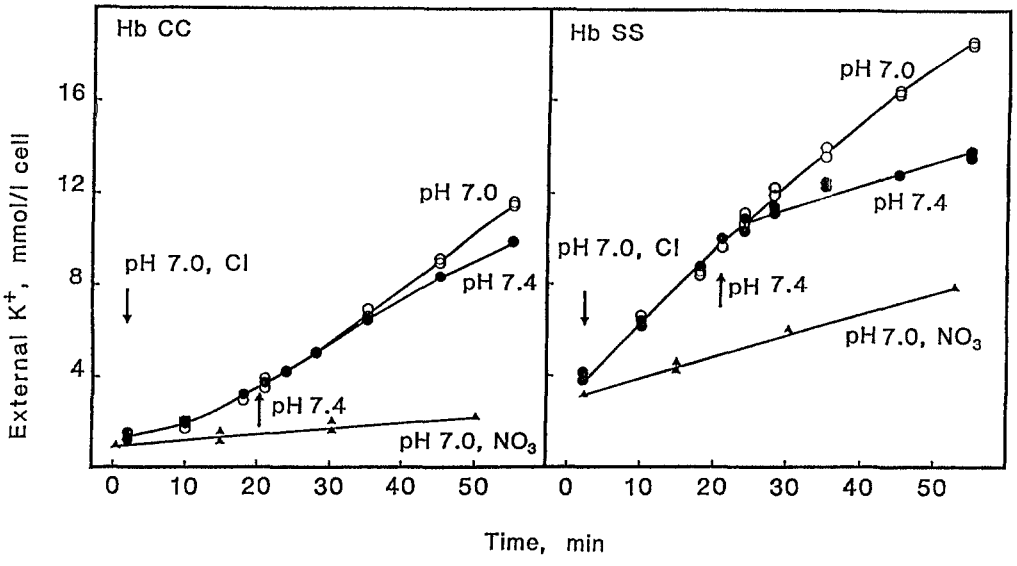

Fig. 7. Activation of K^+ efflux induced by pH 7.0 in RBCs of CC and SS patients. Potassium efflux was induced by incubation in isotonic Cl^- and NO_3 media with pH 7.0. Exposure to pH 7.0 increased the Cl-dependent K^+ efflux from 0.8 to 6.2 mmol/liter cell × hr in CC cells and from 1.0 to 13.1 mmol/liter cell × hr in SS cells. In CC cells incubated in isotonic Cl-media, pH 7.0 (-○-○-○-), the total activated K^+ efflux was 19.9 ± 0.7 mmol/liter cell × hr with a delay of 11.6 ± 1.9 min from the time of exposure to pH 7.0 at 0 min (left arrow). K^+ efflux into NO_3 media, pH 7.0 was 1.5 ± 0.2 mmol/liter cell × hr (-▲-▲-▲-). In flask (-●-●-●-), CC cells were exposed to pH 7.0 at 0 min (left arrow) and after incubation at pH 7.0 in Cl media for 19 min, external pH was brought to pH 7.4 by addition of Tris-OH (right arrow). The lag (τ) from the time of restoration to pH 7.4 predicted for the nonlinear regression fitting was longer than 30 min and with a r^2 value lower than 0.8. K^+ efflux between 19–55 min was 11 ± 0.1 mmol/liter cell × hr, which represents an inhibition by cell shrinking of 27% of the activated flux. SS cells were exposed to Cl media with pH 7.0 (-○-○-○-); at time 0 min (left arrow) there was a delay time of 3 min for activation of potassium efflux to 18.5 ± 0.7 mmol/liter cell × hr. Reduced pH increased Cl-dependent K^+ efflux from 1.0 to 18.5 mmol/liter cell × hr. After 19 min, the external pH was brought to pH 7.4 by addition of Tris OH (right arrow) to deactivate cotransport (-●-●-●-). There was a delay (τ) of 6 min after restoration of pH 7.4 for deactivation of K^+ efflux to a 6.5 ± 0.1 mmol/liter cell × hr. In flask (-▲-▲-▲-), K^+ efflux into isotonic, pH 7.0 NO_3 was 1.5 ± 0.2 mmol/liter cell × hr.

