Patch-clamp technique for studying ion channels in activated platelets

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Received: 14.03.2021 Accepted: 27.03.2021 Published: 30.03.2021

DOI: 10.52455/sbpr.01.202101012

Abstract

In this study, we have revisited the existing and suggested new approaches to the use of patch-clamp methodology for measuring the activity of single ion channels and membrane potential of human platelets in the cell-attached configuration. We recorded single-channel events of platelets in the cell-attached configuration after activation with potent agonists: thrombin and ionomycin. The feasibility of platelet membrane potential measurement in cell-attached mode was investigated both experimentally and with the help of simple electrical circuits, revealing that the single-channel events can alter the recorded potential by invoking oscillations. Here, the simple approach to obtain inside-out configuration was described and calcium-dependent single-channel ion currents were recorded. Taken together, this study introduces new approaches for further investigations of the role of ion channels and membrane potential in platelet physiological and pathophysiological response.

Keywords: platelets, patch-clamp, membrane potential, ion channel

Introduction

Mammalian platelets are small anucleate cells that play a major role in thrombosis and hemostasis. In the past couple of decades, it has been identified that both ion channels and membrane potential play a pivotal role in platelet physiological and pathophysiological functions. Before the introduction of patch-clamp methodology, researchers used fluorescence potential sensitive dyes [1–3] and radioactive isotopes [4–7] to study ion conductance and changes in membrane potential. The introduction of the patch-clamp technique [8, 9] provided tools for direct assessment of the conductance of single ion channels or the whole membrane, as well as the way to precisely measure the value of the membrane potential even of such small cells as platelets.

Patch-clamp is an electrophysiological technique, which allows measurement of ion currents through single channels by clamping voltage of an isolated piece of the cell membrane (or whole-cell). There are several configurations in this technique. Getting a giga-seal between the pipette and the cell membrane leads to cell-attached configuration, allowing the measurement of ion currents through single channels under the patch pipette. Rupture of cell membrane under patch pipette leads to whole-cell configuration, in which both average currents across the entire surface area of the cell and kinetics of membrane potential can be measured. Other widely used patch configurations are the inside-out and outside-out configurations, in which either the intracellular or extracellular surface of the membrane patch is exposed to the extracellular solution.

The application of the patch-clamp technique to platelets shed light on various mechanisms of their functional activity. For example, it was shown that the most abundant ion channel in the platelet membrane is voltage-dependent potassium channel $K_v$ 1.3, and its pivotal role in maintaining resting membrane potential was demonstrated [10, 11]. Patch-clamp helped to identify calcium-dependent potassium channels $K_{Ca}$ 3.1 and to reveal their role in maintaining the driving force for $Ca^{2+}$ during platelet activation [12]. Furthermore, several important modulators of calcium signaling were revealed in platelets and megakaryocytes using this technique: the receptor-operated cationic P2X channels [13, 14] as well as CRAC-channels Orai-1 [15–17]. One of the essential applications of the patch-clamp technique is the measurement of membrane potential. Previous studies, using fluorescent techniques, showed that platelet activation by various agonists leads to different changes in membrane potential. Thus, platelet activation by thrombin (more than 0.1 U/ml) leads to depolarization of the platelet membrane, while ADP (0.3-30 µM) induces hyperpolarization followed by depolarization [18]. More recent experiments using patch-clamp showed that membrane potential of megakaryocytes and possibly platelets can oscillate during activation with ADP [19, 20]. Furthermore, changes of the membrane potential of megakaryocytes can directly modulate intracellular...
calcium spiking [20]. There is also some evidence of direct voltage control of G-protein coupled receptors [21, 22].

Transcriptomic analysis [23] as well as recent studies of platelets and megakaryocytes [24] evidence for the existence of ion channels unidentified earlier in platelets. This suggests that the application of the patch-clamp methodology to platelets is still of great importance for understanding the mechanisms underlying the variety of functions of these minute cells.

However, no matter how helpful this method is, there are many challenges concerning applying it to platelets. These cells are very tiny and fragile, which makes some of the developed patch-clamp techniques unusable. For example, there is no documented evidence that outside-out configuration is possible for platelets; furthermore, there is an opinion that there is no need for it as whole-cell configuration for platelets is essentially outside-out [25]. It is also worth saying, that obtaining whole-cell configuration for platelets is sometimes pretty challenging, and oftentimes leads to the deterioration of the patch. Evidence exists that adding ATP to the pipette solution in the absence of calcium ions helps to obtain this configuration [10]. A more prominent way to achieve whole-cell is to use nystatin (perforated patch-clamp technique) [13], but it also has several limitations.

