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The Effect of Hemoglobin A and S on the Volume- and pH-Dependence of K-Cl Cotransport in Human Erythrocyte Ghosts

D. Vitoux[1], **Y. Beuzard**[2], **C. Brugnara**[3]

[1]Service de Biochimie A et Neurobiologie, Hopital St Louis, 75010 Paris, France
[2]Laboratory of Experimental Gene Therapy, Hopital St Louis, 75010 Paris, France
[3]Department of Laboratory Medicine, The Children's Hospital, Harvard Medical School, Boston, MA 02115, USA

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Abstract. K-Cl cotransport is abnormally active in erythrocytes containing positively charged hemoglobins such as Hb S (SS: β6 Glu → Val) or Hb C (CC: β6 Glu → Lys). The relatively younger age of erythrocytes in these diseases cannot completely account for the increased K-Cl cotransport activity. It has been suggested that these positively charged Hb may interact with the K-Cl cotransport system or one of its regulators and induce changes in its functional activity. We report here data on the volume- and pH-dependence of K-Cl cotransport in ghosts obtained from normal and sickle erythrocytes, and on the effect of addition of either Hb A or Hb S before resealing. In erythrocyte ghosts prepared with the gel column method to contain minimal amounts of Hb, (white ghosts, WG), K-Cl cotransport has similar magnitude in normal and sickle erythrocytes, is not inhibited by alkaline pH and it is volume-independent. Addition of low concentrations of Hb A to WG from normal erythrocytes decreases the magnitude of K-Cl cotransport and restores its volume dependency, but not its pH sensitivity. Addition of Hb S to WG from either normal or sickle erythrocytes restores the volume-dependent component of K-Cl cotransport and increases the magnitude of flux mediated by this transporter. Thus, Hb A and Hb S seem to affect in different manners the functional properties of K-Cl cotransport.

Key words: Erythrocyte — Sickle cell — Hemoglobin — K-Cl cotransport

Introduction

Most animal erythrocytes are able to decrease their volume in response to hypo-osmotic swelling. This regulatory volume decrease (RVD) includes conductive K and Cl channels, K/H and Cl/HCO_3 exchangers, amino acid and taurine transport systems (*see* for review Rasgado-Flores, Penarasgado & Ehrenpreis, 1995; Häussinger, 1996; Sachs, 1996) and the K-Cl cotransport system which contributes to RVD in human and several other animal red cells.

Volume-sensitive K-Cl cotransport system is present in normal young human red cells containing Hb A (AA) and disappears on cell aging (Kaji & Kahn, 1985; Hall & Ellory, 1986; Brugnara & Tosteson, 1987*a;* Canessa et al., 1987). However, it remains abnormally active in red cells containing Hb S (SS; Brugnara et al., 1986) or Hb C (CC; Brugnara et al., 1985). Several studies have shown that activation of K-Cl cotransport system generates K and water loss and induces formation of high density sickle cells (Brugnara et al., 1986; Brugnara & Tosteson, 1987*b;* Franco et al., 1994, 1995). The dehydration produced by the abnormal K-Cl cotransport system has important pathophysiological consequences. Sickle cell dehydration increases Hb S polymerization and sickling, and leads to loss of membrane deformability (Clark, Mohandas & Shohet, 1980). DIOA (Dihydroindenyl Alkanoic Acid), an inhibitor of K-Cl cotransport, prevented acid pH-induced formation of dense cells (Vitoux et al., 1989). Dehydration mediated by K-Cl cotransport is inhibited by increased intracellular Mg, both in vitro and in vivo (Brugnara et al., 1987; De Franceschi et al., 1996, 1997, 1998).

An abnormally active K-Cl cotransport system is also present in red cells containing Hb C (Brugnara et al., 1985; Brugnara, 1989). We have shown that the loss of the negative charge or the presence of positive charge on β6-β7 positions of Hb could modulate the activation of the KCl cotransport system in red cells (Olivieri et al., 1992). There is evidence that the volume-dependence of K-Cl cotransport is altered in cells containing Hb C

Correspondence to: D. Vitoux

(Brugnara 1989; Canessa et al., 1994). However, direct evidence of a Hb role on this modulation has not been demonstrated. It has been suggested that the volume sensor could be the intracellular protein/Hb concentration (Minton, Colclasure & Parker, 1992). This hypothesis is supported by the fact that in dog erythrocytes activation of KCl cotransport in intact erythrocytes and ghosts occurs at the same level of dilution of intracellular constituents (Colclasure & Parker, 1991).

