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

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Short title
Gradient-dependent inhibition of GPCRs

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Abstract

As platelet activation is an irreversible and potentially harmful event, platelet stimulatory signaling must be tightly regulated to ensure the filtering-out of inconsequential fluctuations of agonist concentrations in the vascular milieu. Herein, we show that platelet activation via G protein-coupled receptors is gradient-dependent that is determined not only by agonist concentrations \textit{per se} but also by how rapidly concentrations change over time. We demonstrate that gradient-dependent inhibition is a common feature of all major platelet stimulatory G protein-coupled receptors, while platelet activation via the non-G protein-coupled receptor glycoprotein VI is strictly concentration-dependent. By systematically characterizing the effects of variations in temporal agonist concentration gradients on different aspects of platelet activation, we demonstrate that gradient-dependent inhibition of protease-activated receptors exhibit different kinetics, with platelet activation occurring at lower agonist gradients for protease-activated receptor 4 than for protease-activated receptor 1, but share a characteristic bimodal effect distribution, as gradient-dependent inhibition increases over a narrow range of gradients, below which aggregation and granule secretion is effectively shut off. In contrast, the effects of gradient-dependent inhibition on platelet activation via adenosine diphosphate and thromboxane receptors increase incrementally over a large range of gradients. Further, depending on the affected activation pathway, gradient-dependent inhibition results in different degrees of refractoriness to subsequent autologous agonist stimulation. Mechanistically, our study identifies an important role for the cyclic adenosine monophosphate-dependent pathway in gradient-dependent inhibition. Together, our findings suggest that gradient-dependent inhibition may represent a new general mechanism for hemostatic regulation in platelets.
Introduction
In platelets, G protein-coupled receptors (GPCRs) mediate activation in response to stimulation with multiple important soluble agonists, including thrombin, ADP and thromboxane \( \text{A}_2 \). These signaling events are critical for triggering platelet hemostatic activities such as adhesion, granule exocytosis, aggregation, procoagulant activity and clot retraction. Hence, they must be tightly regulated to ensure efficient hemostasis while concurrently avoiding undue activation, which could potentially lead to excessive clot growth and thus thrombosis, vessel occlusion or embolization. The importance of platelet GPCRs in the pathophysiology of arterial thrombosis is demonstrated by the thrombo-protective effects of inhibitory drugs targeting GPCR-mediated pathways, such as clopidogrel, prasugrel, ticagrelor (ADP-receptor P2Y\(_{12}\)), aspirin (thromboxane synthesis), and vorapaxar (thrombin receptor PAR1).

Vascular damage is associated with a localized rapid increase in the concentrations of soluble agonists acting on platelet stimulatory GPCRs. By contrast, concentrations of such agonists outside the core of a forming hemostatic plug change slowly due to dilution, mechanically restricted diffusion and agonist degradation. Recent studies of intra-thrombus architecture have shown that spatial differences in thrombus porosity result in distinct diffusion rates of solutes, leading to heterogeneous concentration gradients of soluble agonists in different regions inside and outside a developing thrombus. In pathological conditions that affect thrombus consolidation and contraction, diffusion of soluble agonists to regions outside the thrombus core is increased, resulting in altered spatial and temporal distributions of agonists.

In this study, we hypothesized the presence of a gradient-dependent gating mechanism for platelet activation by soluble agonists. Gradient-sensing mechanisms are used in other cell types to regulate dynamic and complex cellular processes such as chemotaxis, and can be predicted to enhance the information processing ability of cells in relation to changes in the ambient stimulation level. For platelets, gradient-sensing could hypothetically enable dynamic modification of hemostatic responses according to the type of precipitating event and the relative position of a platelet in a developing thrombus. Gradient-dependent activation could ensure a robust activation response under conditions of rapidly increasing agonist concentrations, such as those encountered when a platelet is recruited from the blood stream to the core regions of a hemostatic plug. On the other side of the spectrum, gradient-dependent activation could also provide a mechanism for ensuring relative inertia in the face of a slow rise of agonist concentrations, as exemplified by platelets attaching to the peripheral shell regions of a consolidating thrombus. Such a mechanism could conceivably be of particular importance for regulating the platelet response to thrombin stimulation via the protease activated receptors (PAR1 and PAR4), since one thrombin molecule is capable of irreversibly activating an indeterminate number of PAR receptors by enzymatic receptor cleavage. Gradient-dependent modulation of PAR signaling could thus constitute a previously unidentified mechanism for equilibrating a signaling machinery otherwise inherently tilted towards unchecked platelet activation.

