Dehydration of Transferrin Receptor–Positive Sickle Reticulocytes During Continuous or Cyclic Deoxygenation: Role of KCl Cotransport and Extracellular Calcium

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The K^+ efflux that mediates sickle-cell dehydration may occur through several pathways, including two with a high capacity for mediating rapid K^+ loss, KCl cotransport and the Ca^{2+}-dependent K^+ channel [$K(Ca^{2+})$]. The rate and pathway of red blood cell (RBC) dehydration most likely depends on cell age and hemoglobin (Hb) composition, with the presence of HbF playing an important role. Oxygenated sickle RBCs have relatively stable cell volume during incubation in vitro, whereas deoxygenated cells become dehydrated, and therefore more dense, due to activation of one or more K^+ efflux pathways. In this investigation, sickle RBCs were deoxygenated either continuously or in 15-minute cycles for 4 hours, and the density increases of very young, transferrin receptor–positive (TfR+) cells and the remaining TfR− cells were determined. The contribution of KCl cotransport was estimated by replacing Cl^- with NO_3^-. $K(Ca^{2+})$ was inhibited by removal of Ca^{2+} or addition of charybdotoxin (ChTX). For both continuous and cyclic deoxygenation, TfR+ cells had a greater density increase when compared with TfR− cells. The lower percentage of HbF found in the TfR+ population may contribute to this difference. With continuous deoxygenation, the density shift was decreased by inhibition of $K(Ca^{2+})$, but not by inhibition of KCl cotransport. With cyclic deoxygenation, the density shift was decreased in an independent, additive manner by inhibition of both pathways. Thus, cyclic deoxygenation of sickle cells under these conditions appears to activate both $K(Ca^{2+})$ and the KCl cotransporter.

THE EXISTENCE of dense sickle reticulocytes implies that a number of young sickle red blood cells (RBCs) undergo abnormal in vivo cation loss and dehydration. Many of these cells have not shed their transferrin receptors (TfR), an event that normally occurs during reticulocyte maturation, indicating that dehydration occurs before or very soon after release from the marrow. The higher concentration of hemoglobin (Hb)S in these dehydrated cells leads to increased polymerization and sickling, but the clinical importance of the young dense population has not been defined.

The classic deoxygenation-induced (DI) cation transport pathway first described by Tosteson et al,[1] which mediates balanced movements of Na^+ and K^+ in the absence of Ca^{2+} and a slight excess of K^+ efflux in the presence of Ca^{2+},[2,3] may contribute to dehydration either directly or through subsequent cation movements via Na^+-K^+-adenosine triphosphatase (ATPase). These relatively small cation shifts are unlikely to cause significant density changes during short time periods. However, deoxygenation and sickling also mediate an influx of Ca^{2+},[4] which, if sufficient to result in an intracellular Ca^{2+} concentration greater than 50 to 60 nmol/L,[5] may lead to rapid cellular K^+ depletion.

Two high-capacity K^+ efflux routes have been implicated in the dehydration of sickle reticulocytes.[6] KCl cotransport is a chloride-dependent pathway that is present in immature erythroid cells. In normal and sickle reticulocytes, this pathway is activated by low pH or cell swelling,[7] and is completely inhibited by replacement of Cl^- with NO_3^-.[8] The Ca^{2+}-dependent K^+ channel [$K(Ca^{2+})$] is present in both reticulocytes and mature RBC and activated by increased intracellular Ca^{2+}. This channel may be inhibited by removal of Ca^{2+} or by addition of charybdotoxin (ChTX).[9,10] Activation of $K(Ca^{2+})$ is likely to be a complex function of Hb composition, degree of morphologic sickling, magnitude of the deoxygenation-induced Ca^{2+} influx, and the capacity/activity of the Ca^{2+} pump. All of these factors may be dependent on cell age, and experiments to evaluate the contributions of both KCl cotransport and $K(Ca^{2+})$ to cellular dehydration must take into account erythroid maturation state.

