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Title: Systems biology insights into the meaning of the platelet's dual-receptor thrombin signaling

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Short title: Why platelets have two PARs

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Keywords: blood platelets, protease-activated receptors, thrombin, calcium signaling, mathematical model

Essentials

* Roles of the two thrombin receptors in platelet signaling are poorly understood.
* Computational systems biology modeling was used together with continuous flow cytometry.
* Dual-receptor system has wide-range sensitivity to thrombin and optimal response dynamics.
* Procoagulant platelet formation is determined by donor-specific activities of the two receptors.

Summary

Background: Activation of human platelets with thrombin proceeds via two protease-activated receptors (PARs), PAR1 and PAR4, that have identical main intracellular signaling responses. Although there is evidence that they have different cleavage/inactivation kinetics (and some secondary variations in signaling), the reason for such redundancy is not clear.

Methods. We developed a multicompartmental stochastic computational systems biology model of dual-receptor thrombin signaling in platelets to gain insight into the mechanisms and roles of PAR1 and PAR4 functioning. Experiments employing continuous flow cytometry of washed human platelets were used to validate the model and test its predictions. Activity of PAR receptors in donors was evaluated by mRNA measurement and by polymorphism sequencing.

Results. While PAR1 activation produced rapid and short-lived response, signaling via PAR4 developed slowly and propagated in time. Response of the dual-receptor system was both rapid and prolonged in time. Inclusion of PAR1/PAR4 heterodimer formation promoted PAR4 signaling in the

medium range of thrombin concentration (about 10 nM), with little contribution at high and low thrombin. Different dynamics and dose-dependence of procoagulant platelet formation in healthy donors was associated with individual variations in PAR1 and PAR4 activities and particularly by the Ala120Thr polymorphism in the *F2RL3* gene encoding PAR4.

Conclusions. The dual-receptor combination is critical to produce a response combining three critical advantages: sensitivity to thrombin concentration, rapid onset and steady propagation; specific features of the protease-activated receptors do not allow combination of all three in a single receptor.

Introduction

Platelet activation is essential for both hemostasis and thrombosis. It is triggered by interaction of different agonists with their receptors. Most (patho)physiological agonists bind to the G-protein-coupled receptors (GPCRs) except for collagen (GPVI) and podoplanin (CLEC-2). GPCRs lead to phospholipase Cβ activation, inositol-3-phosphate (IP3) generation, and release of calcium ions from intracellular stores (Fig. 1). The increase in cytoplasmic calcium concentration mediates a hierarchy of platelet responses [1] beginning with integrin activation/aggregation [2], proceeding to the release of granules, appearance of plasma membrane pseudopodia and platelet surface spreading. All these responses are mediated by elevated cytosolic calcium [3]. Strong agonists of PARs or GPVI lead to segregation of platelets into two subpopulations with distinct features, including phosphatidylserine (PS) exposure on the outer leaflet of the plasmatic membrane in one of them [4]. High PS levels allow binging of coagulation factors and accelerate blood plasma coagulation reactions on platelet surface [5-7] (hence the name “procoagulant”). Procoagulant platelets are also characterized by collapsed mitochondria, high cytosolic calcium [8] and disrupted cytoskeleton [9]. *In vitro* procoagulant platelets formation could be stimulated with 10-100 nM of thrombin to washed platelets [10]. In blood, procoagulant platelets support prothrombinase and tenase formation on their surface [5] thus acting as a positive feedback loop [11].

