We report experiments on the effect of intracellular divalent cations (Mg, Ca, Mn) on K transport and cell volume in erythrocytes from patients with homozygous hemoglobin S disease (SS cells). When CO-treated SS erythrocytes are exposed to the ionophore A23187, removal of cell Mg markedly stimulates K efflux, whereas increasing cell Mg inhibits K efflux. The K, for the inhibition by internal free Mg is 0.38 ± 0.10 mmol/L, a value comparable to the concentration of free Mg in normal cells (0.3 to 0.4 mmol/L). When swollen SS cells with increased Mg content were incubated in plasma-like medium, they shrunk much less than swollen SS cells with normal Mg content. Thus, elevation of cell Mg produces inhibition of swelling-induced K movement from SS cells. Internal Ca and Mn also inhibit K movement from SS cells. The inhibition of volume regulation by divalent cations suggests that increases in intracellular divalent ions, especially Mg, could induce a persistent degree of cell swelling in SS RBCs and thereby inhibit intracellular polymerization.

POLYMERIZATION of hemoglobin S and cell sickling are extremely dependent on oxygen tension and hemoglobin concentration. At any given oxygen tension, hemoglobin S concentration is the most important factor in determining the rate of polymer formation and morphological cell sickling. Because hemoglobin concentration is determined by the water and cation content of the RBC, it is important to study how water content and cell volume are regulated in sickle cell anemia. Cell volume is determined in RBCs by transport mechanisms moving cations and anions across the RBC membrane. We recently described a K transport system that is very active in oxygenated SS and CC cells and is also present in the least dense (reticulocyte-rich) fraction of normal AA cells. K movement through this system allows SS cells to shrink when they are isotonically swollen in vitro or when they are exposed to acid pH. This K movement is not sensitive to inhibitors of the Na-K pump (ouabain, 0.1 mmol/L) and/or Na-K-Cl cotransport (bumetanide, 0.01 mmol/L) systems, and differs from the Ca-activated K permeability first described by Gardos. This pathway shares many properties with the pathway for K transport described by Lauf in sheep RBCs (SRBCs) (reviewed in ref. 7). A similar chloride-dependent pathway has also been described in "young" human RBCs by Hall and Ellory. Because cell swelling is a logical strategy to ameliorate the clinical course of SS disease, and because this K transport pathway induces a sizable cation and water loss, thus counteracting cell swelling, and may also play a role in the formation of dense dehydrated SS cells, a detailed knowledge of its properties could be helpful in designing future pharmacologic interventions to induce cell swelling in SS cells.

The system for K transport described in low K (LK) SRBCs is modulated by divalent ions. We studied the effect of divalent cations on K transport and cell volume in SS erythrocytes. We found that increasing internal Mg concentration in SS cells has marked effects on cell volume and K transport: An increase in internal Mg directly produces an increase of cell volume and thus a decrease of mean corpuscular hemoglobin concentration (MCHC). Moreover, the net K loss and cellular dehydration induced by acid pH or swelling in SS cells are markedly inhibited by increased concentrations of internal Mg. Other divalent cations, such as Ca and Mn, share a similar inhibitory action, albeit with different affinities.

MATERIALS AND METHODS

Blood was drawn from patients homozygous for hemoglobin S disease. All patients had received no blood transfusions for at least 3 months prior to sampling. Preparation of RBCs, nystatin-loading procedure, and measurement of K efflux and phthalate density profile were performed as detailed in previous publications. The methods we used to control and adjust Mg levels in erythrocytes and to measure total RBC magnesium with atomic absorption spectrometry were those described by Flattman and Lew.

To study the effect of Mg on cell volume, the phthalate density profile was measured in SS cells incubated for 30 minutes in a medium containing 10 mmol/L NaCl, 130 mmol/L KCl, 0.5, 1, 5, and 10 mmol/L MgCl2, 10 mmol/L Tris-MOPS, pH 7.4 at 37°C, 10 mmol/L glucose, 0.1 mmol/L ouabain, 0.01 mmol/L bumetanide, and 90 μmol/L/m cell A23187.