Hb could also play a direct role in affecting RVD via K-Cl cotransport. In fact, pink ghosts prepared from normal human red cells with 1/10 or 1/20 diluted cytoplasmic content demonstrate a volume-sensitive, chloride-dependent K movement (Dunham & Logue, 1986; O'Neill, 1989) which is absent in white ghosts (Brugnara, Van Ha & Tosteson, 1988). However, Sachs (1988) reported the presence of volume-sensitive K movement in white ghosts of normal erythrocytes.

To examine the possible role of Hb S in abnormal activation of the KCl cotransport system in sickle erythrocytes, we measured Cl dependent K efflux in white ghosts from normal erythrocytes (WGAA) and sickle cells (WGSS) resealed in presence of low concentration of Hb A (WGAA-HbA) or Hb S (WGSS-HbS) and in pink ghosts (PG). We show that both Hb S and Hb A can induce a volume-sensitive and chloride-dependent K movement in ghosts. However, only Hb A and not Hb S reduces the magnitude of K-Cl cotransport-mediated K efflux in isotonic conditions. Thus, these two Hb types, or their associated cytoplasmic components, have differential effects on the regulation of K-Cl cotransport in human erythrocytes.

Materials and Methods

Blood was drawn from normal subjects and from patients with homozygous sickle cell disease and low reticulocyte count (<4%), into heparinized Vacutainer tubes. The red cells were immediately washed three times by centrifugation and resuspended in isotonic washing choline chloride solution at 4°C containing (in mM): choline chloride 140, TRIS-MOPS pH 7.4 (at 4°C) 10, $MgCl_2$ 1.

White Ghosts Preparation

White ghosts preparation were performed by the gel filtration method at 0°C (Wood, 1989). Washed erythrocytes were suspended at 10% hematocrit (Hct) in an ice-cold pre-lysing solution containing: choline chloride 140 mM, dithiotreitol (DTT) 2 mM, Tris (hydroxymethyl) aminomethane-3-(N-morpholino) propanesulfonic acid (TRIS-MOPS) 10 pH 7.40 at 0°C with a final volume of 30 ml and applied to the top of a jacketed column containing agarose gel (exclusion limit of 50 million daltons; Biorad A50m dalton, 50–100 mesh). The column temperature was maintained between 0 and 2°C and equilibrated with a lysing solution containing (in mM): choline-chloride 15, TRIS-MOPS 10 pH 7.4 at 0°C, DTT 2, EDTA 0.1. Just prior to the addition of cells to the column, 20 ml of pre-lysing solution were layered into the column. The pre-lysing solution was used as eluent. The fractions of the eluate containing the membranes were pooled and spun down at 9000 × *g* for 10 min. The supernatant was removed and the white ghosts were placed in a resealing solution for 10 min at 0°C (equilibration) and at 37°C for 50 min (resealing) with gentle shaking. The resealing solution contained (in mM): NaCl 10, KCl 140, TRIS-MOPS 10 pH 7.40 at 37°C, $MgCl_2$ 0.15, DTT 2, EGTA 0.1, K phosphate buffer (pH 7.40) 1 and ATP 0–2. DTT has been shown to inhibit K-Cl cotransport in intact sheep erythrocytes (Bergh et al., 1990). Although we were unable to show any effect of DTT on the K efflux from normal white ghosts, we elected to include DTT in our preparation to rule out any possible oxidative effect induced by the incorporation of sickle Hb into normal cells and to assess the effect of Hb S in normal and SS ghosts independently from thiol oxidation.

The white ghosts were collected after spinning at 9000 × *g* for 5 min and the ghosts were washed three times with the choline-washing solution at 4°C and used for K efflux measurement.