To test our hypothesis, we used novel instrumental setups to continuously monitor the platelet response to temporal agonist gradients (Fig. S1), enabling us to verify the presence of a
mechanism for gradient-dependent inhibition (GDI) of platelet activation involving activation of cAMP-dependent signaling mechanisms.

Methods
Blood collection and sample preparation
Whole blood from healthy adult volunteers was collected in hirudin or sodium citrate or acid citrate dextrose containing tubes as per the local Ethics Committee of Linköping University Hospital and platelet rich plasma (PRP) or washed platelets were prepared using standard procedures as described in supplementary information.

Light transmission aggregometry
Platelet aggregation was measured by light transmission aggregometry (LTA) using Chronolog Corporation model 490-X, Haverton, USA aggregometer. A pump controlled agonist infusion system (Fig. S1) was developed to generate constant temporal agonist concentration gradients and allow for continuous monitoring of platelet aggregation. In this system, 1 mL disposable plastic syringes were used in the syringe pumps and connected by fine tubing, of which the other end was directly immersed (≈3 mm) into the platelet-rich plasma (PRP) in the aggregometer cuvette via a custom-made cuvette adapter. A Matlab (MathWorks, Natick, USA) program was created in-house for controlling infusion rates and agonist loading (Fig. S1D). Predefined algorithms were followed for the parameters in the aggregometry experiments (Fig. 1A, 3A and S2A) to avoid the potential for bias associated with ad hoc experimental design. Based on that, aggregation was measured after infusing the same volume and concentration of agonists during either 2, 40, 80, 160, 320, 640 or 1280 seconds (s). The details of experimental conditions including use of various inhibitors and the stability of all the agonists used in the study under experimental conditions are described in supplementary information.

Flow cytometry
The effect of agonist gradients on platelet alpha granule release was 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 as described in supplementary information.

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 using standard procedures as described in supplementary information.

Fluorescence microscopy
Resting, activated and GDI-induced platelets by PAR1-AP were visualized by fluorescence microscopy after staining of F-actin as per manufacturer’s protocol as described in supplementary information.
Electron microscopy
Transmission electron microscopy was used to visualize the differences between resting, activated and GDI-induced platelets by PAR1-AP at subcellular level as described in supplementary information.

