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Volume-Stimulated, Cl^--Dependent K^+ Efflux is Highly Expressed in Young Human Red Cells Containing Normal Hemoglobin or HbS

Mitzy Canessa†, Mary E. Fabry‡, Nadia Blumenfeld‡, and Ronald L. Nagel‡

†Endocrine-Hypertension Unit, Brigham and Womens Hospital, Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, and ‡Division of Hematology, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York 10461

Summary. We report here that a Cl^--dependent K^- (K : Cl) efflux, which is stimulated by N-ethylmaleimide (NEM) and by increased red cell volume, exists in young red cells of individuals with normal hemoglobin A (AA) and in those homozygous for hemoglobin S (SS). We have investigated this K : Cl efflux in several density-defined red cell fractions obtained from Percoll-Stractan continuous density gradients. We found high activity of the NEM-stimulated K : Cl transport in reticulocytes and young red cells from nine sickle cell (SS) patients (43 ± 27 mean ± SD mmol K^+/liter of cells/hr = flux units (FU)) and in the young cell fraction of three AA individuals with high reticulocytosis recuperating from nutritional anemias (41.7 ± 10 FU). In addition, we observed significant interindividual variation of this K : Cl efflux in the discocyte fraction of SS blood. Cell swelling markedly stimulated the K : Cl efflux, in SS whole blood (9.8 ± 7.4 FU, in SS young cells (13 ± 13 FU), and in AA young cells (21.4 ± 11 FU). The activity of the Na-K-Cl cotransport, as estimated by the bumetanide sensitive K^+ efflux was not found to be cell-age dependent in either AA or SS cells.

Measurements of red cell density by isopycnic gradients indicated that 27% of the young cells reduce their volume by a Cl^--dependent process in hypotonic or low pH-induced swelling.

The large volume-stimulated K : Cl efflux in AA young cells raises the possibility that these fluxes may be involved in the maturation of erythropoietic precursors. The high activity in the red cells of sickle cell anemia patients and its interindividual variation may have pathophysiological consequences since it reverses the decrease in the intracellular concentration of hemoglobin which occurs in response to low pH or osmolarity, an unwelcome pro-sickling event.

Key Words potassium efflux · volume stimulated · chloride dependent · sickle cell anemia · reticulocyte · red blood cell

Introduction

We have recently reported (Canessa, Spalvins & Nagel, 1986*c*,*d*) elevated values of volume and N-ethylmaleimide (NEM) stimulated, chloride-dependent K^+ (K : Cl) efflux from the red cells of patients homozygous for the Hb S (SS) and Hb C (CC) gene. This property is only minimally expressed in the red cells of normal individuals (homozygous for hemoglobin A, AA). In those studies we showed that oxygenated SS, SC, and CC cells have high values of a Cl^--dependent, ouabain and bumetanide-resistant K^+ efflux which can be stimulated by an increase in red cell volume induced by hyposmotic or acid medium. Ouabain inhibits the ATP-dependent Na^+/K^+ pump and bumetanide the Na-K-Cl cotransport (Glynn & Karlish, 1975; Palfrey & Greengard, 1981). The K : Cl efflux of SS red cells is stimulated by NEM and cell swelling as previously observed in LK sheep (Lauf & Theg, 1980) and in mature AA human red cells (Wiater & Dunham, 1983; Lauf, Adragna & Garay, 1984; Kaji & Kahn, 1985; Lauf, Perkins & Adragna, 1985; Weder & Torreti, 1985). Stimulation of ouabain and bumetanide-resistant K^+ efflux by swelling induced by hyposmotic or acid media was previously observed in CC (Brugnara et al., 1985) and SS cells (Canessa et al., 1986*c*,*d*; Brugnara, Bunn & Tosteson, 1986).

We have previously shown that SS and SC cells are highly heterogeneous in cell density (Fabry & Nagel, 1982*a*; Fabry et al., 1982). Density heterogeneity is particularly important in sickle cell patients because both the extent and rate of deoxygenation-induced polymerization are strongly dependent on the intracellular hemoglobin concentration (MCHC) (Eaton, Hofrichter & Ross, 1976) which is directly proportional to the red cell density (Lee, Kirk & Hoffman, 1984). In AA individuals there is a clear relation between red cell density and cell age, with reticulocytes having the lowest densities and the oldest cells occurring at the highest density (Hoffman, 1958; Piomelli, Lurinsky & Wasserman, 1967). The densest SS cells are not necessarily the oldest, but the reticulocyte count is highest in the least dense cells (Kaul et al., 1983). We have previ-

ously reported that activity of the red cell age-dependent enzyme G-6-PD (glucose-6-phosphate dehydrogenase) decreases as cell density increases (Fabry & Nagel, 1982*a*).

To define the nature of the high activity of the volume-stimulated K : Cl efflux in sickle cells it is important to determine if these fluxes are affected by red cell age. If the activity increases with red cell age, this would suggest that the cumulative effect of red cell membrane damage induced by the presence of the abnormal hemoglobin is involved. On the other hand, if the K : Cl efflux is present predominantly in young cells it might be a property related to the requirements of erythropoietic precursors and present in these hemoglobinopathies as a consequence of hemolysis.

We will report here the correlation between the expression of the K : Cl efflux and Na-K-Cl cotransport and red cell density (and red cell age) in AA and SS subjects and the interindividual differences observed among SS patients.

This work was first presented at the 1986 Annual Meeting of the Society of General Physiology (Canessa et al., 1986*b*).

Materials and Methods

Patient Material

Blood from patients followed in the Heredity Clinic of the Albert Einstein College of Medicine was obtained after informed consent and characterized by two electrophoretic methods and a solubility test as homozygous for hemoglobin S (SS). Also studied were patients homozygous for hemoglobin A with high reticulocyte counts secondary to the treatment of pernicious anemia, folic acid or iron deficiency.

