Rigshospitalet
Danish Red Blood Cell Centre

AUTOMATED PATCH CLAMP UNRAVELING THE FUNCTIONAL DYNAMICS OF PATHOGENIC PIEZO1 VARIANTS AND FUNCTIONAL INSIGHTS INTO KCA3.1 CHANNELS ON ERYTHROCYTES

Kian Haseli[1], Andreas Glenthøj[1], Jesper B. Petersen[1], Bo Hjorth Bentzen[2], Kim Boddum[3].
[1]Department of Hematology, Rigshospitalet, Copenhagen, Denmark, [2]Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark, [3]Sophion Biosciences, Ballerup, Denmark

Introduction

Purpose

- Understanding the functional dynamics of ion channels in red blood cells (RBCs) is crucial for diagnosing and treating hereditary anemias.

Background

- Piezo1 and KCa3.1 regulate RBC volume and cellular deformability. Pathogenic mutations in *Piezo1* cause **dehydrated hereditary stomatocytosis** (DHSt) and **heriditary xerocytosis** (HX), leading to RBC dehydration through excessive Ca^{+2} ion influx and consequential K^+ efflux.

Challenge

- Traditionally, Piezo1 activity requires mechanical stimulation to evaluate ion-channel activity. We developed a **novel voltage-based assay** using automated patch clamp (APC) technology to evaluate the functional effects of pathogenic Piezo1 variants, achieving high-throughput, reproducible measurements.

Methods – Automated Patch Clamp Assay Developed for Red Blood Cells

Automated Patch Clamp Assay Development

- **Cell Models**:
  Fresh human RBCs expressing wild-type (WT) Piezo1 and two variants:
  - *PIEZO1* R2456H (ACMG class 5; pathogenic)
  - *PIEZO1* P1771L (ACMG class 3; variant of uncertain significance)
- ***Equipment:***
  Qube384 (Sophion Bioscience A/S, Ballerup, Denmark)
- **Protocols**:
  - **Piezo1**: Voltage-ramp protocol with pharmacological modulation using **KC159** (activator) and **$GdCl_3$** (inhibitor).
  - **KCa3.1**: Voltage-step protocol with treatments: Pre-compound → NS309 (activator) → TRAM34 (inhibitor) → Post-compound.
- **Key Goal**:
  To quantify channel currents, evaluate prolonged open states in Piezo1 variants, and confirm functionality of the assay.

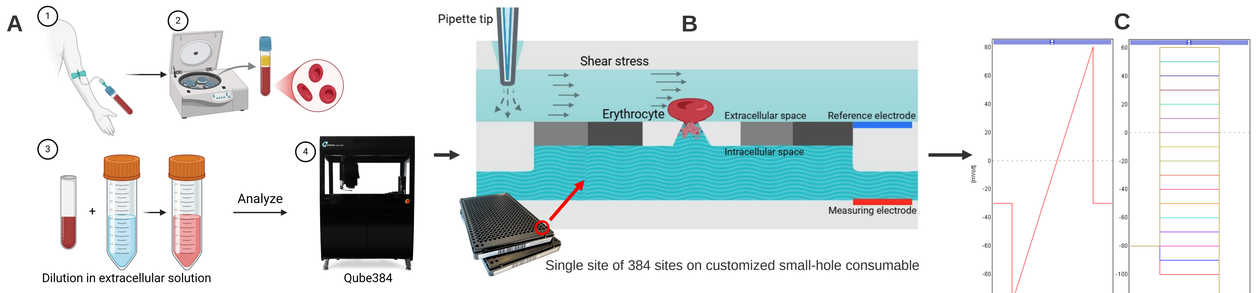


Fig. 1: Methodology for studying ion channel activity in red blood cells (RBCs). (A) RBCs are isolated via centrifugation and washed with Sophion extracellular solution for electrophysiological recordings. (B) Patch-clamp setup in one site of 384 sites in consumable: Shear stress is applied to activate Piezo1, and ionic currents are recorded after whole-cell configuration is obtained. (C) Voltage protocols: A ramp protocol assesses Piezo1 mechanosensitivity, while a stepwise protocol evaluates KCa3.1 voltage dependence. This approach enables detailed characterization of ion channel function in RBCs.

