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Activation of Piezo1 channels in compressed red blood cells augments platelet-driven contraction of blood clots

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Abstract

Background: Piezo1 is a mechanosensitive cationic channel that boosts intracellular $[Ca^{2+}]_i$. Compression of red blood cells (RBCs) during platelet-driven contraction of blood clots may cause the activation of Piezo1.

Objectives: To establish relationships between Piezo1 activity and blood clot contraction.

Methods: Effects of a Piezo1 agonist, Yoda1, and antagonist, GsMTx-4, on clot contraction *in vitro* were studied in human blood containing physiological $[Ca^{2+}]$. Clot contraction was induced by exogenous thrombin. Activation of Piezo1 was assessed by Ca^{2+} influx in RBCs and with other functional and morphologic features.

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AUTHOR CONTRIBUTIONS

J.W.W., R.I.L., and F.I.A. designed the experiments. N.G.E., A.I.K., I.A.A., A.D.P., F.A., S.A., and T.S. performed the experiments. R.I.L., J.W.W., N.G.E., I.A.A., F.A., and E.L.G. interpreted the data. N.G.E., T.S., F.I.A., R.I.L and J.W.W. contributed to the preparation of the manuscript.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

SUPPLEMENTARY MATERIAL

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Results: Piezo1 channels in compressed RBCs are activated naturally during blood clot contraction and induce an upsurge in the intracellular $[Ca^{2+}]_i$, followed by phosphatidylserine exposure. Adding the Piezo1 agonist Yoda1 to whole blood increased the extent of clot contraction due to Ca^{2+}-dependent volumetric shrinkage of RBCs and increased platelet contractility due to their hyperactivation by the enhanced generation of endogenous thrombin on activated RBCs. Addition of rivaroxaban, the inhibitor of thrombin formation, or elimination of Ca^{2+} from the extracellular space abrogated the stimulating effect of Yoda1 on clot contraction. The Piezo1 antagonist, GsMTx-4, caused a decrease in the extent of clot contraction relative to the control both in whole blood and in platelet-rich plasma. Activated Piezo1 in compressed and deformed RBCs amplified the platelet contractility as a positive feedback mechanism during clot contraction.

Conclusion: The results obtained demonstrate that the Piezo1 channel expressed on RBCs comprises a mechanochemical modulator of blood clotting that may be considered a potential therapeutic target to correct hemostatic disorders.

Keywords

blood clotting; clot contraction; Piezo1; platelets; red blood cells

1 | INTRODUCTION

Since the discovery of mechanosensitive receptors, such as Piezo [1], study of the functions of these mechanotransducers in a variety of (patho)physiological processes has become a booming field of research that has now reached thrombosis and hemostasis.

Piezo1 is a type of mechanosensitive membrane ion channel expressed by many cell types, including blood cells. Piezo1 is embedded in the plasma membrane and is sensitive to any mechanical perturbations of the cell with selective permeability for cations upon stimulation [2,3]. Individual Piezo1 is a trimeric protein resembling propeller blades that are sensitive to membrane distortion, with a hole in the middle for passage of ions through the cell membrane in response to conformational activation [4].

Piezo1 cation channels play an important role in many (patho) physiological processes as mechanotransducers [5-8]. In cells, Piezo1 transmits “outside-in” and “inside-out” mechanical force signals of various origins, including compression, membrane stretching, shear stress, stiffness of extracellular matrix, and osmotic pressure [9,10]. The mechanically stressed cell membrane induces a conformational transition of Piezo1 from the closed to the open state, allowing gradient-dependent diffusion of Ca^{2+}, K^+, and Na^+ cations through the membrane and modulating various cellular functions such as protein synthesis, secretion, migration, proliferation, and apoptosis in response to mechanical stimulation [11]. Of particular importance is the ability of Piezo1 to induce the influx of calcium ions into a cell [2,5,12]. Notably, within a resting cell, $[Ca^{2+}]_i$ does not exceed approximately 40 to 60 nM [13] compared with millimolar concentrations of Ca^{2+} in the physiological extracellular milieu, including blood.

Contraction or retraction of blood clots is a process of shrinkage of a clot driven by the contractile forces generated by activated platelets [14,15]. Clot contraction occurs both *in vitro* and *in vivo* inside blood vessels or in a wound after the formation of hemostatic clots or pathologic obstructive thrombi [16-18]. The pathophysiological significance of contraction includes the following: 1) shrinkage of a clot or thrombus improves local blood flow past the obstructive thrombotic mass [19]; 2) compaction of a blood clot at the site of injury leads to the formation of a dense impermeable hemostatic plug [20]; 3) compaction of a clot or thrombus reduces porosity and permeability for fibrinolytic enzymes [21]; and 4) compaction-associated stiffening increases the mechanical stability and resistance of blood clots to rupture, ie, reduces the risk of thrombotic embolization [22,23]. Clinical studies have shown that the ability of blood clots to contract is impaired in patients with (pro)thrombotic conditions, which may reflect a pathogenic mechanism for aggravation of the course and outcomes of thrombotic complications [19,22,24-27].

During contraction of blood clots, activated platelets generate contractile forces that are distributed through the fibrin network, resulting in a decrease in clot volume. In addition to the overall volumetric shrinkage, during contraction, clot components are redistributed in space such that the contracting platelet-fibrin meshwork is accumulated on the periphery of the clot and densely packed red blood cells (RBCs) are amassed in the interior [16]. Under pressure from outside, RBCs in the core get tightly packed, compressed, and take the shape of polyhedra, hence named “polyhedrocytes” or “piezocytes” [16,28]. Since the contraction of blood clots is accompanied by the deformation of blood cells, there is good reason to hypothesize that clot contraction involves the activation of Piezo1, primarily in RBCs and/or platelets. This presumption is supported by the established relationship between Piezo1 activation and various biological properties of RBCs undergoing mechanical perturbations [29-36]. The potential role of Piezo1 in hemostasis and thrombosis has now started attracting the attention of researchers [37-40]. In particular, the plasma membrane of platelets experiences mechanical stresses that are associated with Piezo1 function as a modulator of platelet functionality via fluctuations of the intracellular $[Ca^{2+}]_i$ [37,39,41,42]. With a basis of existing data, the goal of this study was to explore if Piezo1 activation in blood cells may comprise a regulatory mechanism of blood clot contraction.

2 | MATERIALS AND METHODS

2.1 | Blood collection

Blood samples from 62 healthy subjects aged 20 to 35 years (average, 26 ± 3 years) were obtained by venipuncture. The study was approved by the Ethics Committee of Kazan Federal University (protocol # 27 as of December 28, 2020), and written informed consent was obtained from the donors. The subjects were excluded from the study if anticoagulants, thrombolytics, or antiplatelet medications were taken within 14 days before drawing of blood.