Transport Measurements

Blood was drawn in heparinized tubes, and centrifuged at 2000 × *g* for 10 min to remove plasma. Red cells were washed once and suspended in a preserving solution for overnight shipment from New York to Boston, as previously described (Ostrow et al., 1983); density-defined red cells prepared as described below were similarly treated. The next day, an aliquot of the red cells was washed 3 times with a solution containing (mM): 150 choline-Cl, 1 $MgCl_2$, 10 Tris-Mops, pH 7.4 at 4°C.

Cation Loading Procedure

Because SS and AA cells contain variable amounts of Na^+ and K^+, all cells and fractions were loaded by a modified nystatin procedure (Canessa et al., 1986*a*,*d*) to have 11.5 ± 2 Na^+ and 95 ± 9 K^+, mmol/liter cell.

After nystatin removal, the cells were washed 4 times at 4°C with a washing solution containing (mM): 150 glucamine-nitrate, 1 $MgCl_2$, 10 Tris-Mops, pH 7.4 at 4°C, and resuspended in the same solution at a hematocrit of 40% (vol/vol) for measurements of Na^+ and K^+ content, hemoglobin g/dl, and K^+ efflux.

Measurement of the K^+ Efflux

All efflux media contained: 10 mM glucose, 10 mM Tris-Mops of the indicated pH, 0.1 mM ouabain and 1 mM $MgCl_2$. The osmolarity of all solutions was measured.

To measure the ouabain-resistant basal K^+ efflux, red cells were incubated at 1–2% vol/vol hematocrit in *medium A:* (mM) 140 NaCl, pH 7.4 at 37°C; or in *medium B* (mM): 140 $NaNO_3$, pH 7.4 at 37°C or in *medium C* (mM): medium *A* + 0.01 bumetanide. The Cl^--dependent K^+ efflux was determined from the rate in medium *A* minus the rate in medium *B*. The bumetanide-sensitive K^+ efflux was determined from the rate in medium *A* minus the rate in medium *C*.

To measure the volume-dependent K^+ efflux, cell swelling was produced by reducing the osmolarity of the pH 7.4 media from 300 to 220 mOsm/liter (osmotic procedure) or the pH from 7.4 to 7 (acid pH procedure). The cells were incubated (1–2% vol/vol hematocrit) in medium *D* (*medium D* (mM): 100 NaCl, 0.01 bumetanide, pH 7.4 at 37°C). The volume-dependent K^+ efflux was calculated from the difference between medium *D* minus medium *C*. The Cl^--dependent volume-stimulated K^+ efflux was estimated as the difference between medium *D* minus medium *E*; (*medium E* (mM): 100 $NaNO_3$, 0.01 bumetanide, pH 7.4 at 37°C).

The response to acid-pH induced swelling was estimated as the difference in K^+ efflux between medium *F* and medium *C* or *G* (*medium F* (mM): 140 NaCl, 0.01 bumetanide, pH 7 at 37°C and *medium G,* containing 140 $NaNO_3^-$, 0.01 bumetanide, pH 7 at 37°C). To measure the NEM-stimulated K^+ efflux, media *A* and *B* were made 1 mM in NEM using a 1 M solution in DMSO.

The chilled cell suspensions (7 ml) in the different media were distributed into three tubes used for 0 time and into another three for 30 min. The transport reaction was initiated and terminated as previously described (Canessa et al., 1986*d*). Ion fluxes per volume of cells were estimated in mmol/liter cell × hr (flux units = FU) from the changes in external K^+ concentrations and the measured hematocrit of the suspension.

Cell Fractionation by Density Gradient Centrifugation

Preparation of Analytical Density Gradients

Percoll (colloidal silica coated with polyvinylpyrrolidone: Pharmacia Fine Chemicals, Piscataway, NJ) and Stractan (arabinogalactan polysaccharide: St. Regis Paper Co, West Nyack, NY) gradients were formed from a mixture of Percoll and Stractan as described elsewhere (Fabry et al., 1984). Stractan was prepared as described by Corash et al. (1974). The density gradients were photographed with Polaroid 46-L continuous density transparency film. The percent of cells present in each depth of the tube was determined by reading the transparencies in a Corning model 720 densitometer to give ten values.

Preparation of Density-Defined Fractions of Red Cells

For preparation of 0.5 to 1.0 ml of packed cells of density (MCHC) defined classes of sickle cells, 0.5 ml of whole blood adjusted to hematocrit 50% (vol/vol) was added to 5.5 ml per

tube of the gradient mixture. The mixture was centrifuged and the cells were aspirated from the formed gradients to previously established depths in the tube with a pipette. They were washed three times with isotonic saline and either used immediately or stored in autologous plasma. Density classes prepared by this technique are very sharply defined. This is demonstrated by re-centrifuging 50 μl of cells on an analytical gradient, which indicated that less than 5% of the cells exceeded the upper or lower density limits as described previously (Fabry et al., 1984).

Preparation of Hypotonic and Low pH NaCl and $NaNO_3$ Density Gradients

For gradients in which the NaCl was replaced with $NaNO_3$, the Stractan was treated twice with Amberlite MB-3 (Sigma, St. Louis, MO) to reduce the chloride concentrations below 3 mM. The pH was adjusted with HNO_3, and the NaCl in the balanced salts was replaced with $NaNO_3$. Hypotonic gradients were formulated by adding a smaller amount of the 10X balanced salts and replacing the missing volume with water. All gradients used in this experiment had 0.1 mM ouabain added. The osmolarity was determined using a Microosmette (Precision Systems, Sudbury, MA). Cells were incubated at hematocrit 5% (vol/vol) for 30 min at 37°C in the appropriate mixture (220 mOsm or 280 mOsm NaCl or $NaNO_3$ with 10 mM glucose, 3% BSA, and 0.1 mM ouabain). An aliquot of the cell suspension was then adjusted to hematocrit 50% (vol/vol), applied to the appropriate gradient and centrifuged as usual.