Difficulties in working with platelets, their fragileness, and small size led researchers to the use of megakaryocytes and corresponding cell lines as model cells in patch-clamp studies instead of platelets [26, 27]. From one side it helps to achieve results that previously were very hard to obtain with platelets. On the other side, besides the similarities of megakaryocytes and platelets in regards to the receptor profiling, main signaling pathways, and ion channels, one can’t be sure, that obtained results on megakaryocytes can be directly transferred to platelets.

That is why this study aims to assess the difficulties of application of the patch-clamp technique to platelets, and suggest possible solutions, as well as to demonstrate the possibilities of the proposed method in regards to studying the single-channel currents during platelet activation.

Materials and methods

Chemicals

Sodium citrate, HEPES, ADP, glucose, ionomycin, and apyrase were purchased from Sigma-Aldrich (St. Louis, USA). Prostacycline was obtained from Santa Cruz (Dallas, USA).

Solutions

NaCl-based extracellular buffer saline (external BS) contained: 150 mM NaCl, 10 mM KCl, 10 mM HEPES, 10 mM glucose, titrated to pH 7.35 with NaOH. KCl-based pipette buffer saline (pipette BS) contained: 150 mM KCl, 10 mM NaCl, 10 mM HEPES, 2 mM CaCl2, 2 mM MgCl2, titrated to pH 7.2 with KOH. In the ion exchange experiments, KCl was replaced by an equimolar amount of NaCl in pipette BS. The osmolarity of solutions was brought to 300 mOsm for extracellular saline and 270 for pipette saline with the MilliQ water. All solutions were filtered through 0.22 μm syringe filters.

Cell preparation

Venous blood of healthy volunteers was collected into 10 mL tubes with 3.8 % (w/v) Na-citrate at 9 to 1 ratio, 0.5 μM PGI2, 0.3 U/mL of apyrase and centrifuged at 100 g for 7 min. 250 μL of platelet-rich plasma was mixed with 1.25 mL of extracellular saline, 0.5 mL of Na-citrate (pH 5.5), 0.5 μM PGI2, 0.3 U/mL apyrase, and then centrifuged at 200 g for 5 min. Cells were resuspended in NaCl-based external BS. All manipulations were performed in compliance with the Declaration of Helsinki.

Patch pipettes

Patch pipettes were pulled from filamented borosilicate glass capillaries (0.86 mm ID, 1.5 mm OD). (HEKA Instruments) on a Sutter Instruments Brown P-97 pipette puller, and then heat polished on the same puller as described by the manufacturer. The resistance of patch pipettes filled with KCl-based pipette BS was 7-15 MΩ.

Electrophysiological recordings

Cell-attached and inside-out patch-clamp recordings were carried out in voltage-clamp and current-clamp modes using HEKA EPS 8 amplifier (HEKA Elektronik GmbH). Because all experiments were aimed at studying single-channel events, no series resistance compensation or correction for liquid junction potential were made. The experimental chamber was earthed directly through Ag/AgCl wire placed aside of the cells. The pipette was handled with Sutter MP-225 motorized micromanipulator. The plastic chamber was placed on the stage of the upright microscope (Olympus IX51WI, Tokyo, Japan) with an overall magnification of 400x. Data was filtered at 0.7 kHz using a built-in low-pass Bessel filter and digitized directly to the personal computer through B-381 AC/DC converter. Data was recorded at 1 kHz using a custom acquisition program on Matlab 2004 (The MathWorks, Inc.). Obtained data was analyzed using Origin 8.1 (Origin Lab, Northampton, Massachusetts, USA), WinEDR v. 3.9.1 (University of Strathclyde), and Spectragryph Software (F. Menges “Spekwint32 - optical spectroscopy software”, Version 1.2.14, 2020,
Activation of the cells

To avoid early platelet activation during platelet contact with the glass capillary (patch pipette), the process of gigaseal formation was performed in NaCl-based external BS in the absence of Ca\(^{2+}\). After that, 100 μL of extracellular suspension was mixed with CaCl\(_2\) and agonist, and the experimental chamber was perfused with that mix (final CaCl\(_2\) concentration in bath solution was 2 mM unless otherwise specified).

