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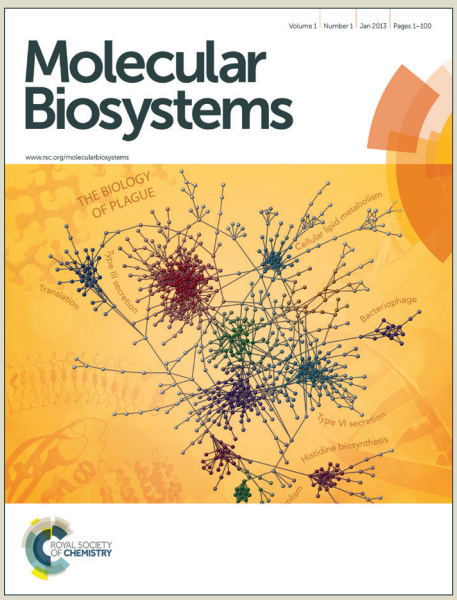





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# Compartmentalized calcium signaling triggers subpopulation formation upon platelet activation through PAR1

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Short title: Calcium signaling in platelet subpopulations

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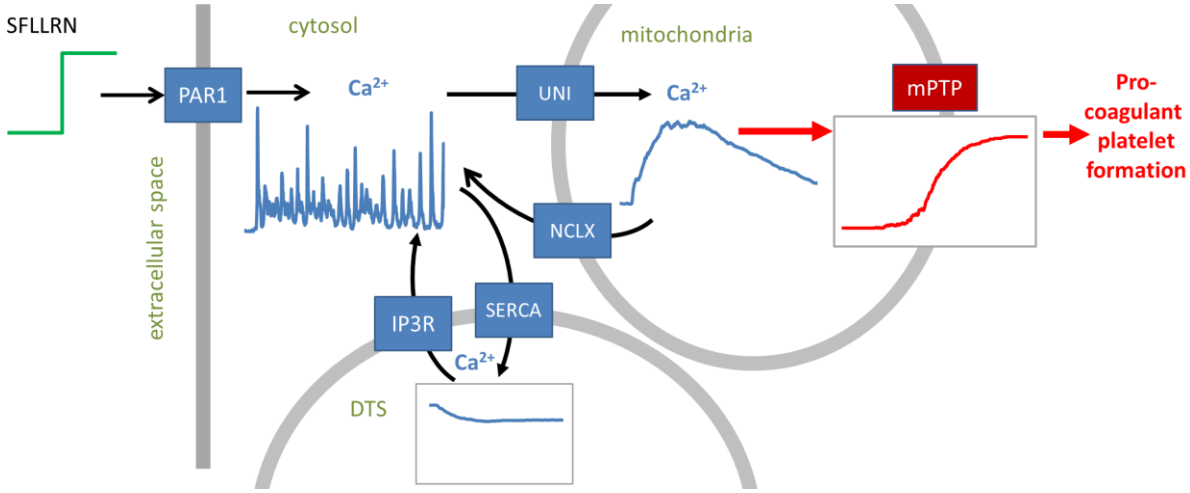


A comprehensive computational systems biology model of PAR1-stimulated platelet calcium signaling is developed to analyze formation of subpopulations upon activation. This occurs via a mitochondria-dependent decision-making mechanism. This is a stochastic phenomenon caused by a small number of proteinase-activated receptors.

## Abstract

Blood platelets need to undergo activation to carry out their function of stopping bleeding. Different activation degree leads to a stepped hierarchy of responses: ability to aggregate, granule release, and, in a fraction of platelets, phosphatidylserine (PS) exposure. This suggests existence of decision-making mechanism(s) in the platelet intracellular signaling network. To identify and investigate them, we developed a computational model of PAR1-stimulated platelet signal transduction that included minimal set of major players of the calcium signaling network. The model comprised three intracellular compartments: cytosol, dense tubular system (DTS) and mitochondria and extracellular space. Computer simulations showed that the stable resting state of platelet is maintained by a balance between calcium pumps and leaks trough the DTS and plasma membranes. Stimulation of PAR1 induced oscillations in the cytosolic calcium concentrations, in good agreement with experimental observations. Further increase in the agonist level activated the mitochondrial uniporter leading to calcium uptake by mitochondria, which caused collapse of mitochondrial membrane potential in a fraction of platelets leading to the PS exposure. Formation of this subpopulation was shown to be a stochastic process determined by the small number of activated PAR1 receptors and by heterogeneity in the number of ion pumps. These results demonstrate how a gradual increase of the activation degree can be converted into a stepped response hierarchy ultimately leading to formation of two distinct subpopulations from an initially homogeneous population.

**Keywords:** platelets; calcium signalling; protease-activated receptors; dense tubular system; mitochondria; platelet subpopulations; mathematical modelling; sensitivity analysis

## Introduction

Platelets are small cell fragments circulating in blood vessels, whose purpose is to plug the injuries in the vessel walls. Platelets can become activated in response to vascular damage: upon activation, they secrete their granule contents, bind coagulation factors and interact with one another to form an aggregate to stop blood loss. Platelet activation occurs through the interaction of different agonists with their corresponding receptors. Most agonists bind to the G protein-coupled receptors on platelet surfaces (except for collagen that binds to a tyrosine kinase receptor) leading to phospholipase C activation, inositol-3-phosphate (IP3) generation, and the release of calcium ions from intracellular stores. Increased concentration of free cytoplasmic calcium leads to the classic platelet activation responses mentioned above[1]. About a decade ago in vitro experiments demonstrated that activation of platelets with high concentrations of potent agonists (thrombin and collagen or their analogs) leads to the formation of a subpopulation of platelets with a totally different pattern: they do not stick to each other, but instead they expose phosphatidylserine (PS) on the outer membrane which enhances the assembly and activity of two major coagulation factor complexes on the platelet surface[2-4].