Low pH gradients were titrated to pH 7.0 with 0.01 M HCl or HNO_3, an equal volume of water was added to the pH 7.4 mix so that the composition of all gradient mixtures was the same. For low pH studies the cells were incubated for 30 min at hematocrit 5% (vol/vol) at 37°C in the appropriate mixture (40 mM HEPES at pH 7.0 or 7.4 adjusted with HCl or HNO_3, NaCl or $NaNO_3$ at 280 mOsm, 3% BSA, 10 mM glucose, and 0.1 mM ouabain). At the end of the incubation period the hematocrit was adjusted to 50% (vol/vol). Intra- and extracellular pH's were measured by packing the cells by centrifugation, removing the supernatant and measuring its pH (pH_e). The cells were hemolyzed by alternate freeze-thaw using liquid N_2, and the intracellular pH (pH_i) was measured with a gun electrode. A separate aliquot of cells adjusted to hematocrit 50% (vol/vol) was applied to the appropriate gradient and centrifuged as usual.

Statistical Analysis

Statistical analysis was performed using the program Statgraphics version 2.0 (Statistical Graphics, Rockville, MD) installed on an IBM-AT computer (M = mean, SD = standard deviation).

RESULTS

Characteristics of Red Cell Density Separated Fractions

Three density-defined fractions were separated from high reticulocyte AA blood: Fraction AA1 contains cells with an MCHC of less than 33 g/dl in isotonic media and has over 90% reticulocytes defined by supravital stain. Fraction AA2 has an MCHC between 33 and 36 g/dl, and fraction AA3 contains the oldest cells and has an MCHC greater than 36 g/dl.

Four density-defined fractions were separated from SS blood: Fraction SS1 contains the younger cells, with a variable reticulocyte count (40.5% ± 29.5), and has cells with a MCHC less than 33 g/dl with initial values of intracellular K^+ of 80 ± 6 mmol/liter cells (mean ± SD). Fraction SS2, contains reversible discocytes, which change shape with deoxygenation; the reticulocyte count of this fraction is also variable (24% ± 22). These cells have MCHC's between 33 and 37 g/dl, and an intracellular K^+ of 80 ± 8 mmol/liter cells. Fraction SS3 consists of unsicklable SS discocytes (which do not change shape appreciably with deoxygenation), and are probably the oldest SS cells, and has cells with MCHC's between 37 and 42 g/dl, and an intracellular K^+ of 62 ± 6 mmol/liter cells. Finally, SS4 has irreversibly sickled cells (ISC's) and some very dense unsicklable SS discocytes. These are not the oldest cells in the sickle cell anemia blood because some reticulocytes are found in this fraction. These cells have an MCHC greater than 42 g/dl and have an intracellular K^+ of 28 ± 6 mmol/liter cells. We have published detailed morphological description of the cells found in each fraction (Kaul et al., 1983).

Cl^--Dependent K^+ Transport in AA Reticulocytes

Three AA patients with high reticulocyte counts secondary to treated nutritional anemias were studied. Reticulocyte-enriched fraction AA1 was obtained preparatively by centrifugation of whole blood using Percoll-Stractan gradients as described in Materials and Methods. Values of K^+ efflux from the AA1 fraction for patient MM incubated in several different media as described in Materials and Methods are shown in Fig. 1. The basal ouabain-resistant K^+ efflux into 140 mM NaCl, pH 7.4 (medium *A*) decreases from 6.1 to 5.6 when bumetanide (medium *C*) is added to block the Na-K-Cl co-transport or to 5.3 when Cl^- is replaced by NO_3^- (medium *B*). Hence, the bumetanide-sensitive K^+ efflux was 0.5 ± 0.18 FU, whereas the chloride-dependent efflux was 0.8 ± 0.3 FU. Addition of 1 mM NEM increased K^+ efflux in chloride medium to 43.3 FU but not in nitrate medium; thus all of the NEM-stimulated K^+ efflux (37 FU) is chloride dependent. Incubation of reticulocyte-enriched AA1 cells in a medium containing bumetanide at pH 7 (medium *F*) stimulated K^+ efflux to 12.5 FU in chloride but not in nitrate (medium *G*). Acid pH-induced swelling stimulated a chloride-dependent K^+

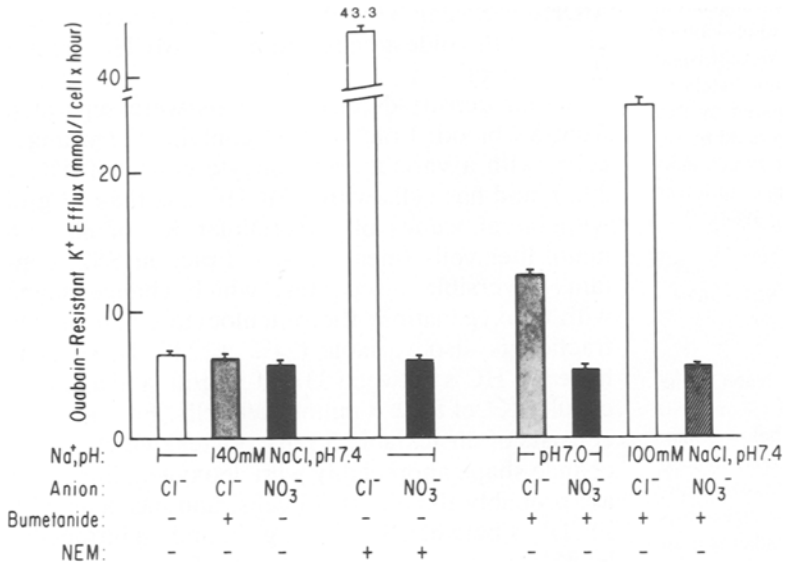

Fig. 1. Properties of potassium efflux from AA1 (reticulocyte-enriched cells from a patient (MM) homozygous for hemoglobin A). Red cells were loaded to contain 13.6 Na^+ and 120 K^+ mmol/liter cell as described in Materials and Methods. The patient had 10% reticulocytes. All K^+ efflux measurements were made in the presence of 0.1 mM ouabain, and the media composition were varied as indicated in the figure and in Materials and Methods

efflux of 6.7 FU. Incubation of AA1 in hypotonic media (medium *D*) stimulated K^+ efflux up to 24.6 FU into chloride (medium *D*) but not into nitrate medium (medium *E*). The volume-stimulated, chloride-dependent K^+ efflux was 19 FU.