Results and Discussion

Patch pipettes and electronic noise

Usually, for small size cells it is recommended to use thick-walled capillaries for patch pipette production (for example, 0.86 mm ID, 1.5 mm OD) [28]. Theoretically, thin-walled pipettes also can be used, but the process of pipette production with a small aperture suitable for platelets is much harder. However, when using thick-walled pipettes, there are several obstacles, the main of which is the increased level of electronic noise that comes from the dielectric properties of glass [8]. Since many of the single-channel currents have amplitudes in the range of 0.5 to 2 pA, increased noise levels can obscure these events. In some patch-clamp studies, the reduction of noise level was achieved by covering the pipette tip with special silicon elastomer Sylgard which reduced pipette capacitance [9]. However, in our experiments, the necessary and sufficient requirements for single ion channel recordings with the amplitudes down to 0.4-0.5 pA, and in some cases even lower were: the proper grounding of the equipment, including Faraday cage, the seal resistance higher than 20 GΩ and gain level of the amplifier of 100 mV/pA or higher.

Obtaining gigaohm seal

2 μL of platelet suspension was placed on the bottom of the plastic chamber made out of 12-well cell culture plates with 700 μL of NaCl-based external BS, containing 1 mM of MgCl\(_2\) (in the absence of Ca\(^{2+}\)). Platelets were allowed to settle to the bottom of the chamber. Patch pipette was moved up to 1-2 mm of the chamber bottom with a motorized micromanipulator. Then 40x water-immersion objective of the upright microscope was moved into the suspension, and the tip of the pipette appeared on the screen. Only floating platelets were patched.

The success rate of the patch relies on several factors. Firstly, the pipette tip should always be clean, thus we made pipettes only before the experiment. And secondly, extracellular and pipette solutions should always be properly filtrated. Other important factors for successful gigaseal formation are the presence of the divalent cation (namely calcium and magnesium) in the pipette solution, and the difference in osmolarity between the pipette and extracellular saline’s at around 10% (30 mOsM), as was suggested by Neher and Sakmann in their pioneer work [9]. Previously it was shown both theoretically and with the help of model membranes, that divalent cations greatly enhance the contact between the cell membrane and capillary glass [29]. Another important factor is the level of pH in the pipette solution, because H\(^{+}\), as well as Ca\(^{2+}\) and Mg\(^{2+}\), also greatly improves the gigaohm seal formation by enhancing the strength of adhesion between cell membrane and glass. When all the above-mentioned requirements were completed, the success rate of gigaohm seal formation increased to about 90%. Although the stability of various patches varied, it was possible to achieve successful gigaseal contacts within 5 hours after obtaining the platelets.

Cell-attached mode for platelets

Due to the small size of platelets and the above-mentioned difficulties only a few ion channels in platelet membrane, including K\(_{ca}\) 3.1, K\(_{v}\) 1.3, P2X, chloride channels, were identified and characterized using the patch-clamp technique. However, recent evidence [23] suggests that despite its small size and absence of nuclei, platelets possess a great variety of ion channels that are poorly characterized. Besides, previously measured experimental results were obtained using megakaryocyte, but not platelets. Therefore identification and characterization of ion channels in platelet membrane are of great importance for extending our knowledge about their role in various cell signaling pathways and physiological response [22, 30-36].

Measurement of single-channel ionic currents during platelet activation

Earlier it was shown that platelets possess a great amount (300-400 per cell) of voltage-gated potassium channels K\(_{v}\) 1.3 [37]. Considering the area of the platelet membrane and the size of the aperture of the patch pipette, at least a couple of these channels will always be present in the patch and will make a significant contribution to the registered ion channel currents, which will make it harder to distinguish other important ion channels. Taking
into account the available data on the biophysical characteristics of this channel, namely the potential of half-activation of about -30 mV, we proposed the method to measure the activity of other ion channels. Usage of cell-attached configuration in voltage-clamp mode and application of a potential to patch pipette from +80 to +150 mV allowed us to avoid activation of voltage-gated ion channels and made it possible to register currents from other ion channels. During this procedure, the membrane under the patch pipette was hyperpolarized, which inactivated $K_{v1.3}$ channels, at the same time there was enough driving force for all ions, which helped with the identification of even the smallest ionic currents in the range of 1-2 pS. Schematic illustration of the technique can be seen in Fig. 1.

The above-mentioned approaches for obtaining a good gigaohm seal allowed us to record single-channel ionic currents in cell-attached mode from platelets activated by thrombin (0.73 μg/ml) without the deterioration of the seal (Fig. 2A, B, C).

