European Journal of Pharmacology 740 (2014) 15–27

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

Reciprocal regulation of human platelet function by endogenous prostanoids and through multiple prostanoid receptors ☆

Katharina Hubertus [a,1], Marcel Mischnik [b,1], Jens Timmer [b,c], Sabine Herterich [a], Regina Mark [a], Maxime Moulard [d], Ulrich Walter [e], Joerg Geiger [a,f,*]

[a] Institute for Clinical Biochemistry and Pathobiochemistry, University of Wuerzburg, Wuerzburg, Germany
[b] Institut für Physik, University of Freiburg, Freiburg, Germany
[c] BIOSS Centre for Biological Signalling Studies, University of Freiburg, Freiburg, Germany
[d] BioCytex, Marseille, France
[e] Center for Thrombosis & Haemostasis, Universitätsklinikum der Johannes Gutenberg-Universität Mainz, Mainz, Germany
[f] Interdisciplinary Bank of Biomaterials and Data Wuerzburg, Straubmuehlweg 2a, 97078 Wuerzburg, Germany

ARTICLE INFO

Article history:
Received 3 March 2014
Received in revised form
17 June 2014
Accepted 18 June 2014
Available online 6 July 2014

Keywords:
Prostaglandin A1
Prostaglandin E2-synthase
Secretion
Cyclic AMP
VASP
P2Y12

Chemical compounds studied in this article:
prostaglandin A_1 (CID 5281912)
prostaglandin D_2 (CID 448457)
prostaglandin E_1 (CID 5280723)
prostaglandin E_2 (CID 5280360)
butaprost (CID 5311035)
iloprost (CID 5311181)
sulprostone (CID 5312153)
cangrelor (CID 9854012)
U46619 (CID 5311493)
BW868C (CID 57340013)
BW245c (CID 119304)
L798,106 (CID 15551229)
CAY 10441 (CID 9839644)
L161,982 (CID 9961192)

ABSTRACT

Platelets are permanently exposed to a variety of prostanoids formed by blood cells or the vessel wall. The two major prostanoids, prostacyclin and thromboxane act through well established pathways mediated by their respective G-protein coupled receptors inhibiting or promoting platelet aggregation accordingly. Yet the role of other prostanoids and prostanoid receptors for platelet function regulation has not been thoroughly investigated. We aimed at a comprehensive analysis of prostanoid effects on platelets, the receptors and pathways involved and functional consequences. We analyzed cAMP formation and phosphorylation of proteins pivotal to platelet function as well as functional platelet responses such as secretion, aggregation and phosphorylation. The types of prostanoid receptors contributing and their individual share in signaling pathways were analyzed and indicated a major role for prostanoid IP_1 and DP_1 receptors followed by prostanoid EP_4 and EP_3 receptors while prostanoid EP_2 receptors appear less relevant. We could show for the first time the reciprocal action of the endogenous prostaglandin PGE_2 on platelets by functional responses and phosphorylation events. PGE_2 evokes stimulatory as well as inhibitory effects in a concentration dependent manner in platelets via prostanoid EP_3 or EP_4 and prostanoid DP_1 receptors. A mathematical model integrating the pathway components was established which successfully reproduces the observed platelet responses. Additionally we could show that human platelets themselves produce sufficient PGE_2 to act in an autocrine or paracrine fashion. These mechanisms may provide a fine tuning of platelet responses in the circulating blood by either promoting or limiting endogenous platelet activation.

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; AC, adenylyl cyclase; cAMP, adenosine 3′,5′-cyclic monophosphate; 6-Cl-PuDP, 6-chloro purine 5′ diphosphate; DMSO, dimethyl sulfoxide; ERK, extracellular signal regulated kinase; Gi, inhibitory G-protein; Gs, stimulatory G-protein; GST, glutathion-S-transferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IGEPAL, octylphenoxypolyethoxyethanol; MAPK, mitogen activated protein kinase; OD, optical density; PBS, phosphate buffered saline; PGI2, prostaglandin I2, prostacyclin; PKA, cAMP dependent protein kinase; PKB, protein kinase B; PPP, platelet poor plasma; PRI, platelet reactivity index; PRP, platelet rich plasma; RalGDS, ral guanine nucleotide dissociation stimulator; Rap1, ras related protein 1; RBD, rap binding domain; SDS, sodium dodecyl sulfate; TBAS, tetrabutylammonium hydrogen sulfate; TMB, 3,3′,5,5′- tetramethylbenzidine; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol; TXA2, thromboxane A2; VASP, vasodilator stimulated phosphoprotein; WP, washed platelets

☆This work was supported by a grant of the German Federal Ministry for Education and Research in the MedSys framework, SARA project [Grant 315395].

* Corresponding author at: Interdisciplinary Bank of Biomaterials and Data Wuerzburg, Straubmuehlweg 2a, 97078 Wuerzburg, Germany. Tel.: +49 931 201 47010; fax: +49 931 201 647 000.
E-mail address: Joerg.Geiger@uni-wuerzburg.de (J. Geiger).

[1] KH and MMi contributed equally to this publication.

http://dx.doi.org/10.1016/j.ejphar.2014.06.030
0014-2999/

1. Introduction

Platelets are regarded a major player in the development of atherothrombosis and cardiovascular diseases originating there from (Linden and Jackson, 2010). Accordingly prevention of platelet activation and aggregation has attracted significant interest as pharmacological target in the prevention and treatment of atherothrombotic events (Patrono and Rocca, 2010). Understanding the complex cross regulation of platelet stimulatory and inhibitory pathways is fundamental to the development of an efficient and specific anti-platelet treatment.

In the intact blood vessel platelets are continually exposed to platelet activating substances present in the circulating blood. However, endogenous factors and enzymes preventing platelet aggregation counterbalance the pro-aggregatory factors (Jin et al., 2005). Two prostanoids are central to this reciprocal regulation of platelet function, prostaglandin I_2 (prostacyclin, PGI_2) and thromboxane A_2 (TXA_2).

Both prostanoids are short-lived molecules which are not stored but produced and released in situ. The short lifespan entails strictly temporally and spatially confined action of the prostanoids, thus offering a fine tuning of cellular function regulation. Stimulation of the platelet prostacyclin receptor IP_1 leads to stimulatory G-protein (G_s) mediated formation of cAMP by adenylyl cyclase (AC). Elevated cAMP levels cause an inhibition of platelet aggregation via activation of cAMP dependent protein kinase (PKA) and subsequent phosphorylation of proteins essential to platelet activation (e.g. VASP) (Smolenski, 2012). In contrast, TXA_2 stimulation induces release of calcium ions from the dense tubular system into the cytoplasm via Gq-protein mediated activation of phospholipase C. By this protein kinases as well as small G-proteins are activated. This leads finally to platelet shape change, secretion, and aggregation (Begonja et al., 2007).

The presence of additional prostanoid receptors on platelets has been investigated by means of functional and binding assays with authentic and synthetic prostaglandins (Fabre et al., 2001; Wise et al., 2002; Heptinstall et al., 2008; Singh et al., 2009) and, in some cases on the molecular level as well (Dovlatova et al., 2008; Kuriyama et al., 2010). To date 9 prostanoid receptors are known in humans: the prostaglandin D receptors DP_1 and DP_2 (CRTH2 receptor), the prostaglandin E receptors EP_1, EP_2, EP_3 and EP_4, the prostaglandin I_2 (prostacyclin) receptor IP_1, the prostaglandin F receptor FP, and the thromboxane A_2 receptor TP (Tsuboi et al., 2002). The expression and function of prostaglandin receptors on blood platelets has been discussed controversially (Armstrong, 1996; Schober et al., 2011). Yet the mode of action, interaction and quantitative contribution of endogenous prostanoids and prostanoid receptors to platelet activation and inhibition has not been shown conclusively. Reasons for the difficulties analyzing platelet prostanoid action originate particularly from the low specificity of prostanoid receptors and the susceptibility of transcription and expression analysis of platelets to contamination by other blood cells or cell debris.

We aimed at a comprehensive analysis of human platelet expression of prostanoid receptors and platelet regulation by prostanoids. By a combination of molecular, biochemical, pharmacological, and mathematical techniques we approached this objective and collected sufficient data of adequate quality to generate a functional model representing platelet prostanoid regulation.

2. Materials and methods

2.1. Reagents and materials

The murine tissues used as positive controls in immunoblots were kind gifts from Cora Reiß (Center for Thrombosis & Haemostasis, Mainz, Germany). The oligonucleotides were commercially synthesized from eurofins nwg operon (Ebersberg, Germany). Human normal tissues cDNA was purchased from BioChain (AMS Biotechnology, Abington, UK). Superscript II reverse transcriptase was obtained from Invitrogen (Darmstadt, Germany). The prostanoid EP_2, EP_3, EP_4 and DP_1 receptor polyclonal antibodies as well as the prostanoid DP_1 receptor agonists Prostaglandin D_2 and 15(R)-15-methyl-PGD_2, the prostanoid DP_1 antagonist BW A868C, the prostanoid EP_2 receptor agonist (R)-butaprost, the prostanoid IP_1 receptor antagonist CAY 10441, the prostanoid EP_3 receptor agonist sulprostone, Prostaglandin A_1, and the PGE_2 and PGE_2M EIA kits were purchased from Cayman Chemicals (Ann Arbor, MI, USA). MAPK, phospho-T180/Y182 MAPK, ERK, phospho-T202/Y204 ERK, PKB, and phospho-S473 PKB antibodies were from Cell Signaling Technology, Inc. (Danvers, MA, USA). CD62P-FITC and Rap1 antibody were from BD Biosciences (Heidelberg, Germany). The phospho-VASP antibody 5C6 (phospho-Ser157) and the total VASP antibody IE273 were gifts from vasopharm GmbH (Würzburg, Germany). The polyclonal actin antibody was from Santa Cruz Biotechnology (Heidelberg, Germany), the goat anti-mouse IgG was from Biorad (Hercules CA, USA). The stable thromboxane A_2 receptor agonist U-46619, the prostanoid DP_1 receptor agonist BW 245c and the prostaglandins PGE_1, and PGE_2 were from Sigma-Aldrich (Schnelldorf, Germany). The prostanoid EP_3 receptor antagonist L798,106 and the prostanoid EP_4 receptor antagonist L161,982 were from Tocris bioscience (Bristol, UK). The prostanoid IP_1 receptor agonist Iloprost (Ilomedin) was from Schering (Berlin, Germany). 6-Cl-PuDP (6-Chloro purine 5′ diphosphate) was obtained from Biolog (Bremen, Germany). The P2Y12 antagonist cangrelor was from Medicines Company (Parsippany, NJ, USA). Fibrinogen, ADP and Fura-2/AM were obtained from Calbiochem (Merck KGaA, Darmstadt, Germany). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and mini complete® EDTA-free (protease inhibitor cocktail tablets) were obtained from Roche (Mannheim, Germany). The cyclic AMP Enzyme Immunoassay Kit was from Assay designs (Ann Arbor, MI, USA). All other chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany) at the highest purity available. Nitrocellulose membranes were obtained from Schleicher & Schuell (Dassel, Germany).

2.2. Platelet preparation

Platelets were used as washed platelets (WP) resuspended in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH = 7.4), depending on the assay applied. WP were prepared from whole human blood as described elsewhere (Geiger et al., 2010) maintaining the platelet functionally intact (Schweigel et al., 2013) with modifications to avoid contamination by other cells (Burkhart et al., 2012). Whole human blood was obtained from healthy volunteers who had not taken any medication affecting platelet function within 2 weeks prior to the experiment after informed consent according to the declaration of Helsinki and our institutional guidelines and as approved by the local ethics committee. The blood was drawn by venipuncture and collected in 1/5 volume of HEPES/citrate buffer (120 mM NaCl, 20 mM sodium citrate, 4 mM KCl, 1.5 mM citric acid, 30 mM D-glucose, 8 mM HEPES, pH = 6.5) and centrifuged at 300*g* for 20 min at 20 °C to obtain platelet rich plasma (PRP). For the preparation of washed platelets the PRP was diluted 1:1 with HEPES/citrate buffer, apyrase (1 U/ml) added and centrifuged again at 100*g* for 10 min at 20 °C. The pellet was discarded and the supernatant was centrifuged at 380*g* for 10 min. The resulting pellet was resuspended in HEPES/citrate, left resting for 5 min and centrifuged again at 380*g* for 10 min. The platelet pellet was resuspended in PBS buffer to a cell density of 3×10^8 platelets/ml

and apyrase (0.1 U/ml) added. Washed platelets were used in 200 μl portions. The samples were incubated with the reagents in the water bath at 37 °C as indicated, stopped and treated appropriately for the respective analyte. The reagents were solved in PBS unless otherwise stated.

