Optical uncaging of ADP reveals the early calcium dynamics in single, freely moving platelets

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Abstract: Platelet activation is considered to be a cornerstone in pathogenesis of cardiovascular disease. The assessment of platelet activation at the single-cell level is a promising approach for the research of platelet function in physiological and pathological conditions. Previous studies used the immobilization of platelets on the surface, which significantly alters the activation signaling. Here we show that the use of photolabile “caged” analog of ADP allows one to track the very early stage of platelet activation in single, freely moving cells. In this approach, the diffusion step and ADP receptor ligation are separated in time, and a millisecond-timescale optical pulse may trigger the activation. The technique allows us to measure the delay (lag time) between the stimulus and calcium response in platelets. We also propose a simple model function for calcium peaks, which is in good agreement with the measured data. The proposed technique and model function can be used for in-depth studies of platelet physiology.

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1. Introduction

The platelets are the paramount element of hemostasis and also contribute to a variety of other normal and pathological processes, including thrombosis, inflammation, and tumor development [1,2]. The hemostatic function of platelets is closely related with their ability to change physical properties in response to vessel wall injury [3]. The first step of this process is platelet activation, which comprises a series of prothrombotic events, triggered by the increase of intracellular calcium [4]. Platelet activation is considered as a cornerstone in pathogenesis of atherosclerotic cardiovascular diseases [5], which are the principal cause of mortality globally. Therefore, it is highly anticipated that in-depth study of platelet activation and development of advanced methods for its assessment could contribute to further progress in cardiovascular medicine.

It is well-known that platelet activation is triggered by a number of regulators, or agonists. These regulators, such as collagen and adenosine diphosphate (ADP), could be released from the injured vascular wall or synthesized by previously activated cells to provide positive feedback loop. Platelet activation signaling involves the sharp increase in intracellular concentration of calcium ions; the downstream effects include the platelet shape change [6], activation of the integrin family receptors, leading to platelet adhesion and aggregation [7], secretion of granules, including dense ADP-containing ones, and presentation of procoagulant factors on the platelet surface [8].

Currently, the light-transmission aggregometry is the standard method for evaluating platelet function in clinical settings [9]. Along with aggregation, this technique is claimed to record
the shape changes. However, the measured signal is only marginally related to activation [10]. The main problem is that unified signal is obtained for the whole suspension of platelets, containing the cells that could be heterogeneous in their size, condition and function. Some distinct populations of platelets were identified by assessment of platelet shape at single-cell level with flow cytometry [11,12]. Besides, the diversity was demonstrated for procoagulant surface formation [8]. The attempts have been made to describe platelet subpopulations by mathematical modeling of calcium homeostasis [13] and cytoskeleton dynamics [14]. The models of platelet activation signaling made a significant contribution to the field [15,16], allowing one to predict the influence of different players on the process. However, the signaling pathway consists of hundreds of species and rate constants, which means that the validation of model is of great importance. For this purpose, ideally, the dynamics of key compounds should be measured in individual platelets. For the cytosolic calcium it can be done by microscopy with fluorescent probes, estimating a real-time concentration of calcium into the cytoplasm.

However, additional methodological problem arises when studying the activation of freely moving single cells. The addition of the soluble agonist can introduce huge uncertainty into experiment. Specifically, if agonist is added near the observation site, cells are shifted away and the information about the initial activation dynamics is lost. In contrast, if the agonist is added in a distant site, its diffusion may contribute significantly to the activation dynamics. One approach to solve these problems is to attach platelets to a fixed surface to prevent moving. Such experiments were first conducted using Fluo-3 calcium probe [17]. To observe the calcium dynamics, platelets were attached to a fibrinogen-coated coverslip and then activated by the addition of ADP. Multiple calcium transients were shown, in contrast to smooth calcium trace in the suspension, which highlights the importance of single-cell approach. Recently, real-time measurements of calcium concentration and mitochondrial potential were performed in the same manner [18]. The only drawback is that fibrinogen significantly alters the platelet signaling, causing activation via GPVI receptor [19]. Therefore, such methods of immobilization make it impossible to assess resting platelets or their changes during activation independently of the effect of immobilizing agent. Alternatively, PECAM-1 antibody with slightly inhibitory action has been proposed to anchor the platelets [20]. Anyway, any contact with artificial materials can activate platelets, for instance, they may undergo transformation into the spread form on the glass. In a recently proposed technique, biotinylated platelets were anchored onto phospholipid bilayer with streptavidin [21]. While this technique is promising, it implies the modification of platelet membrane, which might also influence the activation signaling. Finally, tiny forces arising due to attachment of platelets may cause additional activation [22,23].

