Supplemental material:

A previously unrecognized Ca2+-inhibited non-selective cation channel in red blood cells

Polina Petkova-Kirova[1], Laura Hertz[1], Asya Makhro[2], Jens Danielczok[1], Rick Huisjes[4], Esther Llaudet-Planas[3], Maria del Mar Mañú-Pereira[3], Joan-Luis Vives Corrons[3], Richard van Wijk[4], Anna Bogdanova[2], Lars Kaestner[5]

[1]Research Centre for Molecular Imaging and Screening, Medical School, Institute for Molecular Cell Biology, Saarland University, Homburg/Saar, Germany

[2]Red Blood Cell Research Group, Institute of Veterinary Physiology, Vetsuisse Faculty and the Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland

[3]Red Blood Cell defects and Hematopoietic disorders unit, Josep Carreras Leukaemia Research Institute, Badalona, Barcelona, Spain

[4]Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, The Netherlands

[5]Theoretical Medicine and Biosciences, Saarland University, Homburg/Saar and Experimental Physics, Saarland University, Saarbruecken, Germany

Materials and Methods

Blood

Blood was obtained from healthy donors after giving an informed consent in compliance with the ethical requirements of the Saarland University, Homburg, Germany (Ärztekammer des Saarlandes, approval number 132/08) and the University of Zürich, Zürich, Switzerland, (the Canton's ethics committee of canton Zürich, KEK ZH NR 2010-0237). The study was carried out in accordance with the Helsinki Declaration of 1975, as revised in 2008. A blood collection system by Becton and Dickinson (Vacutainer Blood Collection Set REF 367282, Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and the same batches of 9 ml containers for K3EDTA (referred to as EDTA) and Sodiumheparin (referred to as heparin) (Vacuette, Becton, Dickinson and Co., Franklin Lakes, NJ, USA) were used.

Patch-clamp

Patch-clamp measurements were performed with a NPC-16 Patchliner (Nanion Technologies, Munich, Germany) as previously described[1]. The resistance of the chips was between 5 and 8 MΩ. Gigaseals were considered successful if exceeding 5 GΩ. Gigaseal formation was facilitated by the use of a seal enhancing solution as recommended by the

Patchliner manufacturer and containing (in mM): NaCl 80, KCl 3, $MgCl_2$ 10, $CaCl_2$ 35, HEPES 10, pH=7.3 adjusted with NaOH. Whole-cell configuration was achieved by negative pressure suction pulses between -45 mbar and -150 mbar and its formation judged by the appearance of sharp capacitive transients. Whole-cell patch-clamp recordings were conducted using voltage steps from -100 mV to 100 mV for 500 ms in 20 mV increments at 5 s intervals, the holding potential being set at -30 mV. To reduce inter-cell variability in currents, data are expressed as normalized current which is the ratio of the current under specified experimental conditions i.e. in 0 mM Ca^{2+} external solution or in a solution with Ca^{2+} (2 or 20 mM Ca^{2+}), to the current at +100 mV determined 30–60 s before starting the measurement in 0 mM Ca^{2+}. The Ca^{2+} blocked current is the current obtained as a result of the subtraction of the current recorded in 2 mM Ca^{2+} from the current recorded in 0 mM Ca^{2+} external solution. Before subtraction currents were normalized. All measurements were performed at room temperature. Current recordings in 2 mM Ca^{2+} solutions were all performed at 5 GΩ and above and the seals were maintained throughout the 0 mM Ca^{2+} solutions measurements as follows: 81% (13 out of 16 cells) at 5Gohm and above; 6% (1 cell) at 4.4 GΩ and 13% (2 cells) at 4 GΩ. Data are presented as means ± SEMs and statistical significance evaluated using a paired Student t-test. N is the number of cells and in brackets after “n” is the number of donors. Cell capacitance (n=16 cells) was 0.59±0.04 pF.