We recently presented evidence that KCl cotransport activity may contribute to dehydration of young cells under oxygenated conditions.[11] When dense cells were rehydrated and subjected to a short incubation without deoxygenation, some TfR+ cells demonstrated a KCl cotransport-mediated density increase at pH 7.4. All of the TfR+ cells in the light fraction and many in the rehydrated dense fraction were stable under these conditions. At lower pH values, KCl cotransport-mediated dehydration occurred in many additional cells, especially among the TfR+ cells that had been dense in vivo. Thus, young SS RBCs that were dense in vivo had higher KCl cotransport activity compared with age-matched cells that were normally hydrated in vivo, even when hydration was normalized. These findings suggested that KCl cotransport is an important factor in the dehydration of young SS RBCs.

Cellular Hb composition influences the generation of dehydrated cells, as shown by the lower percentage of HbF in dense sickle RBCs compared with intermediate-density cells.[12] Hb composition also varies with cell age. Young sickle cells, identified as either reticulocytes[13] or TfR+ cells,[14] tend to have a lower content of HbF. This is most likely due to a longer circulation lifespan of F-containing cells and their resultant enrichment in older cells. The densest ($\rho > 1.092$) TfR+ cells, which are as severely K^+-

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depleted as the bulk dense population,[14] contain little or no HbF.[14] Therefore, compared with non-F cells, F cells appear to be relatively resistant to severe dehydration. Since HbF inhibits polymerization and sickling upon deoxygenation, these data support a sickling-dependent component in the dehydration of older SS RBCs[12] and also in the formation of severely K^+-depleted, dense TfR+ cells.[14]

Apovo et al[13] found that both KCl cotransport and $K(Ca^{2+})$ were important in the dehydration of sickle cells upon cyclic deoxygenation. However, hydration changes during continuous deoxygenation depended only on the presence of Ca^{2+}. This study used the entire sickle-cell population, with no attempt to focus on density- or age-defined cells, or to explore the role of HbF. In other investigations that used reticulocyte-enriched light fractions subjected to continuous deoxygenation[2] or to deoxygenation cycles,[16] extracellular calcium was required for net cation depletion and density increase. In these studies,[2,16] the role of KCl cotransport was not investigated.

The experiments presented here focused on the tendency of very young sickle (TfR+) cells to become dehydrated during cyclic or continuous deoxygenation, and compared this behavior with that of older cells (TfR−) in the same incubation. The experiments were designed to evaluate the relative importance of KCl cotransport and $K(Ca^{2+})$ as the pathway for K^+ efflux. Unfractionated sickle cells were subjected to 4 hours of cyclic or continuous deoxygenation. Before and after this incubation, the RBCs were separated into four density fractions, and the TfR+ cells in each fraction identified by flow cytometry. In this way, changes in the density distributions of TfR+ and TfR− cells during cyclic or continuous deoxygenation were determined. Under the conditions of these experiments, TfR+ reticulocytes exhibited a larger density increase than TfR− cells with both cyclic and continuous deoxygenation. With cyclic deoxygenation, this change was mediated by both KCl cotransport and $K(Ca^{2+})$. With continuous deoxygenation, density changes were partially inhibited by removal of Ca^{2+} or inhibition of $K(Ca^{2+})$, but no dependence on KCl cotransport was apparent.

MATERIALS AND METHODS

The following buffers were used: HBIS (135 mmol/L NaCl, 20 mmol/L HEPES, 5 mmol/L KCl, 1 mmol/L Na_2HPO_4, 1.5 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L glucose, and 290 to 300 mOsm/kg, pH 7.40 at 37°C) and HBIN (same as HBIS except Cl^- replaced with NO_3^-).