In this paper, we describe an alternative approach for time-resolved single-cell experiments that does not involve the attachment of platelet to the surface. We demonstrate that automated tracking software allows one to account for the random motion of the cells and reconstruct the calcium dynamics in resting and activated platelets. To solve the problem of cell displacement due to the addition of agonist, we use a technique of “caged compounds”, widely used in the study of the dynamics of biological processes [24,25]. So-called “caged ADP” contains o-nitrobenzyl “protective” group, which blocks the binding of ADP to its receptors. The “protective” group can be removed by ultraviolet irradiation (~350 nm) to generate free ADP in a vicinity of platelets. Thus, the diffusion step and receptor binding turn out to be separated in time. In this method, platelet activation may be triggered optically without creating additional hydrodynamic flows. Although the caged agonist was used in platelet research earlier, the study was limited to potentiating of aggregation in suspension [26]. Here we demonstrate the applicability of this approach to study the early stage of platelet activation (up to 0.01 s after stimulus) on a single-cell level.
2. Methods

2.1. Sample preparation

The fasting blood samples were obtained from the cubital veins of two healthy volunteers, man and woman, 22 and 30 years of age. Written informed consent was obtained from these individuals prior to the study. The study protocol was approved by the Ethics Committee of the Research Institute of Clinical and Experimental Lymphology – Branch of the Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences.

The samples were collected in the vacuum tubes containing sodium citrate as anticoagulant (9 : 1). After collection, each sample was kept at room temperature for two hours to obtain a layer of plasma containing platelets. Sample was obtained from the middle of this layer. We did not use the centrifugation in order to preserve the platelet activation state. During the observation of the sample under the microscope, no other cells were observed except platelets. On the next step, the sample was labeled with fluorescent calcium probe Fluo-4-AM (Thermo Fisher Scientific, USA). The stock solution (1 mM) of Fluo-4-AM was diluted 62.5 times in phosphate buffered saline (PBS) and mixed 1:1 with blood plasma. After incubation for 30 minutes, the sample was 10 times diluted in PBS and allowed to rest for another 30 minutes before experiments. It is important to note that no calcium was added to the sample, so the extracellular calcium concentration was negligible due to the anticoagulant and the final 20-fold dilution of plasma.

To induce platelet activation, we used either ADP (final concentration $\sim 10^{-5}$ M) or caged ADP (final concentration $\sim 10^{-4}$ M). The caged ADP was purchased from Jena Bioscience company (Germany).

2.2. Optical System

Fluo-4 complexes with Ca$^{2+}$ show fluorescence at a wavelength of 516 nm when excited by 488 nm light. Inverted microscope Carl Zeiss AxioVert.AI with appropriate fluorescence filter set was used in the study to observe cell fluorescence caused by calcium release during platelet activation. The microscope was equipped with high-sensitive Axiocam 503 mono camera, which was applied to record the process. The 40x dry objective (0.65 NA) was used in the study to observe many platelets and visualize the focal layer just before the bottom coverslip surface. Platelets were freely moving in this horizontal layer (2D plane) but confined vertically by gravity.

