Cryohydrocytosis: increased activity of cation carriers in red cells from a patient with a band 3 mutation

Anna Bogdanova,[1]* Jeroen S. Goede,[2]* Erwin Weiss,[3] Nikolay Bogdanov,[1] Poul Bennekou,[4] Ingolf Bernhardt,[3] and Hans U. Lutz[5]

[1]Institute of Veterinary Physiology, and the Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland; [2]University Hospital Zurich, Clinic of Hematology, Zurich, Switzerland; [3]Saarland University, Laboratory of Biophysics, Saarbruecken, Germany; [4]Department of Biology, University of Copenhagen, Denmark, and [5]ETH Zurich, Institute of Biochemistry, Zurich, Switzerland

*AB and JSG contributed equally to the work.

Funding: the studies were supported by SNF grants 3100A0-105831 to HUL and 3100B0-112449 to AB.

Manuscript received on April 17, 2009. Revised version arrived on July 15, 2009. Manuscript accepted on July 20, 2009.

Correspondence:
Anna Bogdanova,
Zurich Center for Integrative Human Physiology, University of Zurich, Winterthurerstr. 260, CH 8057 Zurich, Switzerland.
E-mail: annab@access.uzh.ch

ABSTRACT

Background

Cryohydrocytosis is an inherited dominant hemolytic anemia characterized by mutations in a transmembrane segment of the anion exchanger (band 3 protein). Transfection experiments performed in *Xenopus* oocytes suggested that these mutations may convert the anion exchanger into a non-selective cation channel. The present study was performed to characterize so far unexplored ion transport pathways that may render erythrocytes of a single cryohydrocytosis patient cation-leaky.

Design and Methods

Cold-induced changes in cell volume were monitored using ektacytometry and density gradient centrifugation. Kinetics, temperature and inhibitor-dependence of the cation and water movements in the cryohydrocytosis patient's erythrocytes were studied using radioactive tracers and flame photometry. Response of the membrane potential of the patient's erythrocyte membrane to the presence of ionophores and blockers of anion and cation channels was assessed.

Results

In the cold, the cryohydrocytosis patient's erythrocytes swelled in KCl-containing, but not in NaCl-containing or KNO_3-containing media indicating that volume changes were mediated by an anion-coupled cation transporter. In NaCl-containing medium the net HOE-642-sensitive Na^+/K^+ exchange prevailed, whereas in KCl-containing medium swelling was mediated by a chloride-dependent K^+ uptake. Unidirectional K^+ influx measurements showed that the patient's cells have abnormally high activities of the cation-proton exchanger and the K^+,Cl^- co-transporter, which can account for the observed net movements of cations. Finally, neither chloride nor cation conductance in the patient's erythrocytes differed from that of healthy donors.

Conclusions

These results suggest that cross-talk between the mutated band 3 and other transporters might increase the cation permeability in cryohydrocytosis.

Key words: red blood cells, cryohydrocytosis, $Na^+,K^+,2Cl^-$ co-transporter, cation carriers.

Citation: Bogdanova A, Goede JS, Weiss E, Bogdanov N, Bennekou P, Bernhardt I, and Lutz HU. Cryohydrocytosis: increased activity of cation carriers in red cells from a patient with a band 3 mutation. Haematologica. 2010; 95:189-198. doi:10.3324/haematol.2009.010215

Introduction

Cryohydrocytosis (CHC) is an inherited, dominant hemolytic anemia found in patients with the phenotype of either hereditary hyperchromic stomatocytosis or hereditary spherocytosis.[1] Red blood cells (RBC) from patients with CHC have increased membrane permeability to Na^+ and K^+ ions, which is particularly pronounced at 0°C.[1-6] In rare cases, these patients' RBC have a deficiency of stomatin accompanied by mental retardation, seizures, cataracts, and hepatosplenomegaly.[7] However, most patients with cation-leaky RBC have normal stomatin levels and no signs of mental retardation. These patients present with fatigue, mild anemia, and pseudohyperkalemia that is caused by a K^+ leak from the RBC.[8]

Recently the disease has been linked to mutations in the band 3 protein, the anion-exchange protein (AE1).[1,9,10] RBC from such patients generally have a partial band 3 deficiency and carry mutations within a transmembrane segment of band 3, which may cause the defect.[1] Mutated forms of band 3 have been found to be less efficient in transporting anions and have a decreased affinity to stilbene disulfonic acid derivatives.[1] Treatment of the RBC of a patient with CHC with high doses of 4-acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt hydrate (SITS) decreased the membrane permeability to K^+.[1] Furthermore, transfection of *Xenopus leavis* oocytes with cRNA from cloned mutant band 3 cDNA caused an increased content of Na^+ and a decreased content of K^+ ions upon incubation at ambient temperature under conditions that blocked the Na^+/K^+ pump and the $Na^+,K^+,2Cl^-$ co-transporter (NKCC).[11] Collectively, these results suggested that the mutation may convert the band 3 protein from an anion exchanger to a non-selective cation channel.[9,10,12] It has been shown that activity of the NKCC is unaltered and that of the Na^+/K^+ pump up-regulated due to the intracellular Na^+ accumulation in CHC erythrocytes.[1] The activities of the K^+,Cl^- co-transporter (KCC) and of the cation-proton exchangers in the RBC of CHC patients have not been addressed. $K^+(Na^+)/H^+$ exchange (KNHE), probably mediated by one of the 6-9 isoforms of NHE,[13,14] was earlier observed in human erythrocytes. This K^+ flux component was most pronounced at low ionic strength and was inhibited by the amiloride derivative HOE-642.[15,16] We hypothesized that changes in band 3 structure may alter its lateral interactions with other transporters. Similar interactions were reported for wild type trout band 3 that could stimulate NKCC in transfected *Xenopus leavis* oocytes.[17]

In this study we used whole blood and freshly isolated RBC from a CHC patient with mutation H734R in band 3 (CHC6, band 3 Hurstpierpoint, in reference[1]). No-one else in this patient's family suffered from CHC, implying that the patient has a *de novo* mutation in band 3. When the deformability and density distribution of the patient's RBC were assessed at room temperature and in the cold, following incubation in solutions containing either Na^+ or K^+ ions, we found that these RBC showed an abnormal temperature-induced volume regulation pattern, namely cold-induced swelling in KCl^-, but not in NaCl-containing media. We, therefore, studied the cation and water movements across the RBC membrane in more detail, focusing on the possible coupling of K^+ movements to that of anions, sensitivity of the cation fluxes to anion transport inhibitors (stilbene disulfonic acid derivatives), a chloride channel inhibitor NS1652, and the inhibitor of the KNHE, HOE-642. Our data suggest that the increased cation permeability of RBC of this CHC patient is at least in part mediated by increased activities of the KNHE and the KCC.