Results
A gradient-dependent mechanism modulates GPCR-mediated platelet activation
The minimal agonist concentration ($C_{agg}$) required to induce strong aggregation (> 65 %) in all samples ($n \geq 5$) with an infusion time of 2 s was determined (Table 1) using the algorithm shown in supplementary Fig. S2A (results in Fig. S2B). To verify the presence of GDI, we then sought to identify the highest concentration gradient ($\Delta C_{nres}$) at which no significant aggregation (< 25 %) was observed in $\geq 75$ % of samples at a final agonist concentration of $C_{agg}$ (algorithm in Fig. 1A). Raw curves from the aggregometry experiments used to define $\Delta C_{nres}$ for the different agonists are shown in Fig. 1B and a color map to aid visual interpretation is provided in Fig. 1C. Our results clearly show that platelet GPCR-mediated responses to PAR1-AP, PAR4-AP and ADP exhibit gradient dependence. In contrast, gradient dependence was not observed for the cross-linked collagen related peptide (CRP-XL, Figure 1B-D) or for the inhibitory signaling elicited by stimulation of the prostacyclin receptor (IP) with PGE1 (Fig. S3), even when using the longest infusion time of 1280s. For TP receptor agonist (U46619), large inter-individual differences in platelet reactivity to different agonist gradients precluded any general conclusions. Interestingly, lowering the infusion rates produced qualitatively distinct inhibitory effects on aggregation for different agonists. For ADP, GDI produced incremental decreases in aggregation over a wide range of infusion times. In contrast, for the PAR peptides, GDI increased dramatically over a narrow range of infusion times, with infusion times above a certain agonist and individual-specific threshold resulting in a complete inhibition of aggregation (Fig. 1 B-C). For example, in the case of PAR4-AP, the max aggregation observed was either below 20% or above 60%, but not in between or in other words, the aggregation response was “on or off”. To account for this phenomenon, we used logistic regression to model the effects of GDI for each agonist, using a threshold of 25 % reduction in absorbance for discriminating between “aggregation” and “no aggregation” (Fig. 1D, Table 1). In this model, we defined the measure $t_{50}$ as the minimal infusion time at which $\geq 50$ % of the samples ceased to aggregate. Aggregation induced by PAR1-AP was most sensitive to GDI, with a $t_{50}$ of 151s, whereas the effects of GDI on the aggregatory response to stimulation with PAR4-AP required significantly longer infusion times, with a $t_{50}$ of 607s.

To investigate the impact of GDI on thrombin-induced platelet activation, we performed infusion experiments with thrombin (final concentration 1 U/mL) in citrated PRP treated with GPRP to prevent fibrin polymerization (Fig. 2A-B). Our results show that GDI has dramatic effects on platelet aggregatory response to thrombin stimulation even at short infusion times, with almost complete inhibition at an infusion time of 80s. The difference in the effect distribution of GDI between PAR and ADP receptors is illustrated by a comparison of the representative curves for thrombin (Fig. 2A) with those for ADP (Fig. 2C), showing a bimodal effect distribution for thrombin whereas GDI increases incrementally over a large
range of gradients for ADP. Results obtained with thrombin were further confirmed by simultaneous infusion of PAR1-AP and PAR4-AP to mimic the composite stimulus of PARs obtained with thrombin (Fig. S4).

The secondary mediators ADP and thromboxane A2 play an important role in amplifying stimulatory signaling from PAR receptors and have previously been shown to be susceptible to desensitization. To assess how ADP and thromboxane A2 affect GDI of PAR receptor-mediated platelet activation, we performed additional experiments wherein PAR activating peptides were infused in the presence of inhibitors of P2Y1, P2Y12, and thromboxane synthesis. Surprisingly, the effects of GDI on PAR receptor-mediated platelet activation was enhanced in the absence of paracrine signaling, as the t\textsubscript{50} was reduced from 151 s to 77 s and from 607 to 320 s for PAR1 and PAR4, respectively (Fig. 2D-F).

**Gradient-dependent inhibition of platelet alpha-granule exocytosis and intracellular calcium mobilization**

To test whether GDI was a general feature of stimulatory GPCR signaling and not restricted to platelet aggregation, we investigated the gradient-dependent effects on alpha granule exocytosis, measured by flow cytometry as P-selectin exposure (Fig. 2G). Using the highest concentration gradient with an infusion time of 2 s, all agonists except ADP, induced strong P-selectin expression (>80 %), in accordance with in vitro observations from other groups showing that stimulation with ADP is not sufficient to evoke a robust paracrine response in platelets. Interestingly, a striking difference was observed in the effects of GDI between PAR4 and PAR1-mediated activation, as PAR4-AP continued to produce a virtually intact P-selectin exposure (>90 %) until lowering the gradient to an infusion time of 640 s, whereas GDI of PAR1-AP-induced P-selectin exposure was evident already when using the 80 s infusion time (Fig. 2G). In line with the results from the aggregometry experiments described above, glycoprotein VI (GPVI)-mediated platelet activation by CRP-XL showed no signs of GDI, producing a high P-selectin exposure that remained > 80 % even at the lowest concentration gradient tested (infusion time 1280 s). Although the large inter-individual differences observed for U46619 in the aggregometry experiments were still evident to some extent, the effects of GDI on alpha granule release were evident at longer infusion times, i.e., 640 and 1280s.