We equipped the microscope with an additional 340 nm, 500 mW LED source for the “uncaging” of caged ADP. The LED was placed just above the sample. The sample was covered by a coverslip to prevent contact (Fig. 1). We did not specifically control the spacing between two coverslips, which was about 7 $\mu$m (based on the sample volume 20 $\mu$L and the coverslip diameter 4cm). Arduino Due microcontroller board was used to control the flash time. Fluorescence was recorded with a frame rate of 3 fps in order to have good signal-to-noise ratio. The acquisition duration for a given sample was about 3 min.

2.3. Mathematical model and processing algorithm for the assessment of calcium peaks

The theoretical description of the calcium transients in platelets and the profile of calcium peaks is the area of active research [15,18,27]. In this paper, we are interested in the simplest mathematical model to correctly describe the position, amplitude and kinetic parameters of each peak. For this purpose, the model should describe the experimental data having as few parameters as possible. We used Eq. (1) to describe single calcium peaks:

$$[Y] = A_0 + Ae^{-kt},$$

where $[Y]$ is the measured Fluo-4 fluorescence intensity, $A_0$ is the signal level before peak, $A$ is the prefactor, $k$ is the decay constant and $t$ is time. Eq. (1) is the result of empirical assumptions.
Fig. 1. Schematic layout of experimental setup for optical activation of platelets. Inverted microscope (Carl Zeiss AxioVert.A1) is used to monitor the level of intracellular calcium during platelets activation. The activation is caused by “uncaging” the cagedADP upon the UV irradiation.

and data analysis and agrees well with experimental peaks. However, in our experiments the calcium transients were observed at different times, so the function should be modified as follows:

\[ Y = A_0 + \begin{cases} 
0 & \text{if } t < t_0 \\
A \cdot (t - t_0)e^{-k(t - t_0)} & \text{if } t > t_0
\end{cases} \]

(2)

The time shift \( t_0 \) accounts for a particular time when the peak occurs. For the first peak, it is delay between the addition of an agonist and the onset of calcium growth. This delay is due to the intermediate processes between the activation of cell receptors and the opening of ion channels. However, this is true only for the first peak after the stimulus, while the experimental signal usually has multiple peaks. For other peaks \( t_0 \) has just a phenomenological meaning of its start time.

Based on the described model, we have developed an algorithm, which seeks for all the peaks and fit each by Eq. (2). The idea of the algorithm is as follows: first, the procedure determines a mean signal level and the standard deviation (SD). Points that are located above the mean + SD are considered as part of a peak. In order to capture the entire peak, several points before and after are taken. This simple search routine works well for our experimental data after tuning auxiliary parameters like how many points to step before and after etc. Each detected peak is separately fitted using Eq. (2). As a result, for each calcium peak we obtain four parameters: the start time \( t_0 \), the decay constant \( k \), \( A_0 \) and \( A \).

3. Results

First, we tested the applicability of our approach to study activation of single freely moving platelets induced by the addition of ADP. It is well known that ADP binds to purinergic receptors
on the cell surface and triggers the signaling cascade which eventually results in the opening of calcium ion channels on the intracellular stores. The subsequent increase of calcium ions in the cytosol is the universal hallmark of platelet activation.

To track the calcium concentration during activation, we labeled the cells with Fluo-4-AM calcium indicator. Platelets were then placed onto the coverslip in a 20 µL drop and observed with inverted microscope. Our developed isolation and staining protocol (see Sec. 2) allowed to observe simultaneously ~100 cells using 40x objective. Typical field of view is shown in Fig. 2(A). Because of relatively small size, most platelets appeared as dots. The focal plane was set to just above the coverslip where a lot of platelets with the same vertical position were moving along the surface, while some cells spontaneously spread on glass. In these circumstances, the used objective with focal depth ~1 µm makes it possible to have quite clear image of cells even during random movement, which minimizes the inaccuracy in recorded fluorescence signal. Figure 2(B) shows a region where one spread cell and several freely moving cells are shown. We marked the spread platelet by a yellow circle, and one of moving cells by a light-blue one. In Fig. 2(B - C), one can see how the position of this platelet changes along with the fluorescence increase due to activation. To measure the fluorescence signal of moving cells, we used the TrackMate plugin [28], which automatically detect cells and trace their motion. In addition, the software calculates the average fluorescence intensity for each cell by integrating the values of all pixels inside the circumscribing circle. Thus, even if the cell is slightly out of focus (blurred), its average fluorescence intensity is correctly determined as long as the image fits inside the circle. However, in our experiments most cells are in focus with rare exceptions. Figure 2(D) shows the example of the obtained cell trajectory and the fluorescence intensity, which is depicted by a color of points (from black to green).

