STUDYING OF PLATELET ACTIVATION USING PATCH-CLAMP

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Platelets are the smallest cellular fragments circulating in bloodstream, with an average size of around 2.5 × 0.5 μm. They play a pivotal role in the blood coagulation cascade, forming a clot in the location a blood vessel rupture. This provides the functioning of the normal hemostasis (that is, stopping of the bleeding), as well as pathological thrombosis (the overlapping of the vessel). Besides, platelets are also involved in inflammatory response and perform the angiotrophic function. Platelet activation in response to various stimuli involves complex multiple ways of intracellular signaling, among which changes in plasma membrane ion channel permeability play a pivotal role. But still the mechanisms of platelet membrane ion channel functioning in response to various stimuli are pure and further investigations are needed [1].

The best method of studying the ion channels on various cells is patch-clamp (PC). Because the small size of platelets made the use of this method rather difficult, megakaryocytes are widely used to represent the properties of platelets in patch-clamp studies [2]. But still, one cannot be sure that this applies to all of the platelet properties, and important data from the recordings of activation can be lost. In this work we represent the method of platelet function investigation by patch-clamp technique in which we emphasize it various peculiarities with regard to platelets.

Venous blood samples from healthy donors was collected in tubes containing 3.8% (w/v) trisodium citrate as anticoagulant at 9:1 ratio and Prostaglandin I_2 at final concentration of 0.5 μM was added to avoid spontaneous platelet activation. Platelet-rich plasma (PRP) was prepared by centrifugation of blood at 200 *g* for 10 min. Washed platelets were prepared by additional two-step centrifugation of PRP at 400 *g* for 30 s at 20°C, and the cell pellet was resuspended in a NaCl HEPES solution (140 mM NaCl, 10 mM HEPES, 10 mM D-glucose, 5 mM KCl, 1 mM $MgCl_2$, pH 7.4), with final cell concentration of 10^6 cells/ml.

Prior to the experiment, 5 to 7 μl of washed platelets was carefully placed in the bottom of a Petri dish containing 3 ml of NaCl HEPES solution with 1 mM $CaCl_2$. Patch pipettes were prepared from the borosilicate glass on a puller Sutter P-97 (HEKA Elektronik GmbH). For whole cell (WC) configuration measurements fire polished glass pipettes with resistance of 5–10 MΩ were used to reach a tight contact between platelet plasma membrane and pipette surface and to avoid patch resealing. For cell-attach (CA) configuration measurements pipette resistance of 14–18 MΩ was required to prevent spontaneous switching to WC. Using a micromanipulator (MP-225, Sutter Instrument), the pipette filled with KCl HEPES solution (5 mM NaCl, 10 mM HEPES, 145 mM KCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 3 mM EGTA, pH 7.2) was brought close to a single cell and a small negative pressure was applied to the pipette, leading to tight seal formation (5 to 60 GΩ). To monitor the dynamic changes in cell membrane potential, CA and WC patch-clamp recordings were carried out in current-clamp (CC) or voltage clamp (VC) mode using an amplifier HEKA EPC 8 (HEKA Elektronik GmbH), filtered at 0.7 kHz.

Switching to WC from CA was spontaneous after about 10–15 min, when using pipettes with small resistance, while using pipettes with high resistance made CA stable for more than 30 min. It should be noted that CA with seal resistance >5GΩ can be used to measure the kinetics of membrane potential in CC mode (but only with the internal solution closely representing the intracellular one) [3]. According to this in our experiments the kinetics of membrane potential in WC and CA were identical.

When using fire polished pipettes, a short hyperpolarization to values of -100 – -120 mV occurred after gigaseal formation. This can be linked to platelet activation after adhesion to the glass of the patch pipette. And without fire polishing there was no hyperpolarization, most likely because the area of contact with glass was much lower. The average membrane potential was -40±4 mV.

Addition of physiological agonist ADP (10μM) and calcium ionophore ionomycin (1μM) led to the hyperpolarization of plasma membrane for 15±4 mV and 10±3 mV respectively and was due to inwardly-rectifying currents. This is consistent with previous results, obtained for megakaryocytes [4]. Furthermore, PC technique can be used to investigate intracellular signaling mechanisms initiated by various regulatory proteins the effect of which measured by traditional methods (turbidimetric aggregometry, fluorescent microscopy, flow cytometry, etc.) is rather weak.

Thus, PC of platelets may serve as a very sensitive method to study the effects of various signaling molecules on platelet activity, despite of platelet small size.

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