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

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Design and Methods

Ektacytometry and density gradients

Whole heparinized blood was mixed with isotonic buffer containing exclusively either K^+ or Na^+ ions and an anti-coagulant. Na-buffer: 145 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, 0.5 mM EDTA, 0.05% mM D-glucose (pH 7.4, 300-320 mOsM); K-buffer: 145 mM KCl, 10 mM K_2HPO_4/KH_2PO_4, 0.5 mM EDTA, 0.05% mM D-glucose (pH 7.4, 300-320 mOsM). The suspensions were passed at room temperature through a leukocyte filter from Pall to remove white cells. Remaining cells were washed from the filter with the appropriate buffer. 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. In the latter case aliquots comprising 4×10^{10} RBC were centrifuged and the cells resuspended with 36 mL Percoll solution containing exclusively either Na^+ or K^+ ions: Na-Percoll solution (300 mL): 257 g Percoll, 11.4 mL 3 M NaCl, 7.5 mL D-glucose (40 g/L), 15 mL 0.2 M NaH_2PO_4, 0.3 mL 0.5 M EDTA, 60 µL PMSF (50 mg/mL in ethanol) adjusted to pH 7.4 with NaOH (310-320 mOsM, 1.11-1.119 g/L). K-Percoll solution (300 mL): as for Na-Percoll, except that 15 mL of 0.2 M K_2HPO_4 and KOH for pH adjustment were used instead of the sodium salts. Where indicated the Percoll solutions were supplemented with 10 µM DIDS or 100 µM 4-acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt hydrate (SITS). These suspensions were centrifuged, the gradients photographed, and fractionated. 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 the Percoll gradient. Osmoscans were obtained as described previously with an ektacytometer (Technikon Products, Bayer, Germany) except that the three solutions required, the low and high osmolality 18% dextran buffer as well as the sample buffers were prepared to contain exclusively Na^+ ions as cations.

Protein composition of red blood cell membranes

Membranes were prepared, solubilized and run on SDS PAGE following reduction and alkylation of the samples. In order to resolve bands 4.1 a and b better, electrophoresis was continued for 30 min once the tracking dye had reached the front.

Determinations of K^+ leak in whole blood

Heparinzed 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, then the plasma was withdrawn and analyzed for K^+ by ion-selective electrodes.

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 (IL-943, Instrumentation Laboratory). 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.

K^+ influx measurements

The RBC were washed three times with a NaCl solution containing: 145 mM NaCl, 10 mM glucose, 10 mM MES (morpholinoethane sulfonic acid)-Tris-(hydroxymethyl aminomethane), pH 7.4 at room temperature or alternatively 6 times with a corresponding solution in which the NaCl was replaced by $NaCH_3SO_4$ to assess the activity of KCC, which was detected with a method reliably used by us and other groups for about 20 years. The RBC were then washed once in the appropriate medium used for the flux experiment (with or without HOE-642). In all experiments ouabain (0.1 mM), bumetanide (0.1 mM), and EGTA (0.1 mM) were present in the solution during the flux measurement. HOE-642 was dissolved in DMSO and employed at a final concentration of 0.5 mM. The RBC were suspended at a hematocrit of approximately 5% in a total volume of 1 mL of flux medium contained in an Eppendorf microcentrifuge tube. The cell suspension was equilibrated at the flux temperature of 37°C or 0°C (on ice) for 5 min, after which tracer solution ([KCl + ^{86}RbCl]) or ([KCH_3SO_4 + ^{86}RbCl]) was added at a final concentration of 7.5 mM. The duration of the exposure of the cells to isotope

(i.e. the flux time) was 30 min. After termination of isotope uptake, the activity of ^{86}Rb in the supernatant was determined by Cerenkov counting in a liquid scintillation counter (Packard Tri-Carb 2900). The hemoglobin content of RBC suspensions was determined as cyanmethemoglobin using Drabkin's reagent.

Conductance determinations and Co^{2+}-influx

The cells were washed three times in MOPS (3-(n-morpholino)propanesulfonic acid) potassium equilibrium Ringer containing 90 mM KCl, 51 mM NaCl, 5 mM MOPS, pH 7.4, 38°C), followed by three washes in isotonic 150 mM NaCl solution, all at room temperature and stored as packed cells at room temperature. Packed RBC were injected into either an unbuffered salt solution [normal Ringer (NR), 154 mM NaCl, 2 mM KCl] or a sucrose-containing low ionic strength Ringer (SR, 264 mM sucrose, 2 mM KCl) to a hematocrit of 3.1%. In all experiments the suspensions were supplemented with 20 µM carbonyl-cyanide-m-chloro-phenyl-hydrazone (CCCP) and in some cases with 10 µM NS1652 (final concentrations) from stock solutions in DMSO.

The membrane potential of the cells was estimated by continuously monitoring the extracellular pH (pH_{out}) in the presence of the protonophore CCCP and calculated as:

$$V_m = 61.5\ mV \times (pH_{in} - pH_{out}). \qquad \text{Eq. 1}$$

pH_{in} was determined as the pH in the solution after the cells had been lysed by addition of 100 µL Triton X-100 (1% v/v) in 3 M NaCl solution.

