Gradient-dependent inhibition of stimulatory signaling from platelet G protein-coupled receptors

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Supplementary information

Methods

Materials

PAR-activating peptides SFLLRN (PAR1-AP) and AYPGKF (PAR4-AP) and fibrin polymerization inhibitor peptide GPRP were synthesized by JPT Peptide Technologies, Berlin, Germany. Human α-thrombin, ADP, PGI_2, PGE1, apyrase (grade III) and U46619 were purchased from Sigma-Aldrich, St Louis, USA. H89 was from Selleck Chemicals, SMS-gruppen, Denmark. Dynasore was purchased from Tocris Bioscience, Bristol, United Kingdom. Milrinone was purchased from Abcur AB, Helsingborg, Sweden. Cross-linked collagen related peptide (CRP-XL) was provided kindly by prof. Farndale, Cambridge, UK, cangrelor was provided by the Medicines company, Parsippany, NJ, USA, acetylsalicylic acid and NaCl 9mg/mL were from Braun, Melsungen, Germany, tirofiban (Aggrastat®) was from Correvio International Sàrl, Switzerland, HEPES buffer, Fluo-4, Alexa Fluor® 546 Phalloidin and Nupage 4-12% bis tris gels were from Fisher Scientific AB, Stockholm, Sweden, Amersham ECL gel 4-12% was from GE Healthcare, Uppsala, Sweden, Immun-Blot® PVDF Membrane was from Bio-Rad Laboratories AB, Solna, Sweden, anti-CD41a-PE, anti-CD62P-PE or PECy5 with corresponding isotype antibodies were from BD Biosciences, San Jose, CA, WEDE15 monoclonal antibody and anti-CD41-PE-Texas Red-X (ECD) was from Beckman Coulter AB, Bromma, Sweden, Immobilon Western Chemiluminescent HRP Substrate and anti-Phosphoserine antibody (AB-1603) was from Merck chemicals and life science AB, Sweden, VASP (3112), phospho-VASP (Ser157) (3111), Akt (pan) (40D4) (2920), Phospho-Akt (Ser473) (D9E) XP® (4060) and secondary antibodies were from Cell Signaling Technology, USA. Breast cancer epithelial cell line MDAMB231 was kindly provided by Prof. Olle Stål, Linköping University, Sweden. Glutaraldehyde, osmium tetroxide, uranyl acetate and lead citrate were purchased from Polysciences Europe GmbH, Germany. Propylene oxide and Spurr Low-Viscosity Embedding Kit were purchased from Sigma Aldrich, St. Louis, USA.

Blood collection and sample preparation

Whole blood from random healthy adult volunteers was collected in hirudin-containing tubes (Roche Diagnostic, Mannheim, Germany) as per the local Ethics Committee of Linköping University Hospital. For experiments with thrombin, blood was collected in tubes containing 1/10 volume of 3.2% sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifuging the blood at 150 x g for 15 minutes and platelet-poor plasma (PPP) was prepared by centrifugation at 2500 x g for 15 minutes. For platelet isolation, blood was collected in Vacuette® tube, 9 ml ACD-A (Greiner Bio-One GmbH, Frickenhausen, Germany). It was centrifuged at 150 × g for 15 minutes for PRP. The PRP was collected and supplemented with apyrase (0.25 U/mL) and 1 µM prostacyclin (PGI_2) and centrifuged again at 480 × g for 20 minutes. After gently replacing the plasma phase with Krebs-Ringer Glucose (KRG; 120.24 mM NaCl, 5.02 mM KCl, 1.24 mM $MgSO_4.7H_2O$, 8.47 mM $Na_2HPO_4.2H_2O$, 10 mM glucose and 1.73 mM KH_2PO_4, PH 7.3) supplemented with PGI_2, the platelet pellet was resuspended. Platelet density was adjusted to

~2.5×10^8 cells/mL with KRG. Extracellular Ca2+ concentration was adjusted to 1.8 mM and incubated at RT for 30 mins. The platelet suspension obtained was used within 3 hours.