2.2 | Clot contraction assay

The extent of contraction of clots formed in whole blood or platelet-rich plasma was determined by optical detection of the clot size over time using the Thrombodynamics

Analyzer System (HemaCore), basically as described earlier [43]. Prior to use, a 12-mm × 7-mm × 1-mm transparent plastic cuvette was lubricated with 4% (volume/volume) Triton X-100 solution in 150-mM NaCl to prevent sticking of the clot to the walls. To initiate clotting and activation of platelets, in a separate plastic tube, 10 μL of freshly thawed human thrombin (20 U/mL) was added to 200 μL of blood or platelet-rich plasma to reach a final concentration of 1 U/mL. Eighty microliters of the activated blood or plasma sample was quickly transferred into a measuring cuvette preheated at 37 °C. Photo registration of the clot during contraction was performed automatically every 15 seconds. Digital images of the clot were used to calculate the extent of clot shrinkage relative to the initial size until 20 minutes after the addition of thrombin to the sample (Supplementary Figure S1A). Kinetic curves of clot contraction were plotted to extract a number of parameters, such as the extent of contraction, lag time, area under the curve (work done by platelets), and average velocity. The process of clot contraction was followed for up to 1 hour, but it was mostly (by 80%-90%) completed at the 20-minute point, which was chosen to characterize the extent of contraction. The clot contraction assay was performed in the absence or presence of the Piezo1 agonist Yoda1 (5 μM) or antagonist GsMTx-4 (1 μM) preincubated with whole blood or platelet-rich plasma for 1 minute at room temperature.

2.3 | Scanning electron microscopy of blood clots before and after contraction

Clots from venous blood drawn from healthy subjects were formed by addition of thrombin (1 U/mL) and either fixed and processed immediately or allowed to contract for 20 minutes. Then, the clots were fixed and processed for scanning electron microscopy (SEM), as described earlier [16]. The samples were cut open and oriented with the sliced surface upward to visualize the clot interior and imaged using a Merlin scanning electron microscope (Carl Zeiss). Notably, clot contraction was followed by compressive deformation of intact biconcave RBCs to polyhedral cells (polyhedrocytes) (Supplementary Figure S1B).

2.4 | Isolation and flow cytometry of RBCs and platelets

RBCs were pelleted by centrifugation of heparinized blood at 200*g* for 10 minutes, washed, and resuspended in Tyrode's buffer. Platelets were analyzed in platelet-rich plasma. The exposure of phosphatidylserine on the surface of RBCs and platelets was assessed by flow cytometry using fluorescently labeled Annexin V as a marker. The fluorescent cells were analyzed on a FacsCalibur flow cytometer (BD Biosciences) equipped with the BD CellQuest software (BD Biosciences). The FlowJo X program (BD Biosciences) was used for data analysis.

2.5 | Statistical analysis

Statistical analysis was performed using the GraphPad Prism 8 (GraphPad Software) software package. The D'Agostino–Pearson, Kolmogorov–Smirnov, and Shapiro–Wilk tests were used to assess the normality of the sample distribution. The significance of statistical differences for paired comparisons was assessed using the parametric Student's *t*-test, Wilcoxon signed-rank test, or Mann–Whitney U-test. The level of statistical significance was 95% ($p < .05$).

Technical details of blood processing and fractionation, the clot contraction assay, SEM of blood clots, isolation and flow cytometry of RBCs and platelets, isolation of mononuclear cells and neutrophils, the thrombin generation assay, thromboelastography (TEG), measurement of adenosine triphosphate (ATP) in blood serum, as well as quantification of the intracellular $[Ca^{2+}]_i$ and phosphatidylserine exposure in RBCs within contracted blood clots are provided in the Supplementary Materials and Methods.

3 | RESULTS

3.1 | Effects of the chemical activation and inhibition of Piezo1 on contraction of whole blood clots

Since Piezo1 depends strongly on the extracellular $[Ca^{2+}]$, the blood was anticoagulated with a soybean trypsin inhibitor that prevents blood clotting while maintaining physiological $[Ca^{2+}]$ at the level of 1.1 to 1.4 mM [44,45]. Piezo1 in blood cells was activated by adding a specific agonist Yoda1 or inhibited by adding a peptide antagonist GsMTx-4. After incubation with either Yoda1 or GsMTx-4, blood was clotted with thrombin, and the size of the macroscopic clot was tracked optically in parallel with the control samples. As shown in Figure 1A, Yoda1 caused a significant approximately 23% increase in the median extent of contraction, whereas GsMTx-4 inhibited contraction significantly by approximately 17% compared with the control. Kinetic analysis in the absence and presence of Yoda1 and GsMTx4 revealed statistically significant effects on the average velocity and final extent of clot contraction (Supplementary Figure S2). Notably, the rate of contraction with Yoda1 actually lagged the control for approximately the first 15 minutes, after which it exceeded the control (Figure 1B), suggesting a time-delayed enhanced contraction, probably from the time needed to promote platelet contractility with thrombin.

To blunt the effect of Piezo1 activation, we studied the effect of Yoda1 on blood clot contraction in Ca^{2+}-free citrated blood samples (Supplementary Figure S3). Unlike in the blood samples stabilized with corn trypsin inhibitor with retained physiological $[Ca^{2+}]$, Yoda1 had no effect on clot contraction in the blood samples with chelated Ca^{2+} ions, strongly suggesting that activation of Piezo1 with Yoda1 is Ca^{2+}-dependent.

Earlier studies showed strong stimulating effects of calcium ionophores on clot contraction [46,47]. To directly compare the effects of Yoda1 and calcium ionophores, clot contraction in a Ca^{2+}-containing blood sample was initiated by thrombin in the absence and presence of the calcium ionophore A23187. The results presented in Supplementary Figure S4 and Supplementary Table S1 show that the stimulating efficacy of A23187 on the extent of clot contraction (approximately 23% on average) is comparable with that of Yoda1, suggesting that the ultimate functional consequences of the intracellular Ca^{2+} influx do not depend on the mechanisms of Ca^{2+} entry. However, the overall kinetic profile of the enhanced clot contraction in the presence of A23187 was different from that obtained by adding Yoda1 (Figure 1B), suggesting that the stimulating effect of Yoda1 is not simply attributable to increasing the intracellular $[Ca^{2+}]$.

Taken together, the results obtained indicate that chemical stimulation of mechanosensitive Piezo1 ion channels in RBCs and/or platelets enhances the contraction of blood clots and that the effect is Ca^{2+}-dependent.

