Volume-dependent and NEM-stimulated K^+,Cl^- transport is elevated in oxygenated SS, SC and CC human red cells

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Mechanisms involved in cell volume regulation are important in SS, SC cells as they might be involved in determining the extent of sickling and the generation of dense cells and irreversibly sickled cells. We have studied in these cells the response to cell swelling of the K^+,Cl^- transporter. We found that Hb SS, SC and CC red cells have higher values of a ouabain-resistant, chloride-dependent and NEM-stimulated K^+ efflux than AA red cells. In contrast, the Na^+,K^+,Cl^- cotransport estimated from the bumetanide-sensitive component of K^+ efflux was not significantly different in SS, SC and CC red cells. The (ouabain + bumetanide)-resistant K^+ efflux from SS, SC and CC red cells was stimulated by cell swelling induced by reduction of the osmotic pressure (300 to 220 mosmol/l) and pH (8 to 7) of the flux media (140 mM NaCl). The Cl^--dependent K^+ efflux stimulated by osmotic swelling highly correlated with the NEM-stimulated component ($r=0.8$, $p<0.001$, $n=22$) and the acid-pH-induced swelling ($r=0.969$, $p<0.001$, $n=22$), indicating that it is driven by the K^+,Cl^- transporter.

K^+ Efflux *K^+,Cl^- transporter* *Cell volume*

1. INTRODUCTION

Cells from sickle cell anemia (SS: homozygote for HbS) patients are characterized by a wide range in red cell volume as estimated by their densities and mean corpuscular hemoglobin (MCHC). A subfraction of cells whose percentage varies widely from individual to individual is extremely dehydrated and dense [1,2]. Hb CC (homozygote for HbC) cells are characterized by a rather homogeneous increase in red cell density [3]. The double heterozygote for HbS and HbC (SC disease) has red cell properties that combine the abnormalities found in the homozygote of each of these abnormal hemoglobins [4]. The mechanisms by which SS, SC and CC cells attain greater than normal densities are not understood, but may be related.

In addition, a number of epistatic effects on the expression of the SS genotype have been described including the copresence of α-thalassemia [5], variable levels of HbF [6] and different DNA β-gene cluster haplotypes associated with this genotype [7]. As differences in membrane cation transport systems have been described in the black population [8,9], it is conceivable that another source of heterogeneity, with potential effects on the modulation of the clinical expression of the disease, may be polymorphism of red cell cation transport systems.

In recent years, it has emerged that cell volume is regulated by different transport systems which are activated by either an increase or decrease of cell volumes. Cell swelling activates the K^+,Cl^- transporter of LK sheep red cells [10,11] and cell shrinkage activates the Na,K,Cl cotransport in duck red cells [12] and the Na^+/H^+ exchange in *Amphiuma* red cells [13]. Lauf et al. [14] reported that human red cells did not possess a basal Cl^--dependent K^+ efflux but an NEM-stimulated component. Brugnara et al. [15] have recently reported that CC cells are able to diminish the cell volume increase produced in response to hypo-osmolarity and acid pH by means of a (ouabain +

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bumetanide)-resistant K^+ efflux whose Cl^- dependency was not studied.

Here we have investigated the K^+,Cl^- transporter in SS, SC and CC cells involved in the volume reduction secondary to cell swelling and found that oxygenated SS, SC and CC cells have various degrees of greatly increased basal, Cl^--dependent K^+ efflux stimulated by cell swelling and NEM when compared to AA cells from black individuals. The magnitude of the increase varies from patient to patient, suggesting heterogeneity in the expression of this transport system of the type described in red cell density and volume distribution [1,2].

2. MATERIALS AND METHODS

2.1. *Materials*

Blood was obtained with informed consent from a pool of 100 patients followed at the Heredity Clinic of the Albert Einstein College of Medicine. They were identified as having the genotype SS, SC, CC and AA by two electrophoretic methods (cellulose acetate at pH 8.6 and agar electrophoresis at pH 6.4) and by a solubility test [5].

2.2. *Transport measurements*

Blood was drawn in heparinized tubes, and centrifuged at 2000 × *g* for 10 min to remove plasma. Subsequently, the red cells were washed once and suspended in a preserving solution for overnight shipment from New York to Boston, as described [9]. The next day, an aliquot of the red cells was washed 3 times with a washing solution containing (mM): 150 choline Cl, 1 $MgCl_2$, 10 Tris-Mops, pH 7.4, at 4°C. The cell volume was estimated measuring the changes in hemoglobin content as described [9].