Light transmission aggregometry

To eliminate the contribution of ADP and thromboxane pathways in gradient dependent inhibition (GDI), aggregation was checked in presence of MRS 2179 (5µM), cangrelor (1µM) and acetylsalicylic acid (ASA, 100µM) which are inhibitors of P2Y1, P2Y12 and cyclooxygenase (COX), respectively. All inhibitors were added into the PRP together and incubated for 10 mins at RT prior to the experiments. For experiments where thrombin was used as an agonist, PRP was supplemented with 4 mM GPRP. To confirm the effect of thrombin, Protease-activated receptor 1 activating peptide (PAR1-AP) 30µM and PAR4-AP 300 µM were infused together into the PRP at different infusion rates. To check the heterologous activation of platelets after GDI, platelets were infused with either PAR1-AP (320s), PAR4-AP (1280s) or ADP (640s) at GDI gradients and then challenged with other receptor agonists at C_{agg} concentration and 2s rate, 1 min after the infusion was over. Different inhibitors or compounds were checked for their effects on GDI. Briefly, for cAMP pathway analysis, protein kinase A (PKA) inhibitor H89 (30µM), PGI_2 (0.01, 0.1 or 1nM), epinephrine (0.1, 1 or 10µM), or milrinone (phosphodiesterase-3 inhibitor; 3µM) were checked. PGI2 and epinephrine were added 1 min before the infusion, whereas, H89 and milrinone were incubated for 10 mins with PRP at RT before the infusion of agonists. To check the internalization of PAR1 receptor in GDI platelets, the effect of dynamin inhibition was determined by incubating the PRP with dynasore (80µM) for 10 mins before the infusion. Statistical analysis and graphs were prepared by using graph pad prism 7 (GraphPad Software, Inc., La Jolla, USA).

All agonists used in the study were tested for their stability under experimental conditions in PRP. Agonists were incubated in platelet free plasma (PPP) at 37°C for 640 or 1280s and then used in aggregometry experiment to induce aggregation in platelet rich plasma (PRP) and all agonists except Protease-activated receptor 1 activating peptide (PAR1-AP, SFLLRN) were stable for 640 and 1280s (Table S1). PAR1-AP stability was therefore confirmed using washed platelets in KRG-buffer (Table S2) where, it was found to be stable. Briefly, PAR1-AP was incubated for different time intervals in KRG buffer or platelet poor plasma (PPP) at 37°C for different time length and then checked for its ability to induce aggregation in either washed platelets or PRP, respectively (Table S2). Since, PAR1-AP was found to be stable in KRG buffer, aggregation experiments at 640s and 1280s were confirmed using washed platelets which resulted in similar outcome to that observed in PRP (Fig. S11), verifying the results shown for PRP. Additionally, to exclude the possibility of gradual breakdown of ADP by enzymes on or within the platelets during the longer infusion times, control experiments were performed with the metabolically stable ADP analogue 2-Methylthioadenosine diphosphate (MeSADP), which produced similar results to ADP, confirming reduced aggregatory responses at longer infusion times (Fig S12).

Flow cytometry

The effects of agonist gradients on platelet alpha granule release were assessed by taking aliquots from samples identical to those used in the aggregometry experiments except for the inclusion of a step wherein samples were pre-incubated with 1 µM tirofiban for 10 minutes at room temperature to prevent aggregation. Samples were collected 1 minute after completion of agonist infusion, labeled and analyzed by flow cytometry on a Gallios™ flow cytometer (Beckman Coulter Inc., Fullerton, CA, USA). Briefly, aliquots of 3 µL PRP were taken from the aggregometry experiment and added to 30 µL of HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1 mg/mL bovine serum albumin and 20 mM HEPES, pH 7.40) containing anti-CD41a-phycoerythrin (PE) (GPIIb; final concentration 1.5 µg/mL) and anti-CD62P-PE-Cyanine5 (Cy5) (P-selectin; final concentration 1.25 µg/mL). PE-Cy5 isotype antibody (final concentration 1.25 µg/mL) was used as a negative control. After incubation for 10 minutes in the dark, samples were diluted 1:20 with HEPES buffer and run in the flow cytometer. Forward scatter and anti-CD41 fluorescence was used to identify platelets. Platelet P-selectin expression was expressed as the percentage of PE-Cy5 positive platelets, with the fluorescence in the negative control samples set to ≤ 2%. To check the internalization of receptors in GDI platelets, receptor density was measured using the Platelet Calibrator kit from Biocytex, Marseille, France, as per manufacturer's protocol. WEDE15 (final concentration 10 µg/ml) monoclonal antibody from Beckman Coulter, Bromma, Sweden, was used for PAR1 as previously described.[1]

Western blotting

Levels of total serine phosphorylation, total and phosphorylated VASP (at S-157) or total and phosphorylated AKT (at S-473) were assessed by western blotting (WB). For activated samples, 2s infusion rate was used for all agonists whereas for GDI-induced samples, different rates (320 s for PAR1-AP, 640s for ADP and 1280s for PAR4-AP) were used. For resting samples, the same rate as used for GDI samples was used but saline was infused instead of agonist. For VASP phosphorylation studies, PRP was incubated with either 2.6 µM prostacyclin (PGI_2) or 30 µM H89 (PKA inhibitor) for 10 minutes at RT prior to the aggregation experiment using 30µM PAR1-AP. Samples were taken out 1 min after the infusion, supplemented with protease and phosphatase inhibitor cocktail, briefly centrifuged to remove plasma and western blotting was performed using standard procedures. To check the efficacy of H89, PRP was incubated with H89 for 15 mins at RT and then treated with 10 or 100nM PGI_2 for 1 min. VASP phosphorylation at S-157 was then measured by WB (Fig. S3B). To check whether prostacyclin receptor (IP) exhibits GDI, it was stimulated at different infusion gradients as described for aggregometry and the activation was measured by western blotting. Since, PGI_2 is not very stable in aqueous solutions[2], its stable analog PGE_1 was used instead to stimulate the IP receptor. PGE_1 diluted in saline was infused to PRP (100nM final concentration) at three different infusion rates 2, 160 or 1280s and after the infusion, VASP phosphorylation at S-157 was measured by western blotting (Fig. S3A). To confirm the stability of PGE_1 in saline during the time course of the experiment, PGE_1 diluted in saline was incubated for 30 mins at RT and then used to induce VASP phosphorylation in PRP which is shown as control in fig. S3A. For WB, briefly, samples were separated using 4-12% gels and transferred to PVDF Membrane. After blocking,