Upon activation with various agonists platelets show a hierarchy of responses depending on the type and concentration of the activator[5]. As the basic response to the activation, platelets aggregate with each other through integrins on their surface. The ability to aggregate appears even at the lowest agonist concentrations[6]. Another marker of activated platelets is the release of intracellular granules containing various coagulation proteins. Platelets contain different types of alpha-granules, possibly differentially released after activation with various agonists[7], and dense-granules released only after medium or strong activation. Activation of platelets with normal

concentrations of agonists leads to the shape change: appearance of plasma membrane pseudopodia and subsequent spreading on a surface. This response requires modifications in platelet cytoskeleton, mediated by elevated cytosolic calcium concentration[8]. Stimulation with high levels of thrombin, thrombin receptor agonist peptides like SFLLRN, collagen, collagen-related peptide or convulxin leads to the exposure of PS on the surface of a subpopulation of platelets[3, 4, 9]. Therefore, there exists a stepped hierarchy of physiological platelet responses: integrin activation – granule secretion – shape change – procoagulant response. There are different hypotheses about the origin of this hierarchy, usually linking different responses with stimulation of different receptors and/or calcium concentration in the cytosol[10].

The calcium ion is the most important second messenger in the classic activation of platelets and appears to bear the same value for the formation of procoagulant platelets[11]. As the main known difference between procoagulant and normally activated platelets lies in the dynamics of cytosolic calcium concentration ("sustained high" for the former and "oscillations" for the latter[12]), the proper place to look for the trigger of the "procoagulant" phenotype is among the regulators of calcium dynamics.

The complex system of calcium channels in the inner and outer membranes of the cell has been theoretically investigated in several mammalian cell types[13, 14] but, for platelets, the mathematical models of signal transduction are scarce[15-17] and there is no model of calcium oscillation in platelets, and no analysis of the role of mitochondria. Mitochondria are known to influence significantly oscillations of calcium ions concentration[18], and procoagulant platelet formation is clearly related to the state of mitochondria[19, 20]; consequently, addition of this organelle into the consideration when investigating hierarchy of platelet answers is required.

The network of platelet activation signaling is incredibly complex[21]. However, calcium signaling is generally accepted to be the central controlling element in this network, in particular for the platelet subpopulations formation, and additional signaling pathways (such as cAMP signaling, PI3K signaling and others) mostly serve to attenuate it or to work downstream[22, 23]. The minimal network comprising the most critical elements determining the platelet calcium signaling kinetics includes the four major players as depicted in Fig. 1: the cytosol, the DTS, the mitochondria and the plasmatic membrane. The most potent activator of platelets, thrombin, in different concentrations can induce all hierarchy of answers through the activations of PAR1[10;24], so the decision-making mechanisms should be located in the pathway of activation of platelet by agonist of PAR1 receptor (SFLLRN)[24].

We made a computer model of calcium signal transduction after platelet stimulation with a PAR1 agonist building upon previous works[15-17, 25, 26]. Computer simulations have shown existence of three steps of platelet responses: low cytosolic calcium, oscillating cytosolic calcium and oscillating cytosolic calcium with opened mPTP. On each of the intermediate levels of activation, the population of platelets simulated by stochastic method shows heterogeneity of responses. Using a detailed analysis of the model, we have found two decision-making mechanisms inside the platelet calcium signaling system. We have shown that separation of platelet into subpopulations originates from heterogeneity of activation of small number of receptors, amplified by DTS and integrated in mitochondrial calcium concentration. The other cause of the platelet segregation into subpopulation is the heterogeneity of the number of calcium channels per platelet.

## Results

*General view of model solutions: calcium spiking, oscillations, pore opening*. The first step of the investigation was to simulate calcium response in deterministic mode, when concentrations of species are considered to be continues. Three different patterns of calcium dynamics were observed: stationary low calcium (less than 50 nM), oscillations with frequency of 0.3-0.4 $s^{-1}$ and amplitude 200-600 nM and sustained high calcium, prolonged for about 20 s (Fig. S1). This behavior is consistent with known patterns of calcium in single platelets, so the deterministic simulations could be used to assess the qualitative response of the model[27, 28].

To simulate platelet response more precisely, we used stochastic simulations (Hybrid solver, COPASI[29]). This type of simulations allows to take into consideration that one platelet contains very low amount of molecules (for example, there are only 1000 molecules of PAR1 receptor per platelet, and numbers of activated PAR1 are typically much smaller), and every reaction should be considered as a probabilistic process[30]. Each run of the stochastic simulation is considered as an individual cell in an individual environment. Typical runs are shown in Fig. 2.

We can observe several possible responses of platelet to the stimulation of PAR1 receptor. The first one is stationary low calcium (Fig. 2A). The second one, occasional calcium spiking, could be obtained only in stochastic simulations due to its random nature. This type of calcium dynamics has been observed experimentally in unstimulated adherent platelets, or in platelets stimulated with low doses of ADP[27, 31]. The simulations allow to explain this type of behavior: due to the occasional stimulation of several PAR1 receptors, such pattern could be occasionally observed even at low doses of activator. For the concentration of SFLLRN of 0.4 μM, occasional spiking is observed in 50 % of runs.

When activation is strong enough, all platelets show sustained oscillations of calcium. The patterns of calcium concentrations are very similar to those observed in single platelets stimulated with thrombin or ADP[27, 32]. The patterns observed under stimulation with 4 μM or 40 μM of SFLLRN are very similar, but average calcium concentration rises with the concentration of agonist. Some patterns lead to the avalanche-like opening of mPTP (Fig. 2F), while others do not (Fig. 2E). In contrast to the previous step, mitochondrial collapse is observed only in a fraction of runs even for the maximal concentration of the activator. Significant opening of mPTP (of the order of 0.15-0.2, after which it becomes irreversible) and collapse of membrane potential are tightly related in the model, so they coincided in all simulations without exception.