Solutions used to study the Ca^{2+} blocked channel were as follows (in mM):

I. For experiments using a Cs^+-based internal and a TEACl-based external solutions:

- Internal: 50CsCl, 20NaCl, 60CsF, 5MgATP, 10HEPES, 20EGTA, pH=7.2 with CsOH
- External 0mM Ca^{2+}: 125TEACl, 10HEPES, 5$MgCl_2$, 45glucose, pH=7.3 with TEAOH
- External 2mM Ca^{2+}: 125TEACl, 10HEPES, 5$MgCl_2$, 45glucose, 2mM $CaCl_2$ pH=7.3 with TEAOH

II. For experiments using a Cs^+-based internal and an external solution without Cl^- :

- Internal: 50CsCl, 20NaCl, 60CsF, 5MgATP, 10HEPES, 20EGTA, pH=7.2 with CsOH
- External 0mM Ca^{2+}: 125$TEANO_3$, 10HEPES, 5$MgSO_4$, 45glucose, pH=7.3 with TEAOH
- External 2mM Ca^{2+}: 125$TEANO_3$, 10HEPES, 5$MgSO_4$, 45glucose, 2mM Ca gluconate , pH=7.3 with TEAOH

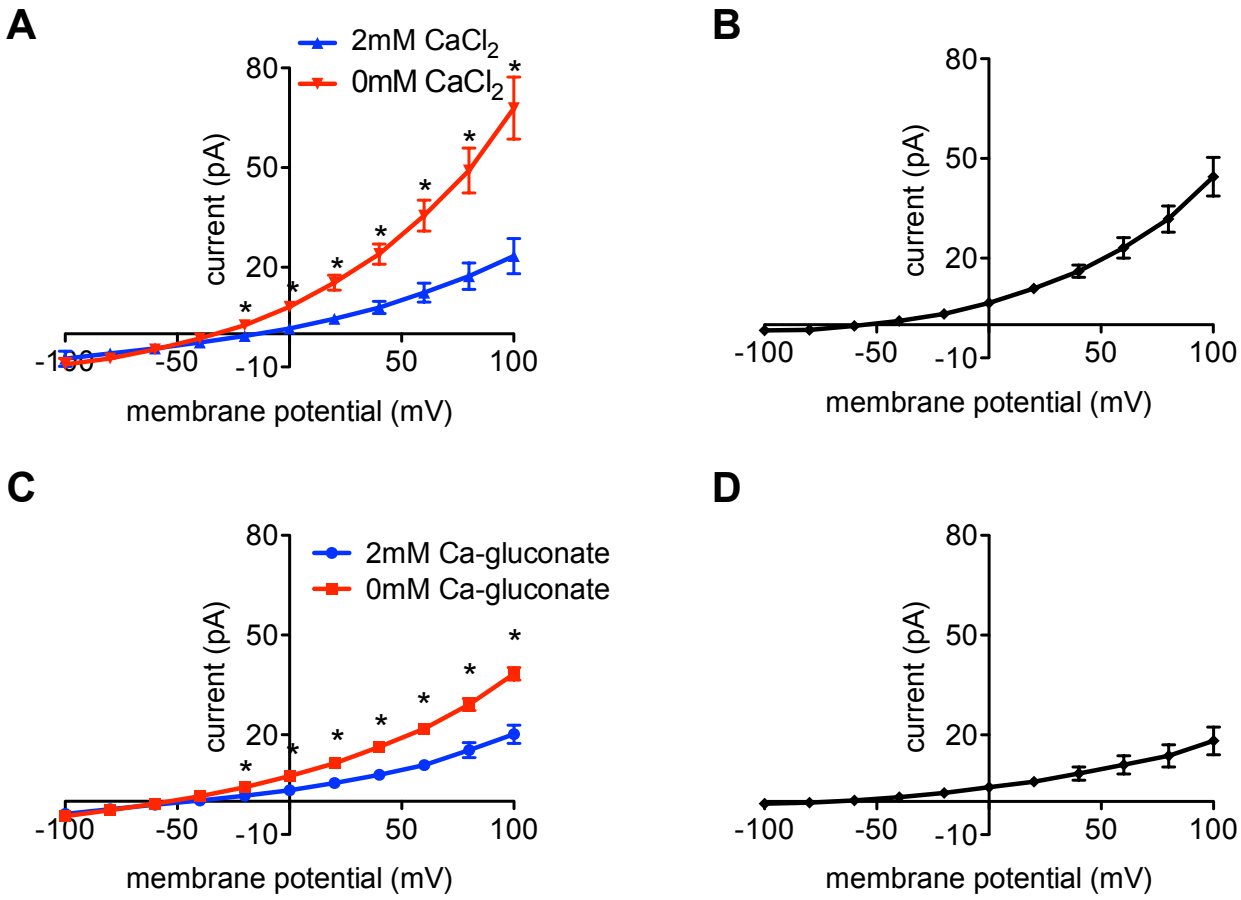
III. For experiments using physiological internal and external solutions:

- Internal: 70KCl, 70KF, 10NaCl, 10HEPES, 3EGTA, 1.2$CaCl_2$, 3MgATP, pH=7.2 with KOH
- External 0mM Ca^{2+}: 140NaCl, 4KCl, 10HEPES, 5$MgCl_2$ 5glucose, pH=7.3 with NaOH
- External 20mM Ca^{2+}: 115NaCl, 4KCl,10HEPES, 5$MgCl_2$, 20$CaCl_2$, pH=7.3 with NaOH
- External 2mM Ca^{2+}: 140NaCl, 4KCl, 10HEPES, 5$MgCl_2$, 5glucose, 2$CaCl_2$ pH=7.3 with NaOH

F- in the internal solution is a necessary requirement to achieve gigaseals when recording from erythrocytes. Inclusion of fluoride has long been known to facilitate and improve patch-clamp sealing with subsequent longer and more stable patch-clamp recordings.[2] Relative to this however a note should be made on the RBC membrane permeability reported to be similar for Cl^- and F^- both in its DIDS sensitive and DIDS insensitive part.[3]

Ion measurements of blood plasma

A blood gas analyzer ABL 700 (Radiometer, Brønshøj, Denmark) was used to measure free Ca^{2+}, Na^{+} and K^{+} content in blood samples and aqueous $CaCl_2$ solutions [4]. Plasma K^{+} and Na^{+} contents were assessed and corrected for Na^{+} and K^{+} present in the vacutainer anticoagulants (Na-heparin and K3EDTA). To perform the correction an aqueous solution of $CaCl_2$ (1.8 mM final concentration) was prepared and filled into the vacutainers in volumes equal to those of the blood samples. Thereafter free Na^{+}, K^{+} and Ca^{2+} were measured. The obtained values were subtracted from those measured for blood plasma. The levels of free Ca^{2+} in the EDTA containing vacutainers were below the detection limit of the ion-selective electrode of the blood analyzer for both the aqueous $CaCl_2$ solution and plasma. All measurements were performed at room temperature and in triplicates within an hour after blood collection.



Supplemental Figure S1 corresponds to Figure 1 in the main body of the manuscript with currents being shown in absolute values (pAs). Whole-cell patch clamp recordings in a Cs^{+}-based internal and a TEACl-based external solutions **(A)** I/V curves with 2 mM $CaCl_2$ (blue) and 0 mM $CaCl_2$ (red) in the external solution (n=5(3)) **(B)** I/V curve of the Ca^{2+} blocked current (CBC) - the current recorded in 2 mM $CaCl_2$-external solution was subtracted from the current recorded in 0 mM $CaCl_2$ -external solution. Whole-cell patch clamp recordings in a Cs^{+}-based internal and a $TEANO_3$-based external solution devoid of

Cl^-. **(C)** I/V curves with 2 mM Ca gluconate (blue) and 0 mM Ca gluconate (red) in the external solution (n=4(1)). **(D)** I/V curve of the Ca^{2+} blocked current (CBC) - the current recorded in 2 mM Ca gluconate-external solution was subtracted from the current recorded in 0 mM Ca gluconate -external solution. Currents were elicited by voltage steps from -100 mV to 100 mV for 500 ms in 20 mV increments at V_h = -30 mV. Measurements were performed at room temperature. Data are presented as mean ± SEM. Significance is assessed with a paired Student´s t test and set at $p<0.05$. For better visualization, a significance anywhere below $p<0.05$ is denoted with one star.

Calculation of the cation flux based on the whole-cell conductance

The cation flux *J* across the RBC membrane caused by the ion channel under investigation can be calculated as:

$$J = J_{SC} \times n \quad (1)$$

with J_{SC} being the flux across the membrane of a single cell and *n* being the number of RBCs per volume of blood ($n = 5x10^6$ μl^{-1}. In similarity to the calculations in the appendix of Kaestner *et al.* 1999 [5], the single cell flux can be estimated as:

$$J_{SC} = \frac{G\,V_m}{q_{el}\,N_A} \quad (2)$$

with *G* being the whole cell conductance, V_m being the membrane potential, q_{el} being the elementary charge and N_A being Avogadro's number.The whole cell conductance can be derived from the slope of the I/V curve in Figure 2F (G = 143 pS). In a first approximation we use for the membrane potential the physiological RBC resting membrane potential V_R (V_R = -10 mV). The above values can be filled in the final equation derived from equations (1) and (2):

$$J = \frac{G\,V_R}{q_{el}\,N_A} n \quad (3)$$

Thus the flux due to CiCC is estimated to be 4.3 mM min^{-1}.

References

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