membranes were incubated overnight at 4°C with gentle shaking with primary antibody and after washing, with their respective secondary antibody for 2 hours at room temperature with gentle shaking. The blots were then developed using chemiluminescent HRP substrate.

Fluorescence microscopy

Resting, activated and GDI-induced platelets for PAR1-AP were visualized by fluorescence microscopy after staining of F-actin as per manufacturer's protocol. Briefly, after aggregometry, platelets were immediately fixed using 3.7% formaldehyde solution in PBS for 10 minutes at room temperature. Samples were then washed thrice with PBS and permeabilized with 0.1% Triton X-100 in PBS for 3 minutes. Samples were stained with Alexa Fluor® 546 Phalloidin for 20 mins, washed and were visualized under 63x/1.4 oil immersion objective in a Zeiss Axio Observer Z1 with a Colibri LED-module and a Neo 5.5 sCMOS camera (Andor Technology Ltd., UK) controlled by μManager software (Vale lab, University of California, San Francisco. USA).

Electron microscopy

Electron microscopy was used to visualize the differences between resting, activated and GDI-induced platelets at a subcellular level. Briefly, after aggregometry, platelets were immediately centrifuged at 1000xg for 2mins to remove plasma, washed once with PBS and fixed for 2 hours at room temperature in 3% glutaraldehyde in sodium phosphate buffer (pH 7.4 and osmolarity 330 mOsmol). The pellet was washed with phosphate buffer and post fixed in 1% Osmium tetroxide for 1 hour at 4 °C. After post fixation the pellet was washed in sodium phosphate buffer and dehydrated in a series of ascending concentration of ethanol, followed by propylene oxide. After a four-step infiltration, specimens were embedded in Spurr Low-Viscosity Embedding Kit. For electron microscopy, ultrathin sections (70 nm thickness) were cut by using a Reichert Ultracut S (Leica, Wien, Austria) ultra-microtome. Sections were collected onto formvar-coated slot grids and were counterstained with uranyl acetate and lead citrate. The observation and examination of the sections took place on a 100kV transmission electron microscope (EM JEM 1230, JEOL Ltd., Tokyo, Japan).

Measurements of intracellular calcium mobilization

Agonist concentration gradient-dependent effects on intracellular calcium mobilization was assessed by a spectrofluorometric method using the calcium binding dye Fluo-4 AM. Detection was performed in an Enspire™ fluorescence plate reader (PerkinElmer, Waltham, US). Each experiment was performed in duplicate with a minimum of five samples from different donors. Three different gradients (low, medium and high) were used for each agonist, based on results from the aggregation experiments (2, 160 & 640s for PAR1-AP and ADP and 2, 320 & 1280s for PAR4-AP & thromboxane A_2 receptor (TPα) agonist U46619. Briefly, 20 μL PRP was diluted to 180 μL with HEPES buffer and pre-incubated with the calcium indicator dye, Fluo-4 AM for 20 minutes at 37°C with mild shaking intervals. Constant agonist gradients were reproduced by 20 incremental additions of 1 μL of concentrated agonist solution with constant time intervals. Gradient-dependent effects on intracellular calcium transients in epithelial cells were assessed similarly using MDAMB231, a human breast cancer cell line. Briefly, 180 μL cells (~0.1 million) in media (DMEM High Glucose (4. 10% FBS, 1 mmol/L glutamine) was taken in the

tissue culture compatible 96 well plate and incubated for 20 mins with Fluo4-AM at 37°C. Calcium mobilization by activation of PAR1 receptor was measured as mentioned for platelets with three different infusion rates (2, 160 & 640s) using 30 µmol/L PAR1-AP. The experiment was performed three times in duplicates.