In the simulation runs of the model we observe different patterns of calcium dynamics very similar to those observed in the experiments with single platelets[27, 31, 32]. Decision between the three different patterns could be made by changing the concentration of agonist, but heterogeneity of answers is observed even at the same concentration of agonist. This means that one or several decision-making mechanisms exist in the calcium signaling system and they could be found by means of the current model.

*Regulation of calcium signaling at low stimulation level: sensitivity analysis.* The first step of the hierarchy of responses is the stationary low calcium increase observed both in deterministic and stochastic simulations. The mechanisms determining low calcium response level could be investigated by means of sensitivity analysis. The sensitivity parameter tells how sensitive the output is to a perturbation of the input or in the parameters of the model[33].

As the output, we have chosen the concentration of cytosolic calcium after stimulation with 1 μM of SFLLRN at the maximum calcium level (achieved at 20 s). The detailed results of sensitivity analysis are shown in the Table S2. The most sensitive parameters are listed in Table 1.

Several groups of parameters made into this list, and two of them (the IP3R-related and SERCA-related ones) belong to the DTS module of the model, while the other two (the PIP2-related and PAR1-related ones) belong to the activation module.

Noteworthy, almost all parameters of the mitochondria module and store operated calcium entrance appeared at the very bottom of the list and none was found among the top influencing parameters. So, we can conclude that the calcium dynamics at low concentrations of SFLLRN is determined by the activation and DTS modules.

*Mechanism of calcium oscillations at medium-level stimulation.*

The calcium oscillations generally are thought to result from the specific dependence of IP3R open probability on calcium[13, 34]. The high calcium concentrations inhibit IP3R activation, and low calcium increases the sensitivity of IP3R in calcium. These properties of IP3R are incorporated in the model of IP3R type 2 constructed by Sneyd et al[35]. To confirm that the IP3R is the source of oscillations we have looked at the concentrations of different forms of IP3R at different activation levels. The IP3R is a multiunit channel, each unit could be assumed to be in several states depending on binding of calcium ions and IP3 molecules from cytosol. The probability of IP3R opening is dependent on concentrations of two IP3R isoforms, "active" and "open"[35].

The changes in concentrations of IP3R isoforms during calcium oscillations are depicted on Fig. 3A. The dynamics of "active" and "open" isoforms are in antiphase, with similar average values (Fig. 3C) confirming oscillations between these two forms. The transition between the isoforms is modulated by the concentration of cytosolic calcium: low levels of calcium stimulate "open→active" transition, and high levels stimulate "active→inhibited" transition (Fig. 3C).

In contrast to the medium-level stimulation, at low levels of SFLLRN the IP3R remains in the "open" and "neutral" form (Fig. 3B,D) leading to a constant calcium flux through the IP3R channel and sustained level of calcium in the cytosol. The described above detailed analysis of the model leads to the conclusion that the first decision-making mechanism is located at the level of DTS, with SERCA pumps responsible for the level of calcium and IP3R responsible for the oscillations.

To gain additional insight into the roles of major calcium signaling mechanisms into regulation of oscillations, we carried out simulations where we partly inhibited phospholipase C activity (Fig. S2), varied number of mitochondrial uniporter molecules (Fig. S3) or parameters of plasmatic membrane calcium exchange (Fig. S4). In agreement with the sensitivity analysis described above, even a 10% inhibition of phospholipase C activity had a detectable effect on cytosolic calcium signaling (Fig. 2SC). In the same time even two-fold increase or complete removal of the plasmatic membrane channels (PMCA or Orai1) or mitochondrial uniporters have little or no effect on cytosolic calcium concentration after PAR1 activation (Fig. S3A-D, S4A-D). However, even these small changes in cytosolic calcium had a profound effect (Fig. S3F, Fig. S4E,F) on the probability of mitochondrial collapse, which is investigated below.

*Opening of mPTP and segregation into subpopulations at high-level stimulation.* The above calculations revealed that mitochondrial calcium uptake and release do not seem essential for the cytosolic calcium response. Upon low or medium stimulation mitochondrial calcium concentration rises but generally not enough for the opening of mPTP (Fig. S5, mitochondrial calcium concentration and mPTP opening upon low-medium stimulation). Increase in average cytosolic calcium leads to the increase of the mitochondrial calcium concentration, resulting in opening of mPTP when this concentration is high enough (Fig. 4A). The uptake of calcium into

mitochondria starts with rise of the calcium level in cytosol (Fig. 4C) and the maximum level of calcium in mitochondria is highly correlated with average calcium concentration in cytosol (Fig. S5). This leads to the conclusion that mitochondria act as an integrator of cytosolic calcium signal. The opening of mPTP is dependent on the level of calcium concentration in mitochondrial matrix and on the mitochondrial membrane potential[25]. Both drop with opening of mPTP. So, in most runs mPTP regulates itself by reducing mitochondrial calcium and only a fraction of the pores open.

However, when the calcium influx into mitochondria is high enough and prolonged enough the $\Delta\psi$ feedback loop leads to the complete opening of mPTP (Fig. 4A) in a fraction of stochastic simulations. The behavior of a population of platelets could be simulated by an array of stochastic runs, and the part of population exposing full opening of mPTP is dependent on the agonist concentration in a step-like manner (Fig. 4B). A similar behavior is demonstrated by the procoagulant platelet subpopulation of platelets in experiments[4;20;34;35]. Another, purely deterministic way to obtain a population of platelets is to consider each platelet to have slightly different number of channels and receptors. We hypothesized that in the population of platelets the number of IP3R is distributed in a normal distribution with standard deviation equal to 10% of the mean value (Fig. 4D). The deterministic simulation shows that, again, at high levels of SFLLRN, mPTP opens in a 4% of population (the platelets with highest IP3R number). The same values were obtained when we modeled population of platelets to have normally distributed number of mitochondrial uniporters (Fig. 4D), sodium-calcium exchangers and SERCA pumps. However, no mPTP opens even at high SFLLRN concentrations in platelets with different numbers of PAR1, PLC or mPTP (data not shown).