For the three individuals studied, the density-defined fractions AA1, AA2, and AA3 had mean (± SD) values of basal ouabain-resistant K^+ efflux of 8.3 ± 3.9, 2.6 ± 0.6, and 1.9 ± 0.5 FU, respectively. The NEM-stimulated K : Cl efflux in oxygenated whole blood of these three high reticulocyte individuals (Table 1) was three times the reported values for normal whole blood (8 FU) (Wiater & Dunham, 1983; Lauf et al., 1984; Kaji & Kahn, 1985; Weder & Torreti, 1985; Canessa et al., 1986*d*). The reticulocyte-enriched fraction (AA1) exhibits an even greater NEM-stimulated K^+ efflux (seven times the normal flux). Fraction AA2, composed of mature red cells, exhibits a NEM-stimulated K^+ efflux very similar to that observed for normal whole blood. This is not surprising since AA2 corresponds to the most abundant class of cells present in whole blood. Fraction AA3, which consists of dense and old normal red cells, exhibited a very low activity of the NEM-stimulated K : Cl efflux. A multifactor analysis of the variance (ANOVA) using a 95% confidence interval revealed that the mean values of the NEM-stimulated K^+ efflux was significantly different for the three density-defined classes (AA1, AA2, AA3) with a $P > 0.0003$.

The volume-stimulated K^+ efflux is markedly stimulated by reduced osmolarity in fraction AA1, but it is not significantly stimulated in fraction AA2 or AA3. The mean values of the volume-stimulated potassium efflux were significantly different at a confidence interval of 95% with a $P > 0.0112$.

The bumetanide-sensitive K^+ efflux, which is an estimation of the Na-K-Cl cotransport, was not significantly different between the fractions under the conditions of these experiments (Table 1). Since the K_m of intracellular sodium for the stimulation of furosemide-sensitive K^+ efflux has been shown to vary between 6–10 mmol/liter cell (Canessa et al., 1986*a*) and the bumetanide-sensitive K^+ efflux has similar values, our estimation of the Na-K-Cl cotransport does not reflect its maximal activity. The low V_{max} of this cotransport in red cells from black individuals may account for their rather low bumetanide inhibitable fluxes (Canessa et al., 1984).

Cl^--Dependent K^+ Transport in Density-Defined SS Cells

Red cells from SS patients have a high NEM-stimulated K : Cl efflux in whole blood (Table 2). We show here that the NEM-stimulated K^+ efflux is large in the reticulocyte-enriched fraction SS1 but also high in fraction SS2, which contains younger cells than fraction AA2 in a normal individual. The NEM-stimulated K : Cl efflux in whole blood (Table 2) is not significantly different from values previously reported (Canessa et al., 1986*c*,*d*) (Table 2; Fig. 2). The cells from fraction SS3 (dense unsicklable SS discocytes) and fraction SS4 (very dense unsicklable SS discocytes and irreversibly sickled cells) exhibit lower levels of this K : Cl efflux and approach values for normal whole blood. Figure 2

Table 1. Distribution of the NEM-stimulated, volume-dependent K^+ efflux in AA red cell density fractions from high reticulocytes containing blood cell fractions

	AA1	AA2	AA3	Whole blood	Reticulocytes (% in WB)
1. Subject MM					
ΔNEM-stimulated[a]	37	9.4	2.9	9.0	10.4
ΔVolume-stimulated[a]	19.0	0	0	1.5	
ΔBumetanide-sensitive[a]	0.4	0.1	0.8	0.4	
MCHC	24.3	33.7	36.8	34.0	
K_i	120	108	93.0	101	
2. Subject DP					
ΔNEM-stimulated	35	8.4	2.2	20.5	17.5
ΔVolume-stimulated	11.6	0.6	0.9	5.7	
ΔBumetanide-sensitive	0.4	0.5	1.3	0.3	
MCHC	25.9	30.9	34.6	31.3	
K_i	122	102	105	109	
3. Subject GR					
ΔNEM-stimulated	53.2	8	1.2	19.7	27.5
ΔVolume-stimulated	33.6	1.3	0.9	12.0	
ΔBumetanide-sensitive	1.4	0	0.2	0	
MCHC	24.8	32.6	36.5	33.3	
K_i	121	103	99	102	
Mean (SD)					
ΔNEM-stimulated	41.7 ± 10.0	8.6 ± 0.7	2.1 ± 0.9	16.4 ± 6.4	*P*1
ΔVolume-stimulated	21.4 ± 11.2	0.2 ± 0.6	0.6 ± 0.5	6.4 ± 5.3	*P*2
ΔBumetanide-sensitive	0.7 ± 0.6	0.2 ± 0.3	0.8 ± 0.6	0.3 ± 0.2	

[a] K^+ efflux measurements, mmol/liter cell/hr. *See* Materials and Methods for details.

K_i = cell K content mmol/liter cell after nystatin loading. WB = whole blood. AA1, AA2, AA3 are density (g of Hb/dl) defined red cell fractions prepared by centrifugation in Percoll-Stractan gradients as described in Materials and Methods. MCHC = mean corpuscular hemoglobin concentration g/dl. $P1 = P > 0.0003$, means of AA1, AA2, AA3 are different at 95% confidence. $P2 = P > 0.0112$, means of AA1, AA2, AA3 are different at 95% confidence.

also reveals the marked inter-individual differences in the magnitude of the NEM-stimulated K : Cl efflux in fractions SS1 and SS2. Analysis of the variance at a 95% confidence interval (ANOVA) revealed that the mean NEM-stimulated K^+ efflux of the density-defined fractions SS1, SS2, SS3 and SS4 were significantly different with $P > 0.0045$. Notice that the NEM-stimulated activity of SS4 is almost three times higher than that of AA3. This is to be expected since the densest SS cells are much younger than the densest AA cells.