Human platelets express two thrombin-activated receptors (PARs), PAR1 and PAR4 [12]. PAR1 interacts with thrombin with high affinity of 0.1-1 nM [12;13], but is rapidly desensitized due to its rapid internalization [14]. In contrast, PAR4 lacks the hirudin-like domain, which allows PAR1 to “sense” low thrombin [15], its EC_{50} for thrombin is much higher (10-100 nM in different studies) [12;13], and it is internalized rather slowly [14].The PAR tethered ligand, produced after its cleavage by thrombin,

could be simulated with an activating peptide (S(T)FLLRN for PAR1, and A(G)YPGKF for PAR4), which gives a valuable tool for separate PAR1 and PAR4 investigation [16]. With PAR-activation peptides and different PAR-antagonists it was shown that PAR1 induces an acute and short-lived rise in cytosolic calcium and integrin activation, whereas PAR4 causes a steady and sustained calcium rise and integrin activation that largely depends on $P2Y_{12}$ [13;14;17;18]. Besides, existence of PAR homo- and heterodimers was shown for platelets in a number of studies [19-21], and PAR1-PAR4 dimerization promotes cleavage of PAR4 by thrombin [22]. Although some difference in intracellular signaling pathways for PAR1 and PAR4 exists [13;17;18;23], main intracellular signaling through PLCβ activation, IP3 appearance and calcium release from the DTS is common to both receptors. Studies with PAR1/PAR4 inhibitors as well with their activating peptides [12;16;24;25] showed that each is capable of invoking all hierarchy of platelet responses including PS exposure [26]. So, despite many important discoveries in the mechanisms of PAR1/PAR4-stimulated platelet response, it is still arguable that the dual-receptor system provides apparent functional redundancy. In order to reconcile the existing scattered experimental data, we aimed here to investigate this system by means of a computational simulation, where a comprehensive scheme can be tested, and where it could be shown whether one receptor would be enough for all responses or not.

The goal of this study was to investigate functioning of the dual-receptor system in platelet activation by thrombin with a special focus on the procoagulant platelet formation using a combination of computational systems biology approach with experiments. We demonstrate that two receptors are necessary to form a rapid, steady, and concentration-sensitive platelet response to thrombin. In addition, our results provided insights into the differential roles of SOCE in PAR1 and PAR4 responses, the meaning of the PAR1-PAR4 heterodimer formation in the middle range of thrombin concentration, and the possible roles of the individual differences in the receptor activity in the development of inter-donor variability in procoagulant platelet formation dynamics and concentration-dependence.

Materials and Methods

Materials. The sources of the following materials were as follows: thrombin (Haematologic Technologies, Essex Junction, VT); prostaglandin E1 (MP Biochemicals, Irvine, CA); calcium-sensitive cell-permeable fluorescent dye Fura Red, Calcium Green-1, Fluo-5N, and

tetramethylrhodamine (TMRM) (Molecular Probes, Eugene, OR); Alexa Fluor 647–conjugated annexin V (Biolegend, San Diego, CA); AR-C 66096, MRS 2179 (Tocris Bioscience; Ellisville, MO, USA), PN IM2085 clone WEDE15 (Immunotech SAS, a Beckman Coulter Company, Marseille, France), TRIzol® Reagent (Life Technologies), HEPES, bovine serum albumin, Sepharose CL-2B, apyrase grade VII (Sigma-Aldrich, St Louis, MO). SFLLRN and AYGPKF were custom-synthesized at the Institute of Bioorganic Chemistry (Russian Academy of Sciences, Moscow, Russia.

Blood collection and platelet isolation. Twenty five healthy volunteers, both men and women aged 18 to 35 years were recruited into the study. Investigations were performed in accordance with the Declaration of Helsinki, and written informed consent was obtained from all donors.

Blood was collected into 4.5 ml tubes containing 3,8% sodium citrate (1:9 vol/vol) and supplemented by apyrase (0.1 U/mL) and prostaglandin E1 (1 μmol/L). Platelets were purified by centrifugation and gel filtration or by double centrifugation as described [5;27]. Briefly, platelet-rich plasma was obtained by centrifugation at 100 g for 8 minutes. Platelet-rich plasma was centrifuged at 400 g for 5 minutes, and platelets were resuspended in buffer A (150 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 20 mM HEPES, 5 mM glucose, 0.5% bovine serum albumin, pH 7.4) and gel filtered on Sepharose CL-2B or washed in acidic (pH 6.5) buffer A..