Heparinized blood samples were obtained with informed consent from patients with homozygous sickle-cell disease who had not been transfused for at least 3 months. A total of 15 experiments were performed using blood from eight patients. After overnight storage in plasma at 4°C, RBCs were washed three times with HBIS and resuspended to a hematocrit of 2% in the appropriate buffers. In addition to HBIS and HBIN, buffers included HBIS containing 300 nmol/L ChTX [an inhibitor of K(Ca)], HBIS in which 1.5 mmol/L Ca^{2+} was replaced by 0.15 mmol/L Ca^{2+}, and Ca^{2+}-free HBIS or HBIN that contained 0.1 mmol/L EGTA. One milliliter of each cell suspension was placed in a 20-mL plastic screw-top scintillation vial that had gas inlet and outlet connections cemented to the side walls. Up to six of these vials could be connected in series for gas equilibration. Cyclic deoxygenation consisted of 10-minute periods of nitrogen flow alternated with 5-minute periods of 50/50 nitrogen/air. Sixteen cycles were delivered over 4 hours, always ending with an extended 10-minute equilibration with nitrogen/air. Continuous deoxygenation consisted of 4 hours of nitrogen, followed by 10 minutes of 50/50 nitrogen/air. High gas flow rates of 300 mL/min for nitrogen and 600 mL/min for nitrogen/air assured that all of the series-connected vials equilibrated quickly at the start of a cycle. Gases were passed through six-stage bubble humidifiers before entering the equilibrators. These humidifiers were preequilibrated with the appropriate gas for at least 30 minutes before the experiment. The equilibrator, the humidifier, and all connecting lines were immersed in a 37°C water bath and shaken at 75 rpm during the incubations. Preliminary experiments indicated that 80% to 90% of cells were sickled during the 10- minute deoxygenation and that less than 10% of sickled cells remained after the 5-minute oxygenation. Buffer osmolality was measured after the incubations and shown to increase by less than 5 mOsm/kg, indicating that gas humidification was adequate.

After cyclic or continuous deoxygenation, RBCs from all incubations, including those with HBIN buffer, were washed three times with ice-cold HBIS and suspended at a hematocrit of approximately 40%. Ten microliters of each cell suspension, including an unincubated sample, were separated at 22°C into four density fractions in a microultracentrifuge (Airfuge; Beckman, Palo Alto, CA) using arabinogalactan densities of 1.086, 1.094, and 1.103 g/mL, and a cushion greater than 1.15 g/mL as previously described.[11] After counting the number of RBCs in each fraction, the cells were reacted with mouse monoclonal fluorescein isothiocyanate (FITC)-conjugated anti-TfR (Dako, Carpenteria, CA) and an appropriate idiotypic control antibody (Sigma, St Louis, MO), and the percentage of TfR+ cells in each fraction was determined by flow cytometry (Coulter XL-MCL, Hialeah, FL) as described,[11] except that the final suspension was in 0.2 mL of phosphate-buffered saline (PBS) and 0.8 mL of 1.25% paraformaldehyde fixative. In a subset of experiments, RBC suspensions were incubated for 4 hours at 37°C in HBIS without deoxygenation and analyzed in an identical manner.

The following equation[11] was used to calculate the percentage of total TfR+ cells in each gradient fraction (P_i) from the number of cells in each fraction (C_i) and the proportion that were TfR+ (F_i):

$$P_i = \frac{100\ F_i C_i}{\sum_{n=1}^{4} F_n C_n}.$$

A density score (DS) with a range of 100 to 400 was defined[11] to characterize the entire density distribution with one number:

$$DS = \sum_{i=1}^{4} iP_i.$$

To quantitate the degree of F-cell enrichment for the eight patients in this study, TfR+ cells were isolated with magnetic beads as previously described,[14] and the percent HbF was quantitated by high-performance liquid chromatography (HPLC).[17] The percent HbF in the isolated TfR+ cells was compared with that of RBCs before isolation of TfR+ cells.

RESULTS

The percentage of TfR+ cells in the blood samples used for these experiments was 6.1 ± 2.4 (1 SD). Incubation of sickle RBCs for 4 hours under oxygenated conditions caused no change in the DS of TfR− cells (235 *v* 235, n = 10,

Table 1. Density Scores for TfR− Cells Subjected to Continuous Deoxygenation

	Cl Media (HBIS)				NO_3 Media (HBIN)	
	1.5 mmol/L Ca			EGTA	1.5 mmol/L Ca	EGTA
Patient	Unincubated	Oxy	Deoxy	Deoxy	Deoxy	Deoxy
H	237	231	260	254	247	256
B	270	264	272	264	265	266
A	232	227	237	234	235	230
G	273	267	294	292	272	262
F	240	251	250	253	252	237
D	230	239	239	217	246	236
Mean	247	246	259	252	253	248