Figures

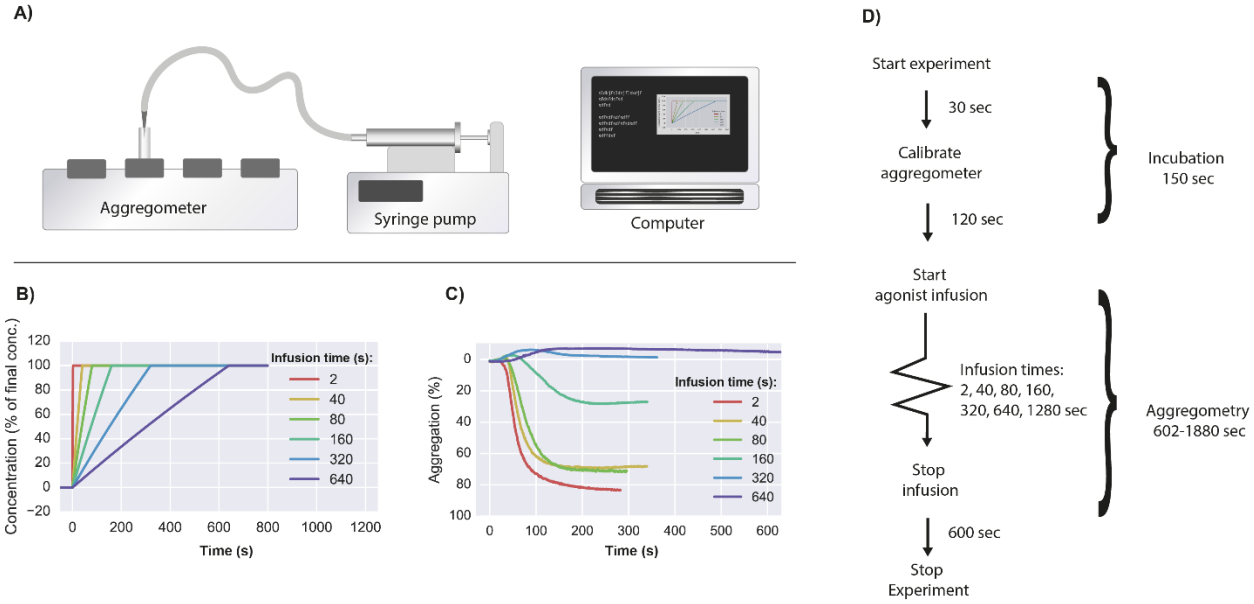

Fig. S1: Instrumental setup and experimental protocol

To investigate the gradient-dependent effects on platelet aggregatory responses, a computer-controlled syringe pump was fitted into a conventional aggregometer (A). Different concentration gradients were obtained by adjusting the infusion time of the tested agonists (B), and the aggregatory response was continuously monitored in the aggregometer (C). The experimental protocol was standardized according to the flow chart in (D) to allow for direct comparisons between runs.