Flow cytometry. For time-course experiments washed platelets were incubated with 10 μM Fura Red/AM and/or Calcium-Green 1/AM, Fluo-5N/AM or 0.5 μM TMRM prior to gel filtration/ second wash for 30 minutes (15 min for TMRM, 2 h for Fluo-5N) at 37°C in the presence of apyrase (0.1 U/mL), pre-incubated in buffer A with 2.5 mM or 1.25 mM $CaCl_2$ or 10 mM EDTA in the presence of annexin V (1% vol/vol) for 1 minute and analyzed using an Accuri C6 (Accuri Cytometers, Ann Arbor, MI) flow cytometer in a continuous regime or using FACS Calibur, LSR Fortessa or FACS Aria II (BD Biosciences, San Jose, CA, USA) in a continuous regime with 20s interruption for the addition of activator or NovoCyte (ACEA Biosciences, San Diego, CA, USA). The possible influence of ADP excreted by platelets upon activation was tested (Fig. S1A,D). Addition of MRS 2179 (the $P2Y_1$ receptor antagonist) at 10 μM and/or AR-C 66096 (the $P2Y_{12}$ receptor antagonist) at 100 μM did not give any noticeable changes in the observed calcium dynamics (Fig. S1A,D). The temperature of the suspension (25 °C or 37 °C) (Fig. S1B,D) or extracellular calcium concentration (1.25 mM or 2.5 mM) also did not have significant influence on the results (Fig. S1C). The cytosolic calcium concentration

was assessed from the ratio of cell's calcium-bound dye fluorescence (Calcium Green-1 exited with 488 nm laser or Fura Red exited with 405 nm laser) and calcium-free dye fluorescence (Fura Red exited with 488 nm laser) using classical "one Kd" calibration model [28] with Kd = 140 nM. For the agonist titration studies (Fig. 5), gel-filtered platelets at 50,000 plts/µl were activated in buffer A with 2.5 mM $CaCl_2$, and various agonists in the presence of annexin V (1% vol/vol) for 5 minutes, diluted 10-fold and immediately analyzed using an Accuri C6 flow cytometer. The acquired data were processed using FlowJo (http://www.flowjo.com/) software.

Single nucleotide polymorphisms (SNP) genotyping. Eight blood samples and thirty three saliva samples from different donors were genotyped for rs168753 (IVSn−14 A/T transition in PAR-1 gene) and rs773902 (Ala120Thr substitution in PAR-4 gene) SNPs. Genomic DNA was extracted from buffy coat using QIAmp DNA Blood Mini Kit and QIAcube™ automatic station (QIAGEN). Saliva was collected in 15 ml tubes after 30 minutes fast. Before DNA isolation 1 ml of saliva was mixed with 1 ml of PBS and centrifuged at 1800 g for 5 munutes. Pellet was resuspended in 1 ml of PBS and centrifuged at the same conditions. After the second centrifugation pellet was resuspended in 180 µl of PBS and processed using QIAmp DNA Blood Mini Kit and the protocol for DNA isolation from blood and body fluids in the QIAcube™ automatic station. PCR was performed with the following primers "SNP/Sense primer 5′-3 sequence′/Antisense primer 5′-3 sequence"": rs168753/TTGTCGCTTTTGCCTTGTTG/GGGAGCACAGACACAAACAG, rs773902/ATGTCCAGCTGTTTCCCACC/AGCCATGCAGAGTCCAAGG [29;30]. Amplicons were purified by ethanol precipitation and sequenced with the same primers, BigDye Terminator v3.1 kit (Applied Biosystems) and 3730xl DNA Analyzer (Applied Biosystems) according to manufacturer's recommendations.

Computational model description. The detailed model description with rate constants can be found in Tables S1 and S2. The scheme of the reactions and signaling pathways incorporated into the model is given on Fig. 1.