Paired *t*-tests (continuous deoxy): Effect of Ca—1.5 mmol/L Ca *v* EGTA in HBIS, n = 6, NS; 1.5 mmol/L Ca *v* EGTA in HBIN, n = 6, NS. Effect of Cl—1.5 mmol/L Ca, HBIS *v* HBIN, n = 6, NS; EGTA, HBIS *v* HBIN, n = 6, NS. Effect of (Ca + Cl)—1.5 mmol/L Ca in HBIS *v* EGTA in HBIN, n = 6, NS.

Abbreviation: NS, not significant.

Table 3. Density Scores for TfR+ Cells Subjected to Continuous Deoxygenation

	Cl Media (HBIS)				NO_3 Media (HBIN)	
	1.5 mmol/L Ca			EGTA	1.5 mmol/L Ca	EGTA
Patient	Unincubated	Oxy	Deoxy	Deoxy	Deoxy	Deoxy
H	168	178	238	215	240	221
B	230	251	267	242	261	239
A	147	166	224	199	232	212
G	210	213	254	241	251	229
F	181	202	229	219	239	216
D	171	187	214	182	243	215
Mean	184	199	237	216	244	222

Paired *t*-tests (continuous deoxy): Effect of Ca—1.5 mmol/L Ca *v* EGTA in HBIS, n = 6, $P < .002$; 1.5 mmol/L Ca *v* EGTA in HBIN, n = 6, $P < .00002$. Effect of Cl—1.5 mmol/L Ca, HBIS *v* HBIN, n = 6, NS; EGTA, HBIS *v* HBIN, n = 6, NS. Effect of (Ca + Cl)—1.5 mmol/L Ca in HBIS *v* EGTA in HBIN, n = 6, $P < .02$.

Tables 1 and 2) and a small increase in the DS of TfR+ cells (181 *v* 193, n = 10, $P < .005$, Tables 3 and 4). Incubation for 4 hours under oxygenated conditions and in both deoxygenation protocols resulted in a 20% decrease in the total number of TfR+ cells, presumably due to shedding or internalization of TfR during the incubation. While this change in TfR status may have contributed to the small DS difference between unincubated TfR+ cells and oxygenated, incubated TfR+ cells noted earlier, it should not effect comparisons made between samples incubated under various conditions.

Continuous deoxygenation resulted in little or no density shift for TfR− cells (Table 1). However, TfR+ cells had a shift (oxy *v* deoxy, n = 6, $P < .005$) that was partially inhibited by removal of Ca^{2+}, but not by replacement of Cl^- with NO_3^- (Table 3). There was a small but significant increase in the density of TfR+ cells induced by continuous deoxygenation that was insensitive to both removal of Ca^{2+} and Cl^- replacement (cells incubated in NO_3^-/EGTA). An example of these experiments is shown in Fig 1, in which the density distributions of TfR+ and TfR− cells are shown without incubation, after a 4-hour incubation in HBIS under oxygenated conditions, and after a 4-hour incubation with continuous deoxygenation in HBIS (1.5 mmol/L Ca^{2+}), HBIS without Ca^{2+}, HBIN (1.5 mmol/L Ca^{2+}), and HBIN without Ca^{2+}. In these experiments, the TfR+ cells exhibited a small density shift under oxygenated conditions, and a larger shift with deoxygenation. This shift was inhibited by removal of Ca^{2+}, but replacement of Cl^- with $NO3^-$ had no effect.