were significantly longer than in SS cells. In contrast, normal AA blood, which has fewer reticulocytes and has an older mean cell age, exhibited a longer delay for activation while AA blood with a high reticulocyte count had a short delay for activation. Both normal and high retic AA blood had the same short delay time for deactivation. This study shows that the very young SS cells not only have a very high K:Cl cotransport activity [8, 16] but also have shorter delay times for activation and deactivation than previously reported values for rabbit [27] and sheep [19] red cells. Previous studies on rabbit RBCs [27] reported a longer delay time for activation (12 min) than for deactivation (1 min) which, using the two-state model (Eq. 1), led to the conclusion that changes in cell volume mainly modulated the deactivation rate constant [27]. Similar results to those reported for rabbit red cells are found in this study for normal mature AA cells in which the delay time for deactivation was shorter than

Table 5. Activation and deactivation of K:Cl cotransport by step changes in pH in SS and CC red blood cells

pH Changes	Hemoglobin SS	Hemoglobin CC
Isotonic, normal volume		
K^+ efflux, pH 7.4		
mmol/liter cell × hr	5.1 ± 0.9	6.1 ± 0.9
Swelling activated at pH 7.0		
Delay time for activation		
(min)	5.2 ± 1.1*	23.4 ± 6.6*
K^+ efflux, mmol/liter cell × hr	16.4 ± 1.2	14.0 ± 2.6
Deactivation by cell shrinkage		
at pH 7.4		
Delay time for deactivation		
(min)	7.3 ± 1.1*	15.0 ± 2.5*
K^+ efflux, mmol/liter cell × hr	5.2 ± 1.1**	8.3 ± 1.2**

Mean ± SE. pH modulation was studied in four CC and three SS patients. $*P < 0.02$; $**P < 0.05$.

the delay time for activation; it seems that for these cells, therefore, changes in volume mainly modulate deactivation. In both CC and SS cells, the time required for deactivation was longer than the time required for activation and the ratio between the on and off delay times is smaller than that for rabbit red cells which implies that the control by cell volume is not strongly localized in either activation or deactivation rate constants. In mature AA and the densest CC cells, as in rabbit red cells, the opposite condition is true; that is, the delay for activation is threefold longer than that for deactivation, and therefore in this case, the deactivation step appears to be volume sensitive [27].

Thus, our studies on human red cells, as well as those reported for rabbit [27] and LK sheep RBC [19] suggest that maturation of young cells has a large impact on cotransport regulatory mechanisms and that species differences may affect the persistence of volume regulatory mechanisms in mature cells.

In agreement with previous reports [8], we find that even small changes in pH (from 7.4 to 7.0 are sufficient to stimulate K:Cl cotransport to levels comparable to exposure to 220 mOsmol. The large differences between SS and CC cells for the delay time for activation and deactivation of cotransport were also found when swelling and shrinking were induced by changes in pH. SS cells have delays fourfold shorter than CC cells. However, in SS and CC cells, both parameters were 2–3-fold longer when transport was activated by changes in pH than by changes in osmolarity. The rate of acidification of red cells is affected by the presence of CO_2/HCO_3^- [26]. In the absence of HCO_3^-, the time required for pH equilibration and probably stimulation of K:Cl cotransport is about 3–4 min; however, near neutrality, even relatively small amounts of HCO_3^- can accelerate pH equilibration by 10- to 15-fold. Therefore, 3 min is an upper limit to the time which needs to be added to the delay time to accommodate pH equilibration.

The Delay Time for Cotransport Activation is Cell-Age Dependent

Previous studies documented that a high cotransport activity was a property of young cells independent of the hemoglobin variant [16, 23, 25]. Measurements of swelling-stimulated K^+ efflux in density-defined fractions of SS and AA whole blood indicated that the lightest fractions containing the highest reticulocyte count also had the highest cotransport activity [9, 13]. The denser SS4 and AA3 fractions did not exhibit sizable cotransport activity, suggesting that this function rapidly declines after the cell ceases to be morphologically identifiable as a reticulocyte.

We find that the delay time for cotransport activation is short when the reticulocyte count of the whole blood is elevated. SS whole blood has the highest proportion of young cells and the shortest delay time for activation (2–3 min) while in AA cells, the delay time decreased from 10 to 4 min when the reticulocyte count increased from 1.5 to 10%. Furthermore, our data also showed that when CC and AA cells are enriched in reticulocytes by density fractionation, the delay times for activation are short. Results of density fractionation of CC cells also imply that volume regulation can vary even for the cells of a single individual. A simple explanation for this diversity of the regulation of cotransport in human cells is that the large differences in red cell lifespan, maturation, and the presence of reticulocytes and stress reticulocytes have a large impact on cotransport regulation.