Here we can conclude that the second decision-making mechanism lies in the calcium uptake by mitochondria and depends on the DTS-mitochondria calcium cross-talk. Formation of two distinct subpopulations results from the combination of stochastic processes at the PAR1 receptor level and small-scale platelet heterogeneity in the number of essential membrane ion pumps.

Taking into consideration previous reports that extracellular calcium is essential for procoagulant platelet formation, in particular upon PAR1 stimulation[36], we carried out simulations of platelet response with and without extracellular calcium (Fig. 5). Removal of calcium did not prevent development of oscillation (Fig. 5A) and the average cytosolic calcium response was decreased only by factor of two (Fig. 5B), which agrees well with experimental reports[12]. However, the procoagulant platelet formation in the absence of extracellular calcium was dramatically impaired (Fig. 5C), which is in excellent agreement with [36].

Interestingly, the relation between the cytosolic/mitochondrial calcium dynamics and the mPTP opening is strongly non-linear; although it is suggested by the fourth-degree equation in Table 1, the actual effect is even greater: see e.g. Fig. S2 where relatively small decrease in cytosolic calcium leads to complete disappearance of mPTP openings. An interesting consequence of this is that perturbations in the number of mitochondrial uniporter molecules result in great changes in the percentage of platelets with collapsed mitochondria although mitochondrial calcium itself is hardly affected (Fig. S3F). A similar model prediction is observed for extracellular calcium: complete removal or two-fold increase in the activity of PMCA or SOCE has little effect on calcium dynamics but a significant one on the pore opening, i.e. on the procoagulant platelet formation (Fig. S4E,F).

## Discussion

The goal of this study was to identify the critical mechanism allowing stepped platelet response to activation by employing computational systems biology techniques. The main result of the present study is identification of the two critical decision-making mechanisms that determine the platelet fate during activation: 1) IP3-DTS system that controls the activation threshold and spiking onset, and 2) accumulation of the calcium by mitochondria that produces platelet divergence into two subpopulations at a high level of activation, because of initial differences between platelets and stochastic nature of the activation process. This was done using a mechanism-driven, thoroughly corroborated by experiments, multicompartmental mathematical model of the PAR-1-activated calcium signaling network in platelets that includes both DTS and mitochondria, and is able to produce experimentally observed phenomena such as calcium spiking and segregation into subpopulations. Major reactions and players in this decision-making were identified by sensitivity analysis of the calcium response. The calcium oscillation mechanism was analyzed. The model was also used to generate predictions for the experiments that would be able to test the proposed concept of platelet activation.

The platelet activation response is dependent on the type and concentration of the agonist, as well as on the local rheological conditions and the position of the platelet in the thrombus. On the other hand, the main intracellular activation cascade consists of the same players for different receptors and agonists. Any stimulation of platelet leads to the activation of PLC (beta or gamma) and subsequent elevation of cytosolic calcium levels. Therefore we focused on modeling of this common pathway, stimulated by SFLLRN, because it is known that after PAR1 stimulation we can have all known cytosolic calcium responses. Still, intracellular triggers found in this pathway will be common for all other PLC-stimulating agonists.

The constructed computer model of the platelet activation differs from the previous ones[15-17] in two essential points. First, we have included mitochondrial calcium homeostasis in the model as an essential part of platelet activation and Second, we model behavior of a single platelet. To take into account the low number of molecules in platelet we simulated its response with stochastic method. Together, these steps allowed us to find to decision-making mechanisms in platelet activation (Fig. 6). The first one, DTS system, amplifies the variations in the activation signal produced by small number of activated receptors. The second one, mitochondrial calcium accumulation, allows integration of cytosolic calcium signals. Although the fact that IP3R is responsible for calcium oscillations in cytosol of various cells is well known[13, 14, 34] as well as the fact that mitochondrial calcium accumulation can lead to the mitochondrial collapse[18, 26, 37], here we show for the first time that these mechanisms can work in platelet and, more than that, they are the possible keys to the regulation of platelet response.

Here we demonstrated that there are two mechanisms leading to the formation of platelet subpopulations: stochastic nature of the PAR1 signaling (which might explain why the number of PAR1 receptors should be small) and platelet heterogeneity in the number of essential receptors and signaling molecules (which might explain why unactivated platelets are very heterogeneous). Both these mechanism require threshold-like opening of the mPTP to function, and it is likely that both contribute to the phenomenon. Formation of the procoagulant platelet subpopulation is one of the most intriguing problems of the platelet field. All other platelet responses (integrin activation, granule secretion, etc.) are gradual, with some heterogeneity between individual platelets, and only phosphatidylserine is

presented only in a fraction of platelets in quantities by orders of magnitude higher that in others. A possible answer is that phosphatidylserine is difficult to be expressed gradually or partially: as soon as a cell stops fighting thermodynamic equilibrium by pumping negatively charged lipids into the inner membrane leaflet, the only stable possibility is equal distribution of lipids between the leaflets. Therefore, in order to obtain a gradual response, Nature devised subpopulations, a method to obtain procoagulant activity that would gradually depend on the degree of activation.

The current mathematical model allows explanation of dynamics of calcium in cytosol during activation with PAR1 receptor agonist. The random spiking of calcium in cytosol is originated from the probabilistic nature of the activation of small number of receptors and therefore we can expect a separation of platelet into subpopulations at low levels of activation, more pronounced for agonists with low numbers of receptors per platelet. This result agrees well with known experimental data.

The other suggestion is that stationary high cytosolic calcium is not necessary for mitochondrial pore opening, as prolonged calcium oscillations can also lead to the mitochondrial collapse in a fraction of platelets. This does not exclude the strong connection between PS-exposure and high concentration of calcium in cytosol, as the rise of cytosolic calcium concentration could be the result of the mPTP opening as well as its origin. The possibility of existence of population of platelets with low cytosolic calcium is suggested by[38].