Cyclic deoxygenation resulted in a shift to increased density of both TfR− (oxy *v* cycle, n = 4, $P < .05$) and TfR+ (oxy *v* cycle, n = 4, $P < .05$) cells. An example of these experiments is given in Fig 2, and the data are summarized in Tables 2 and 4. For TfR− cells, there was a trend toward inhibition of the density shift with either removal of Ca^{2+}

Table 2. Density Scores for TfR− Cells Subjected to Cyclic Deoxygenation

	Cl Media (HBIS)						NO_3 Media (HBIN)		
	1.5 mmol/L Ca			.15 mmol/L Ca	EGTA	ChTX	1.5 mmol/L Ca	EGTA	ChTX
Patient	Unincubated	Oxy	Cycle	Cycle	Cycle	Cycle	Cycle	Cycle	Cycle
A	209	211	238						
A	230	223	245		231		235	227	
F	207		242		243		224	207	
E	246		258		249		252	227	
D	223		222		215		219	217	
C	231		256	244	250			225	
A	239		254	252	242		244	243	
D	219	211	248			238	220		222
D	206	216	226			223	215		211
Mean	223		243		238		229	224	

Paired *t*-tests (cycle): Effect of Ca—1.5 mmol/L Ca *v* EGTA in HBIS, n = 6, $P < .05$; 1.5 mmol/L Ca *v* EGTA in HBIN, n = 5, $P < .1$. Effect of Cl—1.5 mmol/L Ca, in HBIS *v* HBIN, n = 7, $P < .01$; EGTA, HBIS *v* HBIN, n = 6, $P < .1$. Effect of (Ca + Cl)—1.5 mmol/L Ca in HBIS *v* EGTA in HBIN, n = 6, $P < .002$.

Table 4. Density Scores for TfR+ Cells Subjected to Cyclic Deoxygenation

	Cl Media (HBIS)						NO_3 Media (HBIN)		
	1.5 mmol/L Ca			.15 mmol/L Ca	EGTA	ChTX	1.5 mmol/L Ca	EGTA	ChTX
Patient	Unincubated	Oxy	Cycle	Cycle	Cycle	Cycle	Cycle	Cycle	Cycle
A	157	160	252						
A	204	204	261		228		227	214	
F	163		216		198		195	165	
E	172		244		213		212	178	
D	184		212		199		211	190	
C	213		264	241	255			225	
A	201		261	237	229		258	230	
D	178	182	227			212	212		209
D	160	185	207			195	211		203
Mean	181		238		220		218	200	

Paired *t*-tests (cycle): Effect of Ca—1.5 mmol/L Ca *v* EGTA in HBIS, n = 6, $P < .005$; 1.5 mmol/L Ca *v* EGTA in HBIN, n = 5, $P < .005$. Effect of Cl—1.5 mmol/L Ca, HBIS *v* HBIN, n = 7, $P < .05$; EGTA, HBIS *v* HBIN, n = 6, $P < .05$. Effect of (Ca + Cl)—1.5 mmol/L Ca in HBIS *v* EGTA in HBIN, n = 6, $P < .002$.

or replacement of Cl^- by NO_3^-. A combination of Ca^{2+} removal and Cl^- replacement was clearly inhibitory ($P < .002$, Table 2). With TfR+ cells, which tended to have a larger density shift under these conditions, inhibition of either K(Ca) or KCl cotransport resulted in a significant, but incomplete, inhibition of the density shift. Blocking both pathways almost completely prevented the density shift, since cells deoxygenated in HBIN/EGTA had DS values similar to unincubated cells or oxy controls. This is illustrated in Fig 2, in which the density distribution of cells in HBIN/EGTA is