3.2 | Differential roles of Piezo1 in RBCs, platelets, and leukocytes in contraction of blood clots

To distinguish the role of Piezo1 in RBCs and platelets, the effects of activation and inhibition of Piezo1 on clot contraction were assessed in clots formed in platelet-rich plasma in the absence of RBCs. The addition of the Piezo1 antagonist GsMTx-4 significantly suppressed contraction of plasma clots (Figure 2), as it did in whole blood clots (Figure 1). Surprisingly, in the presence of the Piezo1 agonist Yoda1, the extent of contraction of clots formed in platelet-containing plasma decreased slightly compared with the control at the border of statistical significance ($p = .05$; Figure 2), which was distinct from the activating effect of Yoda1 on clot contraction in whole blood (Figure 1). These results point to the critical importance of Piezo1 in RBCs for blood clot contraction, at least under these particular experimental conditions.

To exclude a potential role of Piezo1 in leukocytes, we performed experiments in which isolated mononuclear cells or neutrophils, untreated or treated with Yoda1 in the presence of Ca^{2+}, were added to Ca^{2+}-containing platelet-rich plasma at a physiological amount, followed by thrombin-induced clot formation and contraction. The results presented in Supplementary Table S2 show that treatment of leukocytes with Yoda1 did not increase the extent and kinetics of platelet-driven clot contraction, indicating that the potential contribution of leukocytes in Piezo1-mediated enhancement of blood clot contraction, if any, is negligibly small.

Thus, it is the chemical activation of Piezo1 in RBCs that stimulates clot contraction, apparently mimicking, sustaining, and enhancing mechanical activation of Piezo1 in RBCs that undergo compression deformation during platelet-driven blood clot contraction.

3.3 | Yoda1-induced RBC volume shrinkage as a mechanism for enhanced clot contraction

Activation of Piezo1 cation channels is associated with RBC dehydration and volumetric shrinkage of individual RBCs [29]. To directly address the question of whether Yoda1 decreases RBC size during blood clot contraction, we measured RBC size by performing SEM of the contracted blood clots formed in Ca^{2+}-containing whole blood samples (stabilized with the corn trypsin inhibitor) in the absence and presence of Yoda1. The interior of both clots contained tightly packed compressed polyhedral RBCs (polyhedrocytes) (Figure 3A, B). The precise morphometry of individual RBCs showed that the median size of RBCs was reduced by approximately 25% in the presence of Yoda1 (Figure 3C and Supplementary Table S3), indicating the Yoda1-induced dehydration and volume shrinkage of RBCs that comprises a structural mechanism for enhanced contraction of entire blood clots via activation of Piezo1 in RBCs.

3.4 | Yoda1-induced externalization of phosphatidylserine on RBCs and platelets and generation of endogenous thrombin

Another possible mechanism for stimulation of blood clot contraction after chemical activation of Piezo1 may be a rise in the procoagulant activity of RBCs due to surface exposure to phosphatidylserine. In this case, “activated” RBCs can promote the generation of endogenous thrombin, which, in turn, additionally stimulates platelets, thus enhancing their contractility. This hypothesis has been tested in a number of independent ways.

Flow cytometry of washed RBCs before and after adding Yoda1 in the presence of labeled Annexin V showed that Piezo1 activation by Yoda1 was accompanied by pronounced externalization of phosphatidylserine (Supplementary Figure S5). The ability of Yoda1 to expose phosphatidylserine has been demonstrated earlier on RBCs [34] as well as on platelets in patients with diabetes [38]. To extend the latter observations, we measured phosphatidylserine on the surface of normal platelets, untreated or treated with Yoda1. The results presented in Supplementary Table S4 show that activation of Piezo1 by Yoda1 in platelets was followed by a moderate approximately 2-fold externalization of phosphatidylserine, which is much weaker than an approximately 90-fold effect of a calcium ionophore A23187 used as a positive control. Despite a relatively minor contribution of Piezo1 to the exposure to phosphatidylserine in platelets, the overall platelet procoagulant activity may contribute substantially to the increased thrombin generation potential of blood as a result of Piezo1 activation.

To validate that Yoda1-induced phosphatidylserine exposure on RBCs enhances thrombin generation, the thrombin generation assay and TEG were performed in platelet-free plasma supplemented with control (treated with dimethyl sulfoxide) or Yoda1-activated RBCs (Figures 4 and 5 and Supplementary Tables S5 and S6). Incubation of RBCs with Yoda1 in the presence of Ca^{2+} caused a substantial increase in the peak thrombin concentration, endogenous thrombin potential, and shortening of the time to peak in the thrombin generation assay (Figure 4 and Supplementary Table S5). In addition, TEG revealed significant shortening of the plasma clotting time (R) and an increase in the rate of clot strengthening (α) in the presence of Yoda1-activated RBCs (Figure 5 and Supplementary Table S6), reflecting increased thrombin activity. Taken together, these results confirm that the effect of Yoda1 on clot contraction is due to the RBC-mediated endogenous thrombin generation due to overexpression of phosphatidylserine.

An independent approach to test the relationships between the Piezo1-mediated stimulation of RBCs, formation of endogenous thrombin, and stimulation of platelet contractility was the use of rivaroxaban, a direct inhibitor of factor Xa, which was added to the blood prior to the addition of Yoda1. As follows from the data presented in Figure 6, rivaroxaban abolished the activating effect of Yoda1 on blood clot contraction, which again confirms the role of endogenous thrombin as a mediator of Piezo1 activation in RBCs and a stimulator of platelet contractility.

3.5 | Blood clot contraction causes Piezo1 activation in RBCs, followed by Ca^{2+} influx and phosphatidylserine exposure

To directly illustrate Piezo1 activation during blood clot contraction, we used reconstituted blood samples containing isolated human RBCs preloaded with a Ca^{2+}-sensitive fluorescent dye Fluo-4 AM (Thermo Fisher Scientific). These dye-filled RBCs were added to fresh human platelet-rich plasma up to 30% hematocrit, followed by recalcification and thrombin-induced formation and contraction of macroscopic clots (Supplementary Materials and Methods). The clots that formed under various experimental conditions were washed, disintegrated, and hemolyzed to release the intracellular cytoplasmic content of RBCs, including Fluo-4. The results presented in Figure 7 and Supplementary Table S7 show that the unconstrained blood clot contraction was characterized by a significantly higher intracellular $[Ca^{2+}]_i$ in the clot-embedded RBCs compared with the uncontracted clots formed in the presence of latrunculin A. The involvement of Pizeo1 in the Ca^{2+} influx into RBCs during clot contraction is suggested by the moderate promoting effect of Yoda1, although the inhibitory effect of GsMTx-4 on the content of intracellular Ca^{2+} was weak. Calcium ionophore A23187, used as a positive control, substantially enhanced the entry of Ca^{2+} into compressed RBCs inside contracted blood clots.