In order to correctly take into account the role of extracellular calcium in the platelet procoagulant response, the mathematical model was designed to include all major mechanisms of calcium entry via the plasmatic membrane channels. In agreement with previous reports[12, 36], absence of extracellular calcium led to a modest decrease in the average cytosolic calcium response (with no obvious changes in the general dynamics of calcium oscillations), while the procoagulant platelet formation represented by the collapse of mitochondria was dramatically decreased. This indicates that extracellular calcium entry is an essential component absolutely required for platelet divergence into subpopulations. However, it acts as a general modifier of platelet responsiveness, rather than as a decision-making element of the signaling network. However, this conclusion is obtained here for PAR1-stimulation that is relatively brief because of rapid receptor desensitization; there is experimental evidence that stimulation with high concentrations of thrombin[22], thrombin in combination with glycoprotein VI agonists[39, 40], or thapsigargin[41].

Importantly, the model of the present study does not include less critical modulating mechanisms inside the calcium signaling system itself, like PKC and PI3K kinases and cAMP inhibition, nor positive feedback loops of ADP and thromboxane A2 secretion and signaling. These mechanisms can shift the balance between decisions; however, this simplification does not change the phenomenon qualitatively[23, 42] either. Addition of these mechanisms, as well as that of other receptors, should be the subject of further study.

## Conclusions

Formation of platelet subpopulations upon activation occurs via a mitochondria-dependent decision-making mechanism. This is a stochastic phenomenon caused by a small number of proteinase-activated receptors.

## Experimental

*General model description*. We discern in the calcium signaling pathway three independent modules (Fig. 1): the activation module takes an activator concentration as input and outputs cytosolic IP3 level, the DTS module describes how IP3 causes the release of calcium ions from DTS, and the mitochondrial module describes how rise in cytosolic calcium concentration causes accumulation of calcium ions in mitochondria and opening of mPTP. Each module was tuned to describe correctly experimental data. The model was completed by addition of plasmatic membrane calcium channels. The complete model describes behavior of 30 different species as ordinary differential equations. Detailed descriptions of algorithms and strategies for parameter estimation are given in the online Supporting Information (see Table S1).

*Module 1: receptor activation*. Activation of PAR1 receptor by SFLLRN was considered essentially as in[16] with modifications: turnover of phosphoinositides was added, and all reactions were assumed to proceed in the plasmatic membrane compartment distinct from cytoplasm. The unknown parameters for the new reactions were adjusted based on the experiments from[43] (Fig S6). We considered that 1 nM of thrombin correspond to 0.4 μM of SFLLRN in PAR1 activation [24, 44].

*Module 2: DTS*. The DTS module included the IP3 receptors, the SERCA pumps, and the DTS membrane leak. The model of the platelet (type 2) IP3 receptor was adapted from[35]. The platelet SERCA includes two isotypes (2b and 3a) . To describe them, we used Hill equations with parameters from[45]. The activity of pumps and membrane permeability were adjusted so as to describe calcium oscillations in[27] (Fig. S7).

*Module 3: mitochondria*. The objective was to correctly describe calcium uptake and mPTP opening as a result of calcium overload, so $O_2$ and ATP were fixed as parameters. Calcium inflow through the uniporter was as in[26]. Calcium release through the NCLX exchanger was as in[46]. The mPTP opening was modified from[26] to take into account its non-linear dependence of the matrix calcium concentration[37]. The quantities/activities of the channels, pumps and pores were adjusted so as to describe[37] (Fig. S8).

*Plasmatic membrane channels*. PMCA (plasmatic membrane calcium ATPse) was included in the model using a Hill equation[47] with parameters adjusted so as to maintain steady-state cytoplasmic calcium level. Store-operated calcium entry system was included as described in [25]. Extracellular calcium concentration was 2 mM in all simulations unless specified otherwise.

*Model solution*. The set of ordinary differential equations was integrated using the COPASI software (http://www.copasi.org). For tuning the model, estimation of parameters and sensitivity analysis the deterministic simulations were used. For the integration of whole model the stochastic simulations were used, because the number of calcium ions in the cytoplasm of a resting platelet is less than one hundred and number of each type of channels and receptors in the membrane of platelet is about one thousand; that is why single-molecule effects should be taken into account[30].

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## Tables

**Table 1. Sensitivity analysis of the model**

The model parameters with the highest impact. Parameters belonging to different modules of the signaling network are shown in shades of grey (from light to dark): IP3R-related, SERCA-related, PIP2-related, PAR1-related.

| Parameter of the model | Relative sensitivity |
|---|---|
| SERCA2b Hill coefficient | 4.9 |
| IP3R activation constant *l6* | 3.8 |
| IP3R activation constant *lm6* | 3.8 |
| IP3R equilibrium constant L5 | 3.8 |
| SERCA2b half-activation constant ($K_{0.5}$) | 2.9 |
| PIP2 resynthesis constant | 2.9 |
| IP3R binding IP3 constant *k2* | 2.7 |
| IP3R release IP3 constant *km2* | 2.6 |
| PAR1* binding of GDP rate constant | 2.4 |
| PAR1* deactivation constant | 2.4 |
| Number of SERCA2b copies per platelet | 1.9 |
| Number of IP3R copies per platelet | 1.9 |
| $Ca^{2+}$ flux through the IP3R channel | 1.9 |
| PIP2 degradation constant | 1.8 |

## Figure Legends

*Figure 1. Basic mechanisms of platelet calcium signaling implemented in the model*. The model includes three main compartments: cytosol, mitochondria, dense tubular system (DTS) and plasmatic membrane calcium channels. Without activation low calcium concentration is maintained in cytosol by the PMCA pump action. Agonist binding to PAR1 receptor leads to phospholipase C (PLC) activation and inositol-3-phosphate (IP3) release into cytosol. IP3 binds to its receptors (IP3R) in the DTS membrane (IM) opening the channels for release of calcium that is normally contained in the DTS because of the SERCA pump action. Significant decrease in DTS calcium concentration leads to Stim1 activation and opening of Orai1 calcium channel in the plasmatic membrane (PM). Mitochondria are able to uptake calcium from cytosol through the uniporter (UNI) driven by the mitochondrial transmembrane potential $\Delta\psi$ (~) and to release it through the NCLX exchanger. High mitochondrial calcium concentrations lead to the mitochondria permeability transition pore (MPTP) opening. This results in the $\Delta\psi$ loss and cell death.