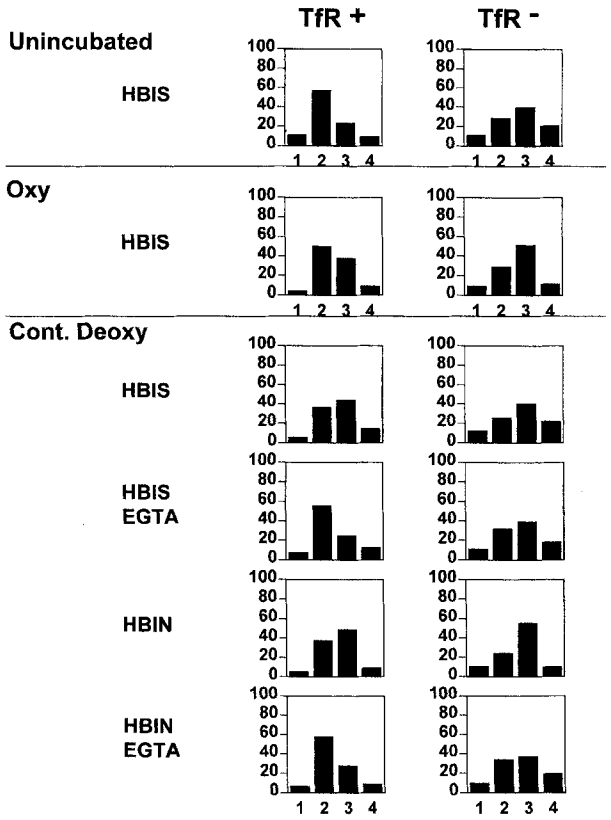

Fig 1. Density distributions of TfR+ and TfR− cells without incubation, after 4 hours of incubation under oxygenated conditions, and after 4 hours of continuous deoxygenation in the buffers indicated. The ordinate is the percentage of the total number of TfR+ or TfR− cells in each density fraction.

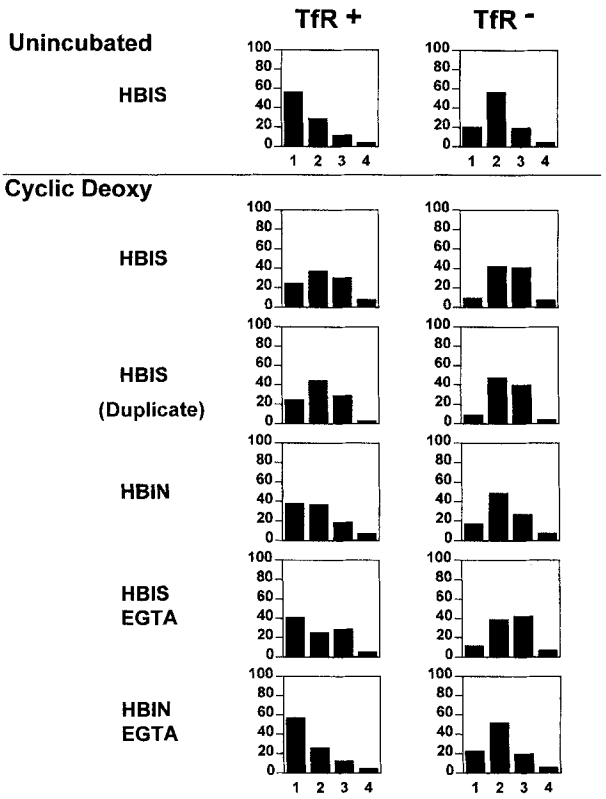

Fig 2. Density distributions of TfR+ and TfR− cells without incubation and after 4 hours of incubation with cyclic deoxygenation. The ordinate is the percentage of the total number of TfR+ or TfR− cells in each density fraction. In this experiment, the incubation in HBIS buffer was performed in duplicate.

Table 5. Percent HbF in Unfractionated and TfR+ Sickle Cells

Patient	% HbF, Unfractionated	% TfR+, Unfractionated	% HbF, TfR+
A	19.6	5.9	4.1
B	8.9	7.5	2.3
C	15.2	3.1	3.5
D	24.5	5.3	7.0
E	1.8	6.6	0.9
F*	24.4	3.6	9.3
G*	8.9	11.1	1.5
H	9.4	10.9	0.9
Mean	14.1		3.3

* HbF data for patients F and G from Franco et al,[14] Table 3.

essentially identical to that of unincubated controls. With cyclic deoxygenation, the Ca^{2+}-dependent and KCl cotransport pathways were each responsible for approximately 50% of the density shift, and appeared to be noninteractive and additive.