While the effects of GDI on GPCR signaling were prominent also when measuring platelet intracellular calcium concentrations (Fig. S5), a comparative quantitative analysis of GDI was not feasible due to differences in the ability of each receptor to generate a robust calcium response when exposed to a high agonist concentration gradient (2 s infusion time). However, a transient and immediate calcium “spike” of progressively smaller amplitude was obtained for PAR1-AP and ADP even with medium and low agonist gradients, whereas longer infusion times generated prolonged calcium mobilization with a temporal shift in [Ca\textsuperscript{2+}]\textsubscript{max} for PAR4-AP and U46619. To examine whether this phenomenon was a unique feature of platelets or if it could be generalized to other cell types, we characterized the effects of GDI on PAR1 signaling in epithelial cells, revealing calcium transients similar to that observed in platelets, with diminishing calcium mobilization with increasing infusion times (Fig. S6).

**Gradient dependent inhibition of GPCR-signaling leads to different levels of refractoriness to subsequent stimulation**
With the presence of a gradient-dependent mechanism for platelet activation verified in the above experiments, we asked to what extent the unresponsive state induced by low agonist gradients made platelets refractory to subsequent stimulation with high gradients of the same agonist. To answer this question, we performed experiments on platelets that had been rendered unresponsive to $C_{agg}$ added with the concentration gradient $\Delta C_{nres}$ (hereinafter called GDI-platelets). We defined $C_{res}$ as the minimal concentration required to achieve aggregation as a response to instantaneous (2 s infusion time) addition of the same agonist in GDI-platelets (algorithm in Fig. 3A). As shown in Fig. 3B and Table 1, GDI-platelets could be activated by immediate addition of $C_{agg} \times 2$ for PAR1-AP and $C_{agg} \times 1$ for PAR4-AP, clearly demonstrating that GDI did not render platelets refractory to subsequent stimulation with the same agonist. In contrast, for ADP, GDI induced a state of pronounced unresponsiveness to subsequent activation, as we were unable to identify a concentration of ADP that could induce platelet aggregation in GDI-platelets, even when reaching concentrations exceeding $20 \times C_{agg}$, a result consistent with previous findings$^{11,12}$. Additional experiments shown in Fig. S7 demonstrate that GDI is strictly agonist-specific, as the aggregatory response to heterologous stimulation of GDI-platelets with another agonist (e.g. PAR4, ADP or U46619 in the case of PAR1-induced GDI) was identical to that of untreated platelets.

**Platelet activation via PAR1 and PAR4 is gradient-dependent and not concentration-dependent**

To confirm that the determinant of the aggregatory response to the instantaneous addition of $C_{res}$ was the agonist concentration gradient and not the final agonist concentration, we investigated if adding $C_{res}$ with the gradient $\Delta C_{nres}$ to GDI-platelets could elicit the same aggregation response as adding $C_{res}$ instantaneously (Fig. 3C). In these experiments, GDI-platelets were exposed either to instantaneous or prolonged gradient infusion to reach the final concentration $C_{res}$. In contrast to the 2s infusion, no aggregation was observed when adding $C_{res}$ with the $\Delta C_{nres}$ gradient using the agonists for which $\Delta C_{nres}$ and $C_{res}$ could be defined (PAR1-AP and PAR4-AP). These results show that the platelet response to these agonists is independent of the final agonist concentration but highly dependent on the agonist concentration gradient.