Patient

A Caucasian born in 1966 was seen at our clinic for the first time in 1995 for pronounced fatigue and casual dizziness. He had been sympathectomized in 1991 at another hospital because of hyperhydrosis. He was on no regular medication. In 1995 physical examination showed an enlarged spleen (7 x 20 cm on ultrasonography) without further abnormalities. His parents and brother were in good health. Laboratory data (Bayer Advia 120) were as follows: Hb 12.6 g/dL, RBC 4.25×10^{12}/L, MCV 83.2 fL, MCHC 35.7 g/dL, WBC 8.60×10^{9}/L, platelet count 194×10^{9}/L, reticulocytes 128‰ (normal range, 6-17‰). The Coombs' test was negative. An erythrocyte histogram showed 14% hyperchromic erythrocytes (normal range 0-1.5%) and a peripheral blood smear revealed microcytic anisocytosis with spherocytes and rare macrocytes and stomatocytes. The osmotic resistance of the RBC was greatly reduced. Hereditary spherocytosis was suspected and cholecystectomy and splenectomy were performed in 1998 in our hospital. Within months after splenectomy a mild hemolytic state persisted (reticulocytosis 40-60‰) and the number of hyperchromic erythrocytes had increased several fold (30-70%). Laboratory data after splenectomy were: Hb 16.5 g/dL, RBC 4.9×10^{12}/L, MCV 88.8 fL, MCHC 40.6 g/dL, WBC 13.15×10^{9}/L (polyclonal lymphocytosis 5.47×10^{9}/L), platelet count 421×10^{9}/L. The patient felt slightly better but with persistent morning fatigue. Given the clear persistence of the hemolytic state and the high numbers of hyperchromic RBC, further investigations have been performed starting in 2002 as shown here and in part published.[1] CHC was diagnosed with an 8-fold higher K^+ leak than that of control RBC and 2-3 times elevated activity of the Na^+/K^+-pump. Immunoblots from RBC membranes revealed an unaltered content of stomatin, while aquaporin was present predominantly in a monomeric form (*data not shown*). Further analysis revealed a single amino-acid substitution in the transmembrane domain of the erythrocyte band 3 protein (band 3 Hurstpierpoint, patient identified as CHC6 in reference[1]). On two occasions we were also able to investigate the patient's parents and his brother. The mother and the brother had completely normal hematologic values. The MCV of the father's RBC was just within the normal range (99 fL) with a slightly increased fraction of macrocytic RBC (4.1%, normal up to 1.5%). There was no obvious reason for this, in particular no alcoholism. Hemolysis could be excluded. Unfortunately, we could not further investigate the slightly macrocytic RBC from the father, because he died in the meantime from a heart attack. All the analyses reported here have been performed at least once with the blood donated at the place where the experiments were carried out. On two other occasions blood was collected in ACD and sent to Saarbruecken or Copenhagen, where it was further processed within 24 h. All studies of the patient and his family were carried out upon mutual agreement and in accordance with the principles of the Declaration of

Helsinki. Ethical approval for the study was granted by the legal service of the Health Department of the canton Zurich.

Design and Methods

Ektacytometry and density gradients

Whole heparinized blood was mixed with isotonic buffer containing exclusively K^+ or Na^+ ions and an anti-coagulant. The suspensions were filtered to remove white cells. Remaining cells were washed from the filter with the appropriate buffer containing 0.5 mM EGTA and 0.05% glucose. The filtered RBC suspensions were kept on ice and where indicated supplemented with 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid, disodium salt (DIDS). Aliquots were taken for ektacytometry or gradient centrifugation.[18,19] In the latter case aliquots containing 4×10^{10} RBC were centrifuged and the cells resuspended with 36 mL Percoll solution containing exclusively either Na^+ or K^+ ions. Where indicated the Percoll solutions were supplemented with 10 μM DIDS or 100 μM SITS. These suspensions were centrifuged, the gradients photographed, and fractionated as outlined.[18] Even RBC from the lightest fraction could be washed and re-centrifuged on a density gradient, implying that a significant portion if not all swollen cells did not lyse during centrifugation in Percoll gradient. Osmoscans were obtained as described previously with an ektacytometer (Technikon Products, Bayer, Germany).[18,19]

Protein composition of red blood cell membranes

Membranes were prepared, solubilized and submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis, following reduction and alkylation of the samples, as described elsewhere.[19] In order to resolve bands 4.1 a and b better, the electrophoresis was continued for 30 min once the tracking dye had reached the front.

Determinations of K^+ leak in whole blood

Heparinized blood from the patient and a control was incubated and aliquots withdrawn at the given times. One part of the sample was submitted to routine analysis (Bayer Advia 120). The other sample was centrifuged, and then the plasma withdrawn and analyzed for K^+ by ion selective electrodes.[20]

Net ion and water movements in NaCl, KCl, and KNO_3 media

RBC were washed and resuspended in the following media containing 150 mM NaCl or KCl or KNO_3, 0.15 mM $MgSO_4$, 1 mM $CaCl_2$ or $Ca(NO_3)_2$, 10 mM glucose, 10 mM sucrose and 10 mM Tris-OH (or HNO_3) adjusted to a pH of 7.4 with either HCl or HNO_3 at either room temperature or 0°C. Aliquots of the suspensions were withdrawn at the given time points into pre-weighed, pre-dried Eppendorf tubes and centrifuged. Aliquots of supernatant were collected and Na^+/K^+ monitored by flame photometry. RBC were washed three times in $Mg(NO_3)_2$-imidazole buffer and the pellet was weighed and dried at 80°C for 48 h to determine the cellular water content. Dry pellets were wet-burned in concentrated HNO_3 and cellular Na^+ and K^+ measured by flame photometry.[21]

K^+ influx measurements

The RBC were washed and resuspended with an isotonic NaCl- or $NaCH_3SO_4$-containing buffer. KCC activity was assessed using ^{86}Rb as a tracer for K^+, as described elsewhere.[22] The activity of KNHE was measured as a unidirectional K^+ influx component sensitive to HOE-642 [cariporide, (4-isopropyl-3-methylsulfonyl-benzoyl)-guanidine methanesulfonate, a gift from Sanofi-Aventis, Germany].[15] In all experiments ouabain (0.1 mM), bumetanide (0.1 mM), and EGTA (0.1 mM) were present in the incubation medium during the flux measurement. The hemoglobin content of RBC suspensions was determined as cyanmethemoglobin using Drabkin's reagent.

Conductance determinations and Co^{2+}-influx

The cells were washed in potassium equilibrium Ringer's solution, followed by three washes in isotonic 150 mM NaCl solution at room temperature. Packed RBC were injected into either an unbuffered salt solution or a sucrose-containing low ionic strength Ringer's solution supplemented with 20 μM carbonylcyanide-m-chloro-phenyl-hydrazone (CCCP) and in some cases with 10 μM NS1652. The membrane potential of the cells was estimated by continuously monitoring the extracellular pH (pH_{out}) in the presence of the protonophore CCCP.[23]

The chloride conductance, under net efflux conditions, which were induced by addition of valinomycin (0.1 μM final concentration), was calculated from $g_{Cl} = i_{Cl} (E_{Cl} - V_m)$, where i_{Cl} was taken to be negative, but identical to the K^+ current (i_K), which was estimated from the increase in the extracellular K^+ content. The cation conductances (g_+) were subsequently estimated.

The Gardos channel was activated by a Ca^{2+} challenge mediated by A23187 (0.5 μM final concentration) at the ambient extracellular Ca^{2+} concentration. Furthermore, the Gardos channel response to an intermediate conductance potassium channel agonist (NS309, final concentration 100 μM) was studied. For Co^{2+} entry measurements ^{57}Co was used as a tracer.