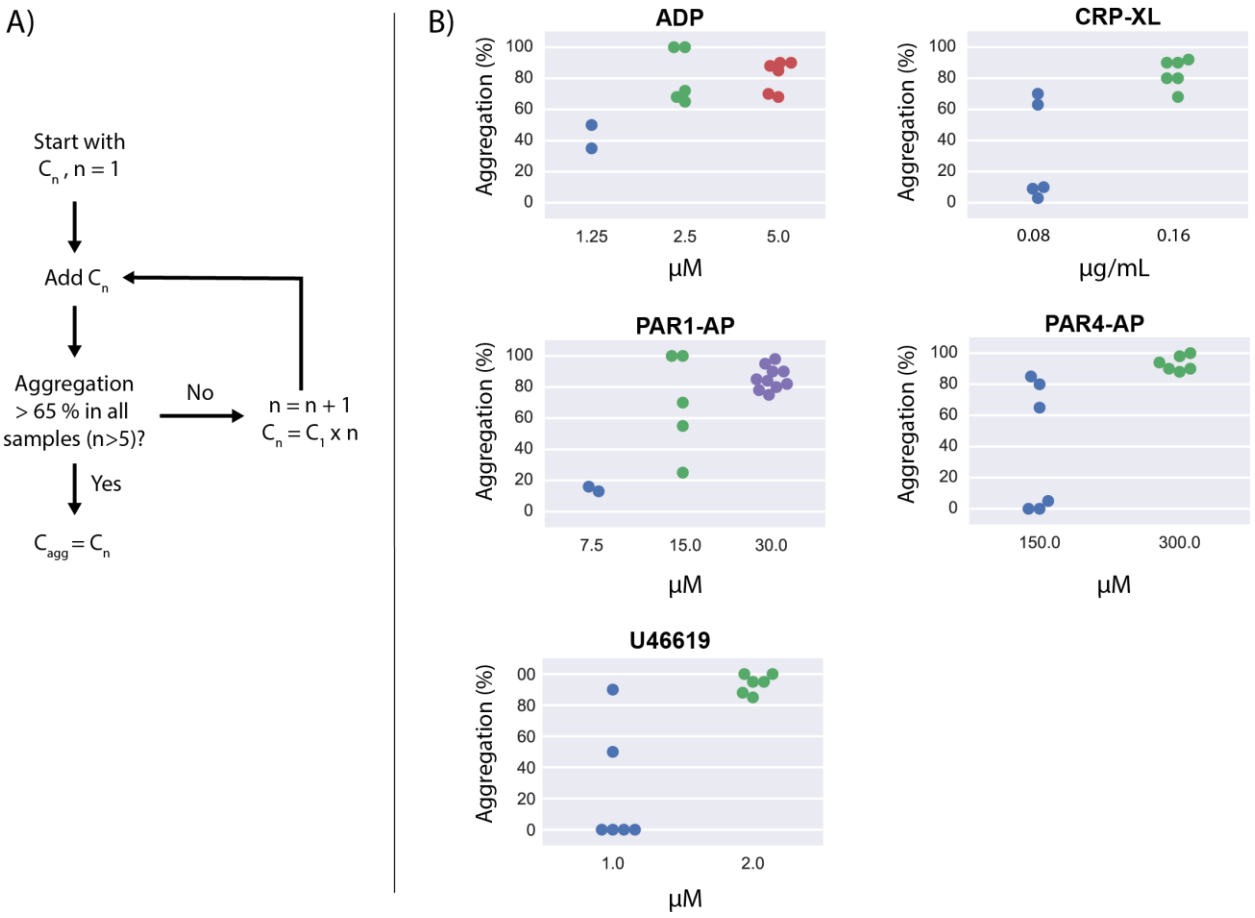

Fig. S2: Evaluation of optimal agonist concentrations
A) Algorithm to determine C_{agg}, the minimal concentrations of each agonist needed to give > 65% aggregation in all samples (n>5) B) Platelet aggregation response to the agonist concentrations tested using the algorithm in (A). The highest agonist concentrations presented in (B) are the C_{agg} agonist concentrations.

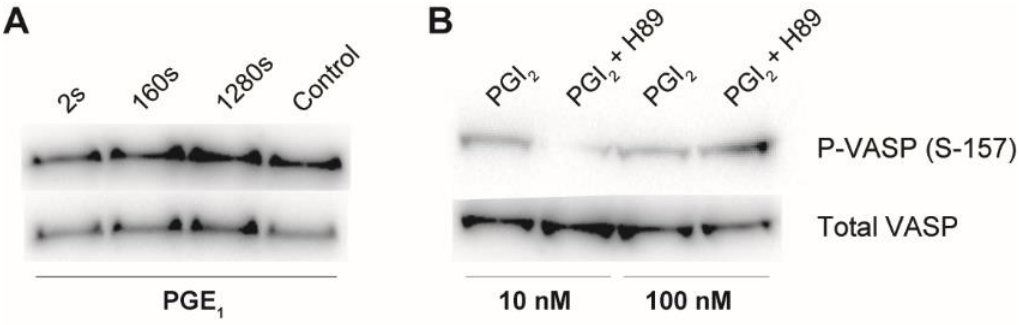

Fig. S3: Effect of GDI on IP receptor and inhibitor efficacy check
(A) To check the effect of infusion gradients on prostacyclin receptor (IP), PGE_1 was infused to PRP at three different rates (2, 160 or 1280s) and VASP phosphorylation was measured by western blotting. PGE_1 diluted and incubated in saline for 30 mins at RT (control) was used to induce VASP phosphorylation to rule out the possibility of PGE_1 degradation. (B) To check the efficacy of the PKA inhibitor, PRP incubated with 30µM H89 for 10 mins was treated with either 10 or 100nM of PGI_2 for 1 min and phosphorylation of VASP at S-157 was measured. Total VASP levels was used as control for both the experiments.

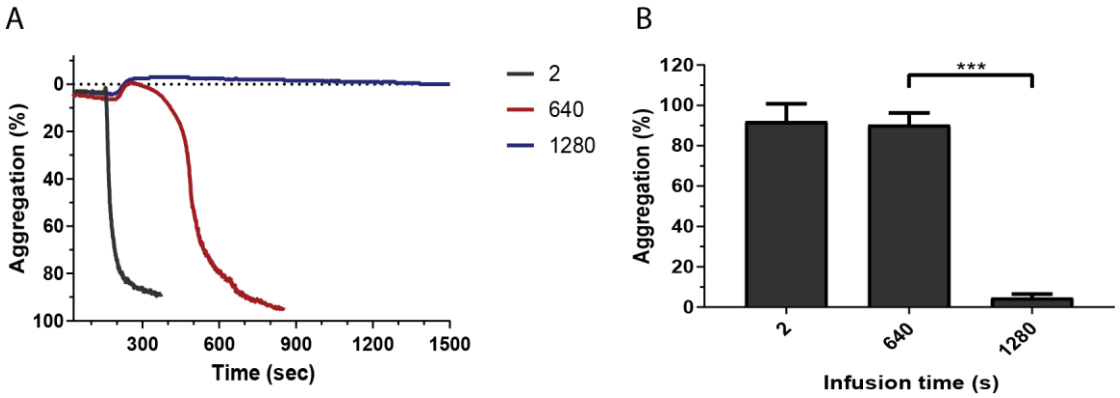

Fig. S4: Effect of GDI on aggregation by PAR1-AP+PAR4-AP
(A) Representative aggregation curves by PAR1-AP+PAR4-AP (30+300µM) at three different infusion rates in PRP from hirudinized blood and (B) the bar graph showing results from n≥3 experiments with error bar indicating SD and stars indicate significance.