Because SS, SC, CC and AA cells contain variable amounts of Na and K, all cell types were loaded by a modified nystatin procedure to have 11.5 ± 2 Na and 95 ± 9 K mmol/l cell; the cell volume was 0.96 ± 0.04 that of the original cells (mean ± SD, n = 22).

2.2.1. Nystatin loading procedure

2 ml packed cells were incubated with 10 ml nystatin loading solution containing (mM): 15 NaCl, 140 KCl, 50 sucrose and 40 μg/ml of nystatin. The cells were processed further as described [16]. 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, resuspended in the same solution at a hematocrit of 40% for measurements of Na and K content and hemoglobin/l cell.

2.2.2. Measurement of the basal K^+ efflux

To determine the Cl^--dependent K^+ efflux: red cells were incubated at 1–2% in medium A (mM) – 140 NaCl, 1 $MgCl_2$, 0.1 ouabain, 10 glucose, 10 Tris-Mops, pH 7.4, at 37°C; in medium B (mM) – 140 $NaNO_3$, 1 $MgCl_2$, 0.1 ouabain, 10 glucose, 10 Tris-Mops, pH 7.4, at 37°C. The Cl^--dependent efflux was determined from (A – B). To estimate the fraction of K^+ efflux transported by the Na^+,K^+,Cl^- cotransport system, the bumetanide-sensitive K^+ efflux was measured into medium C (medium A + 0.01 mM bumetanide).

2.2.3. Measurement of volume-dependent K^+ efflux

Cell swelling was produced by lowering of the osmotic pressure of the efflux media from 300 to 220 mosmol/l ('osmotic procedure') and reduction of the pH media from 8 to 7 ('acid-pH-induced swelling'). The response to osmotic and acid-induced swelling was studied in the presence of ouabain and bumetanide to block the Na^+ pump and the Na^+,K^+,Cl^- cotransport, respectively. At zero time the cell volume increased to 1.15–1.25-times the original volume.

To estimate the response to 'osmotic swelling', the cells were incubated (1–2% hematocrit) in medium D (mM): 100 NaCl, 1 $MgCl_2$, 0.1 ouabain, 0.01 bumetanide, 10 Tris-Mops, pH 7.4, at 37°C. The volume-dependent K^+ efflux was calculated from the differences between mediums D and C. The Cl^- dependence of the volume-stimulated K^+ efflux was determined from the difference in K^+ efflux between mediums D and E (mM): 140 $NaNO_3$, 1 $MgCl_2$, 0.1 ouabain, 0.01 bumetanide, 10 glucose and 10 Tris-Mops, pH 7.4, at 37°C.

The response to pH-induced swelling was studied by incubating the cells in isotonic medium C but buffered at pH 7 and 8. In acid medium, protons titrate the negative charge of hemoglobin and stimulate Cl^- entry to preserve electroneutrali-

ty; the elevation of cell chloride increased cell water content to 1.15–1.25-times the original volume. The acid-pH-induced swelling was estimated as the difference in K^+ efflux between mediums F and G. Medium F (mM): 140 NaCl, 1 $MgCl_2$, 0.1 ouabain, 0.01 bumetanide, 10 glucose, 10 Tris-Mops, pH 7, at 37°C and medium G, same as F but containing 10 mM Tris-Mops, pH 8, at 37°C.

2.2.4. Measurement of the NEM-stimulated K^+ efflux

Mediums A and B were made 1 mM NEM using a 1 M solution in DMSO. The time course of K^+ lost was determined by measuring the appearance of K^+ in the incubation media as a function of time in triplicate samples in the presence of NEM, which always rendered higher flux units. To ensure linearity, triplicate samples were taken at 0 and 30 min. Similar values were obtained by pretreatment with NEM for 5 min and then washing to remove the NEM. The chilled cell suspensions (7 ml) were distributed in 6 tubes, aerated with room air and capped. We tested the effect of CO treatment and aeration with oxygen to avoid cell sickling in red cells of one SS patient and found no significant differences in transport between these procedures. The transport reaction was initiated by warming to 37°C, stopped by cooling for 2 min and centrifuged for 5 min at 6000 × g in a Beckman Accuspin refrigerated centrifuge. The supernatants were transferred with plastic Pasteur pipettes into plastic tubes for determination of K^+ by atomic absorption spectrophotometry (Perkin Elmer 3030 B) as described [9,16].