The ouabain-resistant K^+ efflux into isotonic Na medium was 7.5 ± 2.8, 5.8 ± 3.2, 4 ± 0.8 and 4 ± 0.5 in SS1, SS2, SS3 and SS4 in eight different subjects. The volume-stimulated K^+ efflux varies widely in the different SS density-defined fractions (Table 2). These values reflect stimulation of K^+ efflux by incubation in hypotonic media (medium *D*—medium *C*). The volume-stimulated K : Cl efflux shows a maximum response to low osmolarity in fraction SS2, which has a high NEM-stimulated K^+ efflux; however, a negligible volume-stimulated response was observed in fraction SS4 which has 10 FU of NEM-stimulated K : Cl efflux. In many cases, there was insufficient material to determine volume-stimulated K^+ efflux in SS1 fraction. It can also be seen from Table 2 that volume-stimulated K : Cl efflux and bumetanide-sensitive efflux are not equally distributed in density-defined fractions of SS red cells. The mean values of the volume-stimulated K^+ efflux was significantly different for the four density-defined fractions with $P > 0.05$. The bumetanide-sensitive K^+ efflux does not show significant differences among the SS cell density fractions (Table 2).

The correlation between reticulocyte count in whole blood and individual fractions ($n = 22$) and the K : Cl efflux (as measured by the NEM-stimulated fluxes) was found to be significant at a $P < 0.0002$, but an appreciable dispersion of the points is observed.

Activation of Cl^--Dependent K^+ Transport and Cell Density Changes

We have previously shown (Fabry & Nagel, 1982*b*) that continuous density gradients allow the detec-

Table 2. Distribution of the NEM-stimulated, the volume-dependent and the bumetanide-sensitive K^+ efflux in SS red cell density fractions

Patient		SS1	SS2	SS3	SS4	WB	% Reticulocytes
EM	ΔNEM-stimulated	26	21	16	7	32	8.0
	ΔVolume-dependent	0.9	2	2.3	0	8	
	ΔBumetanide-sensitive	4	1.5	0.8	1	0.9	
	MCHC	34.0	32.8	37.5	39.9	32.2	
TN	ΔNEM-stimulated	73	36.2	19.6	19.8	42.5	17.4
	ΔVolume-dependent	11.6	8.1	4.2	0	7.7	
	ΔBumetanide-sensitive	0.4	0	0	0	0.7	
	MCHC	34.8	30.4	34.5	40.4	41.5	
OE	ΔNEM-stimulated	14.3	10.7	16.4	6.9	24.9	8.8
	ΔVolume-dependent	0	5.4	4.0	0.0	6.8	
	ΔBumetanide-sensitive	0.16	1.3	0	0.24	1.2	
	MCHC	30.1	30.8	32.1	35.4	30.4	
WR	ΔNEM-stimulated	97.5	53.9		24.0	49.95	29.6
	ΔVolume-dependent	—	36.0		6.4	22.0	
	ΔBumetanide-sensitive	—	0.71		0.6	0.2	
	MCHC	26.3	35.3		33.7	30.5	
JN	ΔNEM-stimulated	67	48	20.5	17.5	35	7.1
	ΔVolume-dependent	—	27	7.0	—	19.2	
	ΔBumetanide-sensitive	—	0.11	0.9	—	1.0	
	MCHC	26.4	29.8	34.1	37.2	31.8	
HA	ΔNEM-stimulated	40.6	28.9	8.9	6.7	24.5	9.2
	ΔVolume-dependent	—	4.4	0	0	1.72	
	ΔBumetanide-sensitive	0.40	0	1.55	0	0.32	
	MCHC	33.6	34.8	39.8	37.5	37.7	
MG	ΔNEM-stimulated	22.3	24.8	10.0	5.5	20.4	—
	ΔVolume-dependent	1.13	8.4	0	0	1.8	
	ΔBumetanide-sensitive	0.86	0.10	0	0	0	
	MCHC	32.5	37.7	40.0	41.5	38.8	
Average values (±SD)	ΔNEM-stimulated	48.7 ± 31	31.9 ± 15.2	15.2 ± 4.8	10.5 ± 6.3	32.7 ± 10.6	*P*1
	ΔVolume-dependent	3.4 ± 5.5	13 ± 13	2.9 ± 2.7	0	9.6 ± 8.0	*P*2
	Mean K_i	98.8 ± 11.1	94.1 ± 11.2	88.5 ± 11.6	80.3 ± 6	93.6 ± 6.3	

NEM-stimulated, volume-dependent, bumetanide-sensitive, K efflux are expressed in mmol/liter cell/hr. K_i = cell K content of the nystatin-loaded cells in mmol/liter cell. WB = whole blood. SS1, SS2, SS3, SS4 are density-defined red cell fractions prepared from Percoll-Stractan gradients as described in Materials and Methods. $P1 = P > 0.0045$; means of SS1, SS2, SS3, SS4 are different at 95% confidence interval. $P2 = P > 0.05$; means of SS1, SS2, SS3, SS4 are different at 95% confidence interval.

tion of small changes in cell density. Red cell MCHC is directly proportional to red cell density, a fact illustrated by Lee et al. (1984), and hence an increase in density is indicative of an increase in MCHC. For aliquots of the same population of red cells, exposure to conditions that change the cell water content will alter the red cell MCHC and hence change in cell volume can be detected as changes in red cell density. In addition, the continuous density gradient method, uniquely, allows detection of heterogenous changes in red cell MCHC within a density-defined fraction in response to the challenging conditions. Since stimulation of K : Cl efflux by cell swelling can reduce cell water content and hence increase MCHC, we have examined the change in MCHC of the discocyte fraction of SS red cells (SS2) by density gradient in isotonic (280 mOsm/liter) and hypotonic (220 mOsm/liter) medium containing Cl^- and NO_3^- (Fig. 3*A*). Mature AA cells markedly swell and decrease their MCHC or density when incubated in both hypotonic Cl^- and NO_3^- medium. In contrast, 37% of the SS2 cells exposed to hypotonic chloride media did not decrease their density or swell as much as control cells exposed to hypotonic nitrate media. The isopycnic gradients were calibrated for MCHC as previously reported (Fabry & Nagel, 1982*b*) and the percent cells at a given depth in the centrifuge tube were determined by densitometry as previously described in Materials and Methods and Fabry et al.