The phosphatidylserine exposure on blood cells during clot contraction was followed using fluorescently labeled Annexin V, which was added to the blood before clot formation and contraction in the presence of free Ca^{2+} (Supplementary Materials and Methods). The results presented in Supplementary Figure S6 revealed the presence of phosphatidylserine in the detergent-treated lysates of contracted clots that was reduced significantly when the clots were formed in the presence of GsMTx-4, a Piezo1 inhibitor, and enhanced in the presence of a calcium ionophore A23187.

Taken together, these results strongly suggest that Piezo1 channels are activated during contraction of a blood clot and induce an upsurge in the intracellular $[Ca^{2+}]_i$ and phosphatidylserine externalization in compressed and deformed RBCs.

3.6 | Extracellular adenine nucleotides as possible stimulators of blood clot contraction

A conceivable hypothetical mechanism for increased contraction of blood clots after stimulation of Piezo1 in RBCs is the secretion of ATP and formation of adenosine diphosphate (ADP) in serum followed by stimulation of platelets through the purinergic receptors. To test this assumption, we established the relationship between the content of adenine nucleotides in serum and clot contraction and investigated the effects of inhibition of purine receptors on platelets.

Despite the reported secretion of adenyl nucleotides by RBCs in response to Piezo1 activation [31], the level of ATP in the blood serum during the contraction of clots was small and decreased progressively from 15.4 nM at 10 minutes after the onset of contraction to 13.8 nM at 15 minutes and 4.5 nM at 20 minutes. Prevention of enzymatic hydrolysis of ATP and ADP by an ecto-ATPase inhibitor (ARL-67156, 100 μM) did not change the extent of clot contraction (45 ± 2% vs 46 ± 2% in control; $n = 4$; $p > .05$). The opposite effect, or enhanced ADP hydrolysis with exogenous apyrase (0.5 U/mL), also had no effect

on the extent of clot contraction (44 ± 2% vs 44 ± 1% in control; $n = 3$; $p > .05$). These experiments did not reveal any association between the levels of serum adenyl nucleotides and contraction of blood clots. The rate and extent of blood clot contraction also did not change in the presence of specific inhibitors of the 3 known platelet purinergic receptors (P_2X_1, P_2Y_1, and P_2Y_{12}), which were added to the blood either separately or simultaneously (Supplementary Table S8).

Taken together, the results obtained do not confirm the secretion of ATP and ADP from deformed RBCs and do not support the role of adenyl nucleotides as secondary stimulators of blood clot contraction.

4 | DISCUSSION

This work was performed at the intersection of hematology and molecular mechanobiology, a rapidly developing area of research, including the field of hemostasis and thrombosis. Mechanical forces play an important role in the formation of blood clots and thrombi *in vivo* because, during development and maturation, they experience hydrodynamic forces of blood flow and fluctuations of the vessel wall. An additional strong mechanical effect is compression, or contraction (retraction), of blood clots and thrombi, which occurs under the action of contractile forces generated by activated platelets. The contraction of a blood clot is accompanied not only by a decrease in the clot volume (Supplementary Figure S1A) but also by profound structural reorganization. One of the most significant consequences of blood clot contraction is the compressive deformation of RBCs from biconcave to polyhedral shapes, with a simultaneous increase in cell packing density (Figure 3A, B and Supplementary Figure S1B) [16,19]. These compressed RBCs were initially named polyhedrocytes [16], but the later name, piezocytes [28], may be more appropriate in the context of this study. Since platelets [48,49] and RBCs [50] both have mechanosensitive Piezo1 cation channels, their activation during blood clot contraction may be a modulator of the entire process, with important pathophysiological consequences for hemostasis and thrombosis. A possible role of Piezo1 in the development of thrombosis has been shown in a number of studies [37-40,42]. In particular, Piezo1 activation induced by experimental hypertension led to platelet hyperreactivity, and Piezo1 inhibition prevented thrombosis and stroke in hypertensive mice [37].

Here, we have shown that activation of Piezo1 channels in blood cells enhances contraction of blood clots *in vitro*, whereas inhibition of Piezo1 weakens the contraction, altogether suggesting that the function of Piezo1 can modulate contraction of blood clots and thrombi *in vivo* and, hence, affect contraction-related pathophysiological processes. Given the importance of intravital contraction of blood clots and thrombi in the formation of a dense hemostatic plug [20], regulation of local blood flow during thrombotic occlusion of a vessel [19], changes in the sensitivity of clots and thrombi to fibrinolysis [21], and the risk of thrombotic embolization [22,23], Piezo1 in blood cells may play an important role in hemostasis and thrombosis.

The promoting effect of Piezo1 activation on clot contraction was observed in whole blood (Figure 1) but absent in platelet-rich plasma (Figure 2), suggesting that mechanically

perturbed RBCs are the active internal stimulators of clot contraction. There are several conceivable ways to enhance the contraction of blood clots by RBCs in which Piezo1 has been activated. The most straightforward, direct mechanism is Piezo1-mediated volumetric shrinkage of individual RBCs, which has been shown by the comparison of RBC size in blood clots formed in the absence and presence of Yoda1, a Piezo1 activator (Figure 3C and Supplementary Table S3). This difference in RBC size has been observed likely because activation of Piezo1 cation channels is associated with RBC dehydration via Ca^{2+}-dependent activation of the KCa3.1 Gardos channels, implicating a role for Piezo1 in RBC volume regulation [29]. An alternative mechanism for RBC-dependent enhancement of clot contraction is an increase in the contractile strength of platelets, the only mechanically active element in the system. However, to cause additional platelet stimulation by RBCs with activated Piezo1, there must be a connecting mechanism and/or substance(s) that acts as a mediator(s) between RBCs and platelets within the blood clot.

Based on the literature [34] and our own data (Supplementary Figure S5) showing that Piezo1 activation in RBCs is followed by externalization of phosphatidylserine, we assumed that thrombin might be the active mediator between RBCs and platelets because phosphatidylserine provides a procoagulant matrix on the membrane for assembly of the enzymatic complexes catalyzing the conversion of prothrombin to thrombin [51]. This assumption was confirmed by 2 independent approaches. Direct evidence was obtained using the thrombin generation assay and TEG, in which Yoda1-activated RBCs caused an increase in the rate of thrombin formation and total thrombin activity (Figure 4 and Supplementary Table S5) as well as acceleration of plasma clotting (Figure 5 and Supplementary Table S6) compared with control RBCs. An alternative approach implies the effect of a factor Xa inhibitor, rivaroxaban, that suppresses thrombin formation and eliminates the stimulating effect of Yoda1 on the contraction of blood clots (Figure 6). Thus, Piezo1 activation in RBCs causes the generation of endogenous thrombin, a strong physiological stimulator of platelet contractile function [43,52]. The proposed sequence of reactions leading to self-amplification of contraction of blood clots through the activation of Piezo1 channels in deformed RBCs is shown in Figure 8.