Activation of both PAR1 and PAR4 was considered essentially as in [31;32] with addition of a Michaelis–Menten kinetics for the thrombin cleavage of the receptors. The number of receptors per platelet were assumed to be 2000 and 1000 for PAR1 and PAR4 respectively [33].The unknown

parameters for the new reactions were adjusted based on the experiments from [13;34] (Fig. S2A-C) and on the original experimental data (Fig. S3, S4). Detailed descriptions of algorithms and strategies for parameter estimation were described earlier [32]. The formation of PAR1-PAR4 heterodimer was assumed to proceed by the following scheme (Table S1). First PAR1 becomes activated by thrombin (as thrombin is essential for this dimerization [22]), then it binds to PAR4 with some constant that was tuned to describe the known experimental data [22] and makes an "advanced PAR4" that could be activated by thrombin with a 6-fold lower K_M [21].

Model solution. The set of ordinary differential equations (Table S1) with initial parameters (Table S2) was integrated using the COPASI software (http://www.copasi.org) [35]. For tuning the model, estimation of parameters and comparison between models the deterministic simulations were used (LSODE solver). For the integration of the whole model the stochastic simulations (the Adaptive SSA/tau-leap method [36]) was used, being more appropriate for small cells [37].

Results

1. **PAR1-PAR4 heterodimers.** PAR1 and PAR4 are known to exist in homo- and heterodimers [19;20], and PAR1 was demonstrated to serve as a cofactor for cleavage and activation of PAR4 at low thrombin concentrations [21]. We have included PAR1 and PAR4 heterodimers formation and consequent 6-fold decrease of EC50 for cleavage of PAR1-bound PAR4 by thrombin into the model [21]) (Fig. 2). The rate constant of PAR1-PAR4 dimerization was tuned to describe the two-fold increase in the rate of PAR4 cleavage by 10 nM of thrombin observed in [22]. The comparison of the two models, with PAR1-PAR4 dimerization (black lines and columns on Fig. 2) and without it (grey lines and columns), with each other and with experimental data is given in Fig. 2. Although dimerization increases PAR4 activation at 1 nM of thrombin five-fold (Fig. 2A, left column), this only marginally influenced the degree of platelet activation (i.e. the average calcium concentration and activated PLC concentration, Fig. 2F,G). After stimulation with 10 nM of thrombin, the total amount of activated PAR4 was only two-fold higher in the model with dimerization, yet it shifted the activation response from peak-like into a prolonged one (Fig. 2D, E), which made an important difference for calcium concentration (Fig. 2D). For 100 nM

of thrombin, dimerization did not affect the activation level (Fig. 2B,C) in agreement with [22]. Note that PAR4 was preferentially cleaved from heterodimers even at 100 nM of thrombin (Fig. 2A, right), but PAR4 could be cleaved by its own almost as well by high thrombin, so once more there was no observed difference. Comparison of the calculations with experimental data at 10 nM of thrombin supports the model with dimerization, while experimental data for 1 nM or 100 nM of thrombin could be described by both models.

2. Intracellular signaling. First, we analyzed dynamics of different signals produced upon stimulation of PAR1/PAR4 receptors individually or together. Stochastic simulations were used with thrombin (100 nM) stimulation in all cases. PAR1 stimulation alone led to a sharp but transient increase in the number of active PLC molecules and cytosolic calcium spiking (Fig. 3A). In contrast to that, PAR4 alone produced slow, gradual increase of the PLC activity over the first 100 s of activation up to the same level, and cytosolic calcium response was a series of oscillations that rose slowly over 100 s post-stimulation and persisted (Fig. 3B). Stimulation of both receptors gave a superposition of the PLC activities, meaning a sharp increase of PLC activity followed by plateau (Fig. 3C). Accordingly, calcium oscillations were initiated rapidly and persisted. Comparison of calcium concentrations averaged over 20 stochastic runs with experimental data confirms this prediction (Fig. S3, Fig. S4).