Analysis of data from 6 patches allowed the identification of at least 5 different types of channels, with the conductivity varying from 2 to 20 pS. Considering that most of the identified and known channels have such a small conductivity, recordings with small applied potentials give the little result. When the applied potential is +50 mV, the resulting single-channel events have amplitudes of 0.4 – 0.5 pA, which only slightly exceeds the noise level (Fig. 2A). So we can recommend using potentials in the range of +80 – +150 mV. In that way, currents from even the smallest-conductance channels will be easily registered. Yet, high applied potentials may lead to relatively quick deterioration of the seal.

In Fig. 2B the same recordings but with NaCl-based pipette BS can be seen. Regarding the observed amplitudes in NaCl salts, we obtained a much lower number of channels than in KCl saline, suggesting the involvement of more than 2 types of potassium channels in the process of activation of platelets by thrombin.

To test the stability of the patches obtained by the proposed method, we recorded single-channel ion currents from platelets in response to 1 μM of ionomycin, Ca$^{2+}$-ionophore which induces strong platelet activation. Results of recordings of single-channel events with different pipette BS solutions are shown in Fig. 2C.

A substantial rise in intracellular calcium levels, induced by ionomycin or combined action of collagen and thrombin, usually switches platelets to a new procoagulant state [38]. During this transition, the morphology of the cells changes drastically. They become balloon-shaped [39], many of the plasma membrane proteins and phosphatidylserine move to the so-called cap region [40, 41]. In Supplementary material, fig. S1 and fig S2, patched and floating platelets can be seen before and after this transition. Despite all that, only about 30% of patches deteriorated after activation with ionomycin.

Ion channels play a pivotal role in a variety of processes underlying platelet activation. For example, potassium channels take part in calcium signaling, maintaining the membrane potential and thus increasing the driving force for Ca$^{2+}$. Calcium entry during activation also depends on the functioning of many ion channels, including Orai1, P2X, TRPC [17, 27, 42]. Scramblase Ano6, which plays a pivotal role in platelet procoagulant response, also acts as an ion channel with chloride conductivity and has a very high threshold for activation by calcium [33, 43]. We believe that the proposed approach of registering single-channel currents is a useful tool to establish and investigate various platelet signaling events in

![Figure 2](Image). Single channel events recorded from platelets in cell-attached mode. A, B Activation of platelets with 0.73 μg/ml thrombin in NaCl-based external BS. KCl-based pipette BS (A), NaCl-based pipette BS (B). C, Activation of platelets with 1 μM ionomycin in NaCl-based external BS. 1,2 - KCl-based pipette BS, 3 - NaCl-based pipette BS. Holding potential ($V_{hold}$) is labeled to the left of each current trace. Typical kinetic records of 15 independent experiments from various donors are presented.
which ion channels may play a key role.

Measurement of membrane potential in the cell-attached mode.

Previously, in the work by Mason J. et. al. [19], a new method for studying membrane potential in a cell-attached model was proposed. According to the model experiments and patch-clamp data, the accuracy of the recorded potential in current-clamp depends on the ratio between seal resistance of the patch and input resistance of the membrane, as well as on the composition of the pipette solution, as it should mimic the intracellular composition of the cell. This method enables one to assess the membrane potential of platelets or any other cell type without breaking the membrane as for whole-cell configuration, in which fast perfusion of the intracellular constituents with electrode saline occurs.

To test this method for platelets we recorded the kinetics of membrane potential of platelets activated by human myeloperoxidase (MPO), which was shown previously to modulate platelet aggregation, cytoskeleton reorganization, and store-operated calcium entry [44], as well as to activate neutrophils [45] and to bind to human red blood cells and change their properties [46, 47]. Adding 100 nM of MPO to extracellular bath solution led to a slight hyperpolarization of the plasma membrane at the level of 10-12 mV (Fig. 3A).

In some experiments, we observed strange spikes in recordings of potential in the cell-attached configuration. This effect can be seen in Fig. 3B. These spikes were very rapid and had an amplitude of about 7-10 mV, suggesting that either these spikes arose from the changes of platelet membrane potential itself, or that openings of ion channels were involved.

We tried to investigate this behavior with a simple model. It is known that the input resistance of the membrane of platelets is around 50 GΩ [10]. We’ve modified it by including single-channel conductance of platelets or any other cell type without breaking the membrane as for whole-cell configuration, in which fast perfusion of the intracellular constituents with electrode saline occurs.

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We tried to investigate this behavior with a simple model. It is known that the input resistance of the membrane of platelets is around 50 GΩ [10]. We’ve used the proposed simplified model [19] of measuring membrane potential in cell-attached mode and modified it by including single-channel conductance ($G_{channel}$), imitating single ion channels under patch pipette. The resulting electrical circuit can be seen in Fig. 3C.