2.3. Platelet membrane preparation

Platelet membranes were prepared from WP by lysis with hypotonic buffer (10 mM EGTA, 10 mM EDTA, 2 mini complete protease inhibitor tablets) freezing in liquid nitrogen followed by the three repeated freeze/thaw cycles. The suspension was then subjected to ultrasound treatment, centrifuged at 10,000*g* at 4 °C for 20 min and the pellet was removed. The supernatant was then centrifuged at 100,000*g* at 4 °C for 45 min and the supernatant discarded. The pellet containing the membrane fraction was resuspended in 300 μl of lysis buffer (50 mM Tris, 150 mM NaCl, 1% SDS).

2.4. Platelet cDNA preparation and PCR experiments

Total RNA was isolated from WP using the TRIzol method according to the manufacturer's protocol (Invitrogen, Darmstadt, Germany). WP were centrifuged to produce a pellet containing 3.5×10^8 platelets which was resuspended in 1 ml Trizol. To visualize the pellet, 2 μl 3 M sodium acetate was added. After the addition of 400 μl chloroform the suspension was mixed well and incubated for 5 min on ice. The suspension was centrifuged (4 °C, 9000*g*, 10 min) and the upper phase was transferred to a tube, 600 μl cold isopropyl alcohol added and centrifuged (4 °C, 9000*g*, 15 min) again. The pellet was resuspended in 1 ml 70% ethanol and centrifuged (4 °C, 9000*g*, 15 min). The supernatant was discarded and the pellet was dried and resuspended in 10 μl deionized water.

mRNA was transcribed in cDNA by the reverse transcriptase reaction with SuperScript II (Invitrogen, Darmstadt, Germany). The platelet cDNA was tested against leukocyte and genomic contamination with specific oligonucleotides (Table 1) and only used if tests were negative. The absence of contaminations by genomic DNA was proven with primers for the exon 2 of the human hemochromatosis (HFE) gene and the absence of leukocyte contamination was tested with a primer for the sGC splice variant N1-α1a which is ubiquitously expressed except for platelets. Human normal tissues cDNA (BioChain) was used as positive control. The oligonucleotides were designed against all known isoforms of the receptors. PCR was done with Platinum Taq Polymerase. All PCR products were validated by sequencing.

2.5. Western blot

Phosphorylation of MAPK (p38), extracellular signal regulated kinase (ERK, p42/44) and protein kinase B (PKB/Akt) was determined by western blot analysis according to Gambaryan et al. (2010). Washed platelets were treated as indicated and 50 μl portions of platelet suspension were stopped by the addition of 25 μl 3× SDS stop buffer (200 mM Tris, 6% SDS, 15% glycerol, 0.003% bromophenol blue), boiled and separated on a 10% polyacrylamide gel. The proteins were detected by transfer on a nitrocellulose membrane (4 °C, 1 h, 2 A), incubation with the respective primary antibody and horseradish peroxidase coupled goat anti rabbit IgG as secondary antibody. The proteins were visualized by chemiluminescence with ECL reagent (GE Healthcare). For quantification of protein phosphorylation the phosphoprotein was first detected with the respective phospho-specific antibody and then after stripping of the western blot by 1 h incubation at 50 °C with stripping buffer (62.5 mM Tris–HCl, 2% SDS, 100 mM β-mercaptoethanol, pH=6.7) the membrane was incubated with the corresponding antibody for the protein to determine the total amount of the respective protein. Relative phosphorylation was calculated from the ratio for the band intensity for phosphorylated/total protein. Detection of the total amount of actin was used as loading control.

2.6. RAP1 pull down assay

Platelets were stopped with the same volume of cold 2× lysis buffer (50 mM Tris, 10 mM $MgCl_2$, 200 mM NaCl, 2% IGEPAL CA-300 (octylphenoxypolyethoxyethanol), 10% glycerol, 1 tablet mini complete protease inhibitor, pH=7.5). The samples were incubated for 30 min on ice, centrifuged (4 °C, 20,000*g*, 15 min) and the pellet discarded. For later quantification of Ras related protein 1 (Rap1) activation a 100 μl portion of lysate was taken from each sample.

The Rap1 pull down was carried out similarly as described (Schultess et al., 2005; Subramanian et al., 2013). The Rap-binding domain (RBD) of the Ral guanine nucleotide dissociation stimulator (RalGDS), which binds activated Rap1 was recombinant expressed with a glutathion-S-transferase (GST) tag (GST-RalGDS-RBD). 50 μl glutathione sepharose bead suspension (Amersham Bioscience, Freiburg, Germany), saturated with GST-RalGDS-RBD was equilibrated two times with 200 μl 2× lysis buffer and centrifuged (4 °C, 5000*g*, 2 min). The platelet lysate supernatant was added and incubated for 90 min at 4 °C in an end over end rotator. After incubation, tubes were centrifuged (4 °C, 5000*g*, 2 min) and the supernatant discarded. The beads were washed 3 times with 200 μl PBS containing mini complete protease inhibitor. After washing, 50 μl 3× SDS stop buffer was added and boiled for 7 min at 95 °C. Pull down samples and total lysate samples were analyzed by western blot using nitrocellulose membranes, and detection with the Rap1 antibody. The relative amount of activated Rap1 was calculated from the ratio of protein band intensities in pull down and total lysate samples.

2.7. Aggregation

Aggregation measurement was carried out with an APACT (Haemochrom, Essen, Germany) or PAP-4C (Mölab, Langenfeld, Germany) aggregometer as described (Geiger et al., 2005) in washed platelet suspensions (WP). Briefly, 200 μl WP were placed in a cuvette containing a stir bar and incubated for 5 min at 37 °C with 1 mM calcium chloride and 1 mg/ml fibrinogen added. 100% Aggregation was calibrated with platelet poor plasma (PPP) or 1% BSA (for washed platelets). Agonists and antagonists were added and aggregation was observed for 5 min under stirring (1000 rpm). Maximal aggregation and the area under curve were used for data analysis.

2.8. P-selectin expression

Surface expression of P-selectin was determined by flow cytometry as described (Geiger et al., 2005). In brief, 20 μl portions of platelet suspension were stained with 10 μl FITC coupled anti-human CD62P and stopped by dilution with 1.5 ml PBS after 15 min incubation in the dark. All samples were analyzed at low flow rate on a Becton Dickinson FACSCalibur. The instrument settings were forward scatter: E00, side scatter: 337 V, fluorescence channel 1: 850 V. Platelets were discriminated from other cells on the basis of their scatter characteristics. A total of 15,000 platelet events were analyzed for mean and median of fluorescence intensity using CELLQuest software version 3.1f.

Table 1
Primer sequences for the amplification of human prostanoid DP_1, DP_2, FP, EP_1, EP_2, EP_3, and EP_4 receptors are shown. Control primers for the detection of leukocyte and genomic DNA contamination are also listed (Fw: forward primer, Re: reverse primer).

Gene	Receptor	Fw	Re
Prostanoid receptors			
PTGDR	DP_1	5′-AAA GCC CAC CCA GGA CTT AG-3′	5′-CCC CAG CTG TTC TTT TAC CA-3′
PTGDR2	DP_2	5′-AAT CCT GTG CTC CCT CTG TG-3′	5′-CGG CCA AGA AGT AGG TGA AG-3′
PTGFR	FP	5′-TGC AAT GCA ATC ACA GGA AT-3′	5′-GAC ATG CAC TCC ACA GCA TT-3′
PTGER1	EP_1	5′-ACC TTC TTT GGC GGC TCT-3′	5′-CCT GGC GCA GTA GGA TGT A-3′
PTGER2	EP_2	5′-TGC TGC TTC TCA TTG TCT CG-3′	5′-AGC TTG GAG GTC CCA TTT TT-3′
PGER3	EP_3	5′-TGA GCA CTG CAA GAC ACA CA-3′	5′-CAA ATT CAG GGA AGC AGG AA-3′
PTGER4	EP_4	5′-GCC GAA GAT TTG GCA GTT TC-3′	5′-GTG ACA GCC AGC CCA CAT AC-3′
Leukocyte marker			
N1-2α1		5′-CAA CAC CAT GTT CTG CAC GAA GC-3′	5′-GTA TCA CTC TCT TTG TGT AAT CC-3′
Genomic DNA marker			
HFE Ex2		5′-TCC TGC TCC CCT CCT ACT ACA-3′	5′-GCT CTG ACA ACC TCA GGA AGG-3′
Control primer			
GAPDH		5′-ATC AAG AAG GTG GTG AAG CAG-3′	5′-TAC TCC TTG GAG GCC ATG TG-3′

2.9. Calcium measurement

Intracellular calcium regulation was determined fluorometrically with the fluorescent indicator Fura-2 as described previously (Geiger et al., 1998). Briefly, platelet rich plasma was incubated with 4 μM Fura-2/AM (di-methyl sulfoxide (DMSO) 1% v/v) for 45 min, centrifuged at 350*g* and the resulting platelet pellet was resuspended in HEPES buffer (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 10 mM HEPES, pH 7.4). The calcium transients were observed in a Perkin-Elmer LS50 luminescence spectrophotometer at an excitation wavelength of 340 nm and emission wavelength of 510 nm. Data were recorded as relative changes in the Fura-2 fluorescence signal.

2.10. Nucleotide secretion

Secretion of ATP and ADP was determined by HPLC analysis of the platelet sample supernatant as described (von Papen et al., 2013). Washed platelets were treated with vehicle or appropriate stimulants with 6-Cl-PuDP added as internal standard. To 600 μl aliquot of the sample 60 μl of ice cold EDTA solution (50 mM, pH 7.4) and 150 μl silicon oil were added. The samples were thoroughly mixed for 15 s on a vortex mixer and centrifuged for 30 s at 5000*g*. Supernatants (600 μl) were transferred into eppendorf tubes containing 1.2 ml of cold 100% ethanol and then centrifuged at 10,000*g* for 6 min at 4 °C. The supernatant was removed and dried in the vacuum. The dried samples were dissolved in 50 μl buffer A (0.1 M KH_2PO_4; 4 mM tetrabutylammonium hydrogen sulfate (TBAS); pH 5.5) and separated on a 250 mm/10 μm RP-18 column (Merck) with a 45 min linear gradient [100% buffer A to 40% buffer A/60% buffer B (0.09 M KH_2PO_4; 0.01 M K_2HPO_4; 2 mM TBAS; 40% v/v methanol; pH = 7.2)] and registration of the absorbance at 260 nm. The nucleotides were identified by their retention time and quantified with a calibration curve obtained with nucleotide standards and the internal standard, corrected for recovery as described (von Papen et al., 2013).

2.11. Serotonin secretion

Serotonin secretion was determined analogous to Aktas et al. (2003). Prior to platelet stimulation, 2 μM fluoxetine, a selective serotonin re-uptake inhibitor, was added to the washed platelet suspension. A 350 μl aliquot was taken from the platelet sample treated as indicated. The sample was stopped by adding 35 μl cold EDTA (50 mmol/l, pH 7.4) and 100 μl silicon oil (Merck). Samples and controls for total platelet serotonin were mixed for 15 s but only the samples were subsequently centrifuged for 30 s at 5000*g*. Supernatants of samples and controls (270 μl) were transferred to eppendorf tubes containing 60 μl of 100% trichloroacetic acid and then centrifuged at 5000*g* for 2 min at 4 °C. An aliquot (250 μl) of the resulting supernatant was added to 1 ml of ortho-phthalaldehyde reagent (0.5% ortho-phthalaldehyde (w/w), 30% HCl) in ethanol and incubated at 95 °C for 10 min. Samples and totals (each 200 μl) were washed twice with 5 ml chloroform, the phases separated, and fluorescence emission of the aqueous phase was measured at an excitation wavelength of 355 nm and an emission wavelength of 475 nm on a Victor Wallac 1420 microplate reader (Perkin-Elmer).