To study the effect of Mg, Ca, and Mn on K efflux, nystatin-swollen SS cells were incubated in isosmotic media containing 140 to 130 mmol/L choline chloride, 0 to 5 mmol/L divalents (MgCl2, CaCl2, MnCl2), and 0 to 15 mmol/L sucrose. These three components were reciprocally varied to maintain constant chloride concentration and osmolarity. The media also contained 10 mmol/L Tris-MOPS, pH 7.40 at 37°C, 10 mmol/L glucose, 0.1 mmol/L ouabain, 0.01 mmol/L bumetanide, 0.010 mmol/L carbocyanine and 0.005 mmol/L chlorpromazine. Incubation times for K efflux measurement were 5 and 15 minutes. The incubation times in these and other experiments were chosen to meet conditions of initial rate and have K effluxes values linear with time. The hematocrit of the flux suspension was 1%, and the concentration of the ionophore A23187 was 90 μmol/L/m cell.

To study the effect of internal Mg, at constant external Mg, nystatin-treated SS cells were incubated in a medium containing 10 mmol/L NaCl, 130 mmol/L KCl, 10 mmol/L Tris-MOPS, pH 7.4 at 37°C, 10 mmol/L glucose, 0.1 mmol/L ouabain, 0.01 mmol/L bumetanide, 90 μmol/L/m cell A23187, and either 0.1 mmol/L EDTA or MgCl2 (1 to 2 mmol/L). After 30-minute incubation, the ionophore was removed with four washes with the same medium plus albumin (1 mg/mL). K efflux was measured in a medium containing...
140 mmol/L NaCl, 1 mmol/L MgCl₂, 10 mmol/L Tris-MES (pH 6.0 to 6.5) or Tris-MOPS (pH 6.75 to 8.0) at 37°C, 10 mmol/L glucose, 0.1 mmol/L ouabain, and 0.01 mmol/L bumetanide. The hematocrit of the flux suspension was 1%, and the incubation times were 5 and 25 minutes at 37°C (each one in triplicate).

To study the effect of internal Mg, at constant external Mg, on the volume regulatory decrease exhibited by swollen SS cells, a protocol similar to the one described above was followed and the cells were then incubated in a medium containing 140 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L phosphate, 10 mmol/L Tris-MOPS, pH 7.4 at 37°C, and 10 mmol/L glucose. MCHC, Na, K, and Mg content and phthalate density profile were measured at the beginning of the incubation and after 12 hours at 37°C. Cell Mg and ATP contents were measured at the beginning and end of incubation and were constant.

RESULTS

Effect of divalent cations on cell volume in SS cells. As shown in Fig 1, when the internal concentration of Mg is increased by using A23187 in SS cells, cell volume is also increased. This effect is due to the movement of chloride and thus water into the RBC, to balance the reduction in negative charges of nonpermeant solutes induced by Mg ions, and has been previously described in normal human RBCs by Flatman and Lew.⁶ Cell swelling is not the only consequence of an increase on the internal Mg content: The increase of the chloride distribution ratio (Clᵢ/Clₒ) at constant external pH and external chloride concentration, produces an alkaline shift in internal pH and a reduction of the RBC membrane potential. We also measured the total Mg content in SS and AA cells. Our preliminary data indicate that the total RBC Mg content is 2.5 ± 0.2 (SD, n = 5) mmol/L/cell in SS and 2.3 ± 0.1 (SD, n = 7) mmol/L/cell in AA cells (7.2 and 7.4 mmol/kg hemoglobin, respectively).

Effect of divalent cations on swelling-induced K efflux from SS cells. As in our previous study of CC cells, we found no effect of external Mg on the swelling-induced K flux from SS cells (data not shown). As shown in Fig. 2a, however, when the internal Mg content is varied by varying the external Mg concentration in the presence of ionophore A23187, K efflux from isosmotically swollen SS cells is markedly affected. Removal of internal Mg (by using A23187 and 0.1 mmol/L EDTA), stimulates K efflux from SS cells. When the internal (and external) Mg is increased, K efflux is progressively inhibited. At 0.15 to 0.2 mmol/L external Mg, K effluxes with and without A23187 are equal. This concentration of external Mg, with A23187, yields free and total RBC Mg values similar to those observed in fresh normal human RBCs.⁸ (C. Brugnara, unpublished observations). The Kᵢ for external Mg, with A23187 added, calculated with the Dixon plot was 185 ± 380 μmol/L (n = 5). This value corresponds to a free internal Mg concentration of 380 ± 100 μmol/L.