Results

Red blood cell deformability and density distribution

Ektacytometry was performed on RBC from the patient and his parents. Whole blood was mixed with isotonic buffer containing either Na^+ or K^+ ions and freed of white cells at room temperature. The suspensions were kept on ice, sequentially centrifuged and subjected to ektacytometry (1 h to 2 h) (Figure 1A). The osmoscans of both parents were super-imposable whether cells were pre-incubated with Na^+- or K^+-containing buffer. While the osmoscans of RBC from the mother (BN, BK) displayed a nearly normal pattern [see the corresponding control scan (con)], the two osmoscans from his father's RBC (CN, CK) showed a left shift, implying a higher surface/volume ratio than normally observed, indicative of macrocytosis. The osmoscans from the patient's RBC differed considerably from those of his parents and varied depending on the cation composition of the buffer (Figure 1A: AN, AK). When kept in Na^+-containing buffer (AN), the RBC revealed a pattern similar to that seen for RBC from patients with hereditary spherocytosis with reduced surface/volume ratio, reduced maximal deformability, and greatly increased density (see the left shift of the right arm of the osmoscan and the corresponding density gradient). Upon cold storage in K^+-containing buffer and ektacytometry in the Na^+-containing dextran-mixture as used for all other samples, the osmoscan revealed an extremely pathological pattern (AK), since the entire curve was shifted to the right, meaning that the cells had swollen and the minimum of the curve indicated that 50% hemolysis occurred slightly below the physiologic tonicity.

Since it was impossible to keep the time from blood withdrawal to ektacytometry constant, on three occasions we performed density gradients and incubated the cells for exactly 1 h on ice or at room temperature before starting gradient centrifugation in the cold. Percoll density gradients loaded with control RBC kept in the two types of isotonic buffer showed very similar results (Figure 1B). Neither the presence of K^+ or Na^+ ions nor cold storage altered the RBC distribution patterns significantly. In contrast to this, cold storage had a marked influence on the density distribution of the patient's RBC in the presence of K^+ ions (Figure 1B). Swelling in K^+-containing buffer was substantial at 0°C, but not at room temperature. The density distribution profiles of RBC from both parents (performed only once, since the father died in the meantime) were comparable to those obtained for an independent control, as shown in Figure 1B. RBC from the patient banded at a very high density when kept in Na^+-containing buffer. When suspended in KCl-containing medium the cells banded at lower density when incubated at 0°C but not at room temperature. This cold-induced swelling was not inhibited by either 10 μM DIDS or 10 μM SITS (*data not shown*), whereas the high concentration of 100 μM SITS partially inhibited the swelling (Figure 1C).

The membrane protein composition of density-fractioned RBC from both parents was normal, while that of the patient's RBC revealed a partial band 3 deficiency (18%), which was of comparable extent in light and dense cells (*Online Supplementary Figure S1*). Most surprisingly, however, the cell age distribution within a regular Percoll gradient, containing both Na^+ and K^+ ions[19] (*Online Supplementary Figure S1*) showed exactly the opposite pattern from that of controls with the youngest cells at the highest density and the oldest at the lowest density (top fraction), based on the band 4.1a/4.1b ratio (*Online Supplementary Figure S1*, magnified in inset, compare band 4.1 a/b in fractions P-1 and P4/5). These results suggest that the youngest cells with the highest Na^+/K^+ pump activity (2-3 fold that of control cells[9]) could best compensate the increased permeability and remained dense.

Whole blood responses to low temperatures

RBC isolated from blood freshly drawn from the patient were dehydrated with the cell water content reduced from the 66.4±0.3% found in healthy donors (N=4) to 62.0±0.2% (measured in triplicate on two occasions). Dehydration was coupled to a low cellular salt content as well as to alterations of the Na^+/K^+ gradient. The cellular K^+ concentration of the patient's RBC was 76.1±4.5 mmol/l_{cells} and the Na^+ concentration 29.9±0.4 mmol/l_{cells}, which can be compared with 88-105 mmol/l_{cells} and 5.9-11.3 mmol/l_{cells}, respectively, reported for RBC from healthy subjects.[2]

The patient's RBC in whole anti-coagulated blood showed a dramatic loss of potassium within the first hour of incubation at 0°C (Figure 2A). The loss of K^+ ions from the cells was followed by a slower reduction of the number of hyperchromic cells and an even slower increase in MCV, most probably because of an uptake of Na^+ ions. In contrast to this, storage at 37°C was followed by a minor increase in plasma K^+ during the first hour, as well as later between 8 and 12 h of storage. Plasma K^+ accumulation was not observed when blood was stored at 20°C (Figure 2A). Interestingly, cell swelling (increased MCV) persisted over 8 h of cold storage, whereas plasma steady state K^+ levels were reached within 1-2 h, suggesting that K^+ and Na^+ were transported by two independent routes. We, therefore, decided to take a closer look at the changes in the plasma K^+ and Na^+ content as well as the cellular ion content during the first hour of cold incubation, when the K^+ loss was particularly intense. We observed a gradual increase in cell water from 61.7% to 63.7%. The increase in plasma K^+ could be described by a sigmoidal function (Figure 2B) with a new steady state K^+ concentration of 26.5±1.8 mM, reached within 1 h (estimated $T_{1/2}$ = 28.1±1.4 min). In contrast to this, the Na^+ accumulation by RBC was linear over time (Figure 2C) and exceeded the K^+ loss by about 10 mmol/(l_{cells}×h), when calculated from the decrease in plasma Na^+ concentration (Figure 2C). We also studied the action of two inhibitors in an attempt to block the observed cold-induced alterations in cell volume and ion composition. Either 100 μM DIDS (a known inhibitor of band 3-mediated anion exchange at >10 μM) or 500 μM HOE-642 (an inhibitor of the cation-proton exchanger) were added to whole blood of the patient and a healthy subject 10 min prior to cold exposure. The high concentration of DIDS (the free concentration may be less than the nominal due to DIDS binding to plasma albumin) and HOE-642 decreased the steady-state plasma K^+ concentration in cold-stored blood to 22.8±3.2 and 20.5±2.0 mM, respectively (Figure 2B). The K^+ loss from the patient's RBC was somewhat attenuated in the presence of DIDS ($T_{1/2}$ = 32.4±2.5 min) or HOE-642 ($T_{1/2}$ = 36.2±1.8 min). The inhibitors also reduced Na^+ accumulation by the cells from 24.0 mmol/(kg_{dw}×h) in non-treated whole blood to 16.5 or 15.4 mmol/(kg_{dw}×h) in the presence of DIDS and HOE-642, respectively (Figure 2C). These data are in line with those obtained from the long-term MCV and plasma K^+ measurements (Figure 2A) and indicate that the observed cold-induced response is mediated by at least two independent processes. We, therefore, studied the activity of several K^+ transport pathways in the patient's RBC to find an explanation for the unusual volume regulation pattern of these cells in KCl-containing medium, shown in Figure 1.

K^+ influx measurements

We assessed the function of the KCC and the KNHE in the patient's RBC using $^{86}Rb^+$ as a tracer for K^+. Unidirectional K^+ influx was assessed both in chloride- and methyl sulfate-containing solutions at 0°C in the presence of ouabain (0.1 mM), bumetanide (0.1 mM) and EGTA (0.1 mM) to inhibit the Na^+/K^+ pump, the NKCC, and the Ca^{2+}-activated K^+ channel, respectively. The residual K^+ influx was increased in patient's RBC in comparison to that in RBC from the mother, brother and a healthy control when measured at 37 °C and at 0 °C (on ice) (Figure 3A). This difference was about five times more pronounced when cells were incubated at 0°C rather than at 37°C (Figure 3A). HOE-642 efficiently blocked both basal and temperature-induced K^+ influx into patient's RBC (Table 1). It inhibited K^+ influx into the patient's RBC at 0°C to more than 50%. SITS at a high concentration (100 μM) had no effect on K^+ influx in RBC from healthy subjects but suppressed this influx in the patient's RBC moderately at 37°C and by only 10% at 0°C (Table 1). Incubating the patient's RBC in low ionic strength

medium (details provided elsewhere[22]) resulted in further activation of the cold-induced K^+ influx component (*data not shown*) suggesting that the KNHE[16] is cold-induced and hyper-activated in the patient's RBC.

