Mechanistic ion channel interactions in red cells of patients with Gárdos channelopathy

Julia Jansen,1,2 Min Qiao,1,2 Laura Hertz,1,2 Xijia Wang,3 Elisa Fermo,4 Anna Zaninoni,4 Raffaella Colombatti,5 Ingolf Bernhardt,3 Paola Bianchi,4 and Lars Kaestner1,2

1Theoretical Medicine and Biosciences, Saarland University, Homburg, Germany; 2Experimental Physics and; 3Laboratory of Biophysics, Saarland University, Saarbruecken, Germany; 4Unita Operativa Semplice (UOS) Fisiopatologia delle Anemie, Unità Operativa Complessa (UOC) Ematologia, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Ca’ Granda Ospedale Maggiore Policlinico Milano, Milan, Italy; and 5Clinic of Pediatric Hematology-Oncology, Department of Woman’s and Child’s Health, University of Padua, Padua, Italy

In patients with Gárdos channelopathy (p.R352H), an increased concentration of intracellular Ca$^{2+}$ was previously reported. This is a surprising finding because the Gárdos channel (KCa3.1) is a K$^{+}$ channel. Here, we confirm the increased intracellular Ca$^{2+}$ for patients with the KCa3.1 mutation p.S314P. Furthermore, we provide the concept of KCa3.1 activity resulting in a flickering of red blood cell (RBC) membrane potential, which activates the CaV2.1 channel allowing Ca$^{2+}$ to enter the RBC. Activity of the nonselective cation channel Piezo1 modulates the aforementioned interplay in away that a closed Piezo1 is in favor of the KCa3.1-CaV2.1 interaction. In contrast, Piezo1 openings compromise the membrane potential flickering, thus limiting the activity of CaV2.1. With the compound NS309, we mimic a gain-of-function mutation of KCa3.1. Assessing the RBC Ca$^{2+}$ response by Fluo-4–based flow cytometry and by measuring the membrane potential using the Macey-Bennekou-Eggé method, we provide data that support the concept of the KCa3.1/CaV2.1/Piezo1 interplay as a partial explanation for an increased number of high Ca$^{2+}$ RBCs. With the pharmacological inhibition of KCa3.1 (TRAM34 and Senicapoc), CaV2.1 (v-agatoxin TK), and Piezo1 (GsMTx-4), we could project the NS309 behavior of healthy RBCs to the RBCs of Gárdos channelopathy patients.

Introduction

The Gárdos channel (hSK4, KCa3.1, KCNN4) is a Ca$^{2+}$-activated K$^{+}$ channel and was among the first ion channels described by the patch-clamp technique. In red blood cells (RBCs), the Gárdos channel was initially related to volume homeostasis and cell death. Only recently was its importance recognized for RBCs passing capillaries or constrictions. Gain-of-function mutations in the Gárdos channel have been associated with hereditary hemolytic anemia, either referred to as hereditary stomatocytosis caused by a Gárdos channel mutation or more directly as Gárdos channelopathy. For one of the identified mutations (p.R352H), increased intracellular Ca$^{2+}$ content was reported; for other genetic variants, it was not investigated. Because the Gárdos channel is a K$^{+}$ channel, the mechanism of the Ca$^{2+}$ increase is completely elusive. Here, experimental evidence is provided for the molecular interactions relating Gárdos channel activity to increased intracellular-free Ca$^{2+}$, a key player in accelerated RBC damage and development of anemia.
Figure 1. Ca^{2+} increase in RBCs with Gárdos channel mutations. (A) Ca^{2+} increase in RBCs of a patient with the p.S314P mutation (AIII.1 in Fermo et al^{13}) in comparison with healthy control RBCs. (a-b) Representative fluorescence images of RBCs stained with Fluo-4; scale bar, 10 µm. (c) Statistical analysis of fluorescent images as depicted in subpanel a. The white numbers refer to the numbers of analyzed cells. (d) Analysis of flow cytometric measurements of Fluo-4–stained RBCs. Statistical differences were checked with a Student t test; ***P < .001. (B) Mechanistic hypothesis regarding how Gárdos channel activity may trigger an increase in intracellular RBC Ca^{2+} concentration. The membrane potential changes induced by the opening of the Gárdos channel (cp. explanation in "Results and discussion") activates the...
Methods

