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Full Length Article

The role of agonist-induced activation and inhibition for the regulation of purinergic receptor expression in human platelets

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ABSTRACT

Introduction: Adenosine diphosphate (ADP) as physiological activator of human platelets mediates its effects via three purinergic receptors: P2Y1, P2Y12 and P2X1. The inhibition of P2Y12 is used pharmacologically to suppress aggregation underlining the physiological significance of this receptor. Since the regulation of purinergic receptor expression has not thoroughly been investigated yet, this study analyzed the content of purinergic receptors on the platelet surface membrane upon activation and inhibition.

Materials and methods: The surface expression of purinergic receptors was measured by flow cytometry using two different polyclonal antibodies as basal values and after incubation with thrombin receptor activating peptide (TRAP-6) or with inhibitors DEA/NO, MAHMA/NO or Prostaglandin E1 (PGE1). Western blot analysis was used to confirm inhibitory effects.

Results: Both investigated antibodies revealed a significant increase of purinergic receptor expression upon TRAP-6 stimulation. The NO donors, DEA/NO and MAHMA/NO, did not influence basal or TRAP-6 stimulated values. PGE1 did not affect basal receptor expression, but diminished TRAP-6 stimulated purinergic receptor expression in a dose-dependent manner.

Conclusions: In summary, TRAP-6 induced platelet activation leads to an elevation of purinergic receptor expression. In contrast to other surface ligands, this effect is not suppressed by cGMP-mediated inhibition, but almost completely abrogated by enhanced cAMP-mediated signaling as induced by PGE1.

1. Introduction

Adenosine diphosphate (ADP) is a physiological activator of human platelets, which is stored in dense granules at high concentrations and released upon platelet stimulation. Secreted ADP promotes platelet activation by enhancing agonist-stimulated intracellular signaling [1–3] and mediates its effects via three different purinergic receptors: P2Y1, P2Y12 and P2X1.

P2Y1 is a Gq-coupled ADP receptor, activating phospholipase C and stimulating calcium release from intracellular stores. P2Y12 is associated with the Gαi protein. The activation of P2Y12 results in adenylyl cyclase suppression and switching-off of the cAMP-dependent inhibitory pathway [4–6]. Simultaneous stimulation of both P2Y12 and P2Y1 is required for the initiation of platelet aggregation, whereas the P2X1 receptor is an ion channel, responsible for a rapid calcium influx synergizing P2Y1 effects and thereby contributing to mechanisms of ADP induced platelet aggregation [7].

The P2Y12 receptor is also used as a pharmacological target in patients suffering from cardiovascular diseases to prevent platelet aggregation and thrombus formation. Several chemical compounds, like clopidogrel, prasugrel or ticagrelor, have been developed for the medical treatment [8].

In addition to activating signals, platelet function is conditioned by inhibitory signaling cascades mediated by cyclic adenosine monophosphate (cAMP)- and cyclic guanosine monophosphate (cGMP)-dependent phosphorylation of effector proteins. [9].

The vasodilator-stimulated phosphoprotein (VASP) is an actin-binding protein and the main substrate for both cAMP- and cGMP-dependent protein kinases (PKA and PKG) that are able to phosphorylate this protein at Ser^{157} and Ser^{239} with different affinities. PKA phosphorylates VASP preferentially at Ser^{157} and consecutively at Ser^{239}, whereas PKG prefers Ser^{239} and thereafter Ser^{157} [10]. Once phosphorylated, it retains the fibrinogen receptor GPIIb/IIIa in the resting conformation leading to inhibition of fibrinogen binding, adhesion and

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aggregation [11,12]. Furthermore, cAMP- or cGMP-platelet inhibition modulates intracellular signaling and decreases agonist-stimulated surface expression of platelet ligands like CD62P, CD40 or CD63 and receptors like the GPIIb/IIIa and the GPIb-IX complexes [13–15].

Nitric oxide (NO) is a potent vasodilator and inhibitor of platelet function. In vivo, it is produced by endothelial cells. In platelets, NO stimulates the soluble guanylyl cyclase (sGC), resulting in increased cGMP production followed by PKG activation and VASP phosphorylation [9,15]. Prostaglandin E1 (PGE1) is another inhibitor, stimulating the prostacyclin receptor (IP) [16] and enhancing cAMP synthesis [17] and, as a consequence, cAMP-dependent phosphorylation via PKA [9].

Both mechanisms, inhibition and activation of platelets, may modulate the presence of purinergic receptors on the platelet surface. Surprisingly, the regulation of purinergic receptor expression dependent on the platelet status has never thoroughly been addressed, although, in turn, it may have influence on platelet responsiveness to activators like ADP and on effects exerted by pharmacological platelet inhibitors. This study, therefore, intended to investigate changes of purinergic receptor expression in platelets upon activation and under stimulation of inhibitory pathways.

2. Materials and methods

2.1. Materials

Thrombin Receptor Activator Peptide-6 (TRAP-6) was from BACHEM (Bubendorf, Switzerland). Rabbit polyclonal anti-P2Y1 (product numbers TA328626; #APR-021), anti-P2Y12 (TA328649; #APR-020) and anti-P2X1 (TA329003; #APR-022) antibodies were from OriGene EU (Herford, Germany) or, for comparison, from Alomone Labs (Jerusalem, Israel), each of them declared to be qualified for flow cytometry. According to the product data sheets, the antibodies to P2Y1 recognize the peptide SDEYLRSYFIYSMC, corresponding to amino acid residues 207–220 in the 2nd extracellular loop of the human P2Y1 Receptor. The antibodies to P2Y12 recognize the peptide CTAENTLFYVKES, corresponding to amino acid residues 270–282 in the 3rd extracellular loop of the human P2Y12 receptor. The antibodies to P2X1 recognize the peptide CRPIYEFHGLYEEK, corresponding to amino acid residues 270–283 in the extracellular loop of the human P2X1 receptor. Each antibody was affinity-purified on the corresponding immobilized antigen and specificity was confirmed by Western blot.