A significant portion of the cold-induced K^+ influx in bumetanide-treated patient's erythrocytes remained dependent on Cl^- and could be blocked by replacement of the extracellular chloride by methyl sulfate (Figure 3B). This Cl^--dependent K^+ flux was most likely mediated by the KCC and was rather low at 37°C, but increased greatly in the cold, especially in stored erythrocytes (Figure 3B). Of note, total K^+ influx in stored cells increased only slightly (Figure 3B) suggesting that the Cl^--dependent K^+ flux was particularly sensitive to low temperatures and storage. To estimate whether the KCC mediated the swelling of patient's RBC in cold KCl-containing media (Figure 1), we assessed the changes in the water content of cells resuspended in KCl- or KNO_3-containing medium and incubated them at 0°C. Replacing the Cl^- in the incubation medium with NO_3^- caused inhibition of the KCC, but not of the band 3-mediated anion transport. The changes in the intracellular water content when the RBC were incubated in the cold were +1.67 % per hour in NaCl-containing medium, +3.02 % per hour in KCl-containing medium and –0.26% per hour in

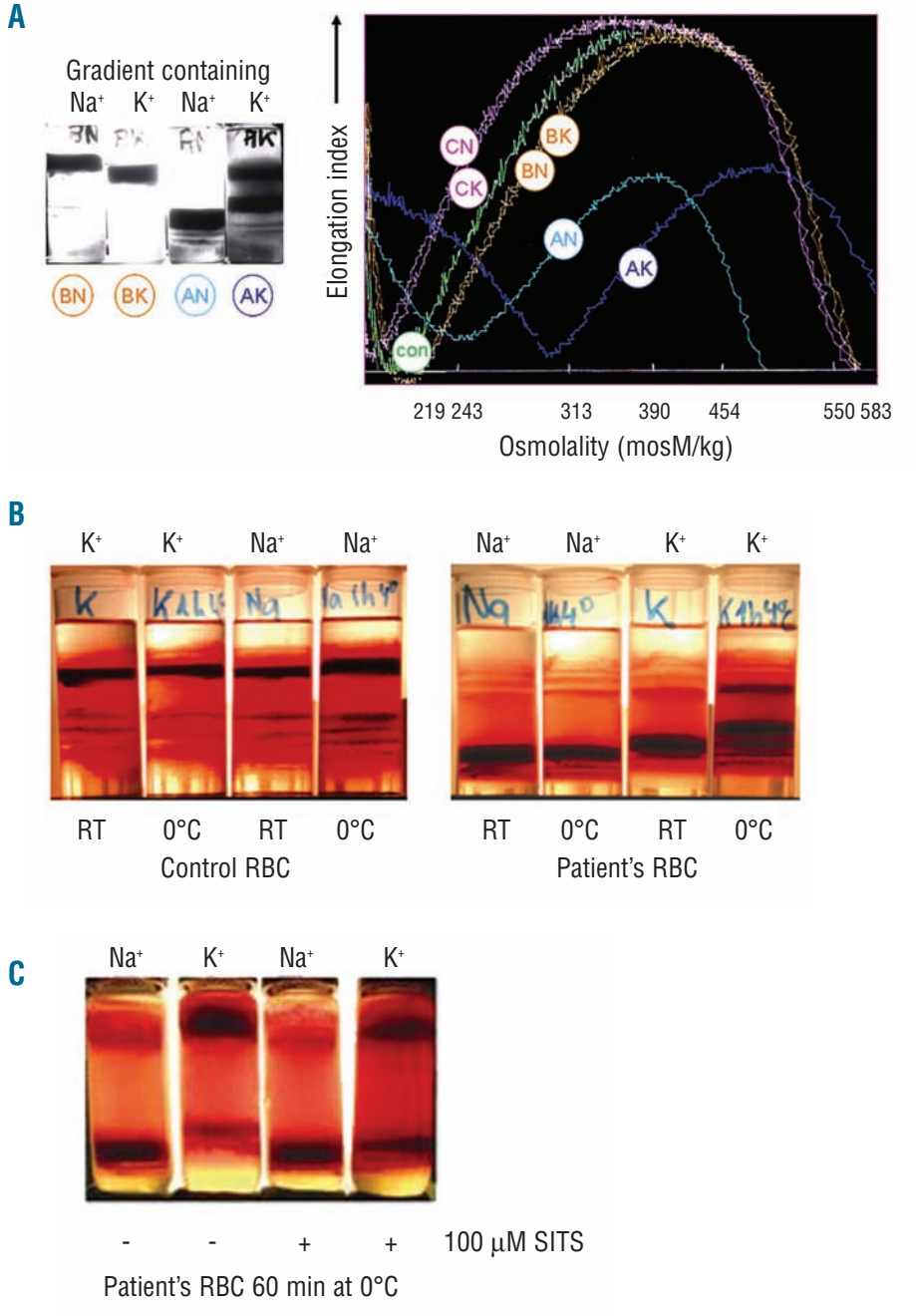

Figure 1. Deformability profiles and density gradients of whole RBC populations kept in either sodium- or potassium-containing isotonic buffer on ice. (A) Deformability profiles at increasing osmolarity were run at room temperature in 18% dextran buffers containing exclusively sodium ions as cations. Osmoscans were recorded from RBC that were resuspended and stored at 0°C in buffers containing the indicated cations, from the patient A (AN: NaCl buffer, AK: KCl buffer), from the patient's mother (BN: NaCl buffer, BK: KCl buffer), the patient's father (CN: NaCl buffer, CK: KCl buffer), and from a random control sample from a healthy donor (con). The corresponding Percoll density profiles for the RBC of the CHC patient and his mother are shown next to the deformability profiles. (B). Density distribution of RBC stored for 1 h as indicated (RT for room temperature or at 0°C) and centrifuged at 0°C in isotonic buffer containing either Na^+ or K^+ as cations. (C) As in (B) except that the buffer and the Percoll solution were supplemented with 100 µM SITS where indicated.

KNO_3-containing medium (average of two independent measurements). This suggests that the abnormal activation of the KCC could account for the changes in cell density and volume observed during density gradient centrifugation and ektacytometry (Figure 1), and, finally for the dehydration and large proportion of hyperchromic cells observed in the patient's RBC (Figure 2A).

Cation currents in the red blood cells from the patient with cryohydrocytosis

The chloride conductance at 38°C was found to be 27.7 μS/cm², which is within the normal range (20–30 μS/cm²). With 10 μM of the chloride channel blocker NS1652 present, conductance was inhibited by about 90%, as in the RBC of healthy donors.[24] Due to the low E_K, and the dull response to both CCCP and valinomycin at lower temperatures, it was not possible to obtain quantitative data at 22.5 °C and at 5.5 °C. Qualitatively, however, the conductivity was similar to that found earlier for RBC from healthy blood donors.[25,26] The patient's RBC responded normally to a Ca^{2+} challenge (A23187) as well as to an activator of the Gardos channel (NS309)[27] at varied temperatures (38°C, 22.5°C and 6.0°C, *data not shown*). Thus, no indication was found for increased Gardos channel activity at low temperatures.