RBC collection

The blood sampling and all investigations were performed in accordance with the Helsinki Declaration of 1975. Patients and healthy donors had given their consent to give blood samples for research purposes. The study was approved by the Ethical Committee at Foundation IRCCS Ca’ Granda Ospedale Maggiore of Milan and the Ärztekammer des Saarlandes (approval #51/18). The patients are described in separate previous reports. Blood samples were taken in Li-heparin tubes and shipped overnight from Milan and Padua (Italy) to Homburg (Germany).

Imaging and flow cytometry

Ca2+ imaging was performed as previously described and confocal images were recorded on a TCS SP5 (Leica, Germany) as outlined previously. For flow cytometry measurements, 30 000 RBCs loaded with Fluo4 were analyzed with the flow cytometer as previously described. The percentage of responding RBCs is the statistically analyzed parameter.

Membrane potential measurements

The membrane potential measurements of a RBC population were performed according to a method initially described further developed and applied by Poul Bennekou (i.e., Baunbæk and Bennekou), and kept alive by the laboratory of Stéphane Egré. Therefore, we refer to it as the Macey-Bennekou-Egée (MBE) method. A detailed description is provided in supplemental Materials and methods.

Results and discussion

RBCs from a patient carrying the KCNN4 p.S314P variant were tested for the free intracellular Ca2+ concentration, which was found significantly increased (Figure 1A) in agreement with a previous report in the p.R352H variant. Although such an increase in intracellular Ca2+ has been proposed as a common component of the mechanisms causing anemia, the causal link to a mutation in Górdos channels is very low (1-5 copies per cell in 75% of the cells), and due to the stochastic behavior of channel openings, one can assume that, upon Górdos channel activation, the membrane potential flickers (i.e., jumps between −12 mV and −60 mV). This is the necessary condition to activate voltage-gated Ca2+ channels, such as Cao2.1, which was reported to be abundant in RBCs. The activation of the Cao2.1 channel would result in increased intracellular Ca2+. A scheme of this concept in the context of the gain-of-function Górdos channel mutations is shown in Figure 1B.

To experimentally test this model, we examined the effect of NS309, a compound able to increase the open probability of the Górdos channel, independent of intracellular Ca2+; experiments with RBCs from healthy donors are shown in Figure 2A. NS309-induced Ca2+ entry is at least partly triggered by Górdos channel activity as application of 2 different Górdos channel blockers, charybdoxin (CTX) and TRAM34, reveal (Figure 2Ab). Both blockers (CTX and TRAM34) alone had no influence on the number of high Ca2+ cells (supplementary Figure 1). In a new set of measurements, Cao2.1 and Piezo1 channel blockers ω-agatoxin TK and GsMTx-4 were applied, respectively. ω-agatoxin TK is a specific channel blocker, whereas GsMTx-4 blocks all kinds of mechanosensitive channels including Piezo1. The block of the Cao2.1 channel with ω-agatoxin TK reduces NS309-induced Ca2+ entry, whereas the block of Piezo1 with GsMTx-4 increases the number of high Ca2+ cells (Figure 2Ac). Both blockers (ω-agatoxin TK and GsMTx-4) alone had no influence on the number of high Ca2+ cells (supplementary Figure 1). Although the reduction of the number of high Ca2+ cells by Cao2.1 channel blocker ω-agatoxin TK looks obvious, the increase caused by the application of GsMTx-4 seems a paradox. However, referring to the concept outlined in Figure 1B, the inhibition of the nonselective cation channel Piezo1 is in favor of the flickering membrane potential: if the nonselective cation channel Piezo1 is in an open state, it depolarizes the RBC and thus disables the function of Cao2.1. To test this interpretation, we performed membrane potential measurements using the MBE method (Figure 2Ad), showing the hyperpolarization when the Górdos channel is active and following depolarization when Piezo1 gets activated. In a previous article, the dependence of NS309-induced Górdos channel activation on external Ca2+ concentration was shown, which we could confirm in terms of the induced membrane potential change (Figure 2Ae). Furthermore, our probe of this Ca2+ dependence for the percentage of RBCs responding with an increased Ca2+ concentration indicated a bell-shaped relation (Figure 2Af). Thus, the concept of the interplay of the Górdos channel with Cao2.1 was further supported: when the open probability of the Górdos channel is steadily increased, a condition is reached when the channels are predominantly open. This in turn results in a decrease of membrane potential flickering and hence less activity of Cao2.1 and a lower number of high Ca2+ cells.