2.12. PGE_2 and PGE_2 metabolite EIA

PGE_2 and as control for assay cross-reactivity the PGE_2 metabolite 13,14-dihydro-15-keto PGE_2 were determined with commercially available EIA kits (Cayman Chemical Company). Washed platelet samples were used either untreated or stimulated as indicated and incubated 2 min at 37 °C under shaking. The reaction was stopped by addition of HCl (20 mM final concentration) and immediate freezing in an ethanol/dry ice bath. The thawed sample was centrifuged 10 min at 10,000*g* at 4 °C, the supernatant was separated on a C18 SPE column (Cayman Chemical Company) activated as described in the manufacturers′ protocol by elution with 5 ml ethyl acetate. The organic phase was dried in the vacuum, dissolved in 50 μl ethanol and diluted with 50 μl of the assay buffer supplied. The concentration was calculated from the calibration curve. If %B/B0 was outside the dynamic range (less than 20% or more than 80%) the sample was measured again at a lower or higher dilution as appropriate.

2.13. cAMP EIA

Cyclic adenosine 5′ monophosphate (cAMP) determination was carried out as described (Geiger et al., 2010). In brief, platelets were lysed with the original volume of 70% (v/v) ice cold ethanol and kept on ice for 30 min. The precipitate was removed by centrifugation for 10 min at 10,000*g* and 4 °C and washed again with the same volume of 70% (v/v) ethanol. The ethanol extracts were combined and dried in the vacuum. The dried samples were dissolved in 200 μl of the assay buffer supplied with the assay and acetylated to increase assay sensitivity as described (Geiger et al., 2010). The samples were measured in a Wallac Victor 1420 (Perkin-Elmer) plate reader at 405 nm. Sample readings below

30% or above 70% %B/B0 are off the dynamic range of the assay and were repeated with an appropriate dilution of the sample.

2.14. VASP phosphorylation

VASP phosphorylation is a highly sensitive indicator for platelet cAMP regulation (Geiger et al., 2010). We determined VASP phosphorylation in a solid-phase assay either by VASP binding on a zyxin matrix (Geiger et al., 2010) or in a sandwich ELISA (BioCytex, Marseille, France) (Barragan et al., 2010).

The zyxin matrix pVASP assay was carried out as described (Geiger et al., 2010). Platelet samples (200 µl each) were lysed by addition of an equal volume of ice cold lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP-40, 10 mM β-glycerolphosphate, 10 mM NaF, pH=7.4), and thorough mixing on a vortex mixer. The samples were diluted with PBS buffer by 1:10. Each sample was measured in triplicate for the VASP phospho-Ser157 antibody (5C6) and total VASP antibody (IE273). As control for background and nonspecific binding 5% bovine serum albumin (BSA) solved in lysis buffer was used. 100 µl sample/well of the zyxin coated microtiter plate were incubated 1 h at room temperature under shaking and washed three times with 300 µl/well PBS-T (0.1% Tween20 supplemented PBS), 1 h incubated with the primary antibodies, washed 3 times with PBS-T, the secondary antibody (horseradish peroxidase coupled goat anti-mouse IgG) added, incubated and washed as mentioned above. The detection reagent ABTS was added, incubated for 20 min at room temperature under shaking and the absorbance of the samples was measured in the microtiter plates with a Wallac Victor 1420 (Perkin-Elmer) plate reader at 405 nm each for 1 s. From the absorbance of each sample the absorbance of the background control sample for the respective antibody was subtracted, and the resulting data multiplied with the dilution factor. From the values obtained with the phosphos-pecific and the total VASP antibodies the phospho-VASP/VASP ratio was calculated.

For the BioCytex assay samples were directly processed in the ELISA-VASP plate wells. After cell lysis, VASP phosphorylation level was revealed using a horseradish peroxidase coupled monoclonal antibody, which only binds to VASP when Ser239 is phosphorylated. Read out was performed using the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine) (TMB. After acidification with H_2SO_4, the absorbance was read at 450 nm. Optical density at 450 nm (OD_{450}) was correlated to the phosphorylated VASP (Barragan et al., 2010). Results were calculated from blank-corrected OD_{450} (sOD_{450}) data.

Data from BW 245c tests (sOD_{450}(BW245c)) were compared to PGE_1-treated sample ($sOD_{450}(PGE_1)$). Results were calculated according to the following formula:

$$\text{pVASP} = \frac{sOD_{450}(\text{BW245c})}{sOD_{450}(PGE_1)} \times 100$$

For sulprostone investigations, the platelet reactivity index (PRI) was calculated as follows:

$$\text{pVASP} = \frac{sOD_{450}(PGI_2) - sOD_{450}(PGI_2 + \text{Sulprostone})}{sOD_{450}(PGI_2)} \times 100$$

2.15. Mathematical modeling of prostaglandin receptor activation

In previous publications we could already show that the simulation of platelet signal transduction pathways by numerical models is feasible and allows a fundamental insight into the entanglement of pathways (Wangorsch et al., 2011; Mischnik et al., 2013). Here a mathematical model was established to simulate the effects of different prostaglandins (PGD_2, PGE_2, PGI_2) on VASP phosphorylation. The model represents a static function depicting the VASP phosphorylation relative to the total VASP amount at steady-state conditions, implying a temporal derivative of zero. The equation depends on three variables, the concentration of the prostanoids PGE_2 c(PGE_2), PGI_2 (cPGI_2) and PGD_2 c(PGD_2). PGI_2 and PGD_2 exert an exclusively positive effect on the phosphorylation of VASP, and PGE_2 has both a positive effect, imparted by the prostanoid EP_2 and EP_4 receptors and a negative effect induced by the prostanoid EP_3 receptor. The shape of the equation was chosen to ensure both a monotonic biphasic behavior within the closest interval (0,1) if only PGE_2 is present, and a parabola-like behavior if PGE_2 is accompanied by PGI_2 or PGD_2. In the latter case and if PGE_2 concentrations are assumed to be low, the inhibitory effect of the prostanoid EP_3 receptor causes a decrease in the phosphorylation of VASP, whereas high concentrations support the action of PGI_2 or PGD_2. In order to keep the function biologically realistic, we introduced six unknown parameters ($a_1 \ldots a_6$), which in a next step were estimated multi-experimentally along the PGE_2-dose–response measurements of phosphorylated VASP in the presence and absence of iloprost. These parameters can be interpreted as the combined effects of receptor concentrations in the plasma membrane, the affinities for the respective ligands, and the effectiveness of the downstream signaling cascades, leading either to PKA activation and the phosphorylation of VASP, or the inhibition of adenylyl cyclase and the subsequent drop in the concentration of cAMP.

$$VASP(c(PGI_2), c(PGE_2), c(PGD_2)) = \frac{a_1(c(PGI_2)+(c(PGD_2))+a_2(c(PGE_2))^3+a_3(c(PGE_2))^2+a_4}{a_5(c(PGE_2))^{a_6}}$$

$$a_1 = 1.7781; \quad a_2 = 0.2224; \quad a_3 = 0.2706$$
$$a_4 = 0.0499; \quad a_5 = 2.2725; \quad a_6 = 2.8799$$

For the implementation of the model we made use of the Matlab programming environment Version R2008b and employed the fminsearch algorithm for parameter estimation. The model delivers a description of the measured data for PGE_2-concentrations ranging from 1 to 5000 nM and allows for predictions of the platelets' response.

2.16. Data analysis

Experimental data were obtained from at least five independent experiments, except for western blot and FACS analysis and the aggregation experiments where each experiment was repeated with three individual platelet preparations. Outliers were identified by the Dixon's test and eliminated. Dose response curves were calculated by Marquardt–Levenberg approximation of the raw data to a Hill function for each receptor and drug. For agonists acting through multiple receptors the contribution of each receptor was assumed to be additive conserving the parameters for individual receptor activation (EC50 and slope). The data sets were analyzed by one-way ANOVA and differences considered significant if $P < 0.05$. All data are expressed as means ± SD of at least three individual experiments.

3. Results

3.1. Prostanoid receptor detection

With primers for the prostanoid DP_1, EP_2, EP_3 and EP_4 receptors we obtained PCR products from platelet cDNA which could be identified by sequencing (Table 1). With primers for the prostanoid EP_1, DP_2, and FP receptors no PCR product was gained, while the positive controls yielded the expected product (Fig. 1A). With isoform specific primers we could identify mRNA for the

prostanoid EP_3 receptor isoforms 4, 5, 6, 7 and 9 (RefSeq: NM_198714.1, NM_198715.2, NM_198716.1, NM_198717.1, NM_198719.1). Western blot detection of prostanoid receptors confirmed the expression of the receptors for which transcripts had been found (Fig. 1B). The receptor proteins could be detected in platelet membrane preparations as well as in whole platelet lysates. Prior to the experiments the antibody specificity was checked with tissue lysates and cell lines expressing the receptor as well as cells and tissues negative for the respective receptors (not shown).

3.2. Prostaglandin D receptor mediated effects

Prostaglandin D_2 and the prostanoid DP_1 receptor agonist BW 245c stimulated cAMP formation with half maximal concentrations of 120 nM and 8 nM respectively (Table 2) and impaired ADP stimulated platelet aggregation at concentrations of 200 nM (PGD_2) or 20 nM (BW 245c) (Table 3). The full aggregation response of platelets to 5 μM ADP or 1 μM U46619 was converted into reversible aggregation after preincubation with 100 nM or 50 nM PGD_2, respectively (Table 3). The prostanoid DP_1 receptor selective antagonist BW A868C fully inhibited cAMP formation stimulated by 0.2 μM PGD_2 or 0.1 μM BW 245c at 20 nM with half maximal inhibition at 1.84 ± 0.216 nM or 6.03 ± 0.422 nM respectively. Prostanoid DP_1 receptor stimulation has been shown to be associated in certain circumstances with mobilization of calcium ions from intracellular stores (Boie et al., 1995), in human platelets neither PGD_2 nor BW 245c could elicit a measurable calcium response (data not shown).

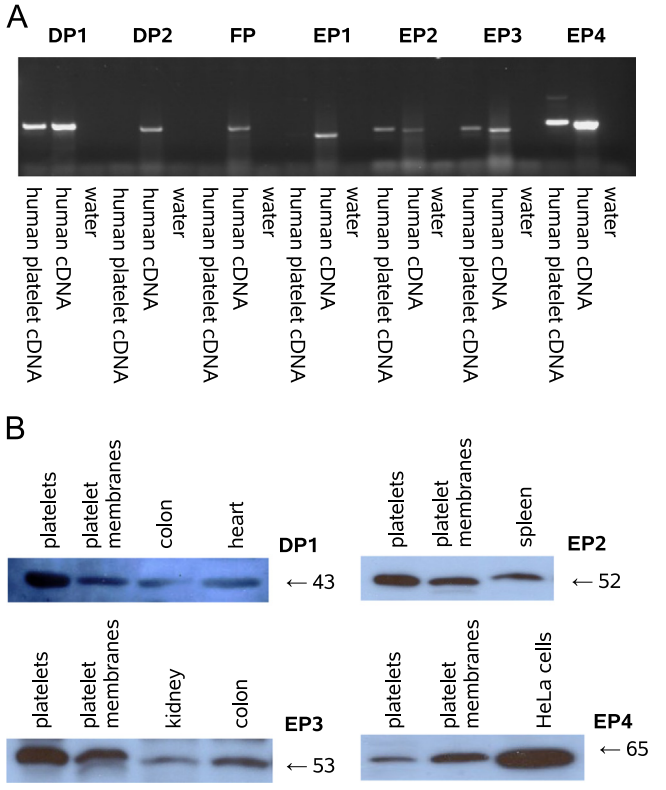


Fig. 1. Detection of prostanoid receptors on platelets by PCR of platelet cDNA and western blot of platelet lysates with appropriate antibodies. Human platelet mRNA reverse transcribed to cDNA was tested for the prostanoid receptor transcripts indicated with appropriate primer pairs. Total human tissue cDNA was used as positive control and water as negative control (A). Platelet lysates and platelet membrane preparations were tested with antibodies for the receptor protein indicated (B). Positive controls were tissue lysates of colon and heart for the prostanoid DP_1 receptor, spleen for the prostanoid EP_2 receptor, kidney and colon for the prostanoid EP_3 receptor and HeLa cell lysates for the prostanoid EP_4 receptor.