Results – KCa3.1 Channels in RBCs Respond to Pharmacological Modulation

- KCa3.1 baseline average currents were **42 pA (95% CI: 28 – 56 pA)**.
- **NS309** increased average currents by **191% (± 39%)** of baseline, while **TRAM34** reduced them to **30 pA (95% CI: 18 – 43 pA)** (**p < 0.001**), n = 38 RBCs.
- These findings highlight it is possible to evaluate KCa3.1's role in RBC ion homeostasis using APC technologies and pharmacological compounds.

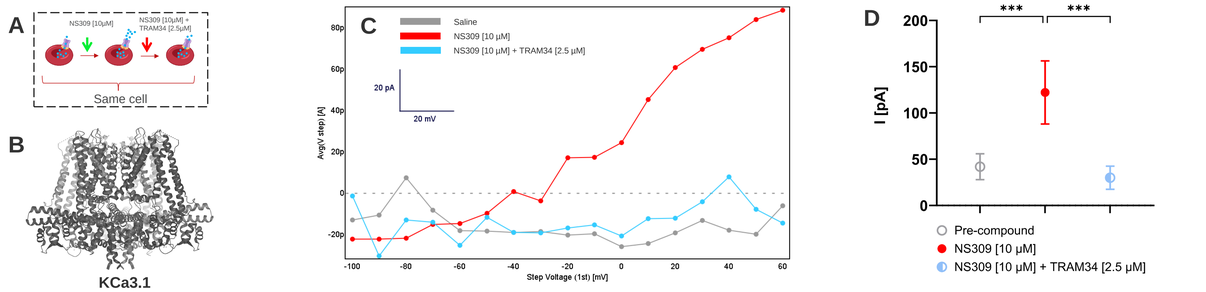


Fig. 2: Electrophysiological characterization of KCa3.1 activity in red blood cells (RBCs). (A) NS309 (10 µM) activates KCa3.1, followed by inhibition with TRAM34 (2.5 µM) on the same cell. (B) Structure of KCa3.1. (C) Current-voltage plot: NS309 (red) robustly activates KCa3.1 compared to saline (gray), while TRAM34 (blue) abolishes the effect. (D) Statistical analysis (One-way ANOVA with Tukey's post hoc): NS309 significantly increases currents (***p < 0.001; error bars: 95% confidence interval), and TRAM34 removes the effect, confirming specific pharmacological modulation of KCa3.1.

Results – Pathogenic Piezo1 Variants Show Increased Currents Compared to Wild Type

- **R2456H** and **P1771L** variants showed significantly higher average currents of compared to healthy wild types.
- These variants exhibited **prolonged open states**, likely causing increased ion influx and RBC dehydration.
- **Pharmacological Modulation**:
  - **R2456H:** KC159 increased Piezo1 currents by **61% (± 17%)** of saline.
  - **P1771L:** KC159 increased Piezo1 currents by **75% (± 30%)** of saline..

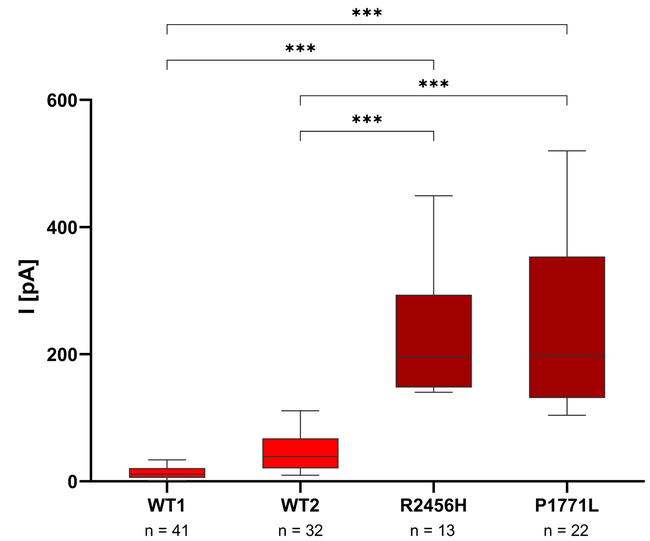


Fig. 3: Electrophysiological recordings of red blood cells (RBCs) treated with KC159, a Piezo1-specific activator, for two wild-type (WT) donors (WT1, WT2) and two pathogenic Piezo1 variants. Box plots show median currents, interquartile ranges (IQR), and whiskers (min-max). Pathogenic variants exhibit significantly elevated currents, consistent with delayed Piezo1 inactivation. Statistical significance (Kruskal–Wallis with Dunn's post hoc): ***p < 0.001. These results highlight distinct electrophysiological profiles between WT and pathogenic variants. n = number of RBCs