Model parameters: $G_{input}$ = 17 pS (59 GΩ), $G_{channel}$ = 5 pS or 24 pS (corresponding to the obtained in present study single-channel conductance's), $G_{seal}$ – variable (corresponding with patch resistance ($R_{seal}$) of 20, 30 и 50 GΩ). $V_{membrane}$ = -60 mV. $F(t)$ – the function of time, that determines the process of channel opening. In our case, it is a simple Gaussian function (eq. 2) with the following parameters: $a = 1$, $b = 3$, $c = 0.07$

The results of modeling (Fig. 3D) indicate that the opening of ion channels led to spikes in membrane potential. The amplitude of spikes is inversely proportional to the contact resistance and directly proportional to the conductance of the opened channels. In the case of a theoretically perfect gigaohm seal (seal resistance approaches infinity), we wouldn’t be able to see any spikes in recorded potential during channel openings. Similarly, in the case of very small input resistance (for example, the addition of nystatin to the patch pipette) there also wouldn’t be any effect.

Earlier the oscillatory nature of membrane potential recordings in the cell-attached configuration was shown by Mason J. et. al. from platelets activated by ADP [19]. The results of the present study indicate that the reason for oscillatory behavior may be the openings of single channels in the patch. This should be taken into account, especially considering that the membrane potential of megakaryocytes can oscillate [20].

Inside-out mode

The inside-out configuration is a useful tool that allows to control the ionic content of solutions on both sides of the patch membrane and to study the activation of ion channels by the direct action of secondary messengers in the absence of various intracellular signaling events.

To get this configuration, we used the method suggested earlier [48]. Briefly, a drop of viscous substance (we used immersion oil for microscopy) is placed on the bottom of the plate, after which the procedure of gigaohm seal formation as described earlier was performed. Then the pipette with the cell on its tip is brought to the edge of the oil drop and the cell is gently dipped into the oil. After contact between platelet and oil occurs, the tip is retracted to break off a patch of membrane from the rest of the cell, which remains in the oil. Following the above-mentioned recommendations we managed to obtain a successful inside-out patch in more than 90 % of cases, and it was stable for up to 10 min. Animation of the process of obtaining inside-out configuration can be seen in Supplementary materials, in Fig. S3.

In this configuration, we obtained recordings of ionic currents through single channels in the absence and presence of Ca$^{2+}$ (Fig. 4) as an example of studying the effects of second messengers on the
Figure 3 Changes in plasma membrane potential of platelets. A, Membrane potential of platelets after treatment with 100 nM MPO was measured in cell-attached configuration with the current-clamp mode of the amplifier. A typical kinetic curve of 15 independent experiments from various donors is presented. B, Kinetics of platelet membrane potential in cell-attached configuration during activation by 1 μM of ADP. Small oscillations of the membrane potential of around 7-10 mV are observed. A typical kinetic curve of 10 independent experiments from various donors is presented. C, Simplified electrical circuit of the recording of membrane potential in cell-attached configuration [19]. \( V_{\text{measure}} \) – recorded potential, \( G_{\text{seal}} \) – seal conductance, \( G_{\text{input}} \) – input conductance, \( G_{\text{channel}} \) – single-channel conductance. In the model, membrane potential \( (V_{\text{membrane}}) \) is -60 mV. D, Results of simple electrical circuit modeling representing the recordings of membrane potential in cell-attached configuration with two different ion channels (24 pS, 5pS) in the patch.
Conclusions

At the moment patch-clamp method is the most powerful instrument for studying ion channels and their role in cell lifespan. During the past couple of decades ion channels, alongside numerous membrane receptors and intracellular messengers, have been realized to be an important constituent of the mechanisms of platelet activation and their physiological response. The present study provides the techniques and approaches that can help other researchers to extend the usage of the patch-clamp technique for the study of small cells and help to reveal new ways by which ion channels govern platelet functions.

Authors’ contributions

A.U.K. performed experiments, analyzed the data, wrote the text, and edited the paper; S.O.Z. and I.I.P performed experiments, analyzed the data and edited the paper; I.V.G. analyzed the data and edited the paper; E.V.S. supervised the project, planned the research, analyzed the data and edited the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

We thank F. Balabin for the constructive discussion of experimental results and for editing the paper. The authors are grateful to Dr. Alexey Sokolov (Institute of Experimental Medicine, St. Petersburg, Russia) for the generously provided MPO and thrombin.

This work was partly supported by Belarusian Republican Foundation for Fundamental Research (grant B20R-215).

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