Pink Ghosts Preparation and Hb Incorporation into White Ghosts

Pink Ghosts Preparation

Washed red cells were lysed directly by addition of a lysing-solution containing choline-chloride (in mM): 15, TRIS-MOPS 10 pH 7.4 at 0°C, DTT 2, EDTA 0.1 at 10% Hct. After 10 min equilibration at 0°C, a concentrated stock resealing solution was added to obtain a final salt concentration of NaCl 10 mM, KCl 140 mM. After 10 min at 0°C, the pink ghosts were resealed by incubation for 50 min at 37°C in the same resealing solution described above for the white ghosts method.

Reconstitution Experiments

Homologous Reconstitution

Hb A or Hb S were incorporated in white ghosts obtained from normal and sickle erythrocytes, respectively. The corresponding definition for these two cell types is WGAA-HbA and WGSS-HbS, respectively.

Heterologous Reconstitution

Hb S was incorporated into white ghosts obtained from normal erythrocytes. This cell type is defined as WGAA-HbS.

Ten volumes of Hb solution (2–4 g/l) were added to one volume of the white ghosts suspension prior to the addition of the concentrated stock resealing solution. After 10 min equilibration at 0°C, salts were added and the ghosts were resealed as previously described. Hb was obtained by lysing red cells in double distilled water and 0.2 volume of CCl_4. Membrane free Hb A and S were purified by ion exchange chromatography according to Abraham et al. (1976) and dialyzed extensively against 10 mM phosphate buffer for 24 hr using a dialysis membrane with a mw cutoff of 10.000 Daltons.

Density Gradient Centrifugation

Density gradient centrifugation in presence of sucrose according to Bodemann and Passow (1972) was used to determine the proper re-

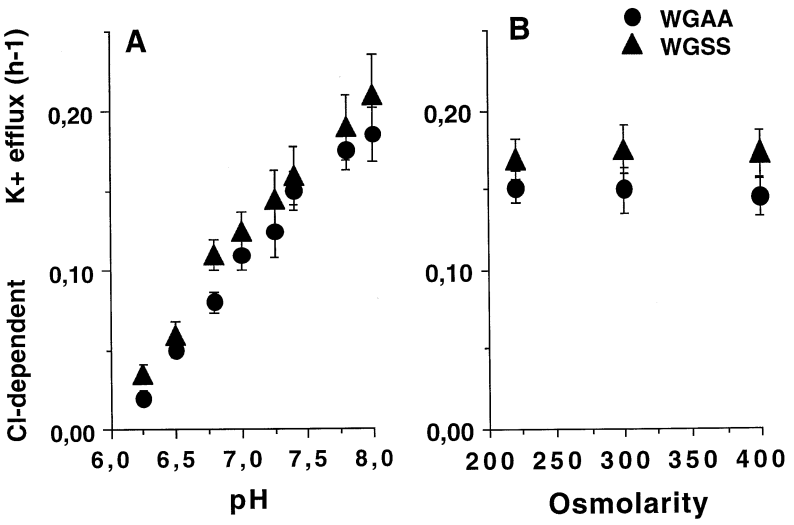

Fig. 1. Effect of pH (A) and volume (B) changes on the Cl- dependent K efflux in white ghosts prepared from normal (WGAA) or sickle cells (WGSS). White ghosts preparation was performed by gel filtration method at 0°C (*see* Materials and Methods). K efflux measurements performed in triplicate; two experiments with 2 different patients with sickle cell disease: reticulocytes count: 4 ± 1% and two normal subjects: reticulocyte count <1%.

sealing of ghosts. A Coulter counter was used to determine the homogeneity of the volume distribution of pink ghosts.

NEM Treatment

Smith and Lauf showed that NEM stimulates K efflux in ''pink'' ghosts obtained from normal human erythrocytes (Smith & Lauf, 1985). NEM was used in white ghosts as specified by Brugnara et al. (1988), with a pretreatment in $NaNO_3^-$ medium. In some experiments NEM was also added directly to the flux medium.