Hypothetically, adenyl nucleotides can also act as mediators between RBCs and platelets for 2 reasons. First, they are secreted by RBCs upon activation of Piezo1 in the form of ATP [31], which can be converted to ADP by blood ecto-ATPases [53]. Second, both ATP and ADP are physiological platelet stimulants acting through the P_2Y_1, P_2Y_{12}, and P_2X_1 purine receptors [54]. However, in our experiments, blood clot contraction was sensitive neither to fluctuations in serum nucleotide levels nor to the inhibitors of all 3 purine receptors, which were applied separately and in combination (Supplementary Table S7). These negative results may be explained by the fact that nucleotides are weak platelet agonists and their effect at nanomolar concentrations is not detectable against a background of powerful platelet activation with exogenous and endogenous thrombin, one of the strongest platelet agonists.

There are other possible mechanisms linking activation of mechanosensitive Piezo1 ion channels in mechanically distorted RBCs and the extent of blood clot shrinkage. One of them is a potential change in the membrane rigidity and deformability of RBCs. Activation

of Piezo1 in RBCs has been shown to boost the generation of nitric oxide, which softens the cell membrane [33]. Then, the extent of contraction of blood clots in response to Piezo1 activation could increase due to a decrease in the mechanical resistance of RBCs, which constitute up to half of the clot volume. However, the opposite effect has also been described: activation of Piezo1 and influx of Ca^{2+} can increase the rigidity of the RBC membrane [55]. An increase in RBC rigidity may have dual consequences: it can either weaken blood clot contraction because of the greater mechanical resilience of the clot [56] or enhance it by increasing platelet contractility via a positive feedback mechanism between substrate rigidity and platelet contractile stress [57,58]. It is possible that the multidirectional effects of Piezo1 activation on the mechanical properties of RBCs can be synergistic and increase the extent of contraction of blood clots. Paradoxically, activation of Piezo1 and influx of Ca^{2+} in RBCs followed by enhanced generation of nitric oxide [33] can reduce platelet contractility because nitric oxide is a potent inhibitor of platelet function [59].

In contrast to whole blood, addition of Yoda1 to platelet-rich plasma does not enhance clot contraction; moreover, the effect of Yoda1 on the contraction of the plasma clots was slightly negative (Figure 2). The lack of a direct positive effect of Yoda1 on platelet contractility is most likely due to competition between the relatively weak artificial chemical activation of Piezo1 channels caused by Yoda1 and their natural intracellular activation by cytoskeletal contractile proteins that activate, accumulate, and localize Piezo1 at focal adhesions [60]. In our experiments, the thrombin concentration was relatively high (1-U/mL exogenous thrombin plus endogenous thrombin generated in the course of clot contraction). Therefore, the effects of thrombin on platelets (including shape change, membrane distortion, activation of Piezo1 and Ca^{2+} influx via various cationic channels, etc.) are so strong that they compete with and overcome the relatively weak activating effect of Yoda1. In addition, one should bear in mind that the expression level of Piezo1 in platelets is relatively low compared with that in RBCs and endothelial cells [39] and that the transmembrane influx of Ca^{2+} into platelets is duplicated through ligand-dependent P_2X_1 cationic channels [61]. Irrespective of the explanations, the main point of the results obtained is that there is a difference in how clot contraction responds to Yoda1-induced Piezo1 activation in whole blood and platelet-rich plasma without RBCs, indicating the critical importance of RBCs in indirectly promoting platelet contractility assessed as the degree of clot shrinkage.

Although Piezo1 has been found in T cells [62] and monocytes [63], the possible role of white cells in blood clot contraction seems unlikely due to their incommensurably low content in clots from normal blood. The normal counts of leukocytes in the blood are approximately 5 to $10 \times 10^3/\mu L$ compared with 1.5 to 4.5×10^5 platelets/μL and 4 to 5.5×10^6 RBCs/μL. The cellular composition of blood clots and thrombi is roughly proportional to their relative blood cell counts [64]. Therefore, the fraction of leukocytes within blood clots formed from normal blood must be relatively small compared with platelets and RBCs. In addition to quantitative arguments, our experiments with Yoda1-treated leukocytes do not support any contribution of leukocytes in the Piezo1-mediated enhancement of blood clot contraction (Supplementary Table S2). These results are consistent with the data showing that removal of leukocytes from platelet concentrates does not affect platelet-driven clot contraction [65].

The important role of Piezo1 in blood clot contraction is strongly supported by the clear effects of its inhibition. In both whole blood and platelet-rich plasma, inhibition of Piezo1 significantly suppressed clot contraction (Figures 2 and 3), suggesting that blood clot contraction is associated with the natural activation of Piezo1 channels both in platelets and RBCs and that this activation contributes to self-amplification of platelet contractility and the extent of blood clot contraction.

The entry of Ca^{2+} ions into Yoda1-treated or mechanically distorted human RBCs [12,29] and platelets [39,42] has been shown earlier. To confirm that compression of RBCs during blood clot contraction is followed by activation of Piezo1 in the absence of chemical stimulation, we showed that clot contraction is followed by Ca^{2+} influx into RBCs and concurrent phosphatidylserine exposure (Figure 7 and Supplementary Figure S6). The phosphatidylserine exposure during clot contraction was suppressed with GsMTx4, an inhibitor of Piezo1. This Piezo1-dependent phosphatidylserine exposure in the contracted clots is likely related to RBCs rather than platelets due to the much higher sensitivity of RBCs to activation of Piezo1 (Supplementary Figure S5 and Supplementary Table S4). Taken together, these data indicate that Piezo1 channels in compressed and deformed RBCs are activated naturally during blood clot contraction.

We believe that the observed relationships between activation of Piezo1 and contraction of blood clots revealed *in vitro* likely translate to *in vivo* effects because the extent of intravital contraction of thrombi and thrombotic emboli (reaching up to half of the initial thrombus volume) is comparable with the *in vitro* volumetric shrinkage of blood clots [23]. This quantitative correspondence supports the general (patho)physiological relevance of the biomechanical mechanisms of compaction of blood clots observed *in vitro*. Moreover, a number of published reports have clearly shown that the activation of Piezo1 in blood cells *in vivo* is real [37-40].