The patient's NSVDC channel showed normal activation at 38°C, both with regard to activation, when the cells were suspended in low ionic strength sucrose-containing Ringer (SR)[28], and with regard to Gardos channel activation, mediated by Ca^{2+} entry through the activated NSVDC channel (*Online Supplementary Figure S2*). As previously shown for normal RBC,[28] the initial hyperpolarization after Ca^{2+} addition, due to Gardos channel activation, was followed by a repolarization caused by Ca^{2+} extrusion through the Ca^{2+} pump (*Online Supplementary Figure S2*). At lower temperatures, the NSVDC channel was activated as found for control cells too (*data not shown*).

Discussion

Our findings demonstrate that KCC and KNHE contribute to the alterations in cellular volume, deformability and density of RBC from the CHC patient we examined. Dehydration of CHC RBC and thus high density RBC is the primary phenomenon of this pathology *in vivo* and when cells are kept at room temperature (Figures 1B, 1C and 3A, hyperchromic cell count). The significantly reduced water content of freshly isolated RBC must have resulted from a substantial loss of K^+ *in vivo*, which was only partially compensated for by Na^+ accumulation. This condition resembles that reported for RBC populations from patients with homozygous HbC- and HbSS-linked anemia, with high reticulocytosis and high KCC activity and acidosis-induced dehydration.[29] This suggestion was further supported by the observation that the density of young cells (age determined by band 4.1a/b ratio, see *Online Supplementary Figure S1*) substantially exceeded that of mature RBC.

In accordance with these properties the patient's RBC remained microcytic in NaCl medium and gained water in KCl medium (Figure 1A and B) although the Nersnst potential for Na^+ in NaCl-containig medium was higher (47 mV) than that for K^+ in KCl-containing medium (27 mV) at 0°C in freshly isolated erythrocytes ($[Na]_i$ =20 mmol/L water, $[K]_i$=50 mmol/L water and cell water content of 66%).

Differences in the kinetics of cold-induced Na^+ and K^+ movements across the RBC membrane (Figure 2B and C) suggest that these two cations may not share the same ion transport pathway in the patient's RBC. Characterization of the K^+ influx via KCC and KNHE in the CHC patient's erythrocytes showed that the unusually high cation permeability in these cells at low temperature is, at least in part, a cumulative effect of an abnormal activation of two independent ion transport systems: KCC and KNHE. As follows from the Table 1, and in agreement with former reports,[22,30] both ion transporters are almost silent in RBC from healthy donors (and the patient's family members), particularly during cold storage.

The $K^+(Na^+)/H^+$ exchanger

Several lines of evidence point towards an abnormally high activity of the cation-proton exchanger in RBC of the CHC patient at 37°C and even more so at 0°C. A minor increase in residual K^+ leak at low temperature had already been reported and was especially pronounced under conditions of low ionic strength.[22,31] This cold-induced response attributed to the activation of the KNHE[16] is amplified many fold in the patient's RBC. When the patient's RBC were suspended in cold, NaCl-containing solution, they showed minor changes in cell volume/density (Figure 1B) because the Na^+ accumulation almost matched the K^+ loss (Figure 2B and C). Very similar observations were made in oocytes expressing a mutated form of band 3 protein from a CHC patient.[11] Furthermore, transport of Na^+ and K^+ in CHC erythrocytes is coupled to that of protons. In fact, abnormally high activity of a pH-dependent Na^+ transport pathway was already reported for another CHC patient.[32] The Na^+ accumulation in the RBC from this CHC patient exposed to the cold was a function of the extracellular pH and increased as the extracellular pH value changed from 6.6 to 8.2.[32] Alkalinization of the extracellular medium triggered H^+ loss in exchange for Na^+ ion uptake in NaCl-containing medium mediated by either NHE or KNHE. Correspondingly, the proton efflux from RBC of the CHC patient into the unbuffered media in exchange for the acquired Na^+ or K^+ ions exceeded that in RBC of healthy donors making up 213-295% of control in NaCl and 134-176% in KCl-containing saline (measured on two occasions). Another indication for the stimulation of the KNHE in response to cooling is the fact that the temperature-sensitive K^+ influx in the patient's RBC was 6-fold higher when cold exposure studies were performed in low ionic strength medium (*data not shown*), a condition known to activate KNHE in erythrocytes from healthy humans.[33] Finally, HOE-642, an inhibitor that was shown to suppress KNHE in RBC from healthy donors,[15] blocked both the temperature-sensitive unidirectional K^+ influx (Table 1) and the K^+ leak from erythrocytes into plasma by about 50% when the patient's blood was exposed to cold (Figure 2B). All these data indicate that an abnormally high activity of KNHE accounts, in part, for the high cation permeability of CHC RBC.

K^+,Cl^- cotransporter

Cold-induced K^+ influx and net movement could be reduced by replacement of Cl^- by methyl sulfate or by NO_3^-,

which are both band 3-permeable but do not support KCC function. This implies that it is most likely mediated by KCC and not by a combination of independent anion and cation conductive pathways. The data obtained also suggest that abnormally high activity of the KCC may be involved in the microcyte appearance of the patient's blood. Activation of this transport pathway caused acute cold-induced swelling of the cells resuspended in the KCl-containing medium (Figure 1). Acute activation of the K^+ fluxes observed in whole blood (Figure 2A) and NaCl- and KCl-containing, but not in chloride-free, medium during cold storage was not correlated with cell volume changes and was not, therefore, triggered by swelling. Long term storage followed by swelling of patient's RBC most likely facilitated further secondary activation of the transporter (Figure 3B). Activation of this transport pathway by cooling is an unusu-