K Efflux Measurements

Resealed ghosts were suspended at 30–40% (vol/vol) in choline washing solution, and 250 μl of this suspension were added to 10 ml of previously chilled flux medium. Briefly the cells were incubated with an incubation medium containing either 10 mM Tris-MOPS in the pH range 6.75–8.00 or Tris (hydroxymethyl) aminomethane-3-(N-morpholino)ethanesulfonic acid (Tris-MES) at pH 6.25–6.50 at 37°C, (in mM): 140 NaCl, 10 glucose, 1 $MgCl_2$, 0.1 ouabain and 0.01 bumetanide. When osmolarity was varied the medium contained (in mM): 100 NaCl, 10 glucose, 0.1 ouabain, 0.01 bumetanide, 10 Tris-MOPS (pH 7.4) and the osmolarity was varied from 220 to 400 mosM by adding cholinechloride. After 5 and 30 min incubation at 37°C in shaking waterbath, aliquots were taken in triplicate. The flux suspension was spun for 1 min in an Eppendorf microcentrifuge, and the supernatant was removed. The incubation times were chosen based on our previous work (Brugnara et al., 1988) and work by others (Sachs, 1988; Dunham & Logue, 1986) showing linearity of fluxes over this time course. The K concentrations in the supernatant were measured in a Perkin Elmer Atomic Absorption spectrophotometer. The percentage of packed ghosts in the suspension was measured by spinning down an aliquot of ghosts suspension for 20 min in an hematocrit microcentrifuge. This was used to calculate both K content and K efflux (expressed as rate constant, hr^{-1}).

Results

Density gradient centrifugation in presence of sucrose according to Bodemann and Passow (1972) was used to determine the proper resealing of white ghosts. In our experimental conditions, type II ghosts (properly resealed) represented 80–85% of the total ghosts population. Type I ghosts (improperly resealed) were less than 2% and type III ghosts (empty) represented about 15–20% of the total ghost population after the last washing. The ghosts populations were homogeneous: filtration measurement showed that 95% of ghosts were filtered at $t = 1.89$ msec and the mean cellular volume varied between 80–90 fl. No Hb was detected in the ghosts when the suspension was analyzed on a Coulter counter or with the Drabkin's solution. Homogeneity of pink ghosts preparations was assessed with a Coulter Counter, which demonstrated a homogeneous distribution of red cell volume, including at least 90% of the total population of cells.

pH and Volume Dependence of KCl Cotransport in White Ghosts from Normal and Sickle Erythrocytes

K efflux was measured in WGSS and WGAA resealed at pH 7.40. In these conditions, K efflux was higher in the Cl media than in the NO_3^- media when pH and osmolarity were varied. The Cl dependent component of K efflux, which was calculated from the difference of K efflux between Cl and NO_3^- media, is presented in Fig. 1*a* and *b*. It can be appreciated that the magnitude of Cl-dependent K efflux was similar in SS and AA ghosts. The pH and volume dependence of this Cl-dependent K efflux were strongly altered compared to that observed in the intact sickle cells (Brugnara et al., 1986). Cl-dependent K efflux was a shallow hyperbolic function of pH, between pH 6 and 8, suggesting pH titration of some residues and loss of inhibition by alkaline pH. A similar pH titration of internal sites has been described in sheep

Table. Comparison of rate constants for Cl-dependent K efflux from intact erythrocytes, pink and white ghosts obtained from normal controls and patients with sickle cell disease

	Intact cells	Pink ghosts	White ghosts
Normal cells	0.015 ± 0.01	0.071 ± 0.005	0.14 ± 0.032
Sickle cells	0.15 ± 0.02	0.191 ± 0.019	0.17 ± 0.02

Cl-dependent K efflux (hr^{-1}) measured in intact cells, pink and white ghosts from normal and sickle erythrocytes in isosmotic Na media (300 mOsm/kg), pH 7.40. Data are presented as mean ± SD of triplicate measurements in 2 patients with sickle cell disease (reticulocytes count: 4 ± 1%) and two normal subjects (reticulocyte count <1%).

erythrocytes with low Mg content (Lauf & Adragna, 1998). There was also no volume-dependent component of the Cl-dependent flux, which was essentially unchanged when osmolarity was varied from 200 to 400 mosM (Fig. 1*b*). These results are similar to those we had reported in normal human red cell ghosts (Brugnara et al., 1988). In addition, in both WGSS and WGAA, there was no effect of NEM treatment on the Cl-dependent K efflux in isotonic medium at pH 7.40 (*data not shown*).

The rate constant for Cl-dependent K efflux was strongly increased in WGAA compared to the intact normal cells. In WGSS, the rate constant for Cl-dependent K efflux was not significantly different from that obtained in intact sickle cells (**Table**).