*Fig. 2. Typical responses in platelet signaling obtained in the model*.
The panels show typical results of independent stochastic simulations for platelets stimulated with SFLLRN at (A, B) 0.4 μM , (C, D) 4 μM, or (E, F) 40 μM in the presence of 2 mM extracellular calcium (the same conditions were used henceforth unless specified otherwise). Low SFLLRN concentration leads to occasional spiking; its increase results in sustained oscillation; further activator increase induces opening of PTP in a fraction of platelets (F).

*Fig. 3. Mechanism of calcium oscillations*. Characteristics of IP3R after stimulation with (A,C) 4 μM or (B,D) 0.4 μM of SFLLRN. (A) Pronounced oscillations of calcium concentration and IP3R forms after stimulation with 4 μM of SFLLRN. Correlation and anticorrellation of concentration dynamics of various IP3R forms is observed. (B) No oscillations occur when SFLLRN concentration is too low. (C,D) Scheme of transitions between different forms of IP3R. The diameters of the circles are shown proportional to the average concentrations of various forms of IP3R. Note that average concentrations of "active" and "open" IP3R forms are similar for 4 μM of SFLLRN, leading to the ability of the system to oscillate.

*Fig. 4. Mechanisms of pore opening and procoagulant subpopulation formation*. (A, C) Typical dynamics of cytosolic and mitochondrial calcium concentrations leading to the opening of the major fraction of the mPTP. Panel C shows correlation between cytosolic calcium spikes and accumulation of calcium ions in mitochondria. (B, D) Fraction of platelets with the opened pore as a function of the activator concentration for different simulation conditions. Heterogeneous platelet subpopulation of platelets with similar distributions can be obtained using any of the following assumptions: Gaussian distribution of the number of IP3R (red line); or similar distribution of the number of uniporters (blue line); or even for initially identical platelets with stochastic simulation (black dashed line with dots). Panel D illustrates the meaning of each calculation point on Panel B: the platelet population, heterogeneous in IP3R number, after stimulation with 40 μM of SFLLRN divides into two subpopulations depending on mPTP opening.

*Fig. 5. Role of extracellular calcium in platelet activation with SFLLRN*. (A) Typical result of independent stochastic simulation for platelets stimulated with SFLLRN at 40 μM after 10 min of incubation without extracellular calcium. (B) Total calcium entrance into cytosol during 200 s after activation as a function of SFLLRN concentration, deterministic calculations. (C) Fraction of platelets with the opened pore as a function of SFLLRN concentration, heterogeneous platelet population was obtained by varying the number of IP3R (Gaussian distribution), deterministic calculations.

*Fig. 6. Decision-making in platelet activation is controlled by different modules*. The PAR1-Gq-PLC-IP3 system is an amplifier. Interaction of DTS with IP3 results in calcium spiking or not: this is the first decision-making element determining the activation threshold. Mitochondrion accumulates calcium acting as an integrator, and plays the role of the second decision-maker for a platelet: to die and become procoagulant, or to survive as a pro-aggregatory.