In conclusion, the mechanosensitive Piezo1 ion channels expressed in blood cells, primarily RBCs and platelets, are new molecular mediators between blood cell biomechanics and blood clotting. Like any newly recognized and understudied structures with associated pathophysiological mechanisms, Piezo1 cationic channels are potential therapeutic targets for correction of hemostasis disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Essentials

- Piezo1 is a mechanosensitive cationic channel expressed in many cell types, including blood cells.
- Relationships between Piezo1 activation/inhibition and clot contraction *in vitro* were studied.
- Activation of Piezo1 on red blood cells (RBCs) follows and promotes clot contraction, whereas Piezo1 inhibition impedes clot contraction.
- Effects of Piezo1 are due to RBC shrinkage and endogenous thrombin generation on deformed RBCs.

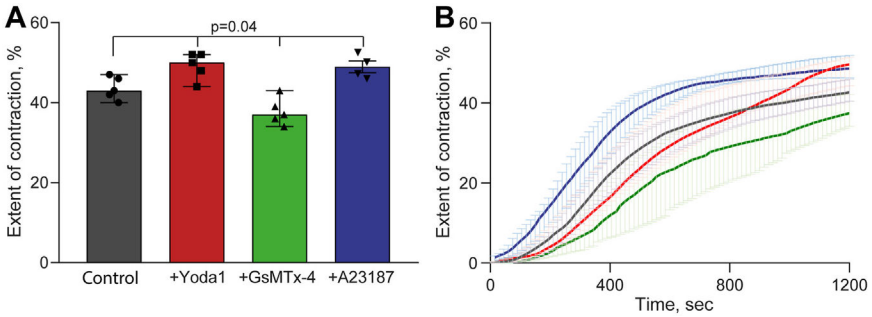

FIGURE 1.

Effects of an activator (Yoda1) and inhibitor (GsMTx-4) of Piezo1 channels and a Ca^{2+} ionophore (A23187) on the contraction of whole blood clots. (A) The extent of clot contraction in the absence (control) and presence of Yoda1 (5 μM), GsMTx-4 (1 μM), or A23187 (15 μM) was measured in blood samples from independent donors stabilized with soybean trypsin inhibitor to maintain physiological [Ca^{2+}]. Results are presented as the median and IQR (25th and 75th percentiles). The Mann–Whitney U-test was used for comparisons with the control. (B) Averaged kinetic curves of blood clot contraction measured in the absence (control, dark gray) and presence of Yoda1 (red), GsMTx-4 (green), or A23187 (blue). The curves' colors match the colors of the corresponding bars shown in Figure 1A.

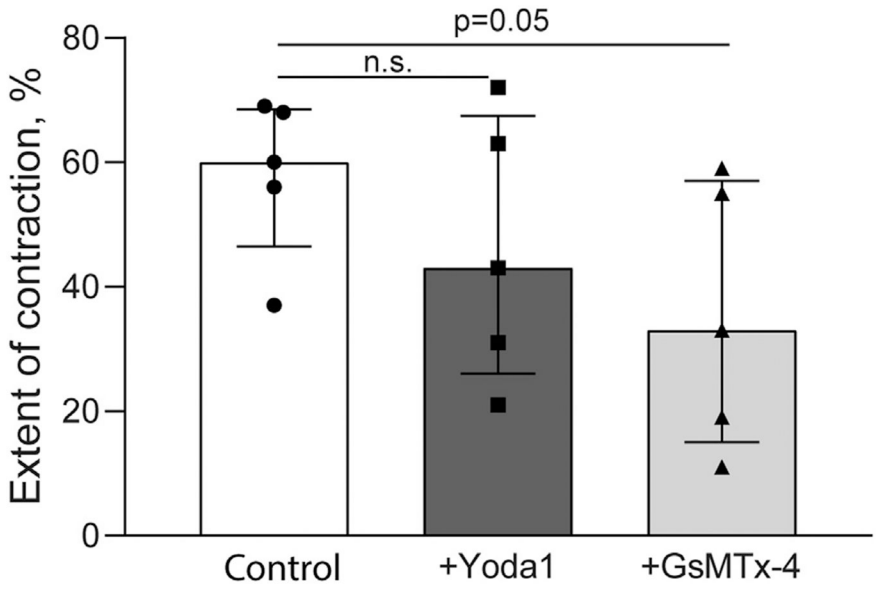

FIGURE 2.

Effects of an activator (Yoda1) and inhibitor (GsMTx-4) of Piezo1 channels on contraction of clots formed in platelet-rich plasma. The extent of clot contraction in the absence (control) and presence of Yoda1 (5 μM) or GsMTx-4 (1 μM) was measured in plasma samples obtained from the blood of 5 independent donors. The blood was stabilized with soybean trypsin inhibitor to maintain physiological [Ca^{2+}]. Results are presented as the median and IQR (25th and 75th percentiles). The Mann–Whitney U-test was used for comparisons with the control. n.s., not statistically significant.

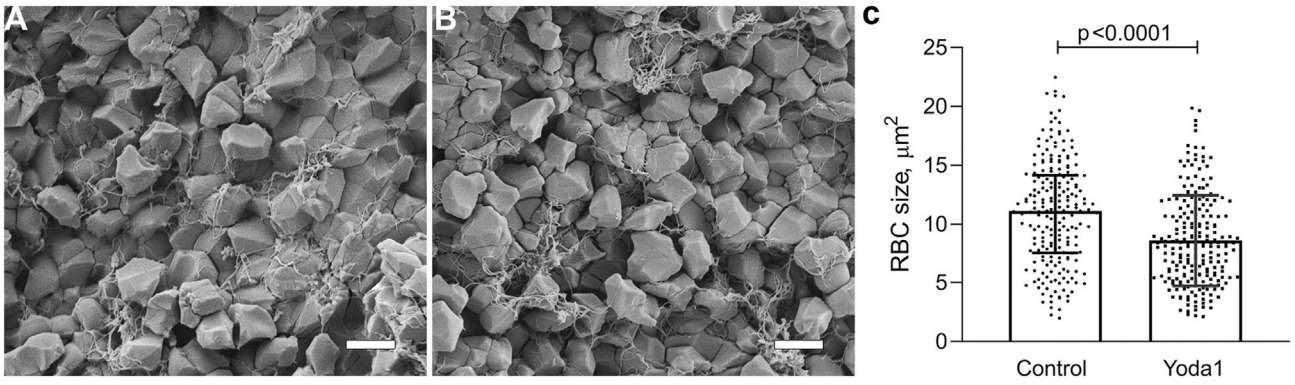

FIGURE 3.