KCl Cotransport Activation by pH and Volume in White Ghosts Resealed with Hb A and Hb S

Homologous Reconstitution

When WGAA and WGSS were resealed with Hb A (WGAA-HbA) and Hb S respectively (WGSS-HbS) Cl-dependent K efflux still progressively increased when the pH of the flux medium was increased from 6.00 to 8.00 (Fig. 2). Thus, re-incorporation of either Hb A or Hb S failed to restore the inhibitory effect of alkaline pH on KCl cotransport. However, Hb A reduced the magnitude of the Cl-dependent K efflux for external pH values of 6.5 or higher to values similar to those observed in pink AA ghosts. In contrast, K efflux was not affected in WGSS by the addition of Hb S (Fig. 2*b*) and no differences could be demonstrated in the magnitude of the flux between WGSS and pink SS ghosts.

Studies of the volume-dependence of K-Cl cotransport also showed differential effects of Hb A and Hb S. Fig. 3*a* and *b* show Cl-dependent K efflux from WGAA-HbA and WGSS-HbS suspended in media at 220, 300 and 400 mosmol/kg, pH 7.40. Increasing the osmolarity from 200 to 400 mosmol/kg significantly inhibited K efflux in WGAA-HbA and WGSS-HbS, respectively, indicating that the presence of either Hb type can restore volume-dependence of K-Cl cotransport. However, the effects of Hb A and Hb S were different. The presence of Hb A produced a significant reduction in Cl-dependent K efflux at any of the three osmolarities studied, whereas Hb S induced a significant stimulation of the Cl dependent K efflux in hypotonic medium. Similar results were obtained for pink ghosts prepared from normal and sickle erythrocytes (PGAA and PGSS, respectively; Fig. 3*a* and *b*). Thus, the volume dependent component of K-Cl cotransport was significantly increased by the addition of Hb S, both in ghosts derived from SS erythrocytes and from normal control erythrocytes (Fig. 3).

Heterologous Reconstitution

Fig. 3*c* presents data on the effect of Hb S on normal erythrocyte ghosts (WGAA-HbS). Incorporation of Hb S resulted in significant stimulation of Cl-dependent flux in hypotonic medium, similar to what was shown in sickle ghosts reconstituted with Hb S (WGSS-HbS, *see* Fig. 3*b*). This effect markedly differs from that of Hb A on normal ghosts preparations (WGAA-HbA), where the incorporation of Hb A results in a significant reduction in the magnitude of the Cl-dependent flux into hypotonic medium (Fig. 3*a*). These results suggest that low amounts of Hb A or Hb S have important and differential regulatory effects on K-Cl cotransport.

Discussion

The regulation of KCl cotransport system is markedly altered in white ghosts as compared to intact cells and pink ghosts (Brugnara et al., 1988; Sachs, 1988; O'Neill, 1989). We have shown here that this applies not only to ghosts obtained from normal erythrocytes but also from sickle erythrocytes. White ghosts of either cell type exhibit a K-Cl cotransport which has lost: (a) the inhibition by alkaline pH and (b) the volume-dependence.

In intact erythrocytes from the least dense fraction of normal cells, and in CC or SS erythrocytes, loss of the inhibition by alkaline pH is observed when cells are exposed to NEM, isosmotically swollen with nystatin technique or Mg-depleted (Brugnara et al., 1988; Lauf et al., 1994; Godard & Ellory, 1996). In sheep erythrocytes, inactivation of K-Cl cotransport by alkaline pH is dependent on internal Mg, and is abolished by Mg removal, or treatment with NEM or staurosporine (Lauf & Adragna, 1998). Although these data suggest the possible involvement of kinases, no direct experimental confirmation of this hypothesis is available. In our study, erythrocyte ghosts were resealed in the presence of 1 mM of Mg. This leads to an internal Mg concentration higher than the physiologic one which should lead to a reduction in