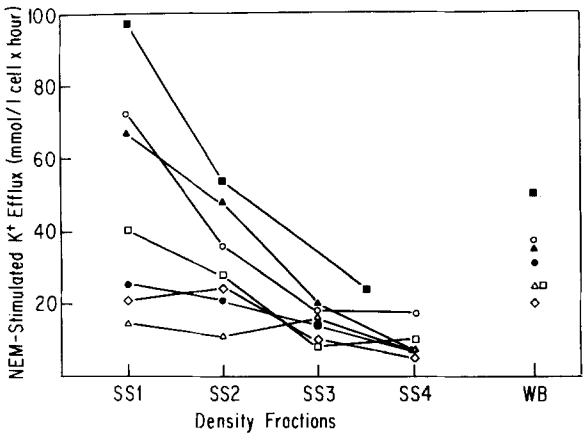

Fig. 2. NEM-stimulated potassium efflux from density-defined sickle cell fractions (*see* text for characteristics of these cells) from seven different patients. Note that, although SS1 usually has a higher efflux than SS2, SS3, or SS4, the relative values differ between patients as do the whole blood values

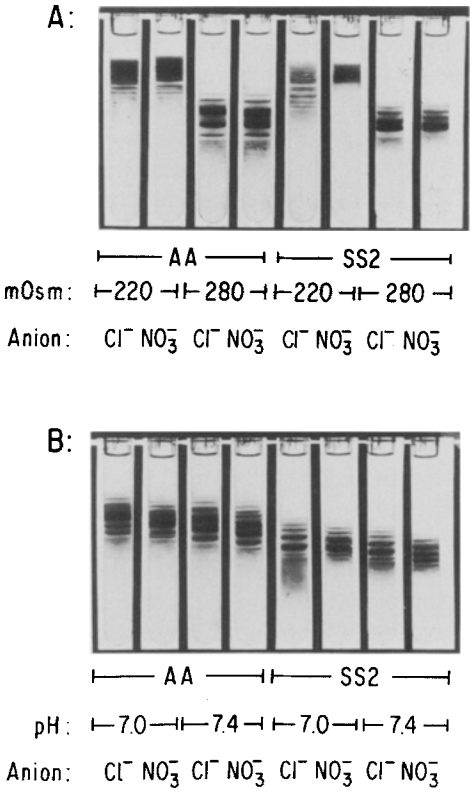

Fig. 3. (*A*) The effect of cell swelling on the density distribution of SS discocytes (SS2) and AA whole blood. Percoll-Stractan continuous density gradients were formulated with either Cl^- or NO_3^- at 280 and 220 mOsm. All tubes are at pH 7.4 and have 0.1 mM ouabain. The first four tubes on the left contain AA whole blood. Note that AA cells have a decreased density (MCHC) in hypotonic media irrespective of whether the anion is Cl^- or NO_3^-. The last four tubes contain the sickle cells from the discocyte fraction (SS2). Note that the SS2 cells in hypotonic NO_3^- media have the same density as AA cells in hypotonic media. However, in the presence of Cl^-, 37% of the SS2 cells exhibited a smaller decrease in MCHC in hypotonic chloride than do the control cells in hypotonic nitrate media. (*B*) The effect of cell swelling induced by acid pH on SS discocyte fraction (SS2) and AA whole blood. Percoll-Stractan continuous density gradients were formulated with either Cl^- or NO_3^-, pH 7.0 or 7.4, 280 mOsm; all tubes have 0.1 mM ouabain. The first four tubes on the left contain AA whole blood; note that the density (MCHC) is not influenced by the anion. The second four tubes contain SS2. Note that in Cl^- media at pH 7.0 the density of 31% of the cells is increased, but a similar effect is not observed in nitrate media. The depth in the tube of AA cells at pH 7.4 and 280 mOsm is different in *A* and *B* because the density profile (density as a function of depth in the tube) has been adjusted in *A* to accommodate the low density of cells in hypotonic medium. The whole blood from which SS2 was isolated was obtained from SS patient MK

(1984). A similar result, not illustrated here, was found for fraction SS1; in this case the percent of cells exhibiting a higher density (lower cell volume) in hypotonic chloride than cells swollen in hypotonic nitrate was larger (50%) than that observed for SS2 cells (Fig. 3).

In acid medium, pH 7.0, 21% of the SS2 cells in Cl^- have a higher MCHC than in NO_3^- medium; in contrast, at pH 7.4 there is little difference between the two media. Note that in nitrate, as anticipated, the cells have a lower MCHC at pH 7.0 than at 7.4. After 30 min of incubation and prior to the loading onto the gradient, the SS2 cells had an intracellular pH (pH_i) of 6.9 at an extracellular pH (pH_e) of 7.0, and at a pH_e of 7.4, SS2 cells had an pH_i of 7.2 in either chloride or nitrate. The AA whole blood did not have Cl^--dependent change in MCHC at either pH. Since incubation and centrifugation occupy a combined time of 1 hr, the time scale of these experiments is comparable to that of the potassium efflux experiments reported above. These results indicate that, even in density-defined fractions of cells, there is a population of cells with very high response to cell swelling.