3. RESULTS

Table 1 depicts the ouabain-resistant, Cl^--dependent K^+ efflux from SS, SC, CC and AA red cells under basal conditions and stimulated by NEM. The absence of Cl^--dependent K^+ efflux in AA cells under iso-osmotic conditions is in agreement with previous findings of Lauf et al. [14,17]. The most striking is the increase in NEM-stimulated K^+ efflux observed in SS, SC and CC cells in comparison with AA cells from black individuals. The increase in K^+ efflux produced by NEM in SS cells incubated in Cl^- medium (24.7 ± 8.7 mmol/l cell) was almost completely inhibited in nitrate medium (3.4 ± 1.7 mmol/l cell). The $MgCl_2$ concentration (1 mM) of the medium cannot account for the residual values. Furthermore, the Cl^--dependent, NEM-stimulated K^+ efflux was elevated and comparable for SS, SC and CC cells. The mean value of NEM-stimulated K^+ efflux from AA cells is not significantly different from the values reported by Lauf et al. [17] in red cells of Caucasians and by Weder et al. in black individuals [18].

Table 1

Basal and NEM-stimulated Cl-dependent K^+ efflux from SS, SC, CC and AA red cells

Cell type	Ouabain-resistant K^+ efflux (mmol/l cell per h)							
	NaCl			$NaNO_3$			Cl^--dependent	
	− NEM	+ NEM	Δ NEM	− NEM	+ NEM	ΔNEM	Basal	NEM-stimulated
AA (n = 9)	2.7 ± 0.8	13.8 ± 3.7	11.1 ± 3.1	2.5 ± 0.9	4.7 ± 0.7	2.2 ± 0.7	0.28 ± 0.46	8.9[a] ± 3.2
SS (n = 10)	5.7 ± 3.1	30.4 ± 11.1	24.7 ± 8.7	3.8 ± 1.4	7.2 ± 2.5	3.4 ± 1.7	2.07 ± 1.76	21.7[a] ± 7.2
SC-1	3.7	26.3	22.6	2.8	5.8	3.0	0.95	19.6
SC-2	2.6	12.5	9.9	2.6	4.4	1.8	none	8.0
CC	4.7	26.2	21.5	3.3	3.9	0.6	1.4	20.9

Values are means ± SD; n = number of subjects; [a] $p < 0.05$

Table 2 shows measurements of the bumetanide-sensitive K^+ efflux from SS, SC, CC and AA cells incubated in iso-osmotic conditions and under conditions which promote cell swelling (hypo-osmolarity and acid pH). Note that the several hemoglobin variants display no significant differences in their bumetanide-sensitive K^+ efflux which is driven by a different transport system, the Na^+,K^+,Cl^- cotransport system. This is in contrast with the significantly higher values of the

Table 2

Stimulation of K^+ efflux by swelling induced by hypo-osmolarity and acid pH in SS, SC, CC and AA red cells

Cell type	K^+ efflux (mmol/l cell per h)								
	Basal		(Ouabain + bumetanide)-resistant swelling induced by						
			Hypo-osmolarity				Acid pH		
	Ouabain-resistant	Bumetanide-sensitive	NaCl	$NaNO_3$	ΔCl	ΔV	pH 7	pH 8	ΔpH
AA (n = 9)	2.6	0.5	2.9	2.1	0.8	0.8	2.4	3.1	− 0.7[a]
	± 0.6	0.3	0.4	0.5	0.5	0.5	0.6	0.7	0.6
SS (n = 10)	5.6	0.3	12.1	3.6	8.6	7.1	9.4	3.5	5.2[a]
	± 3.1	0.3	5.6	1.5	4.8	3.6	3.9	1.6	3.5
SC	2.6	0.1	3.5	2.4	1.1	0.8	3.4	3.2	0.1
SC	5.5	none	17.7	4.5	13.2	12.1	13.7	4.8	8.9
CC	4.7	0.5	12.6	2.9	9.7	8.5	11.5	3.4	8.0