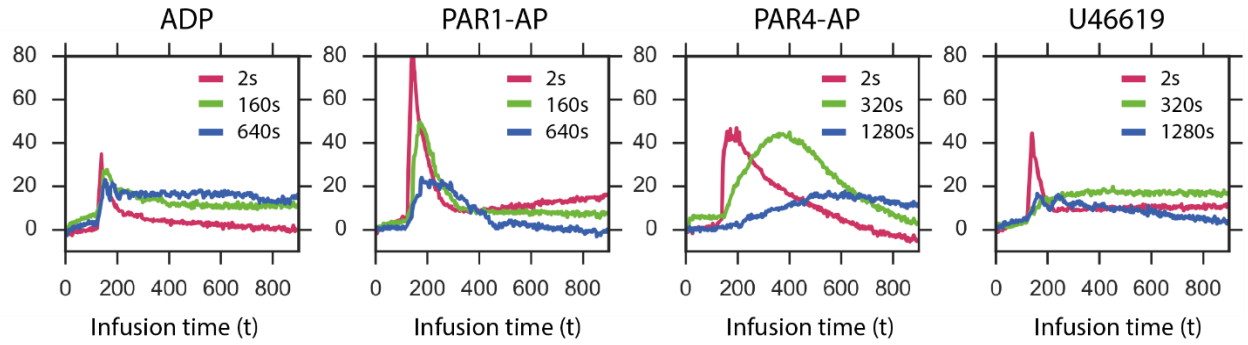

Fig. S5: Effect of GDI on calcium mobilization
Calcium mobilization in PRP was measured using the fluorescent calcium probe Fluo-4. Three different infusion times (representing high, medium and low agonist gradients) were selected for each agonist depending on the extent of gradient-dependent modulation of platelet activation observed at different infusion times in the aggregometry experiments. All the values were normalized to the zero time point, fluorescence from saline (background) was subtracted and the resulting values were presented here as percentage. Each line represents the average of at least 4 different measurements.

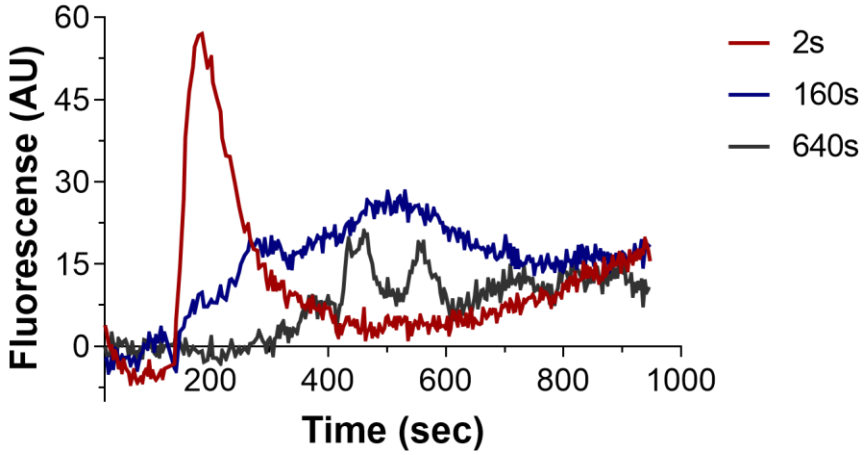

Fig. S6: Effect of agonist gradients on intracellular calcium mobilization in epithelial cells
Calcium mobilization by activation of the PAR1 receptor on epithelial cells was measured as done for platelets with three different infusion rates (2, 160 & 640s) using 30 µM PAR1-AP. The experiment was performed three times in duplicates and representative curves from one experiment are shown.