To investigate whether other divalent cations have a similar inhibitory action on K transport of SS cells, we also increased the erythrocyte concentration of Ca and Mn. Because any increase of the internal free Ca opens Ca-activated K channels (Gardos effect, ref. 6), inducing a large K loss and also activating the Ca pump with associated increased consumption of ATP and metabolic substrates, we included in the incubation medium 10 μmol/L carbocyanine, an inhibitor of the Ca-activated K channel and 5 μmol/L chlorpromazine, an inhibitor of the Ca pump. We did not use trifluoperazine because it produced cell lysis. The presence of these two drugs did not affect the inhibitory action of Mg on the OB-resistant K fluxes. Indeed, similar results were obtained when the internal Mg was varied with and without carbocyanine and chlorpromazine. Figure 2a shows the effect of external Ca and Mn, with A23187 added, on the swelling-induced K efflux from SS cells. When the internal (and external) concentration of these two divalent ions is increased, there is a marked inhibition of K efflux from SS cells. Mn is a more powerful inhibitor of K movement than Ca, since in two experiments the Kᵢ for inhibition of K efflux by external Ca with A23187 were 300 μmol/L, and 200 μmol/L, whereas the comparable figures for external Mn with A23187 were 50 μmol/L and 70 μmol/L. Increasing concentrations of internal divalent cations inhibit the swelling-induced K efflux despite a concomitant increase in the cell water content that by itself would further stimulate K loss.

![Graph](https://www.bloodjournal.org/graphics/graph.png)

**Fig 1.** Effect of increasing the erythrocyte Mg content on density profile of SS cells. To increase cell Mg content, SS cells were incubated in a medium containing: 10 mmol/L NaCl; 130 mmol KCl; 0.5, 1.5, and 10 mmol/L MgCl₂; 10 mmol/L Tris-MOPS, pH 7.4 at 37°C; 10 mmol/L glucose; 0.1 mmol/L ouabain; 0.01 mmol/L bumetanide, and 90 μmol/L cell A23187. After 30-minute incubation at 37°C, the phthalate density profile was measured.
Effect of Internal Mg on the pH dependence of K efflux from SS cells. To rule out the possibility that these effects were due to A23187, and to measure K efflux from cells with different Mg content at constant (1 mmol/L) external Mg, SS cells were exposed to A23187 and different Mg concentrations, and the ionophore was then removed by washing at 37°C with albumin. When the RBC Mg content was increased, K efflux was markedly decreased at any external pH studied (Fig 2b). When the internal Mg was decreased (incubation in 0 Mg + 0.1 mmol/L EDTA), there was a marked change in the pH dependence of K efflux. K efflux was progressively stimulated when the external pH became more alkaline and the inhibition by alkaline pH was abolished (Fig 2b). Different effects were observed when cell volume was changed by altering the osmolarity of the medium at constant pH (7.40). At any given internal Mg concentration studied, K efflux was markedly inhibited when SS cells were shrunken by incubation in hypertonic media (data not shown). Thus, when the free and total cell Mg are markedly reduced in SS cells, there is a dissociation between the inhibitory action of alkaline pH (that is lost) and the inhibitory action of cell shrinkage (that is conserved) on K efflux.

