

1 Supplemental material:

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

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

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

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

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

13 ⁴Department of Clinical Chemistry and Haematology, University Medical Center Utrecht,
14 Utrecht, The Netherlands

15 ⁵Theoretical Medicine and Biosciences, Saarland University, Homburg/Saar and
16 Experimental Physics, Saarland University, Saarbruecken, Germany

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18 **Materials and Methods**

19 *Blood*

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

29 *Patch-clamp*

30 Patch-clamp measurements were performed with a NPC-16 Patchliner (Nanion
31 Technologies, Munich, Germany) as previously described¹. The resistance of the chips was
32 between 5 and 8 MΩ. Gigaseals were considered successful if exceeding 5 GΩ. Gigaseal
33 formation was facilitated by the use of a seal enhancing solution as recommended by the

34 Patchliner manufacturer and containing (in mM): NaCl 80, KCl 3, MgCl₂ 10, CaCl₂ 35,
35 HEPES 10, pH=7.3 adjusted with NaOH. Whole-cell configuration was achieved by negative
36 pressure suction pulses between -45 mbar and -150 mbar and its formation judged by the
37 appearance of sharp capacitive transients. Whole-cell patch-clamp recordings were
38 conducted using voltage steps from -100 mV to 100 mV for 500 ms in 20 mV increments at
39 5 s intervals, the holding potential being set at -30 mV. To reduce inter-cell variability in
40 currents, data are expressed as normalized current which is the ratio of the current under
41 specified experimental conditions i.e. in 0 mM Ca²⁺ external solution or in a solution with
42 Ca²⁺ (2 or 20 mM Ca²⁺), to the current at +100 mV determined 30–60 s before starting the
43 measurement in 0 mM Ca²⁺. The Ca²⁺ blocked current is the current obtained as a result of
44 the subtraction of the current recorded in 2 mM Ca²⁺ from the current recorded in 0 mM
45 Ca²⁺ external solution. Before subtraction currents were normalized. All measurements were
46 performed at room temperature. Current recordings in 2 mM Ca²⁺ solutions were all
47 performed at 5 GΩ and above and the seals were maintained throughout the 0 mM Ca²⁺
48 solutions measurements as follows: 81% (13 out of 16 cells) at 5 Gohm and above; 6% (1
49 cell) at 4.4 GΩ and 13% (2 cells) at 4 GΩ. Data are presented as means ± SEMs and
50 statistical significance evaluated using a paired Student t-test. N is the number of cells and
51 in brackets after “n” is the number of donors. Cell capacitance (n=16 cells) was 0.59±0.04
52 pF.

53 Solutions used to study the Ca²⁺ blocked channel were as follows (in mM):

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

- 55 • Internal: 50CsCl, 20NaCl, 60CsF, 5MgATP, 10HEPES, 20EGTA, pH=7.2 with CsOH
- 56 • External 0mM Ca²⁺: 125TEACl, 10HEPES, 5MgCl₂, 45glucose, pH=7.3 with TEAOH
- 57 • External 2mM Ca²⁺: 125TEACl, 10HEPES, 5MgCl₂, 45glucose, 2mM CaCl₂ pH=7.3 with
58 TEAOH

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

- 60 • Internal: 50CsCl, 20NaCl, 60CsF, 5MgATP, 10HEPES, 20EGTA, pH=7.2 with CsOH
- 61 • External 0mM Ca²⁺: 125TEANO₃, 10HEPES, 5MgSO₄, 45glucose, pH=7.3 with TEAOH
- 62 • External 2mM Ca²⁺: 125TEANO₃, 10HEPES, 5MgSO₄, 45glucose, 2mM Ca gluconate ,
63 pH=7.3 with TEAOH

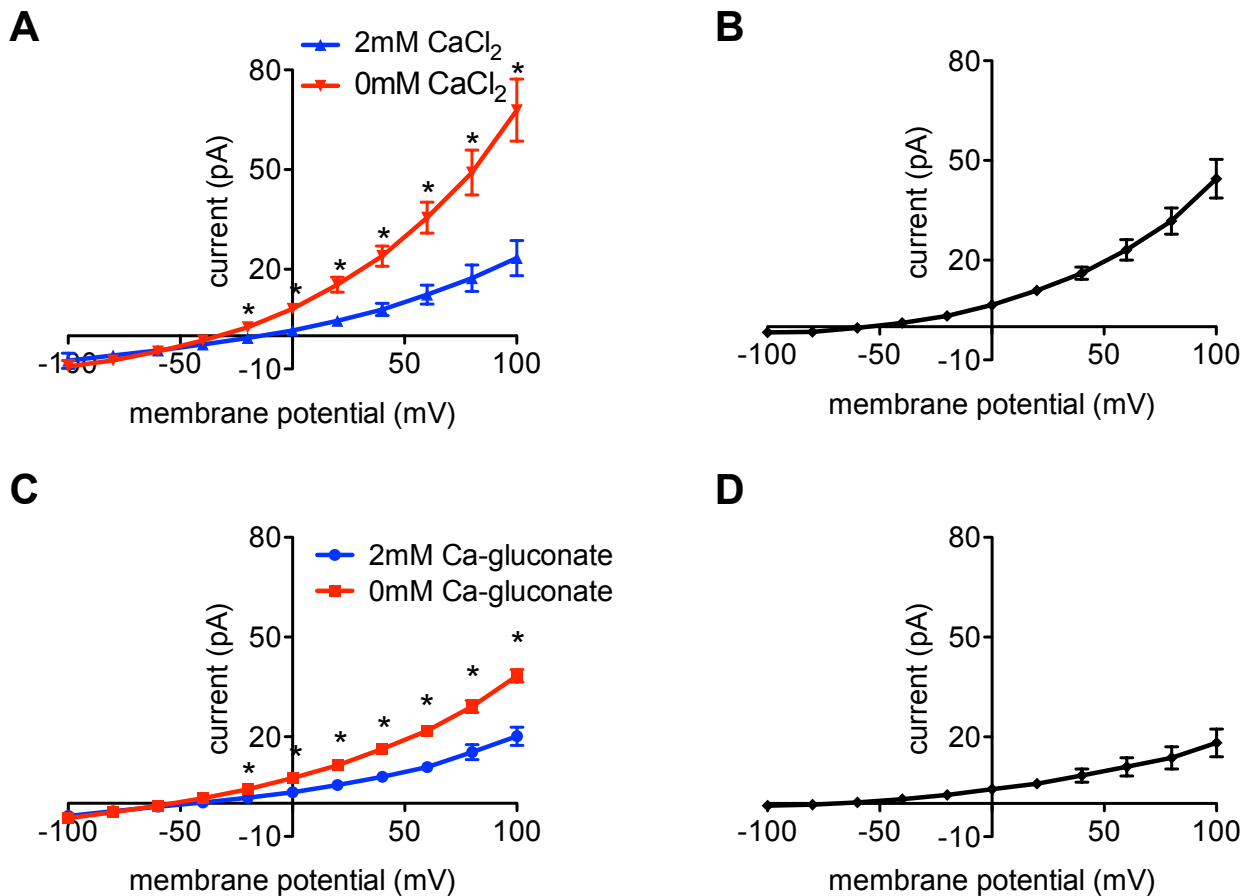
64 III. For experiments using physiological internal and external solutions:

- 65 • Internal: 70KCl, 70KF, 10NaCl, 10HEPES, 3EGTA, 1.2CaCl₂, 3MgATP, pH=7.2 with
66 KOH
- 67 • External 0mM Ca²⁺: 140NaCl, 4KCl, 10HEPES, 5MgCl₂ 5glucose, pH=7.3 with NaOH
- 68 • External 20mM Ca²⁺: 115NaCl, 4KCl, 10HEPES, 5MgCl₂, 20CaCl₂, pH=7.3 with NaOH
- 69 • External 2mM Ca²⁺: 140NaCl, 4KCl, 10HEPES, 5MgCl₂, 5glucose, 2CaCl₂ pH=7.3 with
70 NaOH

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

76 *Ion measurements of blood plasma*

77 A blood gas analyzer ABL 700 (Radiometer, Brønshøj, Denmark) was used to measure free
 78 Ca²⁺, Na⁺ and K⁺ content in blood samples and aqueous CaCl₂ solutions⁴. Plasma K⁺ and
 79 Na⁺ contents were assessed and corrected for Na⁺ and K⁺ present in the vacutainer
 80 anticoagulants (Na-heparin and K3EDTA). To perform the correction an aqueous solution of
 81 CaCl₂ (1.8 mM final concentration) was prepared and filled into the vacutainers in volumes
 82 equal to those of the blood samples. Thereafter free Na⁺, K⁺ and Ca²⁺ were measured. The
 83 obtained values were subtracted from those measured for blood plasma. The levels of free
 84 Ca²⁺ in the EDTA containing vacutainers were below the detection limit of the ion-selective
 85 electrode of the blood analyzer for both the aqueous CaCl₂ solution and plasma. All
 86 measurements were performed at room temperature and in triplicates within an hour after
 87 blood collection.



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89 **Supplemental Figure S1 corresponds to Figure 1 in the main body of the manuscript**
 90 **with currents being shown in absolute values (pAs).** Whole-cell patch clamp recordings
 91 in a Cs⁺-based internal and a TEACl-based external solutions **(A)** I/V curves with 2 mM
 92 CaCl₂ (blue) and 0 mM CaCl₂ (red) in the external solution (n=5(3)) **(B)** I/V curve of the Ca²⁺
 93 blocked current (CBC) - the current recorded in 2 mM CaCl₂-external solution was
 94 subtracted from the current recorded in 0 mM CaCl₂-external solution. Whole-cell patch
 95 clamp recordings in a Cs⁺-based internal and a TEANO₃-based external solution devoid of

96 Cl⁻. **(C)** I/V curves with 2 mM Ca gluconate (blue) and 0 mM Ca gluconate (red) in the
 97 external solution (n=4(1)). **(D)** I/V curve of the Ca²⁺ blocked current (CBC) - the current
 98 recorded in 2 mM Ca gluconate-external solution was subtracted from the current recorded
 99 in 0 mM Ca gluconate -external solution. Currents were elicited by voltage steps from -100
 100 mV to 100 mV for 500 ms in 20 mV increments at V_h = -30 mV. Measurements were
 101 performed at room temperature. Data are presented as mean ± SEM. Significance is
 102 assessed with a paired Student's t test and set at p<0.05. For better visualization, a
 103 significance anywhere below p<0.05 is denoted with one star.

104

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

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

$$108 \quad J = J_{SC} \times n \quad (1)$$

109 with J_{SC} being the flux across the membrane of a single cell and n being the number of
 110 RBCs per volume of blood ($n = 5 \times 10^6 \mu\text{l}^{-1}$). In similarity to the calculations in the appendix of
 111 Kaestner *et al.* 1999⁵, the single cell flux can be estimated as:

$$112 \quad J_{SC} = \frac{G V_m}{q_{el} N_A} \quad (2)$$

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

$$119 \quad J = \frac{G V_R}{q_{el} N_A} n \quad (3)$$

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

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