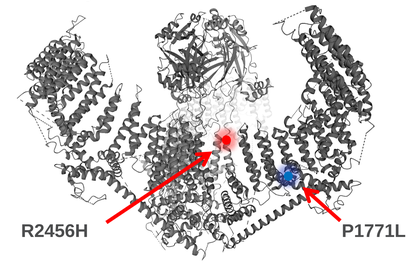


Fig. 4: Structural model of the Piezo1 ion channel showing the locations of the pathogenic variants R2456H (red) and P1771L (blue).

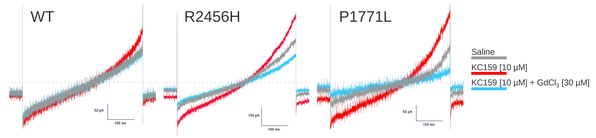


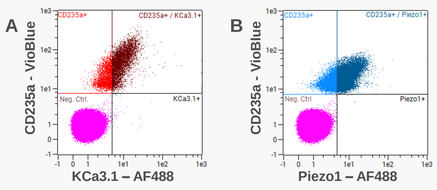
Fig. 5: Representative electrophysiological traces of Piezo1-mediated currents in RBCs from wild-type (WT) and pathogenic variants (R2456H, P1771L) under saline (gray), KC159 (10 µM; red), and KC159 + $GdCl_3$ (10 µM + 30 µM; blue). Pathogenic variants show higher currents than WT, with KC159 activation and $GdCl_3$ inhibition confirming Piezo1 specificity.

Piezo1 currents [pA]	
Sample	**Median (IQR)**
WT1 (n = 41)	12 (15)
WT2 (n = 32)	39 (47)
R2456H (n = 13)	196 (146)
P1771L (n = 22)	198 (223)

Table 1: Median Piezo1 currents (pA) with interquartile ranges (IQR) in RBCs from wild-type (WT1, WT2) and pathogenic variants (R2456H, P1771L), as shown in Figure 3.

Supporting data: Flow cytometry analysis shows variable KCa3.1 and Piezo1 expression levels in Erythrocytes

- The higher variability of KCa3.1 and Piezo1 expression in labeled cells aligns with the variations seen in electrophysiological currents from functional assays.



C

	Neg. Ctrl.	Piezo1-	Piezo1+	KCa3.1-	KCa3.1+
Median RFI	**0.86**	**1.59**	**5.68**	**1.52**	**5.89**
rSD	0.35	0.49	3.63	0.47	4.12
Min	**0.06**	0.47	3.18	0.59	3.02
Max	3.84	2.97	47.34	2.91	44.18

Fig. 6: Flow cytometry plots depict CD235a+ erythrocytes labeled for KCa3.1 (Panel A, red) and Piezo1 (Panel B, blue). The x-axes represent fluorescence intensities for events labelled with anti-KCa3.1 and anti-Piezo1 primary antibodies and a secondary antibody conjugated with Alexa Fluor 488. While the y-axes show CD235a-VioBlue intensity, identifying erythrocytes. Negative controls are erythrocytes only labelled with secondary antibody, shown in magenta. The table (Panel C) shows the values of the median relative fluoroscence intensities (RFI) normalized to the negative control (Neg. Ctrl.) and the minimum and maximum RFIs.

Conclusion

- **Advancement in Assay Development**: Our study demonstrates that **Piezo1 pathogenic variants can be reliably evaluated** using APC technology and voltage-ramp protocols. Additionally, our results highlight the **characterization capabilities of KCa3.1 channels**, showcasing the utility of specific activators to discern functional differences under various experimental conditions.
- **Pathophysiological Implications**: Prolonged open states in Piezo1 variants lead to ion dysregulation and RBC dehydration, linking functional effects to **clinical phenotypes**. KCa3.1 modulation highlights its role in RBC physiology and its potential for targeted therapies of RBC channelopathies.
- **Broader Impact**: This approach bridges the gap between functional ion channel analysis and clinical diagnostics for hereditary anemias.
- **Future Directions**: Further characterization of Piezo1 and KCa3.1 in RBC pathologies and exploration of targeted pharmacological interventions.

Presented @BPS2025