To induce activation, 1 µL of ADP solution was added to a drop of platelets to a final concentration 10 µM (there was no upper coverslip in this experiment). The addition was made as accurately as possible, but it still causes significant displacement of cells, especially in the first seconds. This effect can be seen in Fig. 2(E). Additionally, the images of cells were motion-blurred in first few frames after the activation. Anyway, we determined the time-dependent fluorescence intensity for each detected cell before and after the stimulation.

Figure 3 shows fluorescence intensity versus time for two typical cells: spread (A) and moving (B). The addition of ADP is marked by yellow line; one can see the sharp spike in fluorescence intensity during that time, which is due to the reflection from pipette tip. In moving cell, sequential calcium pulses started just after the ADP addition, whereas in spread cell the response was much weaker. It can be explained by the fact that the cell was pre-activated by the surface, therefore, it was already in a process of spiking and calcium release. Accordingly, the response to ADP was weaker because of the interference of this process with the signaling cascade. It is important that the presence of spontaneously spread and pre-activated cells influences other platelets via the secretion of ADP and thromboxane. To make future experiments more reliable, we plan to use inhibitors that prevent cell spreading.

The changes in fluorescence intensity during peaks for moving cell was ~50% relative to the baseline. To further quantify the calcium spiking for moving cells, we applied the algorithm described in Section 2. It seeks for calcium transients and for each peak determines its time and dynamic parameters. Figure 3(B) shows typical processing results. Five peaks were found for this cell, and their parameters are shown below the graph. The parameter $t_0$ is the absolute delay time from the start of recording, not from the ADP addition.

The fact that ADP causes numerous calcium transients is a well-known phenomenon. Thus, our experimental data are in agreement with results of previous studies performed on surface-tethered platelets. However, it is unclear whether initial fast increase of fluorescence is due to the calcium rise or caused by uncontrolled flows after addition of ADP droplet. Indeed, platelets may sense fast flows because they have special mechanoreceptors [22]. Interestingly, same “preliminary”
increase before the first calcium peak can be seen in Fig. 1 of [17], although platelets were spread on fibrinogen. Moreover, intervals between peaks and decay constants are also in the same range. In our case, due to the addition of ADP many cells floated out of the frame or moved between the layers of focus of the microscope, which result in lost or even erroneous information. Finally, the time of stimulation and the concentration of ADP cannot be certainly said because of diffusion. All of these factors make it impossible to study the initial stage of activation with this technique.

The displacement problem may be solved partially by decreasing the volume of ADP droplet or by using the droplet which is limited by walls. However, when a small volume of ADP is used, its concentration varies significantly from cell to cell. To overcome this effect, we decided to use the photolabile analog of ADP (“caged” ADP). This molecule could be added to platelets in advance and does not cause activation until UV light is applied. Thus, the diffusion step and receptor ligation turn out to be separated in time, and platelet activation may be triggered by UV flash.