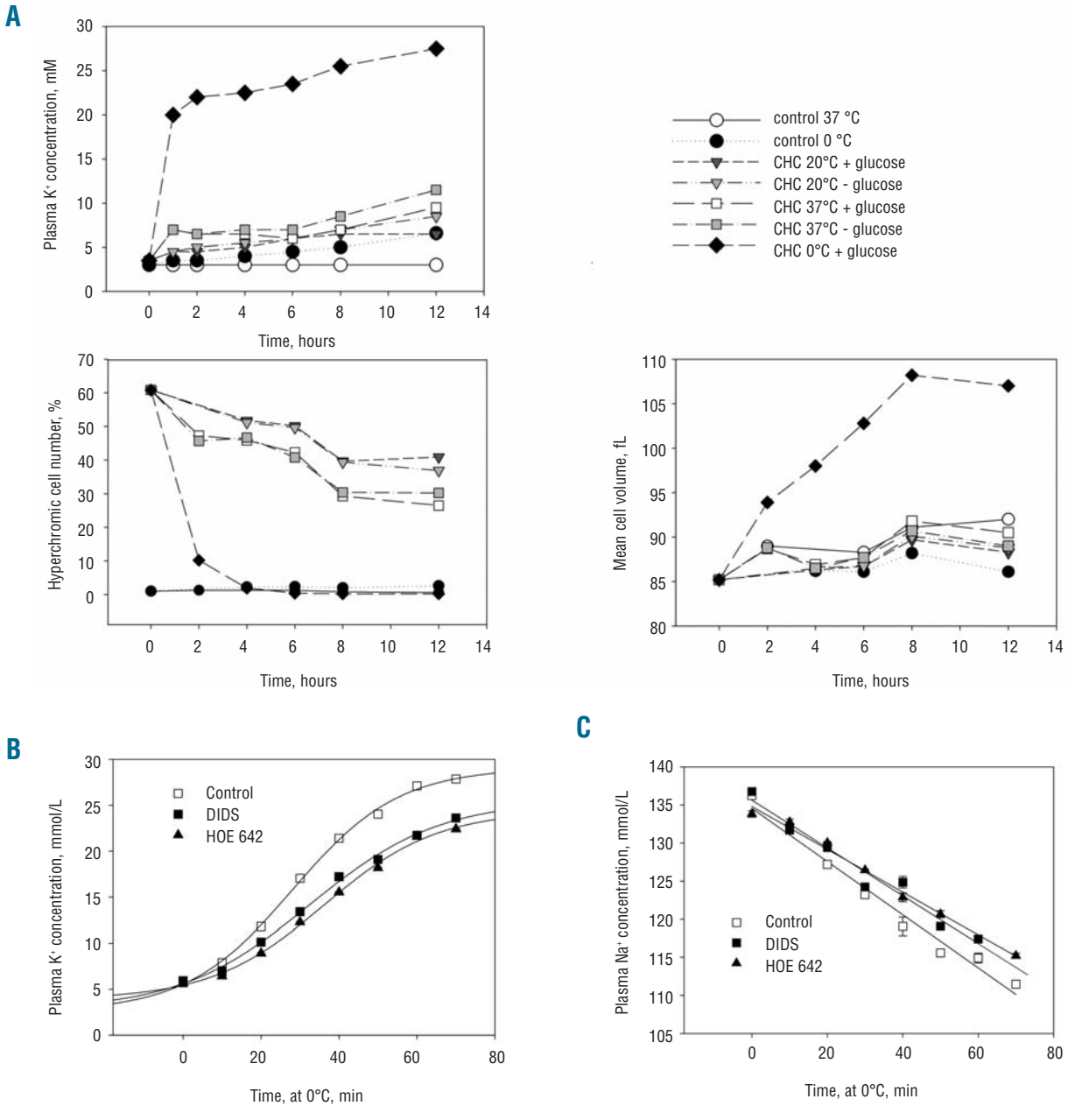

Figure 2. Plasma cation content, MCV, and hyperchromic RBC following incubation at different temperatures. (A) Changes in plasma K^+, hyperchromic cell number and MCV during storage of blood from a healthy donor and the CHC patient at various temperatures with and without glucose. (B) Changes in plasma K^+ concentration in patient's whole blood (Hct 40%) stored at 0°C in the absence (□) and in the presence of 100 μM DIDS (■) or 500 μM HOE-642 (▲). Curve fitting was done using a logistic sigmoid function. (C) Changes in plasma Na^+ concentration in whole blood of the CHC patient during storage at 0°C in the absence (□) or the presence of 100 μM DIDS (■) or 500 μM HOE-642 (▲). Linear regression was used for the curve fitting. Measurements were performed in triplicate and are shown as mean±SEM.

al phenomenon that has never been reported before. The KCC is silent in RBC of healthy donors and can only be detected when activated, e.g. by swelling or Mg^{2+} deprivation.[34] A decrease in cell volume results in suppression of the KCC activity in RBC from healthy donors, but not in the CHC patient's RBC, indicating abnormal regulation of the KCC in these latter cells.[35] Uncontrolled activation of the KCC has been reported in patients with sickle cell anemia and beta-thalassemia in whom pathological RBC dehydration is abundant.[36,37] Our observations show that this is also the case in the CHC patient's RBC. As for most ion transport systems, the activity of the KCC in normal RBC is down-regulated in response to cooling. The suppression of KCC activity with cooling by 10 degrees (Q10) is 2.5 for frog RBC, 6.9 for shark erythrocytes and 1.7 for trout RBC.[38] RBC from the CHC patient we studied represent a unique exception, because a decrease in temperature activates the KCC in these cells. The temperature-induced alterations in activity of this transporter may originate from a modified interaction with the cytoskeleton, as suggested earlier,[39] or directly with the mutated band 3. If this were the case, these features could be explained by changes in the structure of the cluster of proteins including the mutated band 3, ankyrin, and numerous elements of the cytoskeletal network, which bind to this membrane-anchored scaffold.[40,41] Whether an altered interaction with cation transporters is also dependent on unique forms of lipids, as found in pseudohyperkalemia Cardiff,[6] remains to be investigated.

Cation conductance

The data obtained for the activity of the NSVDC channel, the Gardos channel, and the chloride conductance suggest that the ion conductance of CHC RBC is not different from that of control RBC. Correspondingly, oocytes transfected with a CHC type band 3 did not reveal a cation conductance within the physiological potential range for erythrocytes of 0 mV to –50 mV).[11] Furthermore, the fact that SITS, a selective inhibitor of band 3 anion exchange at 10 μM,[42] inhibited the cation flux in oocytes transfected with CHC type band 3 protein at 500 μM[11] and swelling of CHC RBC partially at 100 μM (Figure 1C) is not a strong argument for band 3 serving as a channel, because at these concentrations this inhibitor affects numerous ion transport pathways including anion-dependent cation transporters.[16]

Outlook

We studied RBC from a well-examined CHC patient to determine the possible mechanisms underlying the cold-induced cation transport in these RBC and found that abnormal regulation of KCC and KNHE may contribute to the constitutive dehydration of RBC of this patient. If similar findings on RBC from other CHC patients confirm these observations, both transporters could be potential targets for developing treatment strategies for patients with CHC. Unfortunately, the molecular identity of the KNHE has not been established and very little is known about its regulation. The only inhibitor known to suppress its function, HOE-642, is rather non-specific and also targets the Na/H exchanger present, for example, in the heart.[43] In contrast, the KCC is well-studied and oral Mg^{2+} supplementation was

Table 1. The (ouabain + bumetanide + EGTA)-resistant K^+ influx (in mmol/[I_{cells}×h]) in the absence or presence of 100 μM SITS or 200 μM HOE-642 in NaCl-containing solution at 37°C and 0°C.

Additions	Control	CHC	
	37°C	37°C	0°C
No inhibitor	0.113±0.013	0.701±0.014	3.711±0.081
+ SITS	0.094±0.021	0.548±0.007	3.316±0.030
No inhibitor	0.141±0.003	0.675±0.009	3.199±0.025
+ HOE-642	0.110±0.010	0.454±0.004	1.113±0.030

For the control (3 different donors), values are represented as mean ± SD. The values for CHC blood are represented as mean ± SD from a triplicate measurement of freshly drawn blood.