Table 2
Properties of platelet prostanoid DP_1 and prostanoid EP_2 receptors determined with the specific agonists PGD_2, BW 245c and butaprost by cAMP EIA and phospho-VASP EIA. EC50 values were calculated from dose–response curves approximated to the experimental data of 5 independent experiments.

Receptor	Agonist	EC_{50}	
		cAMP	VASP
DP_1	PGD_2	121 ± 17.9 nM	n.d.
	BW 245c	8.22 ± 1.50 nM	6.8 ± 1.02 nM
EP_2	Butaprost	58.6 ± 25.5 μM	35.8 ± 2.60 μM

Table 3
PGD_2 and butaprost and prostaglandin D_2 (PGD_2) on ADP and U46619 induced platelet aggregation. Aggregation data are given as maximum of aggregation (max %agg), aggregation 5 min after stimulation (5′ %agg) and area under aggregation curve (AUC) ± standard deviation of the mean of 3 independent experiments.

	Control	Butaprost 20 μM
ADP 5 μM		
max %agg	84.3 ± 13.0	17.5 ± 3.3
5′ %agg	79.0 ± 19.5	15.3 ± 2.2
AUC	18,354 ± 4079	1386 ± 240
U46619 1 μM		
max %agg	76.8 ± 6.5	13.6 ± 1.8
5′ %agg	65.5 ± 33.2	9.75 ± 3.3
AUC	15,396 ± 5631	607 ± 498

3.3. Prostanoid EP_2 receptor mediated effects

Prostanoid EP_2 receptor mediated effects are small in platelets and are superimposed by prostanoid IP_1 and EP_4 receptor stimulation. Therefore we used the prostanoid EP_2 receptor specific agonist butaprost to allow observation of isolated prostanoid EP_2 receptor responses. Butaprost elicited a 3 times increase of cAMP (Table 2); using the more sensitive VASP phosphorylation as read out revealed a 10 fold increase of Ser157 phosphorylation by 10 μM butaprost (Table 2). This butaprost concentration strongly diminished 5 μM ADP or 1 μM U46619 induced aggregation in PRP and WP (Table 3).

3.4. Platelet responses mediated by prostaglandin E receptors

The E prostaglandins are the authentic EP receptor stimulants, however due to the lack of specificity not only EP receptors but also other prostanoid receptors can be stimulated by PGE_1 and PGE_2. Hence pharmacological distinction of the EP receptors is only possible with combinations of suitable agonists and antagonists. In addition analysis of EP receptor effects is interfered by the opposing effects of EP receptor stimulation on adenylyl cyclase. We have addressed this complex problem by using PGE_1, PGE_2, or synthetic, specific agonists and inhibitors. AC inhibition was investigated by pretreatment of platelets with the AC stimulants iloprost and forskolin. Prior to the experiments inhibitor concentrations sufficient to prevent receptor activation but low enough to preclude unspecific action were determined experimentally based on the literature data. Prostaglandin E_1 which can activate all EP receptor subtypes as well as the prostanoid IP_1 and DP_1 receptor (Table 4) caused at EC50 = 41 ± 6.2 nM a 5 times increase in cAMP and 7 times in VASP Ser157 phosphorylation increase at EC50 = 44 ± 9.5 nM. The prostanoid IP_1 receptor antagonist CAY10441 (100 nM) strongly affected adenylyl cyclase stimulation by PGE_1 shifting the EC50 of PGE_1 to 520 nM and reducing the

Table 4
Contribution of platelet expressed prostanoid receptors to PGE_1 stimulated cAMP increase. Washed human platelets treated with PGE_1 and inhibitors of the prostanoid IP_1 (CAY 10441), prostanoid DP_1 (BW A868C), or prostanoid EP_4 (L161,982) receptor and combinations were analyzed for cAMP accumulation and VASP phosphorylation. EC50 concentrations for PGE_1 were calculated from dose response curves approximated to the data from 5 independent experiments ± - standard deviation.

EC50 [nM]	Control	CAY10441 100 nM	L161,982 20 μM
cAMP	40.9 ± 6.2	523 ± 59.8	130 ± 24.8
VASP	44.0 ± 9.5	206 ± 48.3	66.9 ± 14.7

maximal cAMP concentration by 15%. The PGD_2 receptor antagonist BW A868C (20 nM) did not affect the maximal cAMP response but increased the EC50 to 190 nM. The prostanoid EP_4 receptor antagonist L161,982 (20 μM) had a weaker effect on the EC50 for PGE_1 (EC50 = 130 nM) but reduced cAMP accumulation by 33% (Table 4). In combination these compounds effectively suppressed cAMP formation to 45% of the control and shifted the EC50 for PGE_1 to 300 ± 49.9 nM. VASP phosphorylation was comparably affected, yet L161,982 and BW A868C effects on VASP Ser157 phosphorylation are more apparent (Table 4).

PGE_2 has a similarly broad pharmacological profile as PGE_1 yet with a more pronounced selectivity for EP receptors. Consequently, PGE_2 stimulation of platelets results in an increase of cAMP and VASP Ser157 phosphorylation (Fig. 2A). Approximation of a logistic function favors a bi-phasic cAMP increase mediated by two receptors with distinct binding properties over a mono-phasic, single receptor stimulation. The assumption is further supported by the observation that inhibition of prostanoid EP_4 and DP_1 receptors results in ordinary dose–response curves with half maximal concentrations comparable to the values obtained in the two receptor models without inhibitors (Fig. 2B). Inhibition of the prostanoid IP_1 receptor by CAY10441 however is without effect (data not shown). Pretreatment of platelets with a prostanoid EP_3 receptor inhibitor significantly enhances cAMP accumulation and VASP phosphorylation induced by PGE_2 (Fig. 2B), indicating prostanoid EP_3 receptor mediated counter-regulation of AC by G_i-protein activation. The considerations are also confirmed by the larger mathematical model comprising all three receptors, including the inhibitory effect conveyed by the prostanoid EP_3 receptor (Fig. 2A).

Treatment of the platelets with cAMP elevating agents like forskolin or the prostanoid IP_1 receptor agonist iloprost prior to PGE_2 stimulation reveals an uncommon dose–response curve for PGE_2 with parabolic characteristic and a minimum at about 20 nM PGE_2 (Fig. 2C). This indicates a superposition of an AC inhibitory and activatory effect with distinct pharmacological properties. Preventing prostanoid EP_4 receptor stimulation with the antagonist L161,982 results in a significant right shift of the parabola minimum to 100 nM (Fig. 2D). As the prostanoid EP_3 receptor is known to be mostly associated with a G_i protein it is likely that the inhibitory effect on AC results from prostanoid EP_3 receptor stimulation. Antagonism of prostanoid EP_3 receptor activation by L798,106 increases cAMP accumulation achieved with 5 nM PGE_2 by 20% indicating reduced AC inhibition (Table 5). Extending the two receptor hypothesis by a further receptor (prostanoid EP_3 receptor) using the parameters determined and adjusting the parameters for the third receptor to fit the data results in a new equation describing the three components adequately (Fig. 2C). Again the theoretical model reflects the behavior of the system reasonably (Fig. 2C). In order to accentuate particular prostanoid EP_3 receptor effects the prostanoid EP_3 receptor specific agonist sulprostone was used instead of PGE_2 in some experiments. Sulprostone causes an inhibition of AC mediated cAMP accumulation and VASP phosphorylation evoked by prostanoids as well as allosteric AC activation by forskolin (not shown).

ADP is known to substantially amplify platelet aggregation by stimulating the G_i coupled P2Y12 receptor thus diminishing cAMP accumulation (Geiger et al., 1998), hence making use of the same pathway as prostanoid EP_3 receptor stimulation. In combination with PGE_2 the ADP mediated AC inhibition is significantly enhanced resulting in a left shift of the ADP response curve (Fig. 3). As the P2Y12 receptor is an important target of antiplatelet drugs (Vivas and Angiolillo, 2010; Bhavaraju et al., 2010) cross-regulation of P2Y12 pathways by other receptors may affect drug efficacy significantly. The P2Y12 receptor inhibitor cangrelor completely abolishes the AC inhibitory effect of ADP, which is efficiently compensated by concomitant stimulation of the prostanoid EP_3 receptor with sulprostone (Fig. 4).

3.5. Prostanoid EP_3 receptor mediated platelet activation

Stimulation of a G_i protein is known to be linked not only to AC but also via the βγ-subunit of the G-protein to platelet responses like kinase activation and secretion (Garcia et al., 2010). We investigated platelet serotonin and ATP secretion as well as P-selectin expression as parameters for platelet exocytosis, protein kinase B (PKB/Akt), p42/44 (ERK) and p38 (MAPK) phosphorylation and activation of the small GTPase Rap1 as activation signals and platelet aggregation as overall indicator for platelet activation. Stimulation with 2 μM ADP or 0.25 μM of the prostanoid TP receptor agonist U46619 induce a transient aggregation response of platelets which is potentiated by concomitant stimulation with 0.1 μM or 1 μM sulprostone to full platelet aggregation (Table 6). Platelet serotonin secretion induced by 0.25 μM U46619 is dose dependently amplified by sulprostone while sulprostone alone up to 1 μM could not significantly induce serotonin secretion (not shown). ATP secretion is slightly but not significantly induced by sulprostone alone but sulprostone clearly enhances secretion induced by 0.25 μM U46619 (Fig. 5). ADP secretion is not increased after sulprostone treatment, but sulprostone can significantly potentiate U46619 stimulated ADP secretion. PGE_2 alone or in combination with U46619 has no significant effect on serotonin and ATP secretion, but on ADP secretion (Fig. 5). Serotonin and ATP δ-granule secretion data are confirmed by α-granule exocytosis monitored by FACS analysis of P-selectin surface expression. Sulprostone addition did not change basal P-selectin expression (control: mean = 11.86 ± 2.93, median = 8.87 ± 2.04; sulprostone 1 μM: mean = 13.07 ± 1.98, median = 9.43 ± 1.52). In combination with U46619 P-selectin expression was increased, however not to a statistically significant degree (U46619 1 μM: mean = 36.29 ± 9.33, median = 28.19 ± 6.5; U46619 1 μM + sulprostone 1 μM: mean = 42.33 ± 12.95, median = 31.78 ± 10.05).

ADP induces phosphorylation of PKB at S473 and p42/44 ERK at T202/Y204 as well as p38 MAPK at T180/Y182, whereas sulprostone evokes PKB and ERK phosphorylation to a lower extent and no increase in p38 MAPK phosphorylation. PGE_2 does only cause phosphorylation of ERK. In combination with ADP sulprostone has a small but not significant effect on protein phosphorylation, while PGE_2 reduces the ADP stimulated phosphorylation to the level achieved with sulprostone (Fig. 6). The small GTP binding protein Rap1 plays a central role in cell activation. Platelet agonists like ADP or thrombin strongly induce Rap1 activation. Sulprostone and PGE_2 alone evoke Rap1 activation slight, but significant which is enhanced by co-stimulation with ADP or serotonin (Fig. 7). However, increasing PGE_2 concentration diminishes ADP induced Rap1 activation. Effects on platelet calcium regulation could not be observed with PGE_2 or sulprostone stimulation, neither in the presence nor absence of ADP or U46619.