In our experiment the UV illumination was performed using 340 nm UV LED connected to an Arduino board for the precise control of flash time. To begin with, we tested the effect of UV light itself on our samples. During a control experiment without caged ADP we found that 100 ms flash did not cause any detectable changes in the fluorescence intensity of platelets. Moreover, calcium transients in spontaneously activated cells were not affected by the flash (Fig. 4(A)). Next, we studied the sample with preliminary added caged ADP (final concentration 100 µM).
Fig. 3. A Snapshots with activation of the spread cell and the graph showing the dependence of fluorescence intensity on time before and after the addition of ADP (yellow line). B. Snapshots of the typical freely moving platelet and the graph showing the dependence of fluorescence intensity on time. There are numerous calcium transients. In addition, the results of our algorithm, which seeks for calcium peaks and fit them with simple model equation, are shown by green lines. The parameters are presented in the corresponding table for each found peak.

Caged ADP was added as an 1 µL drop, then the sample was gently mixed, covered by the second coverslip and placed under the microscope without delay. In this case, 100 ms flash caused significant activation. According to our estimation based on the photophysical properties of caged ADP and the optical setup, this corresponds to a ∼5-10% dissociation of caged ADP; thus, the concentration of free ADP was comparable to previous experiments (∼5-10 µM). Typical signals for several cells are shown in Fig. 4(B). For two typical platelets we also provide detailed graphs where fluorescence versus time and cell snapshots are shown (Fig. 4(C - D)). Although they are in agreement with the results of ADP activation, there was no stepwise calcium increase immediately after the light pulse (marked by violet lines).

The intensity of fluorescence relative to the baseline increases during peaks approximately by 30% (lower than ∼50% in Fig. 3(B)). These differences may be explained by several reasons.
Fig. 4. A. Control experiments showing that two UV flashes do not influence platelet calcium signaling. Note two spontaneously activated cells which show the signaling completely independent on UV. B Several typical signals obtained during experiments with caged ADP. There are numerous calcium transients after the UV irradiation pulse (violet line) which cleaves the caged ADP. C,D: Snapshots with activation of two different typical cells and the graph showing dependence of fluorescence intensity on time. The processing (fitting) results are shown by green line.

First, the concentration of active ADP is different in two experiments. Second, slightly different experimental conditions might affect the activation status of platelets due to their high sensitivity to ADP. Third, the residuals of protective group after the photodissociation might somehow inhibit the platelet activation. Finally, the UV light induces the photochemical transformation of Fluo-4 probe, leading to fluorescence bleaching. However, the fact that the basal fluorescence remains unchanged after the light pulse (Fig. 4(C - D)) indicates that photobleaching is not significant.
Fig. 5. A,B. The histograms of the decay constant for each peak measured in ADP and caged ADP experiments. C,D. The histograms of the peak-to-peak interval obtained in ADP and caged ADP experiments. E. The histogram of appearance time ($t_0$) for all peaks in experiment with caged ADP.
Overall, we believe that the optical activation method is much more reliable, reproducible and resistant to artifacts. Next, we decided to compare two methods of activation in terms of parameters which reflect the platelet physiological state, namely the decay constant and peak-to-peak interval [17]. The peak-to-peak interval was calculated as follows. First, we choose all cells with more than two calcium transients. Then we from the time of each peak except the first we subtracted the time of previous peak. As the majority of cells were freely moving with only few spread on glass, we include all cells in the statistics.

Figure 5(A)-(D) show distributions of all measured cells over the decay constant $k$ and the peak-to-peak interval. The obtained values agree well with values previously reported in literature [15,17]. The distributions also agree between the ADP and caged ADP experiments. Note that the decay constant is calculated for each peak, whereas peak-to-peak interval can be obtained only for cells which possess more than one peak, that is why sample size is different in the right column. Overall there were $\sim 300$ analyzed cells in both experiments.

Figure 5(E) shows the histogram of peak time $t_0$ for all peaks from experiments with caged ADP. One can see that activation occurs from 0.5 seconds after the stimulus to tens of seconds for some cells, most frequently from 8 to 10 s. The experimental data for this histograms is well described by a lognormal distribution with mode $5.6 \pm 0.6$ s. It can also be seen that some peaks appeared before the UV flash, which results from low-level spontaneous activation. Platelets are very reactive cells and can become activated due to the environmental factors such as shear stress, influence of glass surface, or paracrine signaling from neighboring platelets.