Our data suggest that RBCs with long delay times for activation do not effectively regulate the cell volume by K:Cl cotransport. Thus, even though K:Cl cotransport activity can be demonstrated in normal mature AA [8, 13, 16, 20, 33], rabbit [1] and LK sheep [18, 33] RBCs, it is likely that the control of cotransport activation rapidly decreases after the reticulocyte stage as well as the capacity to effectively re-establish the initial cell volume. In fact, none of these cells have shown the volume regulatory decrease response to cell swelling that has been demonstrated for SS [13, 23] and CC [8] cells.

Our results indicate that the cotransport activity of CC cells is proportional to the reticulocyte count of the cell fractions. However, one cannot fully exclude that when erythropoiesis is strongly stimulated, a young cell population which is not morphologically identifiable as reticulocytes may also possess cotransport activity as was demonstrated for SS cells [23]. The swelling-activated K^+ efflux was not only most active in the reticulocyte-enriched fraction, but had a short delay for activation; in the very dense CC bottom fraction, cotrans-

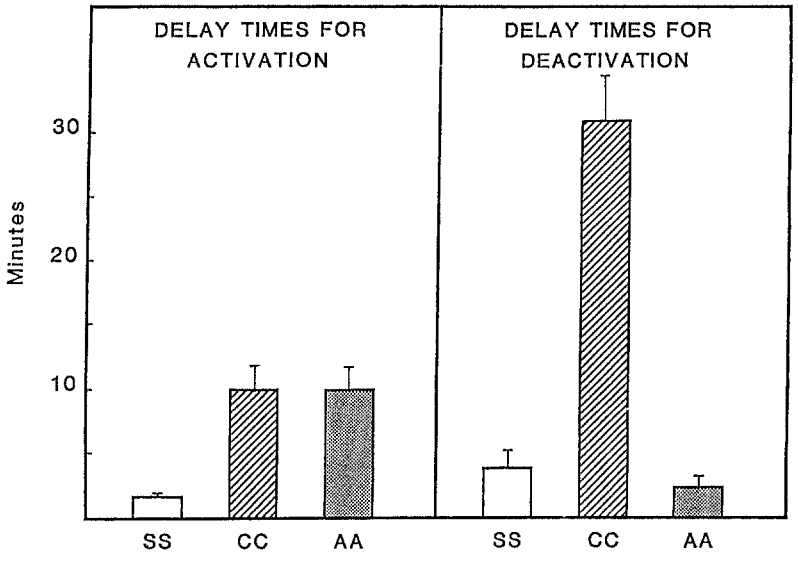

Fig. 8. Comparison of the mean values for the delay times for activation and deactivation of K:Cl cotransport in whole blood of hemoglobin SS, CC and AA subjects. Mean ± SE. For SS cells, $n = 8$. For CC cells, $n = 8$. For normal AA cells, $n = 6$ for activation and $n = 3$ for deactivation.

port activity was low and had a very long delay time for activation. Therefore, inactivation of either regulation or the transport protein occurs with increasing cell density. Although the correlation of age and red cell density is far from exact, studies of cell-age-dependent enzyme activities suggest that in broad strokes cell age and density are related [31, 40]. Thus, we may conclude that inactivation of K:Cl is either cell-age dependent or cell-density (intracellular hemoglobin concentration) dependent. Since the same lengthening of delay times for activation is observed when we compare AA individuals with high reticulocyte counts to normal AA individuals with lower reticulocyte counts, as was observed when we compared high reticulocyte CC density fractions (lower density) to low reticulocyte CC density fractions (higher density), the least complex hypothesis is that regulation of activation of K:Cl cotransport is cell-age dependent. These findings suggest that CC reticulocytes with a short delay for activation and a long delay for deactivation are very prone to becoming dehydrated as a result of a persistent net cellular K^+ in isosmotic media of normal pH.

Cell Shrinking Does Not Rapidly Deactivate K:Cl Cotransport in CC Cells

Another important finding of this study is that in contrast to SS and AA cells, in CC cells the delay time for cotransport deactivation was fourfold longer than that for activation. The finding that the deactivation delay time in CC cells is not cell-age dependent, as are the activation rates, is compatible with the idea that deactivation might be influenced by other factors such as differences in hemoglobin structure or concentration.