Discussion

We have previously reported that the elevated values of K^+ efflux (in the presence of ouabain and bumetanide) from CC, SC and SS red cells can be identified as determined by a Cl^--dependent K^+ efflux which is stimulated by NEM and by low osmolarity or low pH-induced cell swelling (Canessa et al., 1986*c*,*d*). The findings presented here demonstrate that this mechanism is highly expressed in human AA reticulocytes as well as in most of the red cells of sickle cell anemia individuals. After the completion of this work Hall and Ellory (1986) also reported that young cells isolated from normal sub-

jects have a high activity of Cl^--dependent, volume-stimulated K^+ influx. In the case of SS patients, the high percentage of younger cells in circulation is a consequence of their short red cell survival (an average of 14 days compared to the normal of 120 days) (McCurdy, 1962). Our results indicate, therefore, that the high expression of volume-stimulated K : Cl efflux is a characteristic of oxygenated young red cells, which is independent of genetically determined changes in the primary structure of hemoglobin.

It is of interest that, even in AA blood with elevated reticulocytes, the mechanism is almost absent in older red cells as estimated by NEM- or volume-stimulated K^+ efflux (fraction AA3) (Table 1). In comparison, in sickle cell anemia blood, while the K : Cl efflux is more active in SS1 and SS2 (Table 2), the NEM-stimulated K^+ efflux in SS3 is still twice as high than that of AA2. This is not surprising since even the oldest cells in the sickle cell anemia patient are younger than the average red cell of a nonhemolytic individual. The densest SS cells (SS4, including irreversibly sickled cells, which are not necessarily older cells) have an activity of the NEM-stimulated K : Cl efflux similar to that of mature AA2 cells and three times higher than old AA3 cells (Table 2). We observed a high activity of volume-stimulated K^+ efflux in the fractions containing young cells (AA1, SS1, SS2). The lack of volume-stimulated response of the K^+ efflux of the densest cells (SS3 and SS4) as well as of AA2 and AA3 fractions despite the observation that they display significant NEM-stimulated K^+ efflux (Tables 1 and 2) deserves further investigation.

Since volume-stimulated K : Cl efflux decreases as SS cells age and become denser, it is unlikely that HbS is directly involved in activating this mechanism by electrostatic interactions, as suggested by Brugnara et al. (1985) or by directly damaging the red cell membrane.

We have observed marked interindividual variation in the activity of the K : Cl efflux in the discocyte fraction (SS2) of Hb SS patients (Table 2). The small number of samples studied in a recent report (Brugnara et al., 1986) did not permit the assessment of this phenomena.

Although the correlation coefficient between the extent of reticulocytosis and the magnitude of volume-stimulated K : Cl efflux was highly significant, appreciable dispersion of the values was observed. This finding might reflect poor correlation between reticulocyte count and the total number of young cells in addition to the large interindividual variation.

The marked cell-age dependance of the NEM-stimulated K^+ efflux in human red cells is reminiscent of that observed by Lauf in HK and LK sheep (Lauf, 1983).

The presence of this transport mechanism in young human red cells raises the question of its function in human erythropoiesis. Cell volume reduction during the maturation of normal red cells, in addition to membrane loss, might be important in a number of developmental steps, including maturation of precursors and passage through the marrow sinusoids into the general circulation. Further reduction in volume is required to acquire the ideal volume to transverse the microcirculatory bed successfully. The role of K : Cl fluxes in these events deserves further study.

As we pointed out before (Canessa et al., 1986*c*,*d*, 1987), the presence of a volume-stimulated K : Cl efflux in most red cells of sickle cell anemia patients has potential pathophysiological implications. Since SS red cells are likely to encounter environments with low pH (in some tissues and in transient vasoocclusions), the swelling response would be reversed by this transport mechanism. That volume correction actually occurs is illustrated in Fig. 3, which demonstrates that a population of SS discocytes (SS2) exhibit a Cl^--dependent decrease in cell density after being exposed to hypotonic or acid media for 1 hr. This result is in agreement with the observation that this density fraction exhibits the highest activity of volume-stimulated K^+ efflux. This volume regulatory decrease response is, of course, an unwelcome effect since it increases the MCHC and favors sickling (Bookchin, Balazs & Landau, 1976). The results presented here do not agree with recent studies of Brugnara et al. (1986) on volume changes of SS whole blood homogenized in volume by nystatin loading. These authors observed a uniform and progressive decrease of cell volume after 4–6 hr of swelling, but they did not determine whether these long-term changes were chloride dependent. In contrast, our studies clearly indicate that only a fraction of the young cells change their volume after swelling for 1 hr by a chloride-dependent process.

Since significant interindividual variability of this volume- and NEM-stimulated K^+ efflux was observed in discocytes (SS2) (Fig. 2), we suggest that these fluxes might participate in the modulation of the phenotypic expression of sickle cell anemia.

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References

Bookchin, R.M., Balazs, T., Landau, L.C. 1976. Determinants of red cell sickling. Effects of varying pH and of increasing intracellular hemoglobin concentration by osmotic shrinkage. *J. Lab. Clin. Med.* **87**:597–616

Brugnara, C., Bunn, H.F., Tosteson, D.C. 1986. Regulation of erythrocyte cation and water content in sickle cell anemia. *Science* **232**:388–390

Brugnara, C., Kopin, A.S., Bunn, H.F., Tosteson, D.C. 1985. Regulation of cation content and cell volume in hemoglobin erythrocytes from patients with homozygous hemoglobin C disease. *J. Clin. Invest.* **75**:1608–1617

Canessa, M., Brugnara, C., Cusi, D., Tosteson, D.C. 1986*a*. Modes of operation and variable stoichiometry of the furosemide-sensitive Na and K fluxes in human red cells. *J. Gen. Physiol.* **87**:113–151