Effect of internal Mg on regulation of cell volume by SS cells. We next studied the effect of varying the cell Mg content on the volume regulatory properties of CO-treated SS cells. SS erythrocytes were isosmotically swollen with the nystatin technique, their Mg content was increased with A23187 and MCHC, Na K contents and phthalate density distribution were measured before and after 12 hours incubation at 37°C in a plasma-like medium containing no transport inhibitor. As shown in Fig 3, swollen SS erythrocytes (total cell Mg 7.5 mmol/kg hemoglobin) shrink more than swollen SS cells with increased Mg content (total cell Mg 16.7 mmol/kg hemoglobin). The difference in cell volume and cation content at the end of the incubation between SS control and SS cells with increased Mg content is mostly due to a smaller K loss in the high Mg cells (Table 1). There is...
K TRANSPORT AND DIVALENT CATIONS IN SS CELLS

Water follows osmotically, increasing water content and volume\(^{18}\) (Fig 1). The second mechanism involves a specific inhibitory effect of internal Mg on a K transport pathway that has increased activity in SS and CC cells,\(^{2,3}\) but is also present in the least dense fraction of normal AA cells.\(^{4,5}\) This inhibitory effect of Mg counteracts the cell shrinkage produced by this pathway when SS cells are swollen or exposed to acid pH (Figs 2B and 3).

Other variables, namely internal chloride, internal pH, and membrane potential are changed when the internal concentration of divalent ions is increased. The inhibition is almost complete at a concentration of divalent cations in the external medium of 1 mmol/L (Fig 2A). This concentration of external Mg yields in the presence of A23187 a free cell Mg of 1.7 mmol/L (normal value is 0.3 mmol/L) and a total RBC Mg content of 6.5 mmol/L/cell water (normal value is 3.2 mmol/L/cell water). As a consequence, the internal chloride concentration rises, the internal pH increases from 7.25 to 7.29, and the membrane potential decreases from -9.0 to -6.5 mV. These last two changes by themselves could inhibit K movement and this possibility cannot yet be ruled out. The different affinities for divalent cations to inhibit K movement (Fig 2A) suggest, however, that this effect is a specific property of divalent cations rather than a secondary effect due to changes in internal pH and membrane potential.

The regulatory action of internal Mg on K movement is further revealed by the finding that decreasing internal Mg in human SS cells stimulates K efflux (Fig 2A and B). A similar effect has also been reported in LK SRBCs,\(^{7,12}\) and the activity of the KCl cotransporter has been proposed to be determined by a divalent ion binding site, which is in turn regulated by the degree of protonation of its chemical groups.\(^{7}\) Indeed, the stimulation of K efflux by removal of internal Mg is also associated with a loss of the bell-shaped dependence on pH of K movement, and K efflux is not inhibited when the internal pH becomes more alkaline (Fig 2B). Thus, the inhibition of this pathway for K transport requires the presence of internal Mg. It is reasonable to suppose that the inhibition of K transport by alkaline pH in the presence of internal Mg (Fig 2B) is due to binding of Mg at some site in the transport system for which Mg and protons compete. A loss of inhibition of K transport by alkalization is also observed in SS, CC, and AA least dense cells after treatment with N-ethylmaleimide (NEM 1 mmol/L) or after isosmotic cell swelling\(^{6}\) (data not shown). The only difference among these three different conditions is that the increased K efflux due to NEM treatment is not volume sensitive, whereas that due to Mg removal or isosmotic cell swelling is inhibited by cell shrinkage produced by suspension of the cells in hypotonic media.

We have proposed that the high activity of this pathway in CC and SS cells is due not only to the presence of young cells, but also to a direct effect of the positively charged S and C hemoglobins, probably through binding to the RBC membrane.\(^{24}\) Indeed, cells with a moderate reduction of life span, such as those homozygous or heterozygous for C hemoglobin, have a much higher volume and pH-dependent K transport that control AA cells with comparable degrees of reticulocyti-

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toxis. Although this pathway is active only in the least dense, reticulocyte-rich fraction of normal AA blood, which represents 5% of the whole RBC population, it is active in all density fractions of CC and SS cells. Moreover, the K, Cl, and water loss induced by the activation of this transport pathway are not limited to a fraction of young SS cells, since they are easily demonstrable in whole SS blood (Fig 3 and Table 1). Because KCl loss through this pathway produces cell shrinkage, and because this pathway is most active in cells with relatively low density (and thus high cation and water content), it is reasonable to suppose that this pathway is involved in the reduction of K and water content that occurs when normal and SS RBCs become mature in vivo and may also be involved in the formation of dense dehydrated SS cells.