**Figures**

Figure 1

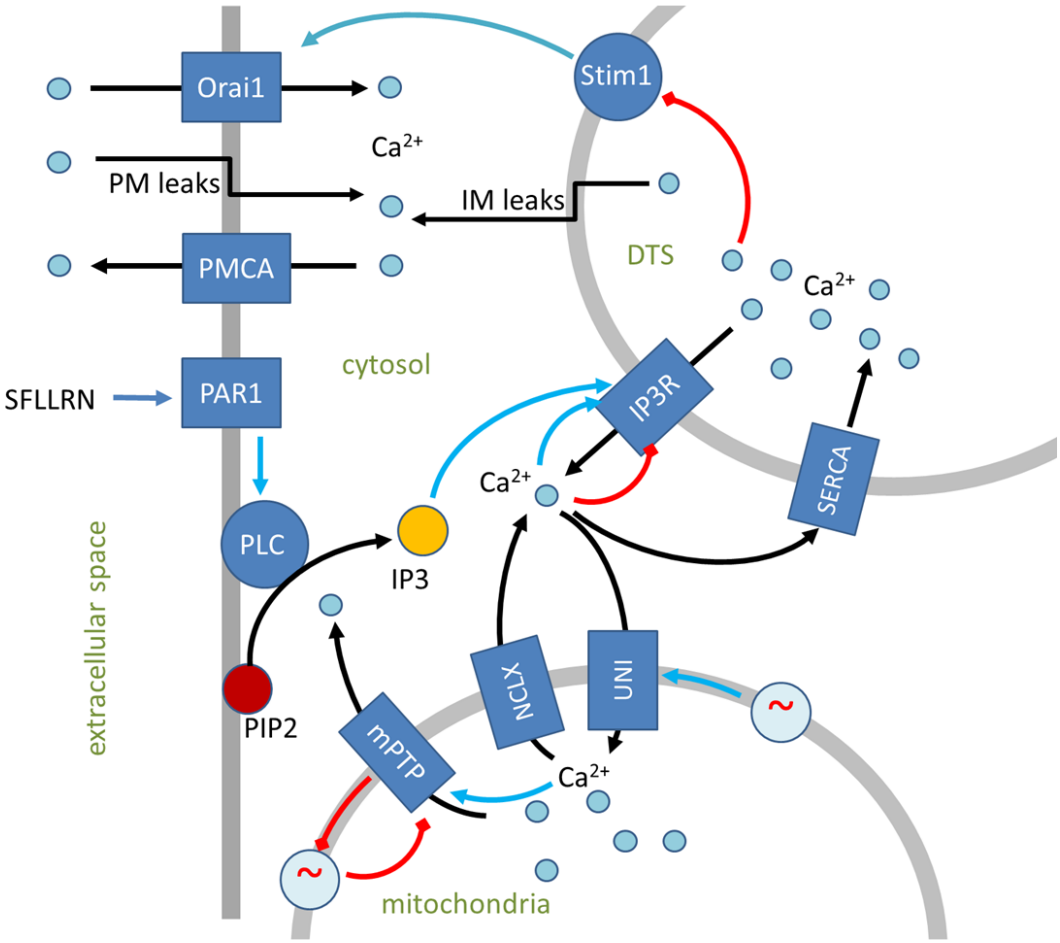
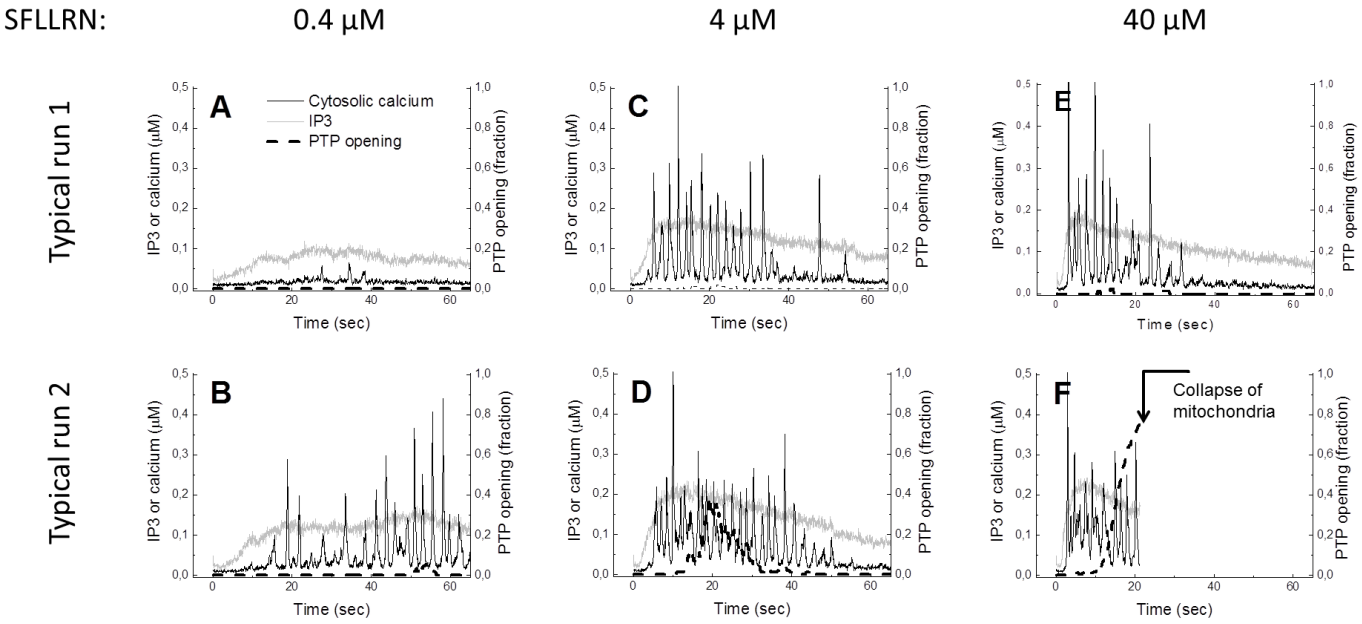
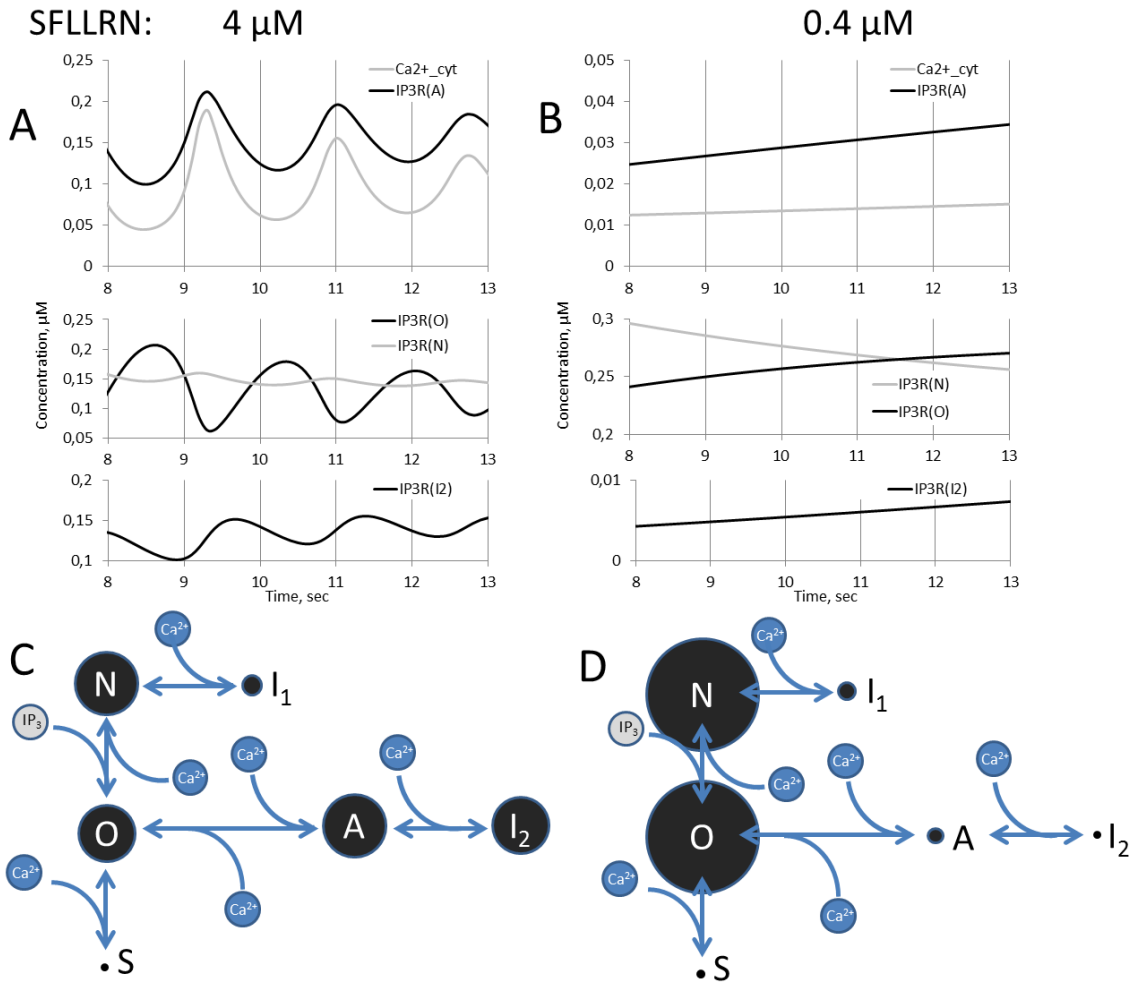