In Figure 2Ba, the different sensitivity of the Górdos channel mutations towards application of NS309 initially reported by Ferro et al. is shown and was also reflected in the number of cells responding with an increased Ca2+ concentration. We aimed to test whether the mechanism induced by stimulation with NS309 in healthy cells (Figure 2Ac) was also applicable to cells with the Górdos channel mutation. NS309 increases the open probability of the Górdos channel; gain-of-function mutations of the channel
Figure 2.
Figure 2. Experimental evidence for a mechanistic explanation. (A) Investigation of healthy RBCs to examine interactions between the Gárdos channel and other RBC ion channels. (a) Representative histogram from a flow cytometry measurement of RBCs stained with Fluo-4 and stimulated with 100 μM NS309. The red and the blue lines mark the populations of nonresponging and responding RBCs, respectively. (b) Number of RBCs responding with a high Ca\(^{2+}\) content as outlined in subpanel a at a concentration of 100 μM NS309 (left column) and the effect (preincubation) of the Gárdos channel inhibitors CTX (0.1 μM) and TRAM34 (1 μM) on NS309 (100 μM)-stimulated RBCs (right columns). (c) A new set of experiments compares the action (preincubation) of ω-Agatoxin TK (1 μM), a specific inhibitor of Cav2.1, and GsMTx-4 (2 μM), a toxin inhibiting the mechanosensitive channel Piezo1, on NS309 (100 μM)-stimulated RBCs. (b-c) All measurements are performed on at least 4 different donors. Potted are mean values and the standard error of mean. Statistical differences were checked with a 1-way analysis of variance (ANOVA) and the Tukey multiple comparisons test. **** P < .0001; ns, not significant (P > .05). (d) Monitoring the membrane potential in a population of RBCs by the MBE method. The blue curve starts at the resting membrane potential (~12 mV). Addition of 15 μM A23187 leads to Ca\(^{2+}\) entry and the subsequent full activation of the Gárdos channel resulting in a hyperpolarization of ~60 mV. Addition of TRAM34 inhibits the Gárdos channel resulting in a depolarization. The red curve also starts with the resting membrane potential and addition of 50 μM NS309 leads to the activation of the Gárdos channel, also resulting in a hyperpolarization but to a lesser extent as for the blue curve. Because we monitor the membrane potential in a cell population of >10⁶ RBCs, it is impossible to see a membrane potential flickering (compare "Results and discussion") that may happen in individual cells. The hyperpolarization to only ~40 mV is therefore caused by, to a lesser extent, Gárdos channel activation, that is, a composition of cells with open and closed Gárdos channels. For both experiments, Triton-X 100 is used to calibrate for 0 mV. The curves present the mean of a triplicate measurement of a healthy donor and are a representative of 3 different donors. (e) Membrane potential upon 100 μM NS309 stimulation in dependence of the extracellular Ca\(^{2+}\) concentration. (f) Percentage of high Ca\(^{2+}\) RBCs upon 100 μM NS309 stimulation in dependence of the external Ca\(^{2+}\) concentration. The corresponding histograms are provided in supplemental Figure 2. The bell-shaped curve can be explained by the Gárdos channel–Ca\(^{2+}\)2.1 interaction (see main text). (e-f) All measurements were performed on 3 different donors. Potted are mean values and the standard error of mean. (B) Testing the principle investigated in panel A on RBCs of patients carrying a Gárdos channel mutation. (a) Differential response (number of cells responding with increased intracellular Ca\(^{2+}\)) of healthy and KCNN4 p.S314P- and p.R352H-mutated RBCs on stimulation with 10 μM NS309. (b) p.R352H-mutated RBCs were incubated for 24 hours with the Gárdos channel inhibitors Senicapoc (5 μM) and TRAM34 (1 μM), the Cav2.1 inhibitor ω-Agatoxin TK (1 μM), and the Piezo1 inhibitor GsMTx-4 (2 μM). The number of cells responding with increased intracellular Ca\(^{2+}\) is shown.