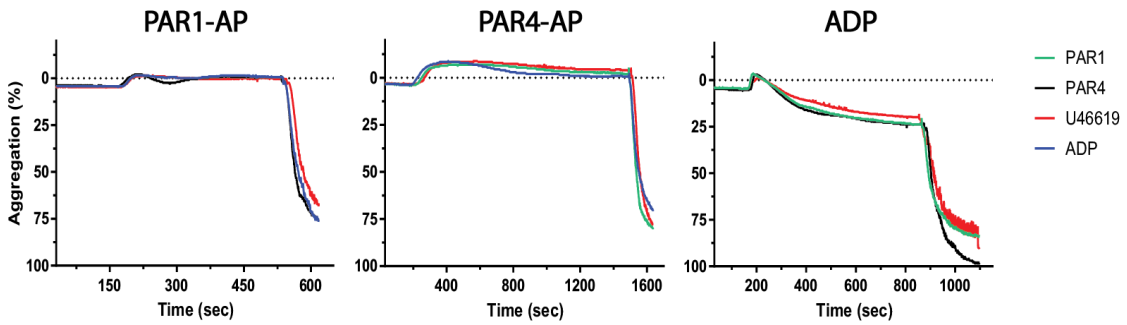

Fig. S7: Heterologous activation after GDI
Effect of heterologous agonists at 2s infusion rate on platelets inhibited by GDI at their respective ΔC_{nres} for PAR1-AP (320s), PAR4-AP (1280s) or ADP (640s). Colors indicate the second agonist used to induce aggregation in GDI platelets.

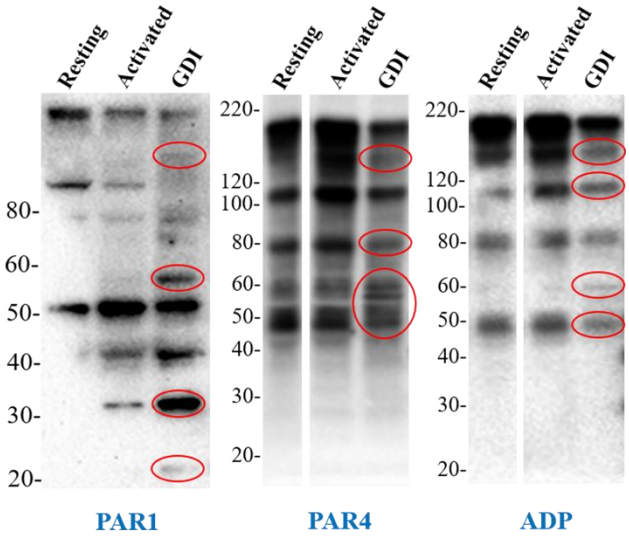

Fig. S8: Total serine phosphorylation in resting, activated and GDI platelets
Changes in phosphorylation patterns at serine residues in resting, activated (2s) or GDI (320s, 1280s and 640s for PAR1-AP, PAR4-AP and ADP, respectively) platelets for different receptors. Protein bands in GDI platelets that differ from their respective resting or activated samples are encircled.

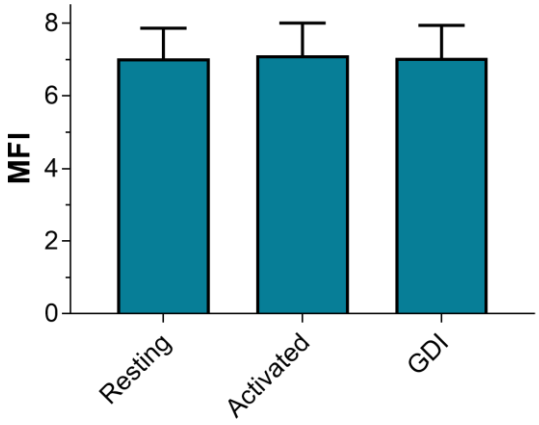

Fig. S9: PAR1 receptor density
Receptor density of PAR1 on the platelet surface in resting, activated and GDI platelets determined by flow cytometry as median fluorescence intensity. Error bars indicate standard deviation (n=3).

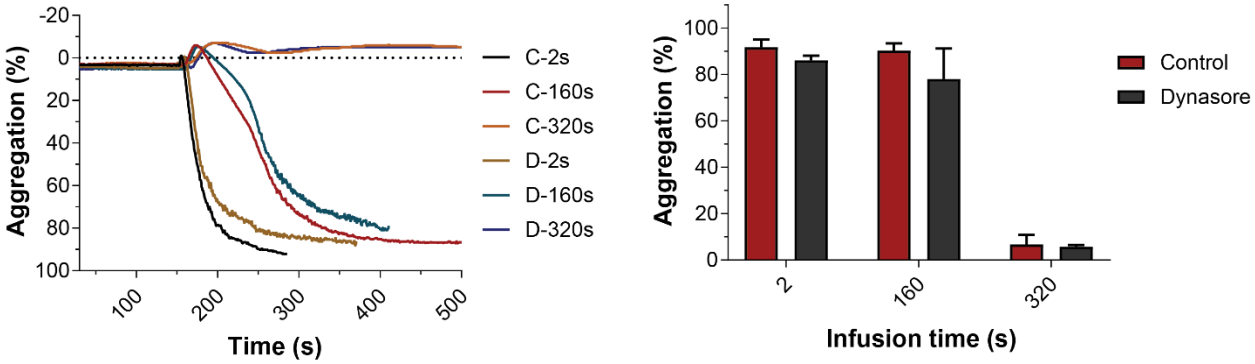

Fig. S10: Effect of dynamin inhibition on GDI
Effect of dynamin inhibitor (dynasore, 80µM) on 30µM PAR1-AP mediated aggregation at different infusion rates are shown as representative aggregation curves and a bar graph showing results from n≥3 experiments with error bars showing standard deviation. C: control, D: dynasore.