**Gradient dependent inhibition is regulated by the cAMP-dependent pathway**

A comparison of total serine phosphorylation levels in GDI-platelets with those of resting and activated platelets for the agonists PAR1-AP, PAR4-AP or ADP (Fig. S8) showed that GDI involves specific phosphorylation events which are not observed in either resting or activated platelets. In further explorations into the mechanisms involved in GDI, we used PAR1 as the model receptor as it was the receptor most prominently affected by GDI in our study. Since receptor internalization has been reported as a common mechanism of desensitization for GPCRs$^{17-20}$, we compared platelet PAR1 receptor density in resting platelet and GDI-platelets using flow cytometry. Receptor density was found to be unchanged in GDI-platelets, indicating that receptor internalization is not a major feature of GDI (Fig. S9). A role for clathrin-mediated receptor endocytosis was also excluded, as GDI was unaffected by pre-treatment with the dynamin inhibitor Dynasore$^{21}$ (Fig. S10). Western blotting revealed that GDI-platelets exhibited a prominent phosphorylation of VASP at serine 157, which was not observed in resting or activated platelets (Fig. 4AB). As PKA is involved in mediating phosphorylation at S157$^{22}$, we examined the effects of PKA inhibition on VASP
phosphorylation and GDI as measured by aggregometry. Inhibition of PKA by 30 µM H89 which inhibited VASP phosphorylation by 10 nM but not 100 nM PGI2 (Fig. S3B), did not in itself cause aggregation in platelets, nor did it affect aggregation induced by rapid (2s) addition of 30 µM PAR1-AP. However, the effects of GDI on PAR1-induced platelet aggregation were partially reversed (Fig. 4C). This effect was also reflected in a decreased level of VASP phosphorylation in PKA-inhibited GDI-platelets treated with PAR1-AP (Fig. 4AB). Further, western blotting revealed markedly decreased AKT phosphorylation in GDI-platelets in comparison with activated platelets (Fig. 4D).

As both PKA and VASP are components of the cAMP/ adenylyl cyclase pathway, we examined the roles of adenylyl cyclase and cAMP in GDI. Pre-treatment of platelets with low doses of prostacyclin (PGI2, concentrations 0.01, 0.1 and 1 nM) to increase adenylyl cyclase activity did not affect aggregation induced by 2 s infusion of 30 µM PAR1-AP, but significantly and dose-dependently enhanced GDI at the 80 s and 160 s infusion time (Fig. 4E). Similarly, pre-incubation of platelets with the phosphodiesterase-3 inhibitor milrinone (3 µM) to inhibit cAMP degradation had no effect on aggregation at 2 sec infusion time, and did not affect platelet aggregation induced by CRP-XL, either at the 2 s or 1280 s infusion time (Fig. 4F). In contrast, significant potentiation of GDI was observed for PAR1-AP, PAR4-AP and ADP, with a similar trend for U46619, although the effect did not reach significance using this agonist. Treatment with epinephrine (0.1, 1 and 10 µM) to inhibit adenylyl cyclase 60 sec before starting agonist infusion with PAR1-AP or thrombin did not in itself cause any aggregation, but produced a significant dose-dependent inhibition of GDI (Fig. 4G-H). This effect was most prominent for thrombin, as 1 µM epinephrine was sufficient to completely block GDI for all tested infusion times.

The role of VASP/PKA pathway in cytoskeleton remodeling has been described previously. Also, VASP has been shown to interact with F-actin and regulation of F-actin rearrangement is modulated by differential phosphorylation of VASP. Therefore, an assessment of the morphological and cytoskeletal changes in platelets induced by GDI was performed using fluorescence microscopy with staining for the cytoskeletal protein F-actin. Compared to resting and activated platelets, GDI-platelets displayed a preferential distribution of F-actin filaments near the cell membrane (Fig. 5A). To confirm this finding and obtain more insights into the structural characteristics unique for GDI-platelets, electron microscopy was also performed, confirming the peripheral orientation of the cytoskeleton in GDI-platelets and additionally indicating that the glycogen bodies were more dispersed throughout in GDI platelets compared to resting platelets where they were mostly present in clusters (Fig. 5B).