Figure 2



# Figure 3



# Figure 4

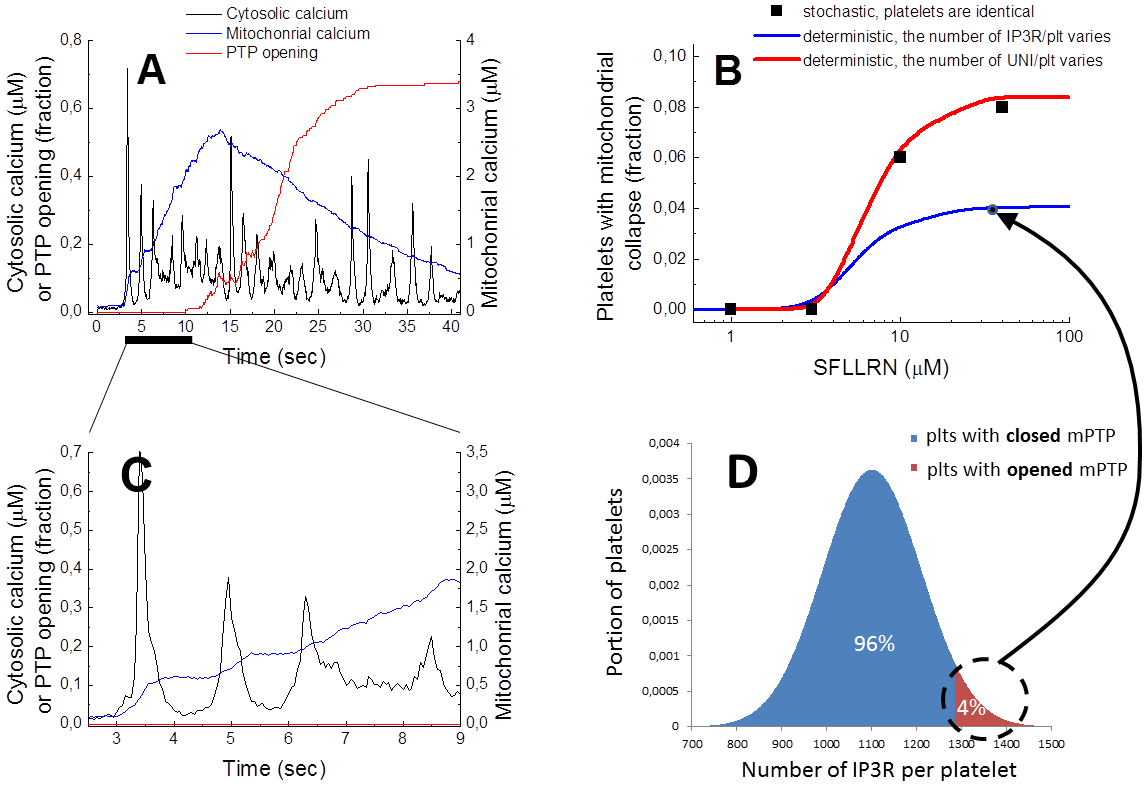


Figure 5

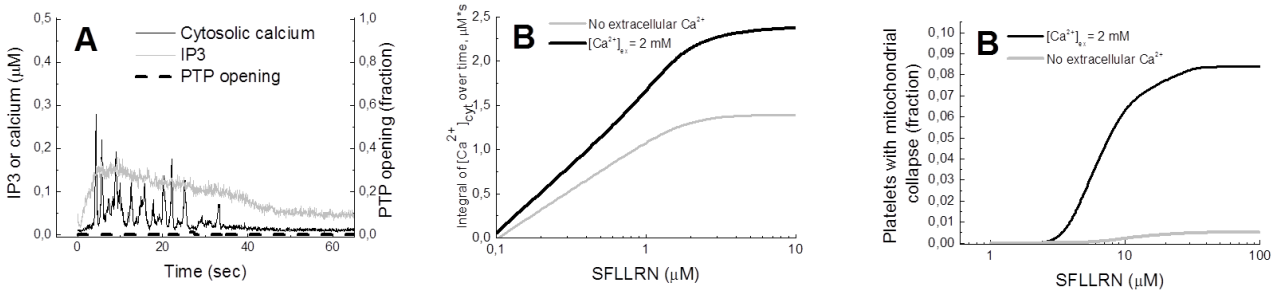


Figure 6