The inhibitory effect of internal Mg on K transport is not an exclusive property of SS cells, but is also observed in the least dense fraction of normal human RBCs. A similar inhibitory action of Mg has been described by Lauf for the volume-dependent K flux of low K SRBCs. Many of the properties of this K transport pathway in human RBCs are similar to those of the KCl cotransport described by Lauf in SRBCs. Like human RBCs, high K sheep reticulocytes have a volume- and chloride-dependent pathway that is then inactivated in mature cells. This pathway is present in all density fractions of low K SRBCs. Volume regulation through this pathway has been recently demonstrated in sheep reticulocytes. In both human (SS and AA) and LK SRBCs, inhibition of K transport is produced not only by internal Mg, but also by other divalent cations, such as Ca and Mn. In both LK SRBCs and SS cells, Mn seems to be the most potent inhibitor, since complete inhibition of K efflux is achieved with internal Mn concentrations below 150 μmol/L.

Mg has also some important effects on other cation transport systems in RBCs: its presence is required for the activity of the Na-K ATPase, of the Ca-ATPase, and of the Ca-activated K channel. External (but not internal) Mg has a marked inhibitory effect on the Na and K fluxes mediated by the bumetanide-sensitive Na-K-Cl cotransport.

The Mg effect and other properties differentiate the K transport system we describe here from the deoxygenation-induced fluxes of SS cells. The volume-, pH-, and chloride-dependent K movement that we have described in carbonmonoxide treated SS cells (and in CC and AA cells) is inhibited by cell Mg, is insensitive to DIDS (4,4'-disothiocyanostilbene-2,2'-disulfonic acid), is not associated with an increased Na influx, and markedly decreases cell volume. Preliminary reports also suggest that chloride-dependent K movement is inhibited when SS cells are deoxygenated.

The deoxygenation-induced K movement in SS cells is chloride-independent and partially inhibited by DIDS, and is associated with a marked increase of the Na permeability, and does not produce changes in cell volume. Deoxygenation of SS cells induces a marked increase of K and Na permeabilities and also of the internal free Mg concentration. These findings seem to indicate that the deoxygenation-induced Na and K fluxes move through transport pathways different from the system we describe and are mediated instead by pathways that are influenced by membrane stretching.

Mg is known to have important biologic regulatory functions. In human RBCs, the total cell Mg content is 2 to 3 mmol/L/cell (3 to 4 mmol/kg/cell water) and the free Mg concentration is 0.3 to 0.4 mmol/kg/cell water. ATP and other nucleotides are the most important RBC buffer system for Mg, and only a small fraction of Mg is bound to cell proteins or 2,3-DPG. Total RBC Mg content is elevated in the least dense fraction of normal RBCs and decreases as cell density increases. Because the least dense fraction contains the youngest circulating RBCs, this observation could partially explain previous reports of elevated RBC Mg in sickle cell anemia and in other anemias characterized by marked reduction of RBC life span. Preliminary reports on total Mg content of density-separated SS cells suggest that the total Mg content is lower in irreversibly sickled cells. Clearly, future measurements of both, total and free RBC Mg contents, will have to be performed in SS erythrocytes separated according to density. The ratio of free to bound Mg in RBCs is markedly affected by the state of oxygenation of hemoglobin. Upon deoxygenation, ATP binds to deoxyhemoglobin, releasing Mg, and the free Mg concentration markedly increases. One study reported that the K and Mg concentrations in normal erythrocytes (and lymphocytes) are positively correlated, so that cells with a higher Mg concentration tend to have a higher K concentration and therefore a higher water content.

Genetic factors play an important role in determining the erythrocyte Mg content. Because the Mg content is genetically determined, and since Mg has such an important effect on cell volume and K transport, it is possible that the genetic regulation of cell Mg is one of the "additional" genetic modulators of sickle cell disease.

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Inhibition of K transport by divalent cations in sickle erythrocytes

C Brugnara and DC Tosteson