FITC conjugated mouse anti-CD62P antibody and corresponding FITC conjugated isotype control were from Acris Antibodies GmbH (Herford, Germany). Phospho-VASP Ser^{239} and phospho-VASP Ser^{157} antibodies were from Nanotools (Teningen, Germany). Rabbit unconjugated polyclonal IgG isotype control was from Biozol (Eching, Germany). Horseradish peroxidase conjugated anti-mouse antibodies were from Bio-Rad Laboratories Inc. (Muenchen, Germany). NO donors, DEA/NO and MAHMA/NO, were purchased from Enzo Life Sciences GmbH (Loerrach, Germany). Ethylene glycol tetraacetic acid (EGTA), Prostaglandin E1 (PGE1), FITC-conjugated goat anti-rabbit polyclonal antibody, Tyrode's salt solution and Ponceau S were from Sigma-Aldrich Chemie GmbH (Muenchen, Germany).

2.2. Blood collection and platelet preparation

The experimental studies with human platelets and the consent procedure were approved by the local ethics committee of the University of Wuerzburg (approval number 101/15). All participants provided their written informed consent. The study was performed according to our institutional guidelines and to the Declaration of Helsinki.

Peripheral blood samples from informed healthy voluntary donors (aged from 22 to 44 years, without any medication 14 days before donation) were collected in polystyrene tubes containing 3.2% citrate buffer (106 mM trisodium citrate, Sarstedt, Nuembrecht, Germany).

3 mM EGTA was added to samples used for the preparation of washed platelets to prevent platelet activation [18]. Platelet-rich plasma (PRP) was obtained by centrifugation at 330*g* for 5 minutes (min). Subsequently, samples of PRP were centrifuged at 430*g* for 10 min. Then pelleted platelets were washed once in CGS buffer (120 mM sodium chloride, 12.9 mM trisodium citrate, 30 mM D-glucose, pH 6.5), and resuspended in Tyrode's salt solution to a final concentration of 3×10^8 platelets/mL. After resting for 15 min in a 37 °C water bath, washed platelets were used for experiments.

2.3. Detection of purinergic receptor and CD62P expression by flow cytometry

15 μL PRP diluted with 15 μL of PBS were stained with 5 μL of anti-P2Y1, anti-P2Y12 or anti P2X1 antibodies, or 3 μL of anti-CD62P-FITC antibody or an appropriate isotype control, for 10 min at 37 °C. After that samples were stimulated with buffer (unstimulated control) or 10 μM TRAP-6 for 2 min at 37 °C as described previously [19,20].

The influence of inhibitory signaling on purinergic receptor expression was analyzed by pre-incubation of PRP samples (stained as described above) with buffer (unstimulated control), with DEA/NO, with MAHMA/NO or with PGE1, respectively, in two concentrations (5 nM or 1 μM) for 5 min at 37 °C, followed by stimulation with buffer or TRAP-6 stimulation for 2 min at 37 °C.

After that the samples were stopped with 1% formaldehyde (final concentration), fixed for 10 min at room temperature. Samples for CD62P detection were diluted with 500 μl of PBS/BSA/Glc buffer and analyzed by flow cytometry. Samples for purinergic receptor detection were centrifuged for 1 min at 14,000*g*. Pellets were re-suspended in 100 μL of PBS/BSA/Glc (Dulbecco's PBS (Ca^{2+}, Mg^{2+} free), 5.5 mM D-Glucose, 0.5% BSA) and stained at room temperature in the dark for 30 min with 1 μL of FITC-conjugated goat anti-mouse antibody. Then, samples were diluted with 500 μL of PBS/BSA/Glc and analyzed by flow cytometry using a FACSCalibur flow cytometer from Becton Dickinson (Franklin Lakes, NJ, USA) using CELLQuest software, version 6.0. The platelet population was identified by its forward and side scatter distribution and 10,000 events were analyzed for mean fluorescence.

2.4. Stimulation of platelets for Western blot analysis

100 μL of washed platelet suspension was supplemented with 1 mM $CaCl_2$ and immediately stimulated with buffer (unstimulated control), DEA/NO, MAHMA/NO or PGE1 (5 nM and 1 μM) for 2 min at 37 °C. The reaction was stopped with sodium dodecyl sulphate (SDS) loading buffer (200 mM Tris-HCl, pH 6.7, 10% 2-mercaptoethanol, 6% SDS, 15% glycerol and 0.03% bromophenol blue).

2.5. Western blot analysis

Washed platelet suspensions were lysed, separated by sodium dodecylsulphate polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes. The membranes were incubated with primary anti-phospho-VASP antibodies (1:100 dilution) overnight at 4 °C. For signal visualisation, anti-mouse IgGs conjugated with horseradish peroxidase were used as secondary antibodies, followed by electrochemiluminescence (ECL) detection kit (GE Healthcare, Piscataway, NJ, USA). Bands were analyzed densitometrically using NIH Image J software (National Institutes of Health, Bethesda, MD, USA) for uncalibrated optical density. Ponceau S (0.1% (w/v) in 5% acetic acid) staining of nitrocellulose membrane was used as loading control.

2.6. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). The n-values refer to the number of experiments, each made with