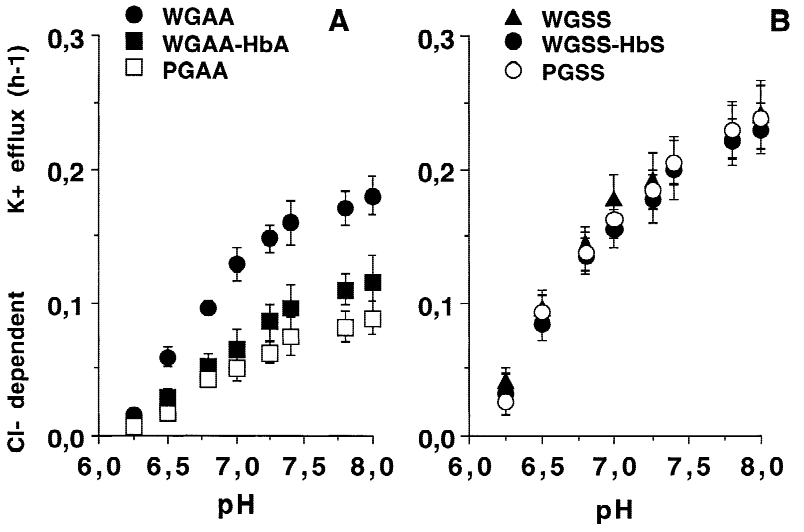

Fig. 2. Effects of hemoglobin A (Hb A) or S (Hb S) on the Cl-dependent K efflux measured at different pH in ghosts prepared from normal or sickle cells. Hb A or Hb S were incorporated in white ghosts (WGAA-HbA and WGSS-HbS respectively) and pink ghosts (PGAA and PGSS) were prepared from washed red cells lysed directly by addition of the lysing-solution at 10% Hct as describe in Materials and Methods. Hemoglobin concentration in WGAA-HbA: 3.2 ± 0.2 g/dl; PGAA: 3.6 ± 0.2 g/dl; WGSS-HbS: 2.4 ± 0.2 g/dl; PGSS: 3.0 ± 0.2 g/dl. K efflux measurements were realized in triplicate; two experiments with 2 different patients with sickle cell disease: reticulocytes count: 4 ± 1% and two normal subjects: reticulocyte count <1%.

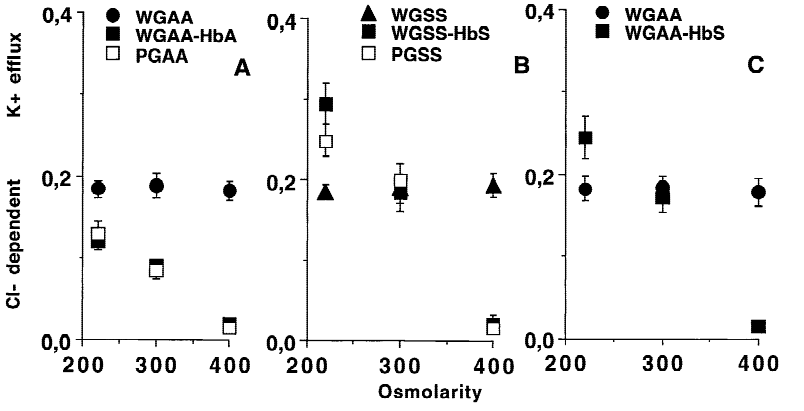

Fig. 3. Effects of hemoglobin A (Hb A) or S (Hb S) on the Cl- dependent K efflux measured at different osmolarities in ghosts prepared from normal or sickle cells. Hb A or Hb S were incorporated in white ghosts (WGAA-HbA, WGSS-HbS WGAA-HbS) and pink ghosts (PGAA and PGSS) were prepared from Washed red cells lysed directly by addition of the lysing-solution at 10% Hct as describe in Materials and Methods. Hemoglobin concentration in WGAA-HbA: 3.2 ± 0.2 g/dl; PGAA: 3.6 ± 0.2 g/dl; WGSS-HbS: 2.4 ± 0.2 g/dl; PGSS: 3.0 ± 0.2 g/dl; WGAA-HbS: 2.8 ± 0.2 g/dl (K efflux measurements were realized in triplicate; two experiments with 2 different patients with sickle cell disease: reticulocytes count: 4 ± 1% and two normal subjects: reticulocyte count <1%).

K-Cl cotransport activity. However, the persistence of the abnormal pH dependence of K-Cl cotransport suggests that changes in cell Mg alone are not sufficient to explain this phenomenon.