The effect of Ca^{2+} on DI density shifts could conceivably be due to either the activation of the $K(Ca^{2+})$ channel by DI Ca^{2+} influx or through the action of external Ca^{2+} to reduce DI Na^+ influx and thereby produce net direct cation loss via the DI pathway.[2,3] Two experimental approaches to this question were taken. In the first, cyclic deoxygenation was performed to compare the effect of 0.15 mmol/L Ca^{2+} to 1.5 mmol/L Ca^{2+}. These concentrations produce similar inhibition of Na^+ fluxes via the DI pathway.[3] However, a 10-fold lower Ca^{2+} level would be expected to greatly reduce the probability of $K(Ca^{2+})$ channel activation[18] by DI Ca^{2+} influx. Table 4 shows that with 0.15 mmol/L Ca^{2+}, the density shift upon cyclic deoxygenation was equivalent to that seen in the absence of Ca^{2+}. In another series of experiments, two incubations were performed in the presence of 300 nmol/L ChTX, a specific inhibitor of $K(Ca^{2+})$, which in preliminary experiments (not shown) gave a 68% inhibition of Ca^{2+}-dependent K^+ flux under these incubation conditions. In both experiments (Table 4) the degree of density shift inhibition was consistent with that seen in the absence of Ca^{2+}. Thus, the Ca^{2+}-dependent component of density shift upon cyclic deoxygenation is consistent with activation of the $K(Ca^{2+})$ channel under these experimental conditions.

Table 5 shows the HbF levels in the unfractionated and TfR+ cells for each patient in Tables 1 through 4. As expected, there was a higher percentage of HbF in the unfractionated cells. The HbF content of TfR− cells should be slightly higher than the values shown for the unfractionated cells in Table 5, since both TfR− and TfR+ cells are included in this population. The average F cell contains about one third HbF and two thirds HbS.[19] Therefore, the percentage of F cells is about three times the percent HbF. From the values for percent HbF in Table 5, it may be estimated that, on average for these eight patients, approximately 40% to 50% of TfR− cells and 10% of TfR+ cells are F cells.

DISCUSSION

Sickle-cell dehydration occurs in vivo, and the presence of young reticulocytes among dense cells suggests this process occurs early in some cells. Nevertheless, the mechanism(s) and precise timing of dehydration remain unclear. TfR+ cells represent a younger subpopulation of reticulocytes. For this reason, and also because survival selection may take place even in these very young cells, their behavior may not be equivalent to the entire reticulocyte population. For some TfR+ cells, KCl cotransport activity may mediate rapid dehydration without the need for deoxygenation. In the peripheral blood, the cells that exhibit this type of behavior are already variably dehydrated, and perhaps at an equilibrium state with respect to KCl cotransport, but may be detected by their rapid density shift without deoxygenation after nystatin rehydration.[11] In the experiments presented here, the cells were not rehydrated, and there was little or no density change observed under oxygenated conditions.

Previous experiments,[3] using a continuously deoxygenated light fraction, demonstrated a relatively small ($\approx$ 0.7 mmol/loc/h) excess of K^+ efflux over Na^+ influx in the presence of Ca^{2+}, a net cation change that would not be expected to cause significant density shifts in short in vitro experiments. This K^+ efflux was not influenced by inhibitors of $K(Ca^{2+})$ and was attributed to a direct deoxygenation-induced pathway. In these experiments,[3] there was no attempt to identify reticulocytes or TfR+ cells, but a high percentage of the cells must have been TfR−. The lack of density shift with continuous deoxygenation for TfR− cells in the current study is consistent with the small net cation efflux in these cells. The contribution of TfR+ cells to K^+ flux could have been overwhelmed by the more numerous mature cells in these earlier experiments.[3] The ability to focus on the cells of interest, even though they are relatively small in number, is an important feature of our experimental approach in the current experiments.

It is difficult to predict, on the basis of in vitro experiments, which pathway(s) are most important for younger and older sickle cells in vivo. Mechanisms that appear relatively unimportant in short-term experiments may dominate over a long period in the circulation. However, short in vitro experiments are more relevant for young cells, in which hydration changes appear to occur more quickly. The controlling pathway(s) for all cell types may be dependent on the in vitro deoxygenation conditions, which are unavoidably different from those in vivo.