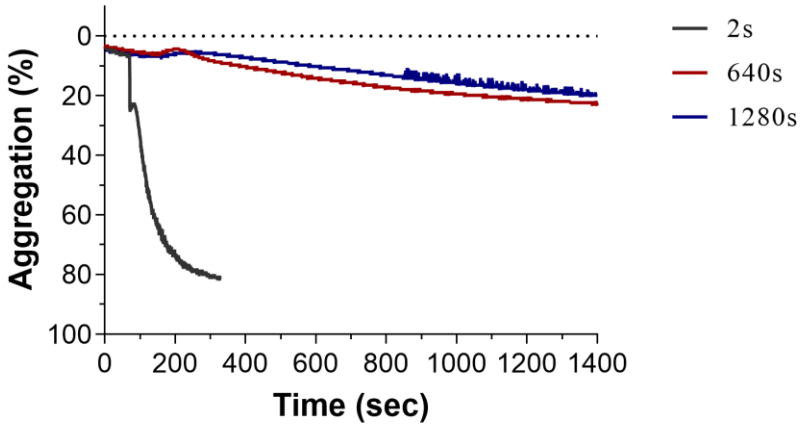

Fig. S11: Stability of PAR1-AP

To confirm, that limited stability of PAR1-AP in PRP has not affected the results, aggregation experiments where longer infusion times (640s & 1280s) had been used were also performed in washed platelets in KRG-buffer, where the peptide agonist stability was not an issue. The results were very similar to the results obtained with PRP. Representative results from n=3 experiments.

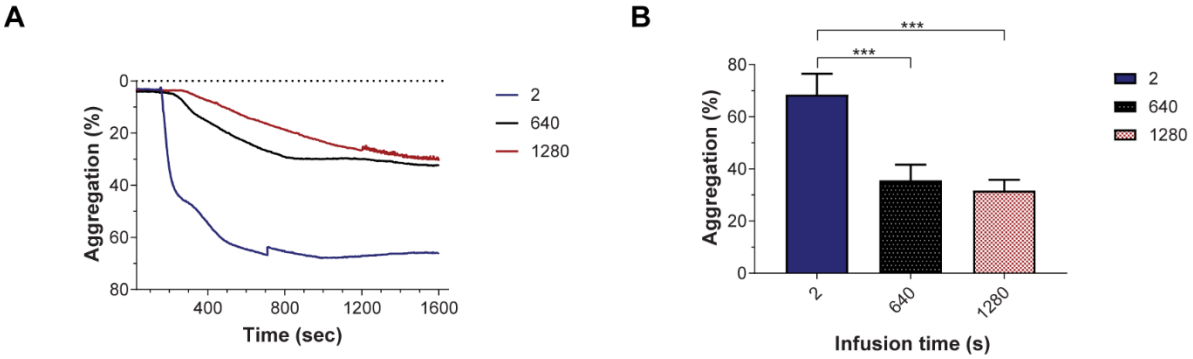

Fig. S12: GDI with MeSADP

To rule out the possibility of degradation of ADP by nucleases of platelets, metabolically stable ADP, MeSADP (30nM) was used to confirm the GDI effect by measuring the aggregation at 2, 640 or 1280 sec infusion rates, which produced similar outcome as physiological ADP. (A) Mean aggregation response curves at different infusion rates and (B) max aggregation at different rates, data represent mean ± s.d., n=4, ***P<0.001.

Tables

Table S1: Stability of agonists
Aggregation by different agonists in PRP after incubation in PPP for 640s or 1280s at 37°C. Saline was used as control.

Agonist	Aggregation					
	after 640s			after 1280s		
	No. of experiments	Yes	No	No. of experiments	Yes	No
Saline	4	0	4	2	0	2
PAR1-AP	6	4	2	3	0	3
PAR4-AP	5	5	0	2	2	0
ADP	2	2	0	2	2	0
U46619	1	1	0	2	2	0
CRP-XL	0	0	0	2	2	0

Table S2: Stability of PAR1-AP
Aggregation induced by PAR1-AP in PRP after incubation in PPP or in washed platelets after incubation in KRG buffer for different time lengths at 37°C. PAR1-AP was found to be stable only until 300s in PPP, however, was stable in KRG buffer for 30 mins.

Incubation (min)	Aggregation by PAR1-AP					
	in PRP			in washed platelets		
	No. of experiments	Yes	No	No. of experiments	Yes	No
0	3	3	0	2	2	0
5	2	2	0			
10	3	1	2			
15	3	1	2	2	2	0
20	1	0	1			
30	2	0	2	2	2	0

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