Does defective deactivation of cotransport influence the overall density of CC cells? In SS and CC cells, swelling induced a similar steady-state-activated K^+ efflux, but upon cell shrinking SS cells rapidly deactivated cotransport while CC cells did not. We have previously demonstrated that only some SS cells are able to reduce their cell volume when K:Cl cotransport is activated by cell swelling [23]. The finding that young CC cells also can rapidly activate cotransport by cell swelling, but have a very slow deactivation with cell shrinkage, indicates that this defect in the modulation of K^+ transport is already present in the reticulocyte. It is unlikely that the Ca-activated K^+ channel plays a role in the dehydration of CC cells because the volume-activated K^+ efflux is Ca independent [8] but Cl dependent [16] in CC cells; in addition, CC cells have a Ca^{2+} content and Ca^{2+} influx similar to AA cells [44]. The increased MCHC of CC cells and their marked reduction in intracellular K^+ content compared to AA and young SS cells (excluding ISCs) [8, 22, 36] pinpoint K:Cl cotransport as a major player in the abnormal cell volume. We can therefore hypothesize that the abnormal cotransport response of CC cells to cell shrinkage could lead in vivo to the reduction in cell volume because our data predict that after a transient activation of net K^+ efflux by a low pH followed by a slow deactivation, cellular K^+ loss will persist and gradually decrease hydration and increase MCHC.

Possible Mechanisms Involved in the Abnormal K:Cl Cotransport Deactivation Rates in CC Cells

Our findings raise the question of what mechanism is involved in the abnormal regulation of volume-sensitive cotransport in CC cells. One factor that could alter K:Cl cotransport regulation is the intracellular level of ionized Mg^{2+}. High intracellular Mg^{2+} is known to inhibit cotransport activity in human and sheep RBCs [10, 32].

Removal of Mg^{2+} stimulates cotransport in AA and SS cells and LK sheep cells [2, 10, 14] and decreases the time lag for activation but not for deactivation [19]; therefore, it is possible that high intracellular Mg^{2+} levels may account for the long delay for activation observed in old cells but not for the incomplete deactivation exhibited by CC cells.

An important role in the regulation of cotransport has been given to phosphatase Type I because its inhibition by okadaic acid reduces cotransport activity in rabbit and human RBCs [24, 28, 30, 38, 46]. This led to the conclusion that an increase in the level of cotransport phosphorylation may deactivate the transporter and, vice-versa, dephosphorylation is required for cotransport activation. The Jennings group has made arguments for the phosphatase associated with activation being insensitive to volume changes and a kinase associated with deactivation being inhibited by cell swelling [27, 28, 46]. However, there is not yet experimental evidence that changes in cell volume modulate the activity of a protein kinase or a protein phosphatase to modify the phosphorylation state of proteins involved in K:Cl cotransport. In addition, recent experiments reported in human red cells by Sachs and Martin [44] concluded that although phosphorylation and dephosphorylation modify the activity of the cotransporter in swollen and shrunken ghosts, neither of these processes is involved in signal transduction between the cell volume sensor and the cotransporter as originally proposed by Jennings and Al-Rohil [27]. The deactivation process has other complexities because okadaic acid and cell shrinking have been shown to stimulate Na^+/H^+ exchange in rat thymocytes, dog RBCs, and human RBCs [4, 39, 41].

Even though there is evidence that Hb C binds tightly to the membrane [42, 43], the nature of its interaction, if any, with transport proteins in the membrane is unknown. The high K:Cl cotransport activity of CC cells has been attributed to an interaction of the mutant hemoglobin with the red cell membrane [5, 8, 37] and our results indicate that both CC and SS whole blood do have approximately the same high K:Cl cotransport activity even though the average reticulocyte count of SS blood is higher than that of CC blood. One possibility is that Hb C may interfere with the regulation of K:Cl cotransport by phosphorylation, or of another transporter involved in the volume-regulatory increase response. Another possibility is that Hb C may affect the rate at which K:Cl cotransport is inactivated as cells age.

In summary, our studies indicate that young AA, SS and CC cells have a very active K:Cl cotransport with short delay for activation of net K^+ efflux by cell swelling. Young SS and AA cells also exhibited a very short delay for cotransport deactivation by cell shrinking once the initial cell volume was restored. In contrast, young and old CC cells showed longer delays for cotransport deactivation which may lead to a persistent cellular K^+ loss and may contribute to increased MCHC and low intracellular K^+ content.

This research was supported by National Institutes of Health grants Shannon Award HL-35664, HL-42120, Sickle Cell Center grant HL-38655, and a Grant-in-Aid of the New York Branch of the American Heart Association. The technical help of Sandra M. Suzuka, M.S. is gratefully acknowledged.

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