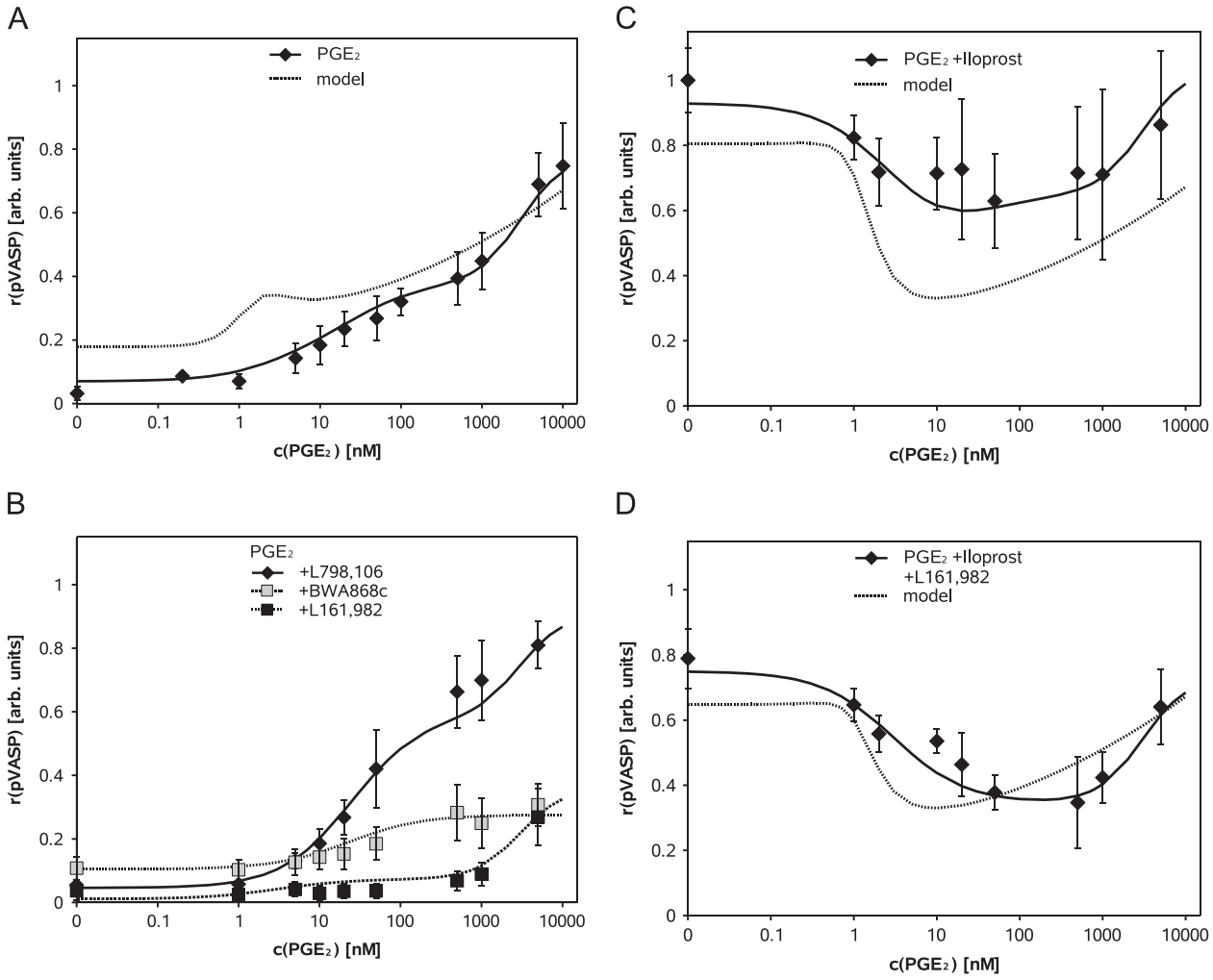


Fig. 2. Experimental data, approximated dose–response curve and a mathematical model of the hypothesized PGE_2 effects are in agreement. Human platelets were treated with PGE_2 alone (A) and in combination with the EP_3 receptor inhibitor L798,106, the prostanoid EP_4 receptor inhibitor L161,982 or the prostanoid DP_1 receptor inhibitor BW A868C (B) and/or the IP_1 receptor agonist Iloprost (C, D) at different concentrations. The activation and inhibition of the AC/PKA pathway was analyzed by detection of VASP Ser157 phosphorylation. (A) PGE_2 stimulated Phosphorylation of VASP Ser157 was determined and a logistic function assuming two distinct binding sites was approximated to the data (solid line). A mathematical model for the presumed prostanoid DP_1, EP_4 and EP_3 receptor stimulation was established and the expected VASP Ser157 phosphorylation calculated (dotted line). (B) Treatment with an prostanoid EP_3 receptor inhibitor (L798,106; filled diamonds) enhances PGE_2 stimulated VASP phosphorylation. Inhibition of the prostanoid EP_4 receptor (L161,982; filled squares) or prostanoid DP_1 receptor (BW A868C; open squares) prior to PGE_2 stimulation abolishes either of the two phases of VASP phosphorylation increase. The logistic functions are shown as solid line (prostanoid EP_3 receptor inhibition), dotted line (prostanoid EP_4 receptor inhibition), or dashed line (prostanoid DP_1 receptor inhibition). (C) VASP phosphorylation of platelets pretreated with 1 nM Iloprost is diminished by PGE_2 at low concentration. With increasing PGE_2 concentration the inhibitory effect is surmounted and VASP Ser157 phosphorylation further increases. A logistic function approximated to the experimental data assuming a three receptor model applying the parameters determined for the two receptor model (prostanoid DP_1 and prostanoid EP_4 receptor) was calculated (solid line) as well as a mathematical model based on the literature data for the hypothesized three receptor model (dotted line). (D) Inhibition of the prostanoid EP_4 receptor with L161,982 (filled squares) of iloprost treated and PGE_2 stimulated platelets causes a right shift of the parabolic curve minimum compared to the curve in the absence of an inhibitor (C). The corresponding approximation of the dose–response curve (solid line) and the result from the model calculation (dotted line) are shown. Experimental data are means of 5 experiments ± standard deviation, the approximation and the mathematical model were calculated as described in Section 2.15.

Table 5
Effect of prostanoid EP_3 receptor inhibition on PGE_2 stimulated cAMP increase. Platelet intracellular cAMP concentration was determined for the conditions indicated. The data in the absence and presence of the inhibitor L798,106; in the absence of PGE_2 are not significantly different ($P > 0.1$) while in the presence of PGE_2 the cAMP concentration is significantly higher in the presence of the inhibitor ($P = 0.03$). Data are means of 5 independent experiments ± standard deviation.

cAMP [μM]	Control	PGE_2 5 nM
control	22.4 ± 1.8	30.6 ± 4.3
L798,106 0.5 μM	22.0 ± 5.8	40.4 ± 4.2

3.6. *Prostaglandin A_1 evoked effects*

The cyclopentenone prostaglandins are formed by dehydration of the cyclopentanol ring system of E- and D-prostaglandins. A multitude of biological functions has been attributed to the cyclopentenone prostaglandins, ranging from anti-inflammatory to anti-tumor activity. The mechanisms mediating the biological action are not yet fully understood (Straus and Glass, 2001). For the anti-inflammatory cyclopentenone prostaglandin PGA_1 inhibitory effects on platelet activation responses, such as calcium mobilization, secretion and TXA_2 formation, and also cGMP extrusion from platelets maintaining increased intracellular cGMP levels have been described (Zhu et al., 2006; Radziszewski et al., 1995). In our experiments PGA_1 causes a strong increase in cAMP and VASP phosphorylation at micromolar concentrations. Treatment with the prostanoid DP_1 receptor antagonist BW A868C or the prostanoid EP_4 receptor antagonist L161,982 diminishes the PGA_1 induced AC activation while the prostanoid IP_1 receptor antagonist CAY 10441 has no significant effect (Fig. 8).

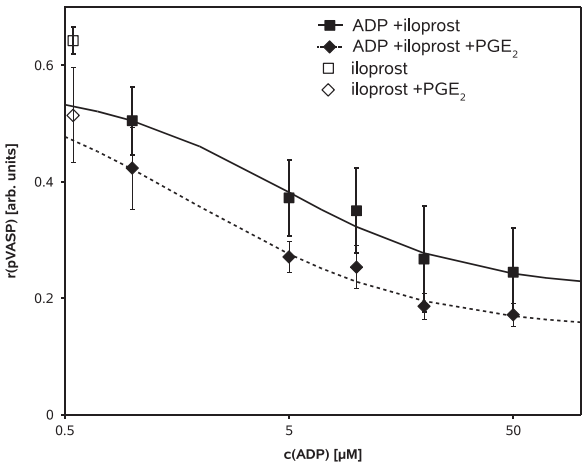
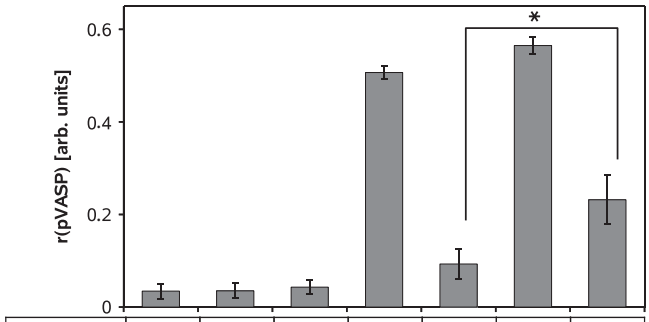


Fig. 3. PGE_2 enhances ADP stimulated inhibition of VASP phosphorylation in platelets. Iloprost (1 nM) prestimulated VASP phosphorylation in human platelets is dose dependently inhibited by ADP (filled squares and solid line). Co-administration of 5 nM PGE_2 causes a significant amplification of the ADP effect indicated by a right shift of the inhibition curve (filled diamonds and dotted line). The VASP phosphorylation in absence of ADP is shown on the left side of the graph for iloprost alone (open squares) and the combination of iloprost and PGE_2 (open diamonds). Data shown are means of 5 independent experiments ± standard deviation and dose response curves calculated as described in Section 2. For the data points indicated by asterisks the differences between iloprost and iloprost/PGE_2 are significant with $P < 0.05$.



ADP	20 μM	-	-	-	20 μM	20 μM	20 μM
cangrelor	-	20 nM	-	-	-	20 nM	20 nM
sulprostone	-	-	1 μM	-	-	-	1 μM
iloprost	-	-	-	1 nM	1 nM	1 nM	1 nM

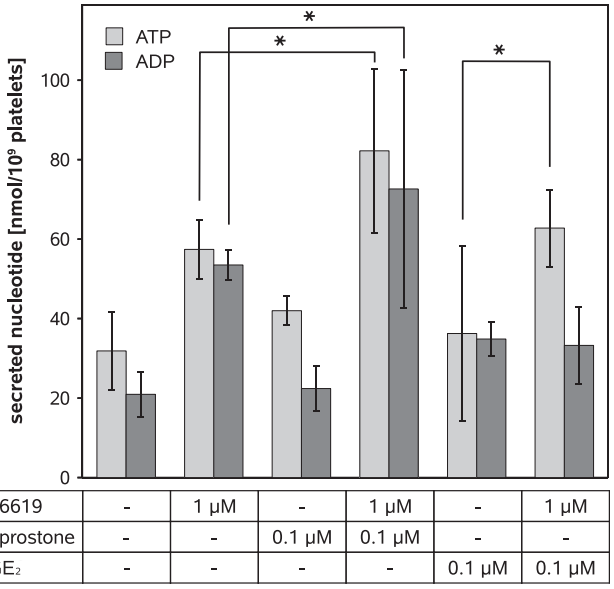
Fig. 4. Sulprostone can restore cangrelor inhibited ADP evoked inhibition of iloprost stimulated VASP phosphorylation. Neither the P2Y12 receptor antagonist cangrelor nor the prostanoid EP_3 receptor agonist sulprostone significantly affect basal VASP phosphorylation. Iloprost induced VASP phosphorylation however is effectively inhibited by 20 μM ADP which is completely abolished by cangrelor. Co-administration of 1 nM sulprostone can partially compensate cangrelor inhibition of VASP dephosphorylation. Data are means of 5 independent experiments ± standard deviation of the mean (*P=0.04).