should lead to a similar result. Because the Gárdos channel gain-of-function mutation could not be “switched on” (as the Gárdos channel can with the application of NS309), the effect of the channel blockers was compared in a 24-hour “long-term” experiment. RBCs carrying the mutation p.R352H were incubated with the respective inhibitors for 24 hours and the number of RBCs with increased Ca\(^{2+}\) was measured (Figure 2Bb). Although statistical analysis was not available due to sample limitation, the result shown echoes the observation in the NS309-stimulated healthy cells (Figure 2Ac).

These data further support the concept of ion channel interplay being causal for the increased Ca\(^{2+}\) concentration in Gárdos channelopathy RBCs. The given interpretation for the increased Ca\(^{2+}\) in the RBCs of KCNN4-mutated patients may have evident bias because the channel inhibitors only block a fraction of the measured effect, both in the NS309 model (Figure 2A) as well as in the patient RBCs (Figure 2Bb). We present experimental indication of >2 direct ion channel interactions in RBCs that are otherwise rather typical for excitable cells. Further research is required to substantiate and extend the results presented in this stimulus report.

Acknowledgments

This work was supported by European Framework Horizon 2020 under grant agreement number 860436 (EVIDENCE) and by Fondazione IRCCS Ca’ Granda Policlinico Milano, project number RC2020 175/05.

Authorship

Contribution: L.K. and P.B. designed the study; E.F., P.B., and A.Z. performed patient diagnostic workup; L.K. and I.B. supervised the experiments; J.J., M.Q., L.H., X.W., and E.F. performed the experiments; R.C. followed-up with patients; L.K. drafted the manuscript; and all authors revised and approved the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: E.F., 0000-0003-2812-8711; A.Z., 0000-0002-8614-3904; R.C., 0000-0001-9797-0457; P.B., 0000-0001-5976-5233; L.K., 0000-0001-6796-9535.

Correspondence: Lars Kaestner, Saarland University, Building E2_6, 66123 Saarbrücken, Germany; e-mail: lars.kaestner@me.com.

References

1. Hamill OP. Potassium and chloride channels in red blood cells. In: Sakmann B, Neher E, eds. Single channel recording. New York, NY: Plenum Press; 1983:451–471.
2. Maher AD, Kuchel PW. The Gárdos channel: a review of the Ca\(^^{2+}\)-activated K\(^+\) channel in human erythrocytes. Int J Biochem Cell Biol. 2003;35(8):1182–1197.
3. Kaestner L, Bogdanova A, Egée S. Calcium channels and calcium-regulated channels in human red blood cells. Adv Exp Med Biol. 2020;1131:625–648.
4. Faucherre A, Kissa K, Nargeot J, Mangoni ME, Jopling C. Piezo1 plays a role in erythrocyte volume homeostasis. Haematologica. 2014;99(1):70–75.
5. Cahalan SM, Lukacs V, Ranade SS, Chien S, Bandell M, Patapoutian A. Piezo1 links mechanical forces to red blood cell volume. eLife. 2015;4(4):e07370.

6. Danielczok JG, Terriac E, Hertz L, et al. Red blood cell passage of small capillaries is associated with transient Ca^{2+}-mediated adaptations. Front Physiol. 2017;8:979.

7. Rapetti-Mauss R, Lacoste C, Picard V, et al. A mutation in the Gardos channel is associated with hereditary xerocytosis. Blood. 2015;126(11):1273–1280.