Effect of the Piezo1 activator Yoda1 on the size of red blood cells (RBCs) within contracted clots made from Ca^{2+}-containing whole blood samples. (A, B) Representative scanning electron microscopy images of the interior of contracted clots formed in the (A) absence and (B) presence of Yoda1 (5 μM), showing polyhedral RBCs (polyhedrocytes) that result from compressive deformation during platelet-driven clot contraction. Magnification bars: 5 μm. (C) The area occupied by individual RBCs (RBC size) was determined from scanning electron microscopy images corresponding to contracted clots in the absence (control, n = 209) and presence of Yoda1 (Yoda1, n = 215). The results are presented as the median and IQR (25th and 75th percentiles). The Mann–Whitney U-test was used for comparisons with the control. For the numerical data, see Supplementary Table S3.

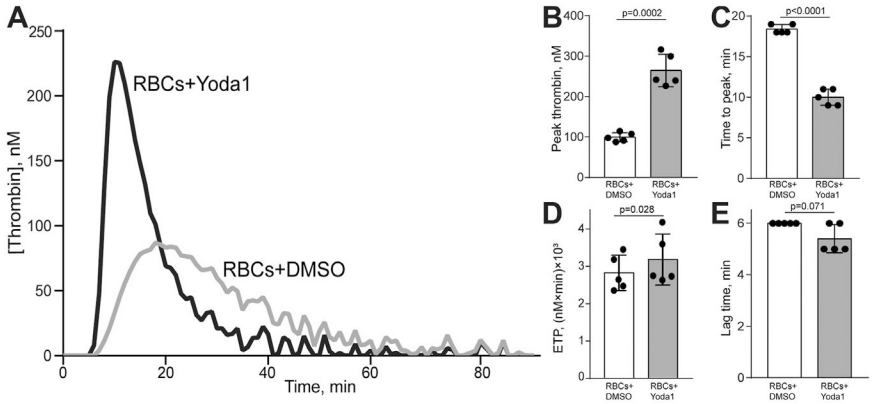

FIGURE 4.

Thrombin generation assay shows increased thrombin formation induced by Yoda1-activated red blood cells (RBCs). (A) Representative thrombin generation curves were obtained in recalcified kaolin-activated platelet-free plasma mixed with RBCs (5×10^6 in 100 μL) pretreated with a vehicle (dimethyl sulfoxide [DMSO], gray curve) or with 5 μM Yoda1 (black curve). (B–E) Average parameters of the thrombin generation assay, namely the (B) peak thrombin concentration, (C) time to peak, (D) endogenous thrombin potential (ETP, area under the curve), and (E) lag time, in kaolin-activated platelet-free plasma mixed with RBCs pretreated with DMSO vs Yoda1 (5 μM). Results are presented as the average and SD ($n = 5$). The Student's paired *t*-test was used for comparisons. For the numerical data, see Supplementary Table S5.

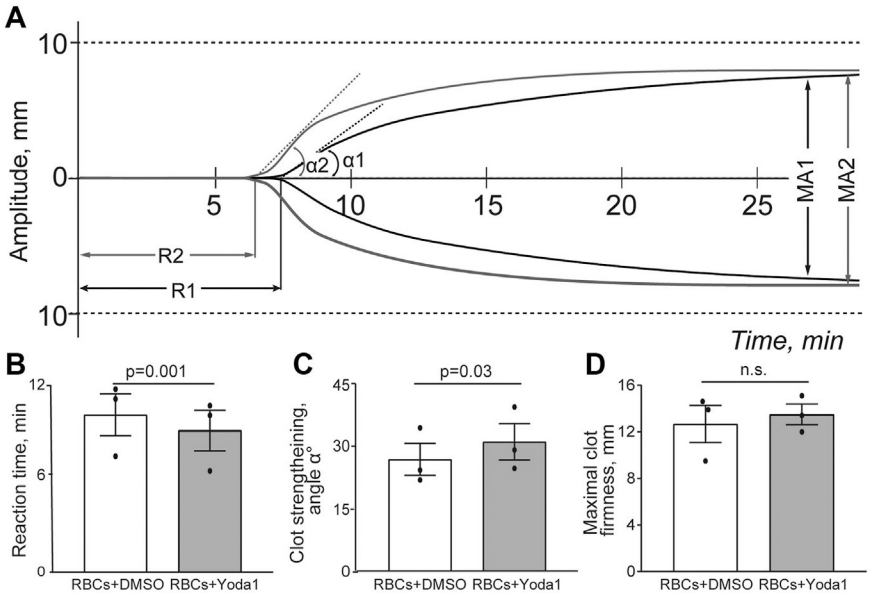

FIGURE 5.

Enhanced procoagulant activity of Yoda1-activated red blood cells (RBCs). (A) Representative thromboelastography curves obtained in Ca^{2+}-containing platelet-free plasma mixed with RBCs pretreated with a vehicle (dimethyl sulfoxide [DMSO], black curve) or with 5 μM Yoda1 (gray curve), illustrating shortening of the reaction time (R1 vs R2) and an increase in the rate of clot strengthening (α1 vs α2) in the presence of Yoda1-treated RBCs; the maximal clot firmness (MA1 and MA2) was almost unaffected. (B,C) Average thromboelastography parameters, namely the (B) reaction time, (C) clot strengthening, and (D) maximal clot firmness, in plasma mixed with RBCs pretreated with DMSO vs 5-μM Yoda1. Results are presented as the average and SD ($n = 3$). The Student's paired *t*-test was used for comparisons. For the numerical data, see Supplementary Table S6. n.s., not statistically significant.

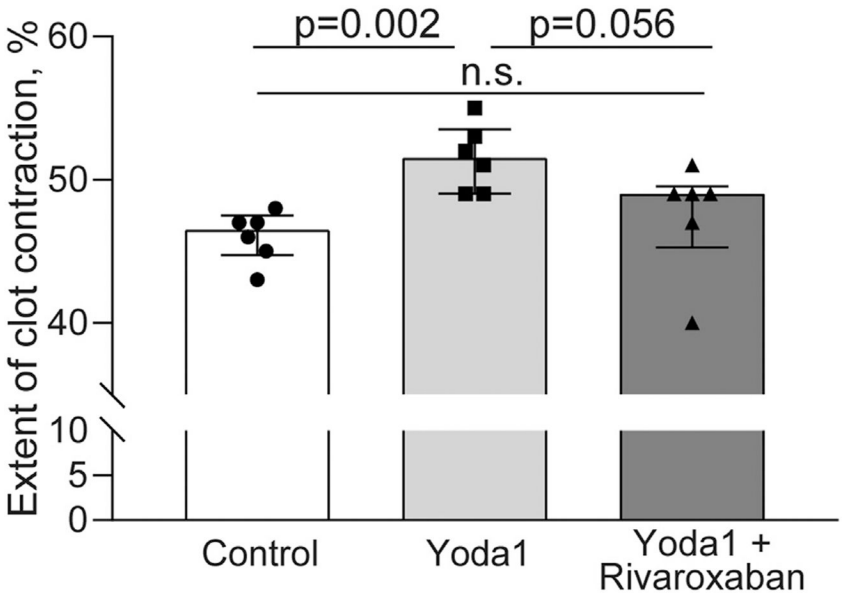

FIGURE 6.