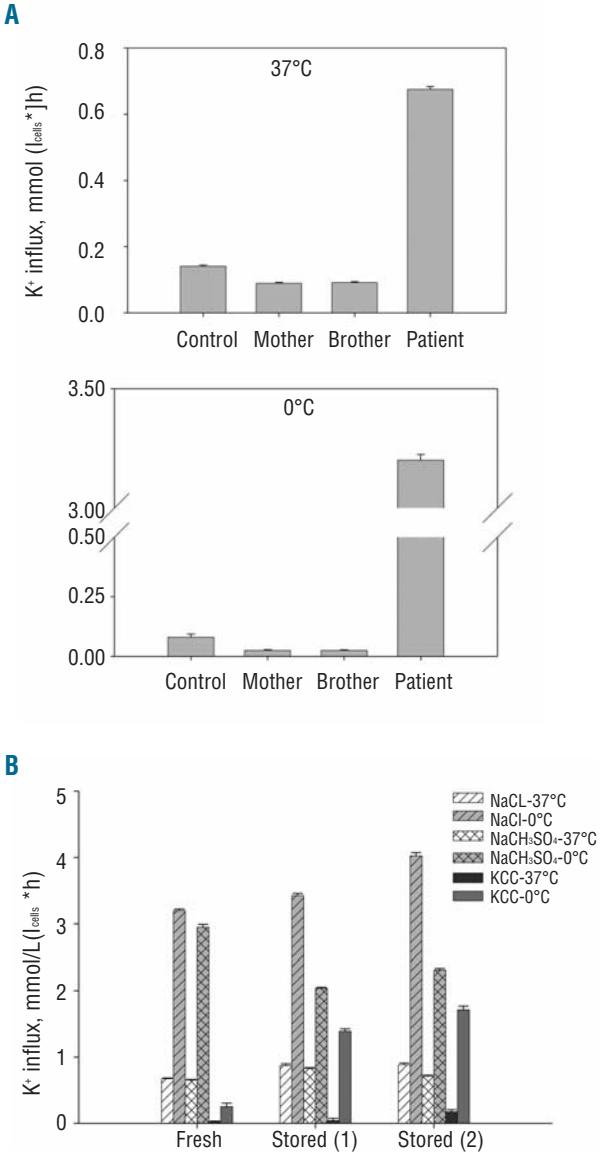

Figure 3. Unidirectional potassium (^{86}Rb) influx into RBC. (A) Residual (ouabain-bumetanide-EGTA-insensitive) K^+ influx in RBC from blood of a control, the patient's mother, his brother and the CHC patient. Erythrocytes were resuspended in NaCl-containing medium and incubated at 37°C or 0°C (on ice) in the presence of ^{86}Rb. (B) Residual K^+ influx in fresh and stored patient's RBC in NaCl- and $NaCH_3SO_4$-containing medium at 37°C or at 0°C. Measurements were done in triplicate on each occasion and are presented as means ± SEM.

proven in clinical studies to efficiently decrease KCC activity in sickle cell anemia.[44] We had the possibility to study the effect of Mg^{2+} supplementation on plasma K^+ and other blood parameters as the patient was prescribed 3 x 200 mg/day magnesium for 6-9 weeks to reduce muscle cramps and fatigue after sport activities. Plasma K^+ levels, which were high before Mg^{2+} supplementation, dropped from 4.72±0.12 mM to 3.9 mM within 3 weeks. When the Mg^{2+} intake was interrupted, the plasma K^+ concentration returned to the high concentration of 4.9 mM. The duration of the observation was not, however, long enough to draw a firm conclusion. According to our *in vitro* data, full normalization of K^+ permeability would require interference with at least two ion transporters (KCC and KNHE).

It remains unresolved how altered band 3 affects the activity of the two ion transporters. Apart from being an anion transport protein, band 3 is also a scaffold for numerous other proteins of the cytoskeleton, hemoglobin, members of the glycolytic pathway, and other ion transporters.[45-47] Most of the mutations reported for CHC patients (including the patient whose blood was used in this study) are in the putative loop between the M9 and M10 membrane-spanning domains or in the putative cytosolic loop between M8-M9. It is likely that these domains are involved in docking to other integral proteins, be they the cation transporters themselves or regulatory proteins affecting their activities. It has been reported that there is cross-talk between NKCC and the band 3 protein in trout erythrocytes.[17] Elucidation of the links between the mutated band 3 proteins and various cation transporters is needed to understand the nature of the abnormally high cation permeability in RBC of CHC patients.

Authorship and Disclosures

AB and NB: experimental work; JSG: experimental work (Figure 2A, Mg^{2+} supplementation effects, patient's supervision and clinical input); IB and EW (Figure 3 and Table 1); PB: experimental work (*Online Supplementary Figure S2*); HUL: experimental work (Figure 1A-C and *Online Supplementary Figure S1*).

All authors participated in discussion of the obtained results as each of the contributors had one's own field of expertise. AB, JSG, NB, PB and HUL put together the manuscript text whereas AB and EW designed the graphics for their experimental contribution.

The authors reported no potential conflicts of interest.

References

1. Bruce LJ, Robinson HC, Guizouarn H, Borgese F, Harrison P, King MJ, et al. Monovalent cation leaks in human red cells caused by single amino-acid substitutions in the transport domain of the band 3 chloride-bicarbonate exchanger, AE1. Nat Gen. 2005; 37(11):1258-63.
2. Delaunay J. The hereditary stomatocytoses: genetic disorders of the red cell membrane permeability to monovalent cations. Semin Hematol. 2004;41(12):165-72.
3. Iolascon A, Perrotta S, Stewart GW. Red blood cell membrane defects. Rev Clin Exp Hematol. 2003;7(1):22-56.
4. Gore DM, Layton M, Sinha AK, Williamson PJ, Vaidya B, Connolly V, et al. Four pedigrees of the cation-leaky hereditary stomatocytosis class presenting with pseudohyperkalaemia. Novel profile of temperature dependence of Na+-K+ leak in a xerocytic form. Br J Haematol. 2004; 125(4):521-7.
5. Jarvis HG, Chetty MC, Nicolaou A, Fisher J, Miller A, Stewart GW. A novel stomatocytosis variant showing marked abnormalities in intracellular [Na] and [K] with minimal haemolysis. Eur J Haematol. 2001; 66(6):412-4.
6. Gore DM, Chetty MC, Fisher J, Nicolaou A, Stewart GW. Familial pseudohyperkalaemia Cardiff: a mild version of cryohydrocytosis. Br J Haematol. 2002;117(1):212-4.
7. Fricke B, Jarvis HG, Reid CD, Aguilar-Martinez P, Robert A, Quittet P, et al. Four new cases of stomatin-deficient hereditary stomatocytosis syndrome: association of the stomatin-deficient cryohydrocytosis variant with neurological dysfunction. Br J Haematol. 2004; 125(6):796-803.
8. Haines PG, Crawley C, Chetty MC, Jarvis H, Coles SE, Fisher J, et al. Familial pseudohyperkalaemia Chiswick: a novel congenital thermotropic variant of K and Na transport across the human red cell membrane. Br J Haematol. 2001;112(2):469-74.
9. Bruce L. Mutations in band 3 and cation leaky red cells. Blood Cell Mol Dis. 2006; 36(3):331-6.
10. Bruce LJ. Hereditary stomatocytosis and cation leaky red cells - recent developments. Blood Cell Mol Dis. 2009;42(3):216-22
11. Guizouarn H, Martial S, Gabillat N, Borgese F. Point mutations involved in red cell stomatocytosis convert the electroneutral anion exchanger 1 to a nonselective cation conductance. Blood. 2007;110(6): 2158-65.
12. Ellory JC, Guizouarn H, Borgese F, Bruce LJ, Wilkins RJ, Stewart GW. Review. Leaky Cl--HCO3- exchangers: cation fluxes via modified AE1. Philos Trans R Soc Lond B Biol Sci. 2009;364(1514):189-94.
13. Orlowski J, Grinstein S. Diversity of the mammalian sodium/proton exchanger SLC9 gene family. Pflugers Arch. 2004; 447(5):549-65.
14. Hill JK, Brett CL, Chyou A, Kallay LM, Sakaguchi M, Rao R, et al. Vestibular hair bundles control pH with (Na+, K+)/H+ exchangers NHE6 and NHE9. J Neurosci. 2006;26(39):9944-55.
15. Weiss E, Lang HJ, Bernhardt I. Inhibitors of the K+(Na+)/H+ exchanger of human red blood cells. Bioelectrochemistry (Amsterdam, Netherlands) 2004;62(2):135-40.
16. Bernhardt I, Weiss E. Passive membrane permeability for ions and the membrane potential. In: Bernhardt I, Ellory JC, eds. Red Cell Membrane Transport in Health and Disease. Berlin: Springer, 2003. p. 83-109.
17. Guizouarn H, Gabillat N, Borgese F. Evidence for up-regulation of the endogenous Na-K-2Cl co-transporter by molecular interactions with the anion exchanger tAE1 expressed in Xenopus oocyte. J Biol Chem. 2004;279(12):11513-20.
18. Lutz HU, Stammler P, Fasler S, Ingold M, Fehr J. Density separation of human red blood cells on self forming Percoll gradients: correlation with cell age. Biochim Biophys Acta. 1992;1116(1):1-10.
19. Reliene R, Mariani M, Zanella A, Reinhart WH, Ribeiro ML, del Giudice EM, et al. Splenectomy prolongs in vivo survival of erythrocytes differently in spectrin/ankyrin- and band 3-deficient hereditary spherocytosis. Blood. 2002;100(6):2208-15.
20. Southgate HJ, Colliss JS, Short SM. Comparison of a colorimetric potassium method with flame photometry and ion-selective electrodes. Ann Clin Biochem. 1991;28(Pt4):412-3.
21. Kaji DM, Thakkar U, Kahn T. Glucocorticoid-induced alterations in the sodium potassium pump of the human erythrocyte. J Clin Invest. 1981;68(2):422-30.
22. Bernhardt I, Hall AC, Ellory JC. Effects of low ionic strength media on passive human red cell monovalent cation transport. J Physiol. 1991;434:489-506.
23. Macey RI, Adorante JS, Orme FW. Erythrocyte membrane potentials determined by hydrogen ion distribution. Biochim Biophys Acta. 1978;512(2):284-95.
24. Bennekou P, Pedersen O, Moller A, Christophersen P. Volume control in sickle cells is facilitated by the novel anion conductance inhibitor NS1652. Blood. 2000; 95(5):1842-8.
25. Bennekou P. K+-valinomycin and chloride

