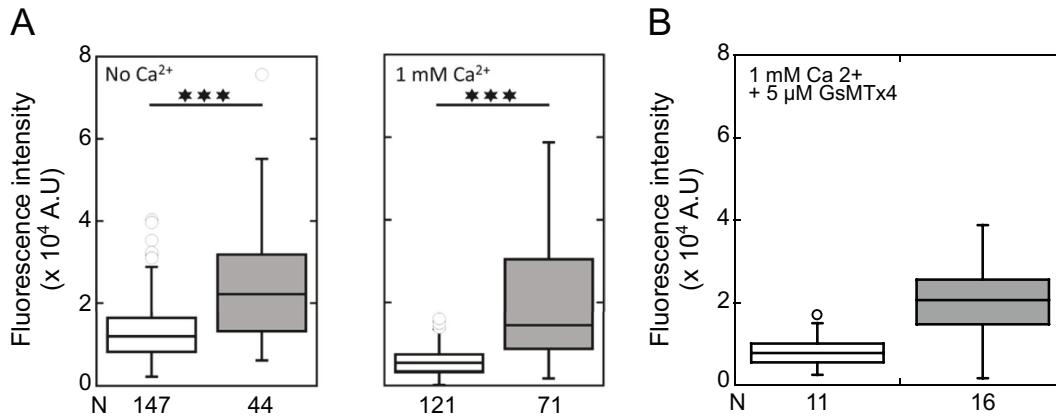


# Reply to Kaestner et al.: Activation of PIEZO1 is not significant for the passage of red blood cells through biomimetic splenic slits

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**Fig. 1.** Intracellular calcium fluorescence intensity upstream (in white) and downstream of the slits (in gray) (A) in the absence or presence of 1 mM calcium in the buffer and (B) in the presence of 1 mM calcium in the buffer and of 5  $\mu\text{M}$  GsMTx4, under  $\Delta P = 500$  Pa. Median values are displayed with 25% and 75% percentiles, and min/max values as whiskers. N: number of analyzed cells. Slit dimension:  $0.80 \times 2.77 \times 4.70 \mu\text{m}^3$ . Portions of this figure have been reused from Moreau et al. (1).

We thank the authors for acknowledging the seminal nature of our work (1).

We have not conclusively addressed the question of Piezo1 activation during splenic filtration *in vivo*. Our aim was to highlight the key mechanisms governing red blood cell (RBC) retention/passage in slits. In the discussion, we were cautious in only suggesting that no PIEZO1 effect is involved in RBC passage.

Kaestner et al. wrote that "Accumulating evidence shows salt solution...contain  $\text{Ca}^{2+}$ " (2). We carefully examined the articles cited supporting this statement.

In ref. 3, the authors use a Gardos channel activator. They observe hyperpolarization and deduce contamination by 4  $\mu\text{mol}$   $\text{Ca}^{2+}$ . However, they give neither the cause (residual  $\text{Ca}^{2+}$  from blood plasma?) nor the dosage. Moreover, at low Gardos activator concentrations, no hyperpolarization is observed at 4  $\mu\text{mol}$   $\text{Ca}^{2+}$ . Finally, hyperpolarization is a slow process (time scale: 500 s).

In ref. 4, the authors use a PIEZO1 activator. They observe hyperpolarization and cite ref. 3 to explain it but provide neither cause nor assay showing contamination. However, they estimate a plausible residual 8  $\mu\text{mol}$ - $\text{Ca}^{2+}$  originating from the plasma of the blood sample.

Our experimental conditions differ radically from those described in these articles:

1) We did not use Piezo1 or Gardos activator that promote activation at subphysiological values.

2) We took special precautions to avoid  $\text{Ca}^{2+}$  contamination. First, we used a  $\text{Ca}^{2+}$ -free commercial buffer prepared from distilled water (See supplier's data sheet available on

the Internet). Second, we diluted the initial 30- $\mu\text{L}$  blood drop in 1 mL (50-fold dilution) prior to centrifugation. The pellet was diluted in 500  $\mu\text{L}$  and centrifuged twice, i.e., two successive 25-fold dilutions before a final 100-fold dilution, so that the residual  $\text{Ca}^{2+}$  concentration is <1 nmol, far too low to activate Gardos.

3) The slit passage time is a fast process <100 ms.

Finally, we performed a limited experiment using the PIEZO1 inhibitor (GsMTx4) and Fluo4, similarly to our paper's figure 4 (reported here as Fig. 1A).

Although the statistics are limited, the trend is similar: i) an increase in intracellular  $\text{Ca}^{2+}$  signal after slit exit; ii) no difference in  $\text{Ca}^{2+}$  signal after exit, regardless of no  $\text{Ca}^{2+}$ , or

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The authors declare no competing interest.

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$\text{Ca}^{2+}$  and PIEZO1 inhibitor in solution (Fig. 1B). Also, RBC transit times and velocities through the slits are unaffected by  $\text{Ca}^{2+}$  and/or PIEZO1 and Gardos inhibitors (Paper's figures S9 and 5B). The similar transit times observed irrespectively of the presence of  $\text{Ca}^{2+}$  or PIEZO1 and Gardos inhibitors show that PIEZO1–Gardos channels do not induce a significant cell volume reduction, in agreement with a previous study (5). Thus, no significant change is expected in hemoglobin concentration and, consequently, in the Fluo4 signal.

Finally, our simulations show that the typical maximum membrane tension is about 421.23 pN/ $\mu\text{m}$  (figure 3F), below the critical tension (6) to activate PIEZO1 [1,440 pN/ $\mu\text{m}$  (7), 2,468 pN/ $\mu\text{m}$  (8), and 7,820 pN/ $\mu\text{m}$  (9)].

To conclude, we think that our experiments and conclusions are correct.

An experiment designed jointly by Kaestner et al. and ourselves would be beneficial in reaching a scientific consensus.

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