Significant cytosolic calcium oscillations in all cases could lead to the uptake of calcium by mitochondria and at least partial opening of mPTP (Fig. 3D-F). However, the pore opening in its turn allowed release of mitochondrial calcium and, once again, its own closure. This mechanism, suggested for PAR1 stimulation in our previous paper [32], is most clearly observed here when PAR4 is added. Only when cytosolic calcium oscillations occurred sufficiently rapidly and frequently, the mitochondria became overloaded with calcium earlier than they could release excess calcium (Fig. S5C). As discussed below, this led to the irreversible pore opening, cell death and formation of the procoagulant platelet subpopulation.

Similar differences were observed in DTS calcium concentrations and SOCE activation (Fig. 3G-I). Depending on the stochastic run, PAR1 activation could induce up to 50% decrease in the DTS calcium with extracellular calcium entry in some runs (Fig. 3G, Fig. S5). Activation of PAR4 alone (Fig. 3H) or in combination with PAR1 (Fig. 3I) in all cases led to a sufficient DTS calcium decrease and

opening of Orai1 channels in line with [38-40] and with observed experimental data (Fig. S5D,E). Also in line with this, platelet response was markedly impaired by removal of Orai1 or extracellular calcium for the case of PAR1-plus-PAR4 stimulation (Fig. S5).

3. Platelet subpopulations formation. Platelet PS-exposure was previously reported to correlate with a drop in mitochondrial membrane potential in flow cytometry experiments [8;41;42], although in individual fibrinogen-attached platelets [42] a 30-60 s delay between these events can be observed. To investigate this assumptions, we performed continuous flow cytometry measurement of TMRM/annexin V fluorescence in thrombin-activated platelets (Fig. 4; see also Fig. S6 for zebra plots and another donor genotype). In all these experiments, platelets first lost their mitochondrial membrane potential and then began to expose PS. In order to investigate roles of PAR1 and PAR4 in the procoagulant subpopulation formation, dynamics and concentration-dependence of procoagulant response (fraction of PS-exposing cells or cells with opened mPTP (drop in mitochondrial membrane potential)) were first investigated experimentally in different donors (Fig. 5). Temporal dependence of the procoagulant platelet fraction in response to SFLLRN was sigmoidal-like (Fig. 5 C,D insets) [32]. Unlike SFLLRN, thrombin produced a variety of donor-specific responses with two extreme phenotypes: in some donors, the overall shape was similar to SFLLRN and could be called “Step-like” (Fig. 5C); in others, there was just steady linear growth of the number of procoagulant platelets with time or with thrombin concentration and could be called “Linear” (Fig. 5B,D). These types of behavior are in agreement with previously reported data [26;39].

Keeping in mind different dynamics of the platelet response to PAR1 and PAR4 stimulation discussed in the previous sections, we hypothesized that this difference could be due to the different contributions of PAR1 and PAR4 in these donors. Computer simulations using steadily increasing PAR4/PAR1 activity ratios indicated that this indeed could be the case: Fig. 5A,C could be described by “normal” PAR activities (those were used to validate the model), and Fig. 5B,D could be described by lowering PAR1 activity by 25% and elevating PAR4 activity by 25%.

To corroborate the model prediction that PAR1 and PAR4 activity could influence procoagulant response, we investigated the genes coding PAR1 and PAR4 (Fig. 6). It is known that the expression of PAR1 receptors on the platelet surface is associated with rs168753 (IVSn-14 A/T) intronic variation with A allele associated with common phenotype and T allele associated with lower PAR1 expression

and lower response to SFLLRN [29]. For PAR4 it was shown that rs773902 SNP in PAR4 gene (F2RL3) leading to Ala120Thr substitution in PAR4 protein could be associated with different PAR4-induced platelet activation, with Alanin (allele G) for common phenotype and Threonine (allele A) for greater PAR4-induced platelet aggregation and reduced inhibition by a PAR4 antagonist [30]. It appeared that the "step-like" phenotype of induced platelet activation (Fig. 5A,C) is associated with PAR1 (A/A) or PAR1 (A/T) and PAR4 (G/G) genotypes (Fig. 6A,B). The "linear" phenotype (Fig. 5B,C) is characteristic for donors, who have at least one A allele (Thr120) of PAR4 gene (Fig 6C-F).