8. Andolfo I, Russo R, Manna F, et al. Novel Gardos channel mutations linked to dehydrated hereditary stomatocytosis (xerocytosis). Am J Hematol. 2015;90(10):921–926.

9. Glogowska E, Lezon-Geyda K, Maksimova Y, Schulz VP, Gallagher PG. Mutations in the Gardos channel (KCNN4) are associated with hereditary xerocytosis. Blood. 2015;126(11):1281–1284.

10. Fermo E, Bogdanova A, Petkova-Kirova P, et al. ‘Gardos channelopathy’: a variant of hereditary stomatocytosis with complex molecular regulation. Sci Rep. 2017;7(11):1744.

11. Andolfo I, Russo R, Rosato BE, et al. Genotype-phenotype correlation and risk stratification in a cohort of 123 hereditary stomatocytosis patients. Am J Hematol. 2018;93(12):1509–1517.

12. Picard V, Guitton C, Thuret I, et al. Clinical and biological features in PIEZO1-hereditary xerocytosis and Gardos channelopathy: a retrospective series of 126 patients. Haematologica. 2019;104(8):1554–1564.

13. Fermo E, Monedero-Alonso D, Petkova-Kirova P, et al. Gardos channelopathy: functional analysis of a novel KCNN4 variant. Blood Adv. 2020;4(24):6336–6341.

14. Hertz L, Huisjes R, Llaudet-Planas E, et al. Is increased intracellular calcium in red blood cells a common component in the molecular mechanism causing anemia? Front Physiol. 2017;8:673.

15. Wang J, Wagner-Britz L, Bogdanova A, et al. Morphologically homogeneous red blood cells present a heterogeneous response to hormonal stimulation. PLoS One. 2013;8(6):e67697.

16. Flormann D, Kuder E, Lipp P, Wagner C, Kaestner L. Is there a role of C-reactive protein in red blood cell aggregation? Int J Lab Hematol. 2015;37(4):474–482.

17. Macey RI, Adorante JS, Orme FW. Erythrocyte membrane potentials determined by hydrogen ion distribution. Biochim Biophys Acta. 1978;512(2):284–295.

18. Baunbæk M, Bennekou P. Evidence for a random entry of Ca^{2+} into human red cells. Bioelectrochemistry. 2008;73(2):145–150.

19. Kaestner L, Wang X, Hertz L, Bernhardt I. Voltage-activated ion channels in non-excitable cells-a viewpoint regarding their physiological justification. Front Physiol. 2018;9:450.

20. Grygorczyk R, Schwarz W, Passow H. Ca^{2+}-activated K+ channels in human red cells. Comparison of single-channel currents with ion fluxes. Biophys J. 1984;45(4):693–698.

21. Wolff D, Cecchi X, Spalvins A, Canessa M. Charybdotoxin blocks with high affinity the Ca-activated K+ channel of Hb A and Hb S red cells: individual differences in the number of channels. J Membr Biol. 1988;106(3):243–252.

22. Andrews DA, Yang L, Low PS. Phorbol ester stimulates a protein kinase C-mediated agatoxin-TK-sensitive calcium permeability pathway in human red blood cells. Blood. 2002;100(9):3392–3399.

23. Seear RV, Lew VL. IKCa agonist (NS309)-elicited all-or-none dehydration response of human red blood cells is cell-age dependent. Cell Calcium. 2011;50(5):444–448.

24. Teramoto T, Kadowa M, Niidome T, Sawada K, Nishizawa Y, Katayama K. A novel peptide from funnel web spider venom, omega-Aga-TK, selectively blocks, P-type calcium channels. Biochem Biophys Res Commun. 1993;196(1):134–140.

25. Suchyna TM, Johnson JH, Hamer K, et al. Identification of a peptide toxin from Grammostola spatulata spider venom that blocks cation-selective stretch-activated channels [published correction appears in J Gen Physiol. 2001;117(4):371]. J Gen Physiol. 2000;(5):583–598.