3.7. Prostaglandin E_2 synthesis and release

Though thromboxane A_2 is the major product of platelet arachidonic acid metabolism we could show that the prostaglandin E receptor agonist PGE_2 is also formed and released by platelets. Stimulation by thrombin or the thrombin receptor activating peptide TRAP6 leads to an increase of PGE_2 levels in the medium from 0.23 ± 0.18 pmol/10^9 platelets before stimulation up to 20.4 ± 0.38 pmol/10^9 platelets. Possible assay cross-reactivity with thromboxane A_2, the major product of arachidonic acid metabolism in platelets, can be excluded as we could also measure formation of the PGE_2-metabolite 13,14-dihydro-15-keto PGE_2 (PGE_2M) correspondingly. ADP does not induce PGE_2 formation up to 50 μM,

Table 6
Effect of sulprostone on ADP and U46619 induced platelet aggregation. Aggregation data are given as maximum of aggregation (max %agg), aggregation 5 min after stimulation (5′ %agg) and area under aggregation curve (AUC) ± standard deviation of the mean of 3 independent experiments.

	Control	Sulprostone	
		0.1 μM	1 μM
ADP 2 μM			
max %agg	49.1 ± 7.7	71.0 ± 18.5	89.1 ± 10.6
5′ %agg	10.6 ± 9.0	66.9 ± 9.5	85.5 ± 11.5
AUC	4611 ± 1727	14,976 ± 2303	19,251 ± 2410
U46619 1 μM			
max %agg	64.4 ± 10.0	84.0 ± 17.0	88.5 ± 22.2
5′ %agg	45.4 ± 27.1	81.9 ± 13.5	85.8 ± 21.0
AUC	10,722 ± 4938	18,227 ± 1426	19,341 ± 1207



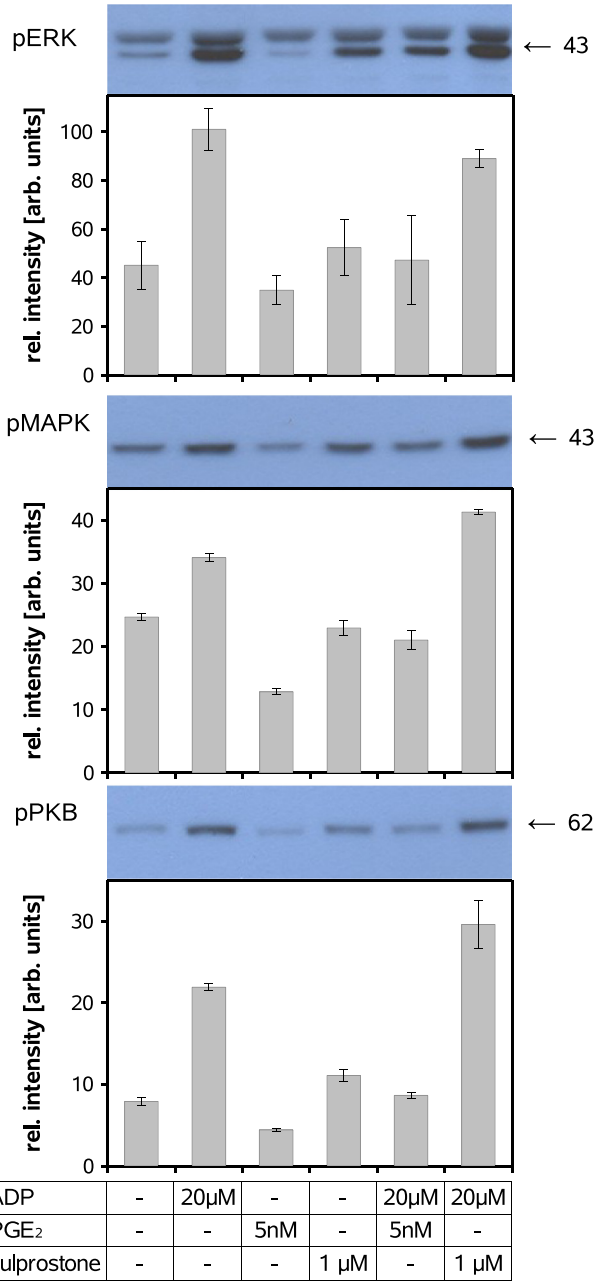
U46619	-	1 μM	-	1 μM	-	1 μM
sulprostone	-	-	0.1 μM	0.1 μM	-	-
PGE_2	-	-	-	-	0.1 μM	0.1 μM

Fig. 5. Platelet prostanoid EP_3 receptor stimulation by sulprostone but not by PGE_2 causes platelet exocytosis and potentiates U46619 stimulated platelet secretion. Supernatants of platelets treated either with sulprostone, PGE_2 and U46619 or combinations were analyzed for ATP and ADP content. ATP and ADP data are absolute values in nmol/10^9 platelets. Data shown are means of 3 independent experiments ± standard deviation. The data pairs marked with an asterisk differ significantly with a $P < 0.05$.

however significantly enhances TRAP6 stimulated PGE_2 synthesis. Formation of PGE_2 in platelets is inhibited by the COX-inhibitors acetylsalicylic acid and indometacin (Fig. 9).

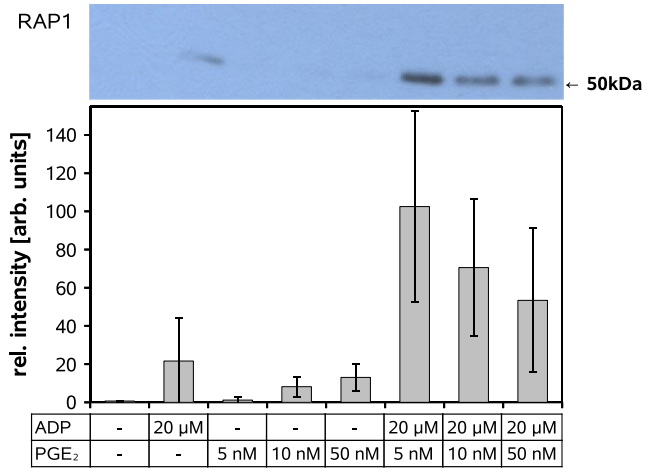
4. Discussion

In current understanding prostanoid action on platelets is governed by two major pathways: (1) the inhibitory effect of prostacyclin released from endothelial cells preventing platelet aggregation cAMP dependently and (2) thromboxane A_2 formed by the platelets and acting as a paracrine platelet activator thus recruiting platelets for thrombus formation. Yet which other prostanoid receptors are expressed in human platelets and how platelet function is regulated by them is still controversial. Analysis of protein expression in platelets is a delicate task demanding proper methods for the collection and preparation of material (Watson et al., 2005) avoiding any significant contamination by other blood cells, cell debris or plasma protein. Improper



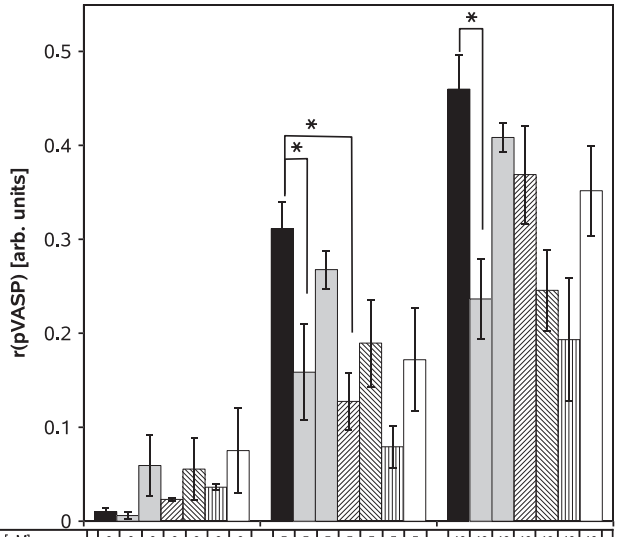
ADP	-	20μM	-	-	20μM	20μM
PGE_2	-	-	5nM	-	5nM	-
sulprostone	-	-	-	1 μM	-	1 μM

Fig. 6. The prostanoid EP_3 receptor agonist sulprostone induces platelet activation by phosphorylation of ERK (p42/p44), MAPK (p38) and PKB and enhances ADP stimulated phosphorylation while PGE_2 alone has only an effect on ERK phosphorylation and reduces ADP stimulated phosphorylation. Phosphorylation of ERK, MAPK and PKB is shown with western blots representative for 3 individual experiments. Bar graphs are means and standard deviation of band intensities determined from western blots normalized for gel loading with the appropriate antibodies against the total protein.



ADP	-	20 μM	-	-	-	20 μM	20 μM	20 μM
PGE_2	-	-	5 nM	10 nM	50 nM	5 nM	10 nM	50 nM

Fig. 7. Activation of the small GTPase Rap1 is induced by PGE_2 and ADP, and potentiated by concomitant stimulation with PGE_2. The blot of the Rap1 pull-down assay is representative of 3 individual experiments. The bar graphs represent the means and standard deviation of 3 experiments normalized for total Rap1 as determined from whole platelet lysates.



PGA_1 [μM]	0	0	0	0	0	0	0	5	5	5	5	5	5	5	10	10	10	10	10	10	10
CAY 10441 0,1 μM	-	+	-	-	+	+	-	-	+	-	-	+	+	-	-	+	-	-	+	+	-
BWA868c 20nM	-	-	+	-	+	-	+	-	-	+	-	+	-	+	-	-	+	-	+	-	+
L161,982 20 μM	-	-	-	+	-	+	+	-	-	-	+	-	+	+	-	-	-	+	-	+	+

Fig. 8. Prostaglandin A_1 induces VASP phosphorylation predominantly through prostanoid IP_1 and prostanoid EP_4 receptors. PGA_1 effects on platelet VASP phosphorylation are shown with 5 and 10 μM PGA_1 treatment. The prostanoid IP_1 antagonist CAY10441 effectively diminishes PGA_1 induced VASP phosphorylation at both PGA_1 concentrations while the prostanoid EP_4 antagonist L161,982 prevents VASP phosphorylation only at 5 μM PGA_1 while no significant effect is observed at 10 μM PGA_1. The prostanoid DP_1 receptor antagonist BW A868c has no significant effect on PGA_1 stimulation. Data shown are means of 5 individual experiments ± standard deviation ($^*P < 0.05$).

preparation and handling of platelets can significantly affect platelet function. Analysis of mRNA transcripts which is occasionally used to provide evidence for protein expression is also not necessarily reliable as transcripts are unstable and do not reflect actual protein expression or may be present with the protein being absent (Geiger et al., 2013). Mass spectrometric analysis of the proteome provides reliable positive evidence while negative results are insufficient as proof of the absence of a particular protein (Burkhart et al., 2012). Functional analysis with pharmacological tools alone also cannot provide clear cut evidence as prostanoid receptors show poor agonist and antagonist selectivity (Abramovitz et al., 2000; Breyer et al., 2001). We endeavored to reliably demonstrate the expression of the prostanoid receptors on platelets and their individual contribution to platelet inhibition and activation by a combined approach, on RNA and protein level as well as confirmation by pharmacological experiments using a range of agonists and antagonists. Significant contamination by other cells was ruled out by appropriate controls for transcripts and protein indicative for other blood cells as well as tests for contamination by genomic DNA. Our data clearly proved the presence of the prostaglandin D_2 receptor DP_1 and the prostaglandin E receptors EP_2, EP_3, and EP_4 while DP_2, EP_1, and FP prostanoid receptors could not be detected. This observation is in