Activation of $K(Ca^{2+})$ appears to be important in vivo, at least for older cells, as evidenced by changes in the density profile of circulating RBCs after administration of the $K(Ca^{2+})$ inhibitor clotrimazole to transgenic sickle mice[20] and to patients with sickle-cell disease.[21] The in vivo importance of KCl cotransport is less well defined, since no inhibitors of this pathway have been used for in vivo studies.

These studies highlight the importance of identifying RBCs by age. Reticulocytes, and the even younger subpopulation of TfR+ cells, may differ from mature RBCs in many ways important to the dehydration process. Certainly, the activity of age-dependent transport pathways, including KCl cotransport, will change with age. Intracellular Hb concentration and composition, degree of membrane oxidation, and level of a number of proteins shed during reticulocyte membrane remodeling[22] will also vary. As previously shown for

reticulocytes,[2] it appears that young TfR+ cells are more sensitive to dehydration upon deoxygenation in vitro than older TfR− cells. This is true not only for the KCl cotransport pathway, which is expected to be more active in younger cells, but also for K(Ca^{2+}).

The TfR+ cells contain a lower percentage of F cells than the TfR− cells,[14] most likely as a result of a longer circulation survival. For the patients in this study (Table 5), the percentage of F cells, estimated as three times the percent HbF, was approximately 10% for TfR+ cells and 40% to 50% for the entire RBC population. Since F cells would be expected to sickle less when deoxygenated, this may contribute to the increased sensitivity of the TfR+ population. In support of this is the recent demonstration that sickle reticulocytes become more deformed upon deoxygenation than mature RBCs.[23] These factors make it difficult to compare directly the hydration changes that occur in TfR+ and TfR− cells during deoxygenation. However, it is clear from these studies that very young TfR+ are sensitive to DI dehydration, and that the mechanisms are not entirely the same for cyclic and continuous deoxygenation.

The Ca^{2+}-dependent component of dehydration is present with both continuous and cyclic deoxygenation, and therefore does not require a period of oxygenation. However, the KCl cotransport component does require periodic oxygenation. This may be due to kinetic differences in metabolic changes that occur during deoxygenation cycles. For example, deoxygenation induces a generalized dephosphorylation,[24] which may activate KCl cotransport.[25] However, this is counterbalanced by the increase in intracellular free Mg^{2+} (due to preferential 2,3-DPG binding to deoxyhemoglobin), which is known to inhibit KCl cotransport.[26] Therefore, deoxygenation alone may have little or no effect on KCl cotransport activity. Upon reoxygenation, free Mg^{2+} returns to a lower concentration quickly, but rephosphorylation may take longer. Under these assumptions, a transient period of increased KCl cotransport activity would occur following reoxygenation.

Activation of KCl cotransport as outlined here is different than a proposed mechanism of reticulocyte dehydration,[2,27] in which a DI influx of Ca^{2+} causes an initial K^+ efflux, which leads to a decreased intracellular pH and acid activation of KCl cotransport. This activation may continue even in the absence of further Ca influx and K(Ca^{2+}) channel activation. Thus, KCl cotransport activation by this mechanism would require Ca^{2+} during the stimulating deoxygenation. Furthermore, prior activation would continue during a subsequent oxygenated state (in fact, would be optimal due to lower Mg^{2+}). The data presented here do not support these predictions, since we found that KCl cotransport was stimulated by cyclic deoxygenation in the absence of Ca^{2+} (Table 4). With continuous deoxygenation, the dehydration was mediated only by the Ca^{2+}-dependent pathway, with no Cl-dependent component detectable. In the oxygenated controls, there was little or no density shift.

In summary, this study confirms that young sickle RBCs have a greater tendency to dehydrate upon deoxygenation than more mature cells.[2] Evidence has been presented to support the role of KCl cotransport in this process when the cells are subjected to cyclic deoxygenation with a 15-minute period. Under these conditions, activation of KCl cotransport did not require Ca^{2+}. We speculate that such activation may result from an interplay between changes in free cellular Mg^{2+} levels and membrane phosphorylation[24] associated with deoxygenation.

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