Canessa, M., Fabry, M.E., Spalvins, A., Blumenfeld, N., Nagel, R.L. 1986*b*. A volume-dependent KCl transporter is highly expressed in young human red cells with AA and SS hemoglobin. *J. Gen. Physiol.* **88**:14*a*

Canessa, M., Fabry, M.E., Spalvins, A., Blumenfeld, N., Nagel, R.L. 1987. Activation of a KCl transporter by cell swelling in HbSS red cells. *In:* Pathophysiological Aspects of Sickle Cell Vaso-occlusion. R.L. Nagel, editor. Alan R. Liss, New York (*in press*)

Canessa, M., Spalvins, A., Adragna, N., Falkner, B. 1984. Red cell Na counter-transport in normotensive and hypertensive blacks. *Hypertension* **6**:349–351

Canessa, M., Spalvins, A., Nagel, R.L. 1986*c*. Volume-dependent and NEM-stimulated K^+Cl^- transport is elevated in SS, SC and CC human red cells. *Biophys. J.* **49(2)**:580*a*

Canessa, M., Spalvins, A., Nagel, R.L. 1986*d*. Volume-dependent and NEM-stimulated K^+, Cl^- transport is elevated in oxygenated SS, SC and CC human red cells. *FEBS Lett.* **200**:197–202

Corash, L.M., Piomelli, S., Chen, H.C., Seaman, C., Gross, E. 1974. Separation of erythrocytes according to age on a simplified density gradient. *J. Lab. Clin. Med.* **84**:147–151

Eaton, W.A., Hofrichter, J., Ross, P.D. 1976. Editorial hypothesis. Delay time of gelation: A possible determinant of clinical severity in sickle cell disease. *Blood* **47**:621–627

Fabry, M.E., Kaul, D.K., Raventos-Suarez, C., Chang, H., Nagel, R.L. 1982. SC cells have an abnormally high intracellular hemoglobin concentration: Pathophysiological consequences. *J. Clin. Invest.* **70**:1315–1319

Fabry, M.E., Mears, J.G., Patel, P., Schaefer-Rego, K., Carmichael, L.D., Martinez, G., Nagel, R.L. 1984. Dense cells in sickle cell anemia: The effects of gene interaction. *Blood* **64**:1042–1046

Fabry, M.E., Nagel, R.L. 1982*a*. Heterogeneity of red cells in the sickler: A characteristic with practical clinical and pathophysiological implications. *Blood Cells* **8**:9–15

Fabry, M.E., Nagel, R.L. 1982*b*. The effects of deoxygenation on red cell density: Significance for the pathophysiology of sickle cell anemia. *Blood* **60**:1370–1377

Glynn, J.M., Karlish, S.J. 1975. The sodium pump. *Annu. Rev. Physiol.* **37**:13–55

Hall, A.C., Ellory, J.C. 1986. Evidence for the presence of volume-sensitive KCl transport in young human red cells. *Biochim. Biophys. Acta* **858**:317–320

Hoffman, J.F. 1958. On the relationship of certain erythrocyte characteristics of their physiological age. *J. Cell. Comp. Physiol.* **51**:415–423

Kaji, D., Kahn, T. 1985. Kinetics of Cl-dependent K influx in human erythrocytes with and without external Na: Effect of NEM. *Am. J. Physiol.* **249**:C490–C496

Kaul, D.K., Fabry, M.E., Windisch, P., Baez, S., Nagel, R.L. 1983. Red cells in sickle cell anemia are heterogeneous in their rheological and hemodynamic characteristics. *J. Clin. Invest.* **72**:22–31

Lauf, P.K. 1983. Thiol-dependent passive K/Cl transport in sheep red cells: II. Loss of Cl^-- and N-ethylmaleimide sensitivity in maturing high K^+ cells. *J. Membrane Biol.* **73**:247–256

Lauf, P.K., Adragna, N.C., Garay, R.P. 1984. Activation by NEM of a latent K^+Cl^- flux in human red blood cells. *Am. J. Physiol.* **246**:C385–390

Lauf, P.K., Perkins, C.M., Adragna, N.C. 1985. Cell volume and metabolic dependence of NEM activated K^+-Cl^- fluxes in low-K^+ sheep erythrocytes modulation by A23187 and bivalent cations. *Am. J. Physiol.* **249**:C124–C128

Lauf, P.K., Theg, B.E. 1980. A chloride dependent K^+ flux induced by N-ethylmaleimide in genetically low K^+ sheep and goat erythrocytes. *Biochem. Biophys. Res. Commun.* **92**:1422–1428

Lee, P., Kirk, G., Hoffman, J.F. 1984. Interrelations among Na and K content, cell volume, and buoyant density in human red blood cell populations. *J. Membrane Biol.* **79**:119–126

McCurdy, P.R. 1962. Erythrokinetics in abnormal hemoglobin syndromes. *Blood* **20**:686–699

Ostrow, D.G., Canessa, M., Sparks, S., Davis, G.M. 1983. Preservation of human erythrocytes for sodium-dependent lithium efflux rate measurements. *Clin. Chim. Acta* **129**:39–44

Palfrey, H.C., Greengard, P. 1981. Hormone-sensitive ion transport systems in erythrocyte as models for epithelial ion pathways. *An. N.Y. Acad. Sci.* **372**:291–308

Piomelli, S., Lurinsky, G., Wasserman, L.R. 1967. The mechanism of red cell aging. I. Relationships between cell age and specific gravity evaluated by ultracentrifugation in a discontinuous density gradient. *J. Lab. Clin. Med.* **69**:147–151

Weder, A.B., Torreti, B. 1985. Racial differences in bumetanide-sensitive cotransport and N-ethylmaleimide-stimulated potassium efflux. *Life Sci.* **36**:1813–1818

Wiater, L.A., Dunham, P.B. 1983. Passive transport of K^+ and Na^+ in human red blood cells: Sulfhydryl binding agents and furosemide. *Am. J. Physiol.* **245**:C348–C357

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