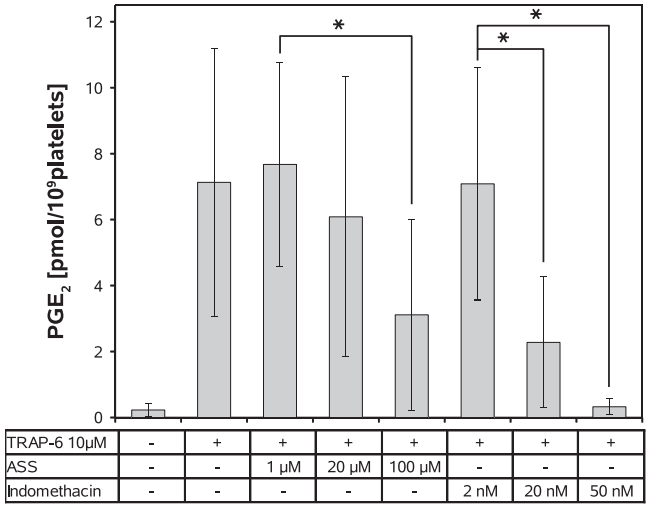


Fig. 9. Stimulation of platelet thrombin receptors with a thrombin receptor activating peptide (TRAP-6) induces PGE_2 synthesis which is abolished by the COX-1 inhibitors indometacin and acetyl salicylic acid (ASS). Generation of PGE_2 by human platelets was determined with a commercial immunoassay. Data shown are means of 5 individual experiments ± standard deviation ($^*P < 0.05$).

accordance with the platelet RNA sequencing data provided by Rowley et al. (2011). Prostanoid IP_1 and TP receptor transcripts were the prevalent prostanoid receptor transcripts, while prostanoid DP_1 and EP receptor subtype transcripts were less frequent. This is in contrast to earlier publications (Phillipose et al., 2010; Petrucci et al., 2011) where prostanoid EP_1 receptor expression was claimed but is in line with recent observations (Iyu et al., 2011a). In addition we could provide unequivocal proof for prostanoid DP_1 receptor expression on platelets which has not been shown on transcript and protein level yet.

Experiments with prostanoid receptor agonists and antagonists corroborate the evidence for prostanoid receptor expression. The prostanoid DP_1, EP_2 and EP_4 receptors share a common signaling pathway with the prostanoid IP_1 receptor by interacting with a stimulatory G protein causing an increase of intracellular cAMP. In contrast to earlier publications (Trist et al., 1989) we could not confirm a biphasic stimulation of cAMP formation by PGD_2. Our data clearly show that PGD_2 acts exclusively via the prostanoid DP_1 receptor and subsequent stimulation of a G_s-Protein. Petrucci et al. (2011) showed that the prostanoid EP_2 receptor agonist butaprost can inhibit the second wave of platelet aggregation induced by ADP. We could show that this inhibition is mediated by cAMP increase and VASP phosphorylation. In contrast to strong adenylyl cyclase stimulation which prevents platelet aggregation entirely, weak stimulation only reduces the aggregation response to reversible aggregation. The slight effect of prostanoid EP_2 receptor stimulation underlines the marginal role of the prostanoid EP_2 receptor in platelets. Selective inhibition of PGE_1 evoked platelet cAMP formation provides evidence for a major role of prostanoid EP_4 and IP_1 receptor stimulation while prostanoid DP_1 and EP_2 receptor stimulation appears less relevant. These observations are in contradiction to the assertion that prostanoid EP_4 would not be involved in PGE_1 stimulated cAMP accumulation (Iyu et al., 2011b). The PGE_1 effect remaining in the presence of prostanoid EP_4, DP_1 and IP_1 receptor inhibitors indicates the presence of a further receptor stimulated by PGE_1 which is most probably the prostanoid EP_2 receptor. Unfortunately no direct evidence could be provided due to lack of a prostanoid EP_2 receptor inhibitor of sufficient specificity. The particular role of prostanoid DP_1 and EP_4 receptors in sensing prostaglandins other than prostacyclin is further underlined by the effects of PGA_1 on platelets which are apparently mediated by these receptors. Hence the inhibitory effect of PGA_1 which has been described (Zhu et al., 2006) can, apart from the claimed inhibition of cGMP extrusion from platelets (Radziszewski et al., 1995), at least partially be attributed to AC activation and subsequent cAMP mediated inhibition of platelet function analogous to the inhibition by endothelium derived prostacyclin (Geiger et al., 1994).

PGE_2 features multiple physiological effects. Among others it plays a major role in inflammation which has been recognized being intimately connected to platelet function (Wagner and Burger, 2003). PGE_2 stimulates all EP receptors with distinct affinity but may also interact with prostanoid IP_1 and DP_1 receptors. The dose response curve approximated to the experimental data proposes two major binding sites which are evidently the prostanoid EP_4 and the DP_1 receptor. In mouse platelets the inhibitory effect of PGE_2 on platelet aggregation could exclusively be attributed to prostanoid IP_1 receptor activation (Fabre et al., 2001), though in human platelets apparently prostanoid EP_4 and DP_1 receptor stimulation contribute considerably while prostanoid IP_1 receptor contribution is at least less significant. The absence of prostanoid IP_1 receptor mediated effects of PGE_2 is confirmed by a pharmacological study (Iyu et al., 2011a) and experiments with prostanoid EP_3/IP_1 receptor knock-out mice (Kuriyama et al., 2010). In a number of papers (Vezza et al., 1993; Armstrong, 1996; Fabre et al., 2001; Heptinstall et al., 2008; Schober et al., 2011) prostanoid EP_3 receptor expression on human platelets has been discussed and platelet activation by PGE_2 suspected, yet could only be shown with synthetic, selective prostanoid EP_3 receptor agonists and antagonists. Only recently effects of PGE_2 have been investigated with regard to platelet activation (Iyu et al., 2011b), however mainly based on evidences from prostanoid EP_3 receptor inhibition. We can show that PGE_2 itself exerts a pro-aggregatory signal which, however is surmounted or prevented under usual experimental conditions by the strong inhibitory effect of AC stimulation through other prostaglandin receptors. Yet with the highly sensitive VASP phosphorylation assay (Geiger et al., 2010) the reciprocal regulation of platelet AC is revealed. While adenylyl cyclase inhibition is invisible under baseline conditions, it is unveiled by concomitant prostanoid IP_1 receptor stimulation. This is reflected by a parabolic dose–response curve composed of 3 individual dose–response curves with distinct EC50 and a negative slope for prostanoid EP_3 receptor stimulation. This remarkable pharmacological property may provide a highly sensitive fine tuning of platelet function depending on the PGE_2 concentration. While PGE_2 acts inhibiting on adenylyl cyclase at low concentrations, and by this promotes aggregation, this effect is inverted with increasing PGE_2 concentration by activation of AC and increasing cAMP levels. As the prostanoid EP_3 pathway is mediated by G_i protein it merges with the ADP P2Y12 receptor initiated pathway which is recognized being a pharmacological target for anti-platelet treatment of outstanding importance (Vivas and Angiolillo, 2010; Bhavaraju et al., 2010). PGE_2 potentiates this ADP stimulated AC inhibition and can – at least partially – compensate for ADP receptor inhibition by a P2Y12 antagonist. In consequence PGE_2 may contribute to attenuation of P2Y12 receptor inhibition drug efficacy.

In addition to the initial G_i mediated effect prostanoid EP_3 receptor stimulation evokes phosphorylation signals associated with platelet activating pathways which are further potentiated by threshold concentrations of secondary platelet agonists like ADP or thromboxane. Prostanoid EP_3 receptor mediated ERK-phosphorylation has been described being dependent on the prostanoid EP_3 receptor isoform (Israel and Regan, 2009). Since up to now no quantitative data on the expression of the respective isoforms in platelets are available the role of the isoforms in platelets cannot be assessed. From the evidence accumulated, the platelet prostanoid EP_3 receptor can be regarded as a platelet

activation potentiating receptor like the P2Y12 receptor (Gresele et al., 2008). Our results indicate that PGE_2 is – analogous to ADP – released from activated platelets. The PGE_2 concentration achieved by stimulation of the proteinase activated receptor PAR1 at physiological platelet density is about 5–10 nM, sufficient to stimulate prostanoid EP_3 receptors but not for adenylyl cyclase stimulation via prostanoid EP_4 or DP_1 receptors. Close to the platelet surface or in the narrow interspace between aggregating platelets (Guy and Fogelson, 2002) the PGE_2 concentration may be considerably higher due to limited diffusion. Thus PGE_2 can presumably provide an auto-inhibitory mechanism through stimulation of the G_s coupled prostanoid receptors surmounting G_i stimulation and, hence limiting agonist induced platelet activation. The reciprocal and concentration dependent action of PGE_2 on platelets may thus rely on distinct autocrine and paracrine effects. The observed compensation of platelet inhibition by prostanoid EP_3 receptor stimulation may contribute to reduced efficacy of anti-aggregatory drugs targeted at the P2Y12 receptor. Combined treatment with COX-1 inhibitors, such as acetylsalicylic acid, may enhance platelet inhibition not only by preventing TXA_2 but also PGE_2 synthesis. Consequently concomitant administration of a P2Y12 receptor inhibitor with a prostaglandin E synthase inhibitor or prostanoid EP_3 receptor antagonist should also increase the efficacy of P2Y12 receptor inhibitors.

5. Conclusions

In sum the prostaglandins I_2, D_2, E_1 and A_1 jointly prevent platelet activation through stimulation of AC and subsequent PKA activation while PGE_2 features a double-sided effect on platelets: pro-aggregatory by inhibition of AC and stimulation of platelet activating pathways and anti-aggregatory by stimulation of AC and subsequent disruption of platelet activating pathways. The hypothesized regulation by multiple receptors could be accurately described with a mathematical model by means of a single mathematical function. Analogous reciprocal platelet regulation has been described already for other pathways (Gambaryan et al., 2010). In sum, these observations indicate an intricate and complex signaling network in platelets providing means for a sensitive fine-tuning of platelet activation and inhibition by either preventing spontaneous, undesired platelet aggregation or promoting platelet activation by increasing platelet susceptibility to stimulatory signals through diminishing platelet inhibitory signaling.

Acknowledgment

The authors are indebted to Sabine Gätzner, Petra-Hönig Liedl, Erhard Kress, Claudia Schütz, and Maria Schwarz for their expertise and excellent technical help.

References

Abramovitz, M., Adam, M., Boie, Y., Carrière, M., Denis, D., Godbout, C., Lamontagne, S., Rochette, C., Sawyer, N., Tremblay, N.M., Belley, M., Gallant, M., Dufresne, C., Gareau, Y., Ruel, R., Juteau, H., Labelle, M., Ouimet, N., Metters, K.M., 2000. The utilization of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs. Biochim. Biophys. Acta 1483, 285–293.

Aktas, B., Utz, A., Hoenig-Liedl, P., Walter, U., Geiger, J., 2003. Dipyridamole enhances NO/cGMP-mediated vasodilator-stimulated phosphoprotein phosphorylation and signaling in human platelets – in vitro and in vivo/ex vivo studies. Stroke 34, 764–769.

Armstrong, R.A., 1996. Platelet prostanoid receptors. Pharmacol. Ther. 72, 171–191.

Barragan, P., Paganelli, F., Camoin-Jau, L., Bourguet, N., Boulay-Moine, D., Moulard, M., Bonello, L., 2010. Validation of a novel ELISA-based VASP whole blood assay to measure P2Y12-ADP receptor activity. Thromb. Haemost. 104, 410–411.

Begonja, A.J., Geiger, J., Rukoyatkina, N., Rauchfuss, S., Gambaryan, S., Walter, U., 2007. Thrombin stimulation of p38 MAP kinase in human platelets is mediated by ADP and thromboxane A_2 and inhibited by cGMP/cGMP-dependent protein kinase. Blood 109, 616–618.