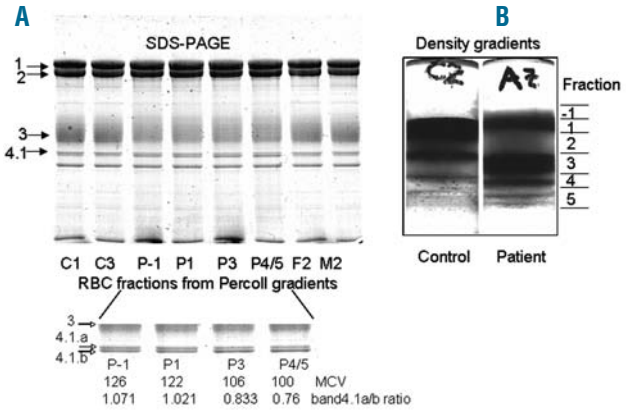
The chloride conductance, under net efflux conditions, which were induced by addition of valinomycin (0.1 µM final concentration), was calculated from $g_{Cl} = i_{Cl}\ (E_d - V_m)$, where i_{Cl} was taken to be negative, but identical to the K^+ current (i_K), which was estimated from the increase in the extracellular K^+ content, determined by flame photometry (Radiometer FLM3). The cation conductances (g_+) were subsequently estimated from:

$$g_+ = g_{Cl}\frac{E_{Cl} - V_m}{V_m - E_{K^+}}, \qquad \text{Eq. 2}$$

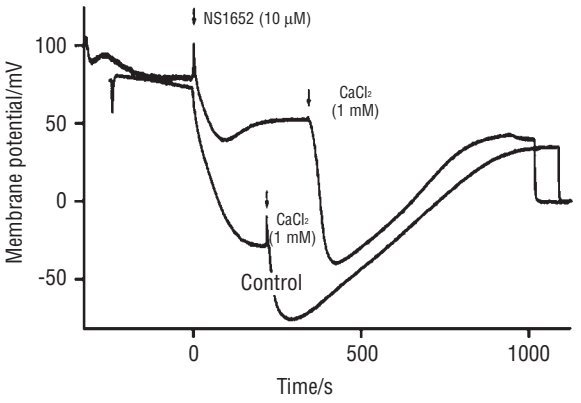
where g_{Cl} is the chloride conductance, V_m the membrane potential, and E_{Cl} and E_K the Nernst potentials for Cl^- and K^+, respectively.

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 (about 4 µM due to contamination from other salts/sucrose in nominally Ca^{2+}-free solutions). Furthermore, the Gardos channel response to an intermediate conductance potassium channel agonist (NS309, final concentration 100 µM) was studied.

For Co^{2+} entry measurements, the cells were suspended in MOPS potassium equilibrium Ringer. At t = 0,50 µM $Co^{2+}/^{57}Co$ was added. At varied time intervals a sample of the suspension was centrifuged through phthalate. The cellular cobalt content was determined from the γ-activity of the cell pellet, isolated below the phthalate layer, relative to the total activity of the sample.



Online Supplementary Figure S1. SDS PAGE of membrane proteins from density-separated RBC of the patient, his family and a control. (A) A Coomassie blue stained 8% SDS PAGE is shown, which was run with 7 µg membrane protein per lane as outlined. The RBC membranes were from the patient (P), his mother (M), father (F) and a control (C). The numbers refer to the density fractions from which the RBC were taken. The relevant protein bands are indicated on the left: 1,2: spectrin band 1 and 2; 3: band 3; 4.1: band 4.1. A blow-up insert underneath shows bands 4.1a and b for the patient's RBC fractions with different densities, the corresponding densitometry results on the band 4.1a:b ratio for each fraction, and the MCV of washed RBC from the indicated fractions. The corresponding Percoll density gradients, from which these fractions were obtained, are shown on the right (B). Regular Percoll gradients, containing Na^+ and K^+ ions, were run with RBC from a control and the patient. For control cells five fractions (1-5) were aspirated; in the case of the patient an additional fraction was collected, which migrated above fraction 1 (-1).



Online Supplementary Figure S2. Cation conductance of the patient's RBC membrane. NSVDC channel response at 38°C. Upper trace, patient's cells, lower trace control cells. After an initial voltage clamp period at the normal chloride conductance (of 6 and 5 min) to enhance the voltage activation of the NSVDC channel, NS1652 was added, which blocks the chloride conductance, leading to hyperpolarization. The cation conductance for CHC RBC is about 2 µS/cm², which is within the normal range, compared to about 3 µS/cm² for control cells. Addition of 1 mM $CaCl_2$ led, in both cases, to Gardos channel activation and to a conductance of about 13 µS/cm², followed by deactivation due to active Ca^{2+} extrusion. Note that the apparent difference between the traces is mainly due to the difference in the Nernst K^+ potential between the cells. Estimation of the intracellular K^+ content passed on the Nernst potential for K^+ (E_K) in the presence of valinomycin gave a value of 70 mM in agreement with the data obtained using flame photometry. Furthermore, the patient's RBC had a rather high chloride content of about 130 mM Cl^- estimated from a membrane potential of about –4.5 mV in the presence of 156 mM external Cl^-, suggesting either low hemoglobin content or low intracellular pH.