4. Discussion

In this study, we demonstrate for the first time that ADP-induced activation of platelets could be assessed \textit{in vitro} at single-cell level in freely moving cells. We reconstructed the dynamics of calcium response in each platelet using automated tracking software. The obtained profiles consist of multiple sequential peaks, in accordance to the previous studies. Next, we developed a simple algorithm, which is able to detect each peak, fit it by a model formula and derive such parameters as peak position, amplitude and decay constant. The peak model was not published earlier and is in a perfect agreement with experimental data.

To assess the initial stage of platelet activation, we applied “caged” ADP which contains $\alpha$-nitrobenzyl “protective” group, preventing the binding of ADP to its receptors on cell surface. The “protective” group can be easily removed by ultraviolet irradiation. It allows one to trigger the process by a millisecond-timescale optical pulse. Using this approach, we performed the accurate measurements of the early phase of platelet activation. The optical activation opens new possibilities in platelet research, including the study of intercellular signaling using well-focused light pulses and agonist synergy using sequential pulses and/or more than one caged compounds.

It is interesting to compare the obtained results with previous works. First, the peak-to-peak interval distributions are in a full agreement, showing the same two modes as reported in [17]. It justifies our method, which does not require attachment of platelets to the surface and any modification of the latter. In the same paper, it can be seen from figures that the delay time is in order of 3-4 s. It is comparable with our results, especially given the fact that the concentration of ADP was four times higher in that study ($40 \mu M$) and platelets were spread on fibrinogen. Activation time as low as 200 ms was obtained using the stop-flow fluorimetry [29] in the absence of extracellular calcium. However, platelets were placed in a rapid flow before mixing with ADP and therefore experienced significant shear stress. In the presence of extracellular $Ca^{2+}$ the delay time was even shorter (10 ms), which additionally points to that because shear-stress-induced activation is mediated by calcium influx into platelets [30]. On the contrary, ADP-induced signaling goes through calcium release from intracellular stores and \textit{then} to store-operated calcium entry [31]. In other study which used flow cytometry the maximum calcium response was achieved 5 s after the addition of 1 $\mu M$ ADP [32], while the maximal calcium response in a
suspension of platelets reported in [15] was about 30 s after the addition of the same amount of ADP. In summary, our results are in range of the platelet activation time course reported in different papers, however, none of them have studied single platelets in suspension.

We believe that the dynamics of ADP-induced activation in the absence of extracellular Ca\(^{2+}\) (reported here) is of interest for understanding of platelet signaling, although it would be also interesting to measure the platelet response with Ca\(^{2+}\) added. There are evidences that light exposure may alter calcium influx in neuronal cell types [33], therefore additional control experiments are needed to exclude this mechanism in platelets. The other remaining issue is that prolonged action of endogenous ADP during sample preparation may result in the desensitization of platelet receptors and hence lowered response [34]. This effect can be minimized by the addition of exogenous apyrase. However, our goal here was to demonstrate the activation of platelets by an optical pulse, and we leave the above-mentioned issues for the future work. There are many interesting questions which can be investigated further with this approach. For instance, the concentration of uncaged ADP can be precisely controlled by varying the flash length, which enables quantitative studies. Moreover, sequential pulses may cause waves of ADP to study the potential enhancement or even resonance effect. Local irradiation may be used to study intercellular signaling. All these studies should be naturally accompanied by simulation of platelet signaling to validate models or test hypotheses. Finally, it is important to note that the proposed activation approach is relatively simple and low-cost (provided that the fluorescent microscope is present in the laboratory). We believe that the described technique would advance the study of platelet function in various physiological and pathological conditions and would help in diagnosis of cardiovascular diseases.

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