Interestingly, the same difference could be observed in the cytosolic calcium dynamics in the same donors (Fig. 7): a peak-shaped one for the platelets from Donor D (PAR4 (G/G)) and a more step-like for platelets from Donor G (PAR4 (A/A)). Note that differences in calcium dynamics after stimulation of platelets from the same donors with thrombin at 10 nM both in theory and in experiments are much less pronounced indicating that the observed phenomenon mostly concerns strong platelet stimulation.

4. Roles of PAR1 and PAR4. The experiments and simulations in the previous sections supported, with some additions and corrections, the accepted picture of the PAR1 and PAR4 receptor action, with former mediating rapid response and mostly important at low thrombin concentration, and the latter responsible for prolonged activation and being more important at high thrombin concentration [12-14;16;17;43]. However, a natural question arising here is why do we need two receptors with almost identical signaling pathways for this?

In order to solve this, we simulated platelet activation with thrombin via PAR1 alone, PAR4 alone, or their combination without receptor dimerization, and plotted IP3 concentration as a function of time for different thrombin concentrations (Fig. 8). Combination of the receptors produced an optimal response with all three parameters improved: rapid onset (characteristic for PAR1, Fig 8A), slow decay (characteristic for PAR4, Fig. 8B), and sensitivity throughout the whole range of concentrations (Fig. 8C). The concentration-response curve (Fig. 8D) shows a bi-phasic dependence of IP3 on thrombin concentration that is almost a clear sum of the dependences for PAR1 and PAR4, while the time-response curve (Fig. 8E) illustrates that IP3 dynamics in time is a similar superposition, with the onset identical to PAR1 rise, and slow decay with the same kinetics as PAR4 signaling. We carried

out simulations of platelet activation via a hypothetical PAR receptor and tried receptors activation and deactivation parameters (Fig. S7) to show that an attempt to obtain a "rapidly activated PAR4" produced only "just another PAR1"; rapid onset in the qualitative dynamics was always co-incident with rapid decay.

Discussion

Here, we developed a first comprehensive, mechanism-driven, thoroughly corroborated by experiments, multicompartmental mathematical model of the thrombin-dependent dual-receptor activated calcium signaling network in platelets that includes both DTS and mitochondria, and is able to reproduce experimentally observed phenomena such as calcium spiking and segregation into subpopulations. We showed how the combination of PAR1 and PAR4 functions to ensure rapid onset of the response, sustained signaling and sensitivity to thrombin concentration within a wide range, and demonstrate that all these three goals would not be reachable with a single PAR receptor.

It is now well accepted that PAR1 and PAR4 have differential roles in platelet activation. The main result of the systems biology analysis in the present paper is that these phenomena are united together, interpreted and put in the correct order. It is our speculation that if a receptor works in the range of 100-1000 nM of its agonist, then 0.1 nM would produce a negligible signal (Fig. 8B). It should be slow and thus we obtain slowly-activated-and-slowly-decaying PAR4. Likewise, for the low range of thrombin concentration, we necessarily come to a rapidly-activated-and-rapidly-inhibited PAR1 (Fig. 8A), and together they form a continuous range of reasonable sensitivity to thrombin concentration (Fig. 8C), which is additionally smoothed by PAR1/PAR4 dimers at the intermediate concentrations.

Formation of the procoagulant platelets, those promote thrombin generation [5], is potently induced by collagen, thrombin or their combination. Although collagen is not present in our model so far, the regulation of this phenomenon by thrombin concentration is a question of interest in itself. The fraction of PS-exposing cells is well known to closely correspond with the fraction of platelets a drop in the mitochondrial membrane potential [8;41;42] (Fig. 4, S6). Earlier we have demonstrated theoretically that platelet heterogeneity leads to different calcium accumulation in mitochondria and calcium-overload-induced mitochondrial dependent platelet necrosis [32]. In the current study we used this mechanism to predict the fraction of procoagulant platelets after different levels of activation (Fig. 5). As it appeared that the relative activity of PAR1 and PAR4 receptors determine this fraction, we

corroborated the finding by determination of PAR1 and PAR4 genes polymorphisms (Fig. 6). The donor mRNA levels could influence the dynamics without switching between phenotypes (Fig. S8).