With our experimental protocols, a cytoplasmic kinase (or phosphatase) would be diluted to the same extent as Hb (1:10) in pink ghosts, and would be greatly reduced or disappear in white ghosts. Thus, a reduction in this yet to be identified cytoplasmic kinase could explain why there is no K-Cl cotransport inhibition in ghosts by either increased concentration of internal free Mg, or alkaline pH and no K-Cl cotransport activation by NEM. The role of kinases in K-Cl cotransport stimulation has been highlighted in sheep erythrocytes by the work of Lauf et al. (1995). However, there are different interpretations of their role in K-Cl cotransport regulation. Jennings and Schulz (1991) proposed the existence of a volume dependent kinase, which is inhibited by NEM. Others have proposed that NEM may act on a Mg-sensitive kinase which regulate protein phosphatase 1 (PP1) and not directly on the volume dependent kinase (Lauf et al., 1995; Flatman et al., 1996). It is also possible that reduction of intracellular Mg stimulates KCl cotransport system indirectly via inhibition of a kinase or stimulation of a phosphatase.

We also observed that Cl-dependent K efflux is highly activated in WGAA compared to that measured in intact cells. In sickle erythrocytes, the magnitudes of Cl-dependent K efflux in intact sickle cells and in WGSS are similar (Table). Cl-dependent K efflux was slightly higher in WGSS compared to WGAA (*see* Fig. 1). It has been reported that WGAA possessed a K-Cl cotransport system that depended on the density of the cells from which the ghosts were made (Brugnara et al., 1988). Since we used in this report only blood from sickle patients with moderate reticulocytosis (~4%), we attribute the slightly increased K efflux in WGSS to their reticulocytosis and not to a specific membrane abnormality of sickle cells.

We have suggested that Hb C and S may play a specific role in the activation of the K-Cl cotransport system, perhaps via an abnormal interaction of this Hb

with the cell membrane (Olivieri et al., 1992). This abnormal interaction is not a property of all positively charged Hb variants but is mainly associated with the loss of the negative charge at the β6 or β7 residues of Hb (Olivieri et al., 1992). We have now compared K-Cl cotransport properties in WGAA with WGSS resealed in presence of low concentration of Hb A and Hb S respectively. The use of low Hb concentrations in these experiments permitted to compare WGAA and WGSS with pink ghosts which were resealed with their cytoplasmic contents diluted 10-fold, to a final Hb concentration of 2–4 g/dl of ghosts. This low concentration of Hb was sufficient to restore the volume dependency of K-Cl cotransport. In all the experimental conditions studied for K efflux, incorporation of low concentrations of Hb A induced a reduction in the Cl-dependent K efflux rate (Figs. 2*a* and 3*a*) suggesting that either Hb A, or the cytoplasmic content of Hb A cells, downmodulate K-Cl cotransport in ghosts. Although it is possible that a diluted kinase present within the Hb suspension could account for the reduction in K-Cl cotransport, this possible mechanism does not explain the differences observed between Hb S and Hb A suspension, unless one postulates the presence of different kinases in sickle erythrocytes.

Similar results were obtained in pink ghosts suggesting that it is unlikely that an additional cytoplasmic factor other than Hb may account for the recovery of the volume-dependence of K-Cl cotransport. Several authors reported volume dependence of K efflux in normal erythrocyte ghosts (Dunham & Logue, 1986; O'Neill, 1989). It is interesting to note that these authors prepared ghosts by a modification of the method of Bodemann and Passow (1972) at acid pH. At low pH, the erythrocyte ghosts obtained are pink and resealed with their cytoplasmic content just diluted 10-fold (Dunham & Logue, 1986) or 24-fold (O'Neill, 1989). Therefore, the volume dependence of K effluxes described in these studies may reflect the presence of residual Hb in erythrocyte ghosts.