Effects of Yoda1 (5 μM) on blood clot contraction in the absence and presence of rivaroxaban (1 μM), an inhibitor of endogenous thrombin generation. Comparative experiments were performed in parallel on identical samples of whole blood obtained from 6 independent donors and stabilized with soybean trypsin inhibitor. Results are presented as the median and IQR (25th and 75th percentiles). The Mann–Whitney U-test was used for comparisons. n.s., not statistically significant.

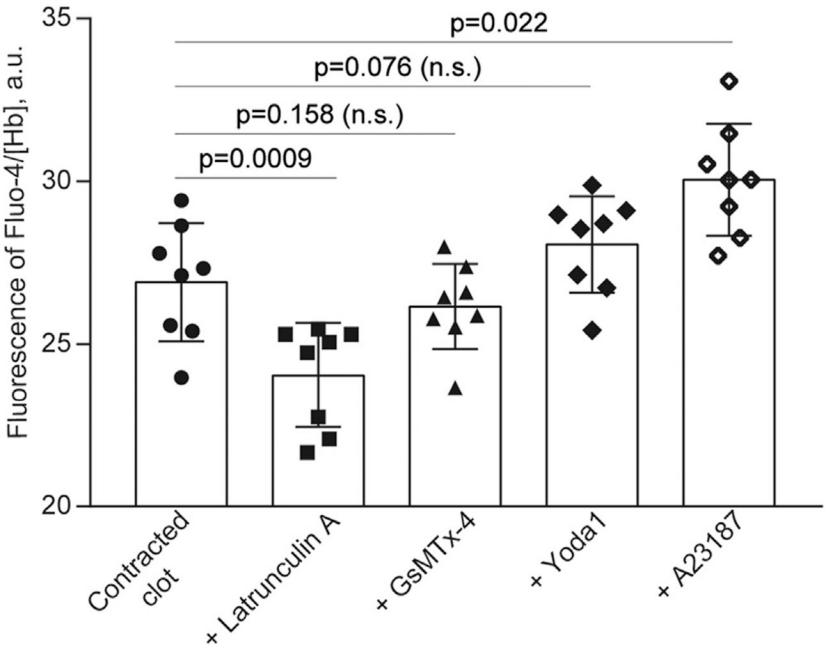

FIGURE 7.

Piezo1-mediated Ca^{2+} influx into compressed red blood cells during clot contraction. The plot shows comparative fluorescence intensity normalized by the concentration of hemoglobin (Hb) in the lysates of reconstituted blood clots containing red blood cells preloaded with Fluo-4, a Ca^{2+}-sensitive dye (see Supplementary Materials and Methods for details). The clots from the same blood samples were formed in the following conditions: 1) a freely contracting clot; 2) an uncontracted clot in which platelet contractility was suppressed with 5 μM latrunculin A, an inhibitor of actin polymerization; 3) a contracting clot formed in the presence of 2 μM GsMTx-4, a Piezo1 inhibitor; 4) a contracting clot formed in the presence of 5 μM Yoda1, a Piezo1 activator; and 5) a contracting clot formed in the presence of 20 μM calcium ionophore A23187. The various types of clots were formed from the same blood samples and analyzed in parallel. Results are presented as the average and SD ($n = 8$). The Student's paired *t*-test was used for comparisons. For the numerical data, see Supplementary Table S7. n.s., not statistically significant.

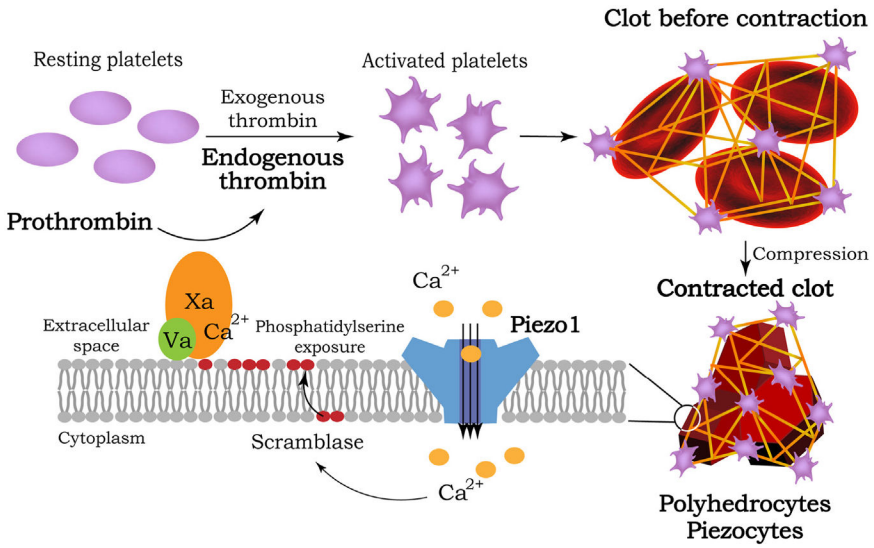

FIGURE 8.

Proposed mechanism of self-amplification of blood clot contraction through activation of mechanosensitive Piezo1 ion channels in compressed and deformed red blood cells (RBCs). Platelet activation and blood clot formation were induced by the addition of exogenous thrombin. Activated platelets generate contractile forces that are transmitted to and distributed by the fibrin fibers. Platelet contraction and compaction of the fibrin network are accompanied by compressive deformation of RBCs to polyhedral shape (polyhedrocytes or piezocytes) and reduction in size. Mechanical deformation of RBCs causes activation of Piezo1 channels and Ca^{2+} influx. An increase in intracellular $[Ca^{2+}]_i$ activates scramblase, which mixes membrane phospholipids, causing externalization of phosphatidylserine. Phosphatidylserine forms a membrane matrix for the assembly of intrinsic tenase and prothrombinase, which catalyzes the conversion of prothrombin to thrombin under the action of factor Xa. The resulting endogenous thrombin diffuses inside the clot and additionally activates platelets by binding to PAR1 and PAR4 receptors. Enhancing platelet contractility further augments the compression of the blood clot.