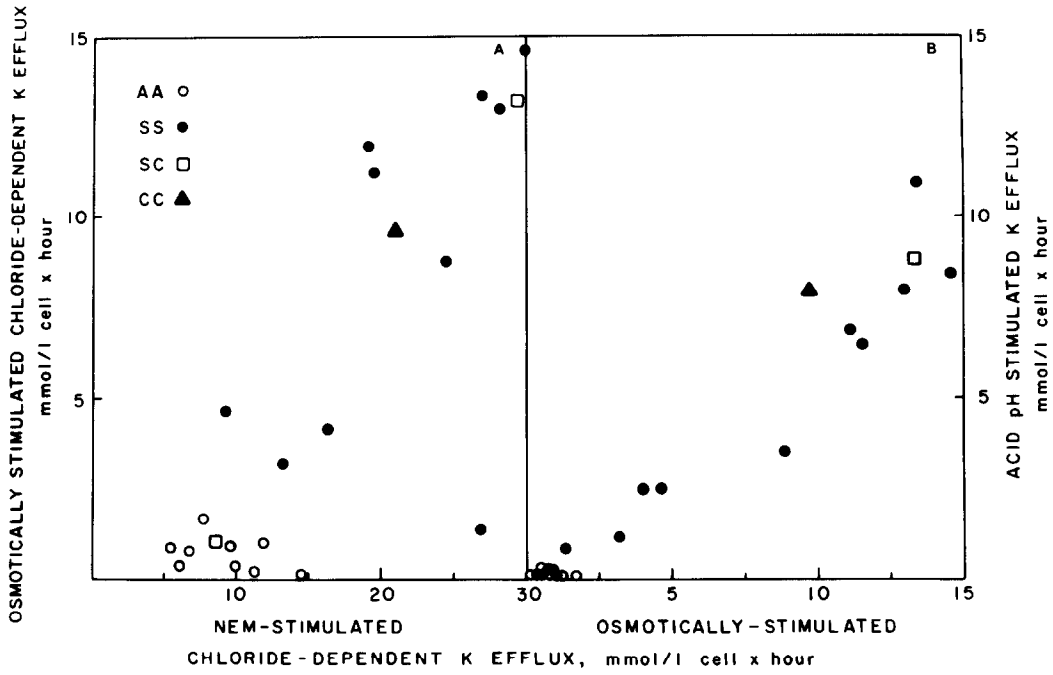

Fig.1. (A) Relationship between Cl^--dependent K^+ efflux stimulated by NEM and by osmotic swelling in AA (○), SS (●), SC (□) and CC (▲) red cells. (B) Relationship between K^+ efflux stimulated by hypo-osmolarity (osmotically) and acid-pH (pH 7–8)-induced swelling in AA, SS, SC and CC red cells.

bumetanide-insensitive, Cl^--dependent K^+ efflux stimulated by cell swelling. K^+ efflux from SS, SC and CC cells incubated in a hypo-osmotic NaCl medium containing bumetanide was significantly higher ($p < 0.05$) than in AA cells. Note that the stimulation of K^+ efflux does not take place in hypo-osmotic medium containing nitrate. The Cl^--dependent efflux from cells incubated in hypo-osmotic medium rendered very similar values to the volume-dependent K^+ efflux (medium D − C). Thus, almost all the swelling-stimulated K^+ efflux is also Cl^--dependent.

Table 2 also shows that the (ouabain + bumetanide)-resistant K^+ efflux (basal and swelling stimulated) from CC cells is shown to be Cl^--dependent, a property not defined in previous studies [15,19].

Fig.1A displays the relationship between the Cl^--dependent, NEM-stimulated K^+ efflux and the Cl^--dependent K^+ efflux activated by osmotic swelling. Both parameters are highly correlated ($r = 0.8$, $p < 0.001$, $n = 22$). A large inter-individual variance can be observed in both parameters in the 10 patients with SS Hb. The values recorded for the 2 SC and 1 CC are within the range observed in SS cells. Note that one SC patient has values near AA cells.

Fig.1B depicts the relationship between K^+ efflux stimulated by osmotic and acid-pH-induced swelling in the several Hb variants. It can be seen that both parameters are highly correlated ($r = 0.969$, $p < 0.001$, $n = 22$) suggesting that cell swelling activates a similar ion transport pathway. No correlation was found between reticulocyte count and the level of the K^+,Cl^- transporter.