We have been unable to explain the difference between our data showing lack of volume dependence of K-Cl cotransport in white ghosts (*see* this paper and Brugnara et al., 1988) and those of Sachs (1988), which showed volume dependence of K-Cl cotransport used an identical gel filtration system for ghosts preparation. Sachs (1988) used a resealing solution containing albumin (from 104 to 42 mg/100 ml), creatine phosphate (from 10.4 to 4.2 mM), and creatine kinase (from 10.4 to 4.2 IU/ml). These substances were not present in our resealing solution. Creatine kinase has been shown to induce volume-dependent K-Cl cotransport in dog erythrocytes (Colclasure, Parker & Dunham, 1995). However, the different effects we have observed on K-Cl cotransport by reincorporating either Hb A or Hb S into white ghosts indicate a role for the specific Hb type, unless one assumes that the creatine kinase of normal erythrocytes differs in quantity or quality from that of sickle erythrocytes.

The results previously described on normal erythrocytes ghosts (Dunham, 1986; Sachs, 1988; Brugnara et al., 1988; O'Neill, 1989) and the results obtained in this study suggest that Hb may play a sensor role in human erythrocyte volume regulation, as hypothesized in the molecular crowding model for volume-dependent regulation of transport, (Minton et al., 1992; Colclasure & Parker, 1991; Parker, 1993).

The present studies highlight differences between Hb S and Hb A regarding their effect on K-Cl cotransport regulation in erythrocyte ghosts. We did not observed downmodulation of K-Cl cotransport by alkaline pH, either by incorporation of Hb S into WGSS or in pink SS ghosts (Figure 2*b*). Moreover, ghosts containing Hb S demonstrated a marked increase in Cl-dependent K efflux in hypotonic conditions compared with Hb A containing ghosts (Fig. 3*b*). As a consequence, the volume-dependent component of K-Cl cotransport (defined as the difference between Cl-dependent K efflux at 200 and 400 mosM) was markedly increased in Hb S containing ghosts. This effect is specific for Hb S since it can be replicated when Hb S is incorporated into normal ghosts (Fig. 3*b*).

The volume dependency of the K-Cl cotransport system may require the presence of a cytoplasmic factor that is lost during the ghost preparation (Brugnara et al., 1988). This factor may be the volume-dependent kinase postulated by Jennings and Schulz (1991) or the volume-dependent protein phosphatase 1 described by Bize et al. (1998). However, Kelley and Dunham (1996) have shown the presence of volume-dependent K movement in inside-out vesicles obtained from sheep erythrocytes. Their findings are consistent with a three-state model of K-Cl cotransport, which has also been proposed for human erythrocytes (Kaji & Gasson, 1995). It is also possible that in white ghosts the dilution of the cytoplasmic kinase controlling K-Cl cotransport may lead to dephosphorylation of the transporter and increased functional activity. Sachs and Martin (1993) have shown that swelling can further activate K-Cl cotransport by a mechanism which does not require kinases or phosphatases.

It has been shown that kinases and phosphatases play an important role in the mechanism of erythrocyte RVD regulation. However, there are significant differences regarding the role of these enzymes in controlling the volume-dependence of K-Cl cotransport and the possible functional configuration of the transporter. Inhibition of protein phosphatases by specific inhibitors such as okadaic acid or calyculin A (Jennings & Schulz, 1991; Kaji & Tsukitani, 1991; Sachs & Martin, 1993) results in

complete inactivation of KCl cotransport activated by cell swelling and suggests that a type I phosphatase is implicated in KCl cotransport regulation. A cytoplasmic kinase (Krarup & Dunham, 1996) could play a role in this regulation because KCl cotransport system is activated by staurosporine (Bize and Dunham, 1994). These findings suggest that at least two states of the KCl cotransport protein may exist in the erythrocyte membrane: a phosphorylated state which represents the resting form, and a dephosphorylated state being the active form of the protein (Jennings & Schulz, 1991). The two-state model is supported by the dependence of calyculin inhibition of K-Cl cotransport on the phosphorylation state of the transporter (Ortiz-Carranza et al., 1997). A three state model has been proposed by Kelley and Dunham (1996) and (Kaji and Gasson, 1995). Sachs (1994) has proposed the presence of a fourth state in K-Cl cotransport, based on the effect of soluble polycations and cationic amphiphiles in human erythrocyte ghosts.

We conclude that Hb plays an important role in the regulation of erythrocyte volume, most probably as a volume sensor and that Hb S, by increasing the sensitivity of the KCl cotransport to volume activation, plays a specific role in the deregulation of RVD in sickle erythrocytes.

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