conductance of the human red cell membrane. Influence of the membrane protonophore carbonylcyanide m-chlorophenylhydrazone. Biochim Biophys Acta. 1984; 776(1):1-9.
26. Bennekou P, Stampe P. The effect of ATP, intracellular calcium and the anion exchange inhibitor DIDS on conductive anion fluxes across the human red cell membrane. Biochim Biophys Acta. 1988; 942(1):179-85.
27. Baunbaek M, Bennekou P. Evidence for a random entry of Ca(2+) into human red cells. Bioelectrochemistry. 2008;73(2):145-50.
28. Bennekou P, Kristensen BI, Christophersen P. The human red cell voltage-regulated cation channel. The interplay with the chloride conductance, the Ca(2+)-activated K(+) channel and the Ca(2+) pump. J Membr Biol. 2003;195(1):1-8.
29. Lew VL, Bookchin RM. Ion transport pathology in the mechanism of sickle cell dehydration. Physiol Rev. 2005;85(1):179-200.
30. Lauf PK, Bauer J, Adragna NC, Fujise H, Zade-Oppen AM, Ryu KH, et al. Erythrocyte K-Cl cotransport: properties and regulation. Am J Physiol. 1992;263(5 Pt 1): C917-32.
31. Stewart GW, Ellory JC, Klein RA. Increased human red cell cation passive permeability below 12 degrees C. Nature. 1980; 286 (5771):403-4.
32. Jarvis HG, Gore DM, Briggs C, Chetty MC, Stewart GW. Cold storage of 'cryohydrocytosis' red cells: the osmotic susceptibility of the cold-stored erythrocyte. Br J Haematol. 2003;122(5):859-68.
33. Kummerow D, Hamann J, Browning JA, Wilkins R, Ellory JC, Bernhardt I. Variations of intracellular pH in human erythrocytes via K(+)(Na(+))/H(+) exchange under low ionic strength conditions. J Membr Biol. 2000;176(3):207-16.
34. Ellory JC, Robinson HC, Browning JA, Stewart GW, Gehl KA, Gibson JS. Abnormal permeability pathways in human red blood cells. Blood Cell Mol Dis. 2007;39(1):1-6.
35. Gibson JS, Ellory JC. K+-Cl– cotransport in vertebrate red cells. In: Bernhardt I, Ellory JC, eds. Red Cell Membrane Transport in Health and Disease. Berlin: Springer, 2003. p. 197-220.
36. Muzyamba MC, Campbell EH, Gibson JS. Effect of intracellular magnesium and oxygen tension on K+-Cl+ cotransport in normal and sickle human red cells. Cell Physiol Biochem. 2006;17(3-4):121-8.
37. De Franceschi L, Ronzoni L, Cappellini MD, Cimmino F, Siciliano A, Alper SL, et al. K-Cl co-transport plays an important role in normal and b thalassemic erythropoiesis. Haematologica. 2007;92(10):1319-26.
38. Agalakova NI, Lapin AV, Gusev GP. Temperature effects on ion transport across the erythrocyte membrane of the frog Rana temporaria. Comp Biochem Physiol A Physiol. 1997;117(3):411-8.
39. Orlov SN, Kolosova IA, Cragoe EJ, Gurlo TG, Mongin AA, Aksentsev SL, et al. Kinetics and peculiarities of thermal inactivation of volume-induced Na+/H+ exchange, Na+,K+,2Cl– cotransport and K+, Cl– cotransport in rat erythrocytes. Biochim Biophys Acta. 1993; 1151(2):186-92.
40. Bennett V, Baines AJ. Spectrin and ankyrin-based pathways: metazoan inventions for integrating cells into tissues. Physiol Rev. 2001;81(3):1353-92.
41. Willardson BM, Thevenin BJ, Harrison ML, Kuster WM, Benson MD, Low PS. Localization of the ankyrin-binding site on erythrocyte membrane protein, band 3. J Biol Chem. 1989;264(27):15893-9.
42. Lepke S, Fasold H, Pring M, Passow H. A study of the relationship between inhibition of anion exchange and binding to the red blood cell membrane of 4,4'-diisothiocyano stilbene-2,2'-disulfonic acid (DIDS) and its dihydro derivative (H2DIDS). J Membr Biol. 1976;29(1-2):147-77.
43. Masereel B, Pochet L, Laeckmann D. An overview of inhibitors of Na(+)/H(+) exchanger. Eur J Med Chem. 2003;38(6): 547-54.
44. Brugnara C. Therapeutic strategies for prevention of sickle cell dehydration. Blood Cell Mol Dis. 2001;27(1):71-80.
45. Birkenmeier CS, Barker JE. Hereditary haemolytic anaemias: unexpected sequelae of mutations in the genes for erythroid membrane skeletal proteins. J Pathol. 2004; 204(4):450-9.
46. Low PS, Zhang D, Bolin JT. Localization of mutations leading to altered cell shape and anion transport in the crystal structure of the cytoplasmic domain of band 3. Blood Cell Mol Dis. 2001;27(1):81-4.
47. Low PS. Structure and function of the cytoplasmic domain of band 3: center of erythrocyte membrane-peripheral protein interactions. Biochim Biophys Acta. 1986; 864(2):145-67.