Bhavaraju, K., Mayanglambam, A., Rao, A.K., Kunapuli, S.P., 2010. P2Y(12) antagonists as antiplatelet agents – recent developments. Curr. Opin. Drug Discov. Dev. 13, 497–506.

Boie, Y., Sawyer, N., Slipetz, D.M., Metters, K.M., Abramovitz, M., 1995. Molecular cloning and characterization of the human prostanoid DP receptor. J. Biol. Chem. 270, 18910–18916.

Breyer, R.M., Bagdassarian, C.K., Myers, S.A., Breyer, M.D., 2001. Prostanoid receptors: subtypes and signaling. Annu. Rev. Pharmacol. Toxicol. 41, 661–690.

Burkhart, J.M., Vaudel, M., Gambaryan, S., Radau, S., Walter, U., Martens, L., Geiger, J., Sickmann, A., Zahedi, R.P., 2012. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. Blood 120, e73–e82.

Dovlatova, N., Wijeyeratne, Y.D., Fox, S.C., Manolopoulos, P., Johnson, A.J., White, A.E., Latif, M.L., Ralevic, V., Heptinstall, S., 2008. Detection of P2Y(14) protein in platelets and investigation of the role of P2Y(14) in platelet function in comparison with the EP(3) receptor. Thromb. Haemost. 100, 261–270.

Fabre, J.E., Nguyen, M., Athirakul, K., Coggins, K., McNeish, J.D., Austin, S., Parise, L.K., FitzGerald, G.A., Coffman, T.M., Koller, B.H., 2001. Activation of the murine EP3 receptor for PGE_2 inhibits cAMP production and promotes platelet aggregation. J. Clin. Invest. 107, 603–610.

Gambaryan, S., Kobsar, A., Rukoyatkina, N., Herterich, S., Geiger, J., Smolenski, A., Lohmann, S.M., Walter, U., 2010. Thrombin and collagen induce a feedback inhibitory signaling pathway in platelets involving dissociation of the catalytic subunit of protein kinase A from an NFkappaB–IkappaB complex. J. Biol. Chem. 285, 18352–18363.

Garcia, A., Kim, S., Bhavaraju, K., Schoenwaelder, S.M., Kunapuli, S.P., 2010. Role of phosphoinositide 3-kinase beta in platelet aggregation and thromboxane A_2 generation mediated by Gi signalling pathways. Biochem. J. 429, 369–377.

Geiger, J., Nolte, C., Walter, U., 1994. Regulation of calcium mobilization and calcium entry in human platelets by cyclic nucleotide-elevating and endothelium-derived factors. Am. J. Physiol. 267 (Cell Physiol.) 36, C236–C244.

Geiger, J., Hönig-Liedl, P., Schanzenbächer, P., Walter, U., 1998. Ligand specificity and ticlopidine effects distinguish three human platelet ADP receptors. Eur. J. Pharmacol. 351, 235–246.

Geiger, J., Teichmann, L., Grossmann, R., Aktas, B., Steigerwald, U., Walter, U., Schinzel, R., 2005. Monitoring of clopidogrel action: comparison of methods. Clin. Chem. 51, 957–965.

Geiger, J., Brandmann, T., Hubertus, K., Tjahjadi, B., Schinzel, R., Walter, U., 2010. A protein phosphorylation based assay for screening and monitoring of drugs modulating cyclic nucleotide pathways. Anal. Biochem. 407, 261–269.

Geiger, J., Burkhart, J.M., Gambaryan, S., Walter, U., Sickmann, A., Zahedi, R.P., 2013. Response: platelet transcriptome and proteome—relation rather than correlation. Blood 121, 5257–5258.

Gresele, P., Falcinelli, E., Momi, S., 2008. Potentiation and priming of platelet activation: a potential target for antiplatelet therapy. Trends Pharmacol. Sci. 29, 352–360.

Guy, R.D., Fogelson, A.L., 2002. Probabilistic modeling of platelet aggregation: effects of activation time and receptor occupancy. J. Theor. Biol. 219, 33–53.

Heptinstall, S., Espinosa, D.I., Manolopoulos, P., Glenn, J.R., White, A.E., Johnson, A., Dovlatova, N., Fox, S.C., May, J.A., Hermann, D., Magnusson, O., Stefansson, K., Hartman, D., Gurney, M., 2008. DG-041 inhibits the EP3 prostanoid receptor—a new target for inhibition of platelet function in atherothrombotic disease. Platelets 19, 605–613.

Israel, D.D., Regan, J.W., 2009. EP3 prostanoid receptor isoforms utilize distinct mechanisms to regulate ERK 1/2 activation. Biochim. Biophys. Acta 1791, 238–245.

Iyú, D., Glenn, J.R., White, A.E., Fox, S.C., Dovlatova, N., Heptinstall, S., 2011a. $P2Y_{12}$ and EP3 antagonists promote the inhibitory effects of natural modulators of platelet aggregation that act via cAMP. Platelets 22, 504–515.

Iyú, D., Jüttner, M., Glenn, J.R., White, A.E., Johnson, A.J., Fox, S.C., Heptinstall, S., 2011b. PGE_1 and PGE_2 modify platelet function through different prostanoid receptors. Prostaglandins Other Lipid Mediat. 94, 9–16.

Jin, R.C., Voetsch, B., Loscalzo, J., 2005. Endogenous mechanisms of inhibition of platelet function. Microcirculation 12, 247–258.

Kuriyama, S., Kashiwagi, H., Yuhki, K., Kojima, F., Yamada, T., Fujino, T., Hara, A., Takayama, K., Maruyama, T., Yoshida, A., Narumiya, S., Ushikubi, F., 2010. Selective activation of the prostaglandin E_2 receptor subtype EP2 or EP4 leads to inhibition of platelet aggregation. Thromb. Haemost. 104, 796–803.

Linden, M.D., Jackson, D.E., 2010. Platelets: pleiotropic roles in atherogenesis and atherothrombosis. Int. J. Biochem. Cell Biol. 42, 1762–1766.

Mischnik, M., Boyanova, D., Hubertus, K., Geiger, J., Philippi, N., Dittrich, M., Wangorsch, G., Timmer, J., Dandekar, T., 2013. A Boolean view separates platelet activatory and inhibitory signalling as verified by phosphorylation monitoring including threshold behaviour and integrin modulation. Mol. Biosyst. 9 (6), 1326–1339.

Patrono, C., Rocca, B., 2010. The future of antiplatelet therapy in cardiovascular disease. Annu. Rev. Med. 61, 49–61.

Petrucci, G., De Cristofaro, R., Rutella, S., Ranelletti, F.O., Pocaterra, D., Lancellotti, S., Habib, A., Patrono, C., Rocca, B., 2011. Prostaglandin E_2 differentially modulates human platelet function through the prostanoid EP2 and EP3 receptors. J. Pharmacol Exp Ther. 336, 391–402.

Philipose, S., Konya, V., Sreckovic, I., Marsche, G., Lippe, I.T., Peskar, B.A., Heinemann, A., Schuligoi, R., 2010. The prostaglandin E_2 receptor EP4 is expressed by human

platelets and potently inhibits platelet aggregation and thrombus formation. Arterioscler. Thromb. Vasc. Biol. 30, 2416–2423.
Radziszewski, W., Chopra, M., Zembowicz, A., Gryglewski, R., Ignarro, L.J., Chaudhuri, G., 1995. Nitric oxide donors induce extrusion of cyclic GMP from isolated human blood platelets by a mechanism which may be modulated by prostaglandins. Int. J. Cardiol. 51 (3), 211–220.
Rowley, J.W., Oler, A.J., Tolley, N.D., Hunter, B.N., Low, E.N., Nix, D.A., Yost, C.C., Zimmerman, G.A., Weyrich, A.S., 2011. Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. Blood 118, e101–e111.
Schober, L.J., Khandoga, A.L., Dwivedi, S., Penz, S.M., Maruyama, T., Brandl, R., Siess, W., 2011. The role of PGE(2) in human atherosclerotic plaque on platelet EP (3) and EP (4) receptor activation and platelet function in whole blood. J. Thromb. Thrombolysis 32, 158–166.
Schultess, J., Danielewski, O., Smolenski, A.P., 2005. Rap1GAP2 is a new GTPase-activating protein of Rap1 expressed in human platelets. Blood 105, 3185–3192.
Schweigel, H., Geiger, J., Beck, F., Buhs, S., Gerull, H., Walter, U., Sickmann, A., Nollau, P., 2013. Deciphering of ADP-induced, phosphotyrosine-dependent signaling networks in human platelets by Src-homology 2 region (SH2)-profiling. Proteomics 13, 1016–1027.
Singh, J., Zeller, W., Zhou, N., Hategen, G., Mishra, R., Polozov, A., Yu, P., Onua, E., Zhang, J., Zembower, D., Kiselyov, A., Ramirez, J.L., Sigthorsson, G., Bjornsson, J. M., Thorsteinsdottir, M., Andresson, T., Bjarnadottir, M., Magnusson, O., Fabre, J.-E., Stefansson, K., Gurney, M.E., 2009. Antagonists of the EP3 receptor for prostaglandin E_2 are novel antiplatelet agents that do not prolong bleeding. ACS Chem. Biol. 4, 115–126.
Smolenski, A., 2012. Novel roles of cAMP/cGMP-dependent signaling in platelets. J. Thromb. Haemost. 10, 167–176.
Straus, D.S., Glass, C.K., 2001. Cyclopentenone prostaglandins: new insights on biological activities and cellular targets. Med. Res. Rev. 21 (3), 185–210.
Subramanian, H., Zahedi, R.P., Sickmann, A., Walter, U., Gambaryan, S., 2013. Phosphorylation of CalDAG-GEFI by protein kinase A prevents Rap1b activation. J. Thromb. Haemost. 11, 1574–1582.
Trist, D.G., Collins, B.A., Wood, J., Kelly, M.G., Robertson, A.D., 1989. The antagonism by BW A868C of PGD_2 and BW245C activation of human platelet adenylate cyclase. Br. J. Pharmacol. 96, 301–306.
Tsuboi, K., Sugimoto, Y., Ichikawa, A., 2002. Prostanoid receptor subtypes. Prostaglandins Other Lipid Mediat. 68–69, 535–556.
Vezza, R., Roberti, R., Nenci, G.G., Gresele, P., 1993. Prostaglandin E_2 potentiates platelet aggregation by priming protein kinase C. Blood 82, 2704–2713.
Vivas, D., Angiolillo, D.J., 2010. Platelet $P2Y_{12}$ receptor inhibition: an update on clinical drug development. Am. J. Cardiovasc. Drugs 10, 217–226.
von Papen, M., Gambaryan, S., Schütz, C., Geiger, J., 2013. Determination of ATP and ADP secretion from human and mouse platelets by an HPLC assay. Transfus. Med. Hemother. 40, 109–116.
Wagner, D.D., Burger, P.C., 2003. Platelets in inflammation and thrombosis. Arterioscler. Thromb. Vasc. Biol. 23, 2131–2137.
Wangorsch, G., Butt, E., Mark, R., Hubertus, K., Geiger, J., Dandekar, T., Dittrich, M., 2011. Time-resolved in silico modeling of fine-tuned cAMP signaling in platelets: feedback loops, titrated phosphorylations and pharmacological modulation. BMC Syst. Biol. 5, 178.
Watson, S.P., Bahou, W.F., Fitzgerald, D., Ouwehand, W., Rao, A.K., Leavitt, A.D., 2005. Mapping the platelet proteome: a report of the ISTH platelet physiology subcommittee. J. Thromb. Haemost. 3, 2098–2101.
Wise, H., Wong, Y.H., Jones, R.L., 2002. Prostanoid signal integration and cross talk. Neurosignals 11, 20–28.
Zhu, Y., Gu, Z.L., Liang, Z.Q., Zhang, H.L., Qin, Z.H., 2006. Prostaglandin A_1 inhibits increases in intracellular calcium concentration, TXA(2) production and platelet activation. Acta Pharmacol. Sin. 27, 549–554.