The obtained data indicated that PAR1 activity determined opening of mPTP in the first minute after activation and PAR4 determined if any platelets will become necrotic after the first minute. As indicated polymorphisms are very common in human population [29;30] and number of PAR receptors per platelet greatly varies [44] it is possible that the activity of PAR receptors can describe variations in platelet response to thrombin activation between different donors, but this question needs further theoretical and experimental investigation.

Recently platelet PAR4 receptor has received much attention as a possible target for antiplatelet therapy milder than vorapaxar [45-47], and PAR4 gene – as a source of functional polymorphisms [48]. Particularly an essential role of PAR4 in procoagulant activity was demonstrated [49]. Our study provides a theoretical basis for this findings, and we have demonstrated crucial role of gene polymorphisms in the development of platelet subpopulations. Yet it should be noted that platelet response to PAR4 activating peptide (Fig. S4C) is more rapid in dynamics than platelet response to thrombin with inhibited or desensitized PAR1 (Fig. S4A, [13]), possibly due to different receptor activation molecular mechanisms. Therefore caution should be exercised in the interpretation of data with strong platelet activation by PAR4 activation peptide.

The ability of GPCRs to form homo- or heterodimers or higher order oligomers is an established fact now [19;50]. It was shown in a number of papers that PAR1-PAR4 heterodimers are formed as a response to thrombin activation and that PAR4 becomes more sensitive to thrombin after formation of such complex [20-22]. We included this mechanism in our computational model and observed an elegant fill of the response gap between 1 nM of thrombin, where the PAR1 mostly works, and 100 nM of thrombin where PAR4 works with full force in good agreement with the experimental data [22]. To our knowledge this is the first computational model describing functioning of the system with two different receptor dimerization in response to activator.

Platelet response to thrombin stimulation could be regarded as a prolonged response to PAR1 activating peptide if not for the steady decline in the level of DTS calcium (Fig. 2). In agreement with previous reports [13;38;39;51], we have demonstrated that the prolonged cytosolic calcium oscillations after stimulation with thrombin is mainly a consequence of prolonged influx of external

Ca^{2+} due to the opening of the store-operated calcium channels. Theoretical investigation of roles of different calcium channels [39;52] in platelet activation will be subject of our future work.

The mathematical model presented here has several simplifications, the most important one being lack of consideration of modulating mechanisms inside the calcium signaling system itself [53], like PKC and PI3K kinases, and cAMP signaling (G_i – coupled receptors). These mechanisms can shift the balance between decisions and level of cytosolic calcium; however, this simplification does not change both responses qualitatively [26;54]. The absence of $P2Y_1$ and $P2Y_{12}$ receptors and secondary platelet activation due to release from platelet granules is not included in the model as all experimental studies was done at very low platelet concentrations (1000 plts/μl) and addition of $P2Y_{12}$ and $P2Y_1$ antagonists does not influence the results (Fig. S1A,D). Addition of all these secondary mechanisms, as well as that of other receptors, should be the subject of further study.

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Authors’ contributions

A. N. Sveshnikova planned research, developed the model, performed simulations, designed and performed experiments (flow cytometry), analyzed the data and wrote the paper. A. V. Balatskiy planned and performed genotyping mRNA quantification. A. S. Demianova, S. S. Shakhidzhanov, T. O. Shepelyuk, M. N. Balatskaya, and A. V. Pichugin performed experiments and analyzed the data (flow cytometry). F. I. Ataullakhanov supervised the project and planned research and analyzed the data. M. A. Panteleev planned model development and research, analyzed the data, wrote and edited the paper.

The authors declare that they have no conflict of interest.

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