4. DISCUSSION

The results presented here establish conclusively that oxygenated SS cells exhibit a volume-dependent (induced either by hypo-osmolarity or acid pH), NEM-stimulated K^+,Cl^- transport system which is only slightly active in normal cells. This situation is shared to different extents by Hb SC and CC cells. While the K^+,Cl^- transporter shares with the Na^+,K^+,Cl^- cotransport system its dependency on Cl^-, it can be distinguished from the latter by its bumetanide insensitivity and lack of *cis* stimulation by Na^+ [16]. The K^+,Cl^- transporter has been confused [20] with the K_i^+/K_o^+ exchange pathway of the Na^+,K^+,Cl^- cotransport system which is independent of external Na^+, bumetanide- and furosemide-inhibitable, Cl^--dependent but activated by intracellular Na^+ [16]. Our results indicate that AA cells have lower but significant values of a bumetanide-insensitive, Cl^--dependent K^+,Cl^- cotransport system as reported by Kaji [21]. The present results also indicate that SS, SC and CC cells clearly differ from AA cells in their bumetanide-insensitive K^+ efflux but not in their bumetanide-sensitive Na^+,K^+,Cl^- cotransport system (tables 1 and 2).

Another important feature of the K^+,Cl^- transporter is the significant heterogeneity in the extent of its expression in SS and SC individuals. Some of them have values similar to those characteristic of CC cells while others, although still quite different from normal cells, are in the lower end of range.

It seems therefore, that in oxygenated AA cells, the K^+,Cl^- transporter is activated by NEM but not (or very sluggishly) by hypo-osmotic and acid-pH-induced swelling. In many of the subjects with SS, SC and CC cells, the K^+,Cl^- transporter is not silent under iso-osmotic conditions and is markedly stimulated by hypo-osmotic and acid-pH-induced swelling ($p < 0.01$).

The K^+,Cl^- transporter explored here must be distinguished from the K^+ efflux stimulated by prolonged deoxygenation, first observed by Tosteson et al. [23] in SS cells. The relationship between the two phenomena, as well as the several pathways for Na^+ entry, requires further experimentation.

The presence of a K^+,Cl^- transporter in SS cells could be the consequence of two phenomena: (i) in contrast to AA individuals, most cells in sickle cell anemia patients are young and the youngest are stress reticulocytes that differ in maturity and shape from normal reticulocytes. The percentage of reticulocytes present in SS individuals is large and variable (5–50%). It is of interest that Lauf [22] has shown that in sheep red cells there is a genetic dimorphism of the NEM-stimulated K^+,Cl^- transporter which is absent in mature HK cells but is present in their reticulocytes and in the mature LK genetic variant. In this study the percentage of reticulocytes does not correlate with the activity of the K^+,Cl^- transporter but this does not exclude that it might be present in young cells

not identifiable by the supravital stain. (ii) Intracellular polymerization of HbS or HbC aggregation may modify the membrane either by direct interaction or by the byproducts of oxidative stress. We are in the process of dissecting the relative importance of these two pathogenic mechanisms.

The presence of the K^+,Cl^- transporter in SS blood is of interest in either case. If it is found in reticulocytes and young cells exclusively (the most abundant cells in SS blood), it opens the question of its physiological role in erythropoiesis. If it is abnormal-Hb-dependent, the exact mechanism of its stimulation becomes an interesting question. More importantly, the presence of the K^+,Cl^- transporter in HbS-containing cells has pathophysiological consequences independent of the presence of this transporter in the young and/or mature cells: it will favor the sickling of those cells, given the steep dependence (30th power) of the rate of sickling with initial Hb concentration. Erythrocytes devoid of volume-dependent K^+ efflux will swell when they encounter a decreased extracellular pH (an event common in the microcirculation); cell swelling will be beneficial in an HbS-containing cell, because it would reduce the MCHC and decrease the rate of sickling, a parameter dependent on MCHC. In turn, in HbS-containing cells exhibiting high levels of the K^+,Cl^- transporter, the low-pH-induced swelling will be rapidly corrected back to the original volume, a circumstance unfavorable for the sickle cells because the rate and extent of polymerization also increased with high MCHC and low intracellular pH. Since the presence of this transport mechanism might not involve all red cells equally (for which we have preliminary data) it is possible that its distribution could be under genetic control. The exciting possibility arises that the volume-stimulated, Cl^--dependent K^+ efflux could be another source of epistatic genetic modulation of the clinical expression of sickle cell.

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