**Discussion**

Under healthy conditions, platelet hemostatic activity is confined to areas near an acute vessel injury. The intracellular mechanisms responsible for this spatiotemporal regulation of platelet activation are incompletely understood. By systematically characterization gradient-dependent effects on stimulatory GPCR signaling in platelets (Table 1), our study shows that GDI represents a previously unknown mechanism for dynamic regulation of GPCR signaling, adaptively modifying platelet pro-hemostatic activity as a response to different spatiotemporal distributions of agonist concentrations. Additionally, we identify significant
differences in susceptibility to GDI among the receptors mediating responses to thrombin, ADP and thromboxane A2 (Table 1), a finding with potential consequences for the physiological roles of these agonists in vivo.

Although GDI and desensitization share many features and probably represent partially overlapping phenomena, there are important differences motivating a distinction between the two concepts. Desensitization can generally be defined as the attenuation of a response due to prolonged or repeated stimulation, whereas the more specific term “homologous receptor desensitization” refers to down-regulation of signal transduction after prolonged or repeated stimulation of a receptor with an agonist\textsuperscript{25}. These definitions imply that desensitization induces a state of unresponsiveness to further homologous stimulation. In contrast, in the case of PAR receptor stimulation, our findings show that GDI is strictly gradient-dependent, as platelet reactivity to instantaneous additions of agonists was found to be unaffected by GDI in our study. One important exception from this rule was observed for ADP, as ADP-induced GDI rendered platelets unresponsive to further stimulation in our experiments. This finding suggests that desensitization is an important mechanism of ADP-induced GDI, in agreement with previous findings showing that desensitization of P2Y\textsubscript{12} receptors occurs rapidly enough to impact ADP signaling during the time-scales relevant for this study\textsuperscript{12}. In contrast, desensitization of PAR receptors has previously only been reported to occur upon prolonged exposure (typically 10-60 min) to subthreshold concentrations of agonists\textsuperscript{17,26,27}. This is contrasted by the rapid and dynamic effects of GDI on thrombin-induced platelet activation observed in our study, as an infusion time of 80 s was sufficient to completely inhibit platelet aggregation as a response to stimulation with 1 U/ml thrombin. The finding that PAR1 is the receptor most prominently affected by GDI is not surprising when considering that the unique enzymatic activation mechanism of the PAR receptors, where one thrombin molecule theoretically could activate a large number of PAR receptors, puts high demands on balancing inhibitory signaling machinery, most particularly in the case of a high-affinity receptor like PAR1, requiring only sub-nanomolar concentrations of thrombin to effect significant receptor cleavage over time\textsuperscript{17,27,28}.

Whereas GPCR desensitization typically involves slow cellular processes such as altered protein translation and receptor internalization\textsuperscript{17-20,29}, we provide evidence against internalization or decoupling as a primary cause of GDI. Firstly, in accordance with previous results from our group\textsuperscript{26}, PAR1 receptor density was found to be unaffected in GDI-platelets (Fig S9). Secondly, inhibiting dynamin had no effect on GDI (Fig S10), excluding a role for clathrin-mediated receptor endocytosis. As additional lines of evidence supporting a conceptual distinction between GDI and desensitization, we present multiple observations strongly supporting a mechanistic link between GDI and activation of cAMP-dependent signaling (Fig. 6). This link provides mechanistic insight into how GDI can be abolished by stimulation with inhibitors of adenylyl cyclase such as ADP or epinephrine, emphasizing the important role of these agonists in GPCR signaling. The unexpected observation that inhibition of paracrine signaling potentiates GDI lends further support to our notion that GDI is mechanistically related to the cAMP-dependent pathway, as ADP is known to decrease cAMP levels via inhibition of adenylyl cyclase\textsuperscript{30}. Lastly, GDI-platelets exhibited altered levels of total serine phosphorylation and altered cytoskeletal organization in comparison
with resting and activated platelets, indicating that GDI involves the activation of unique kinase-dependent signaling pathways.

Our results may, at least in part, explain previous findings of non-responsive, “exhausted”, platelets in different diseases characterized by excessive diffusion of soluble platelet agonists, for example in cancer, sepsis, and intensive care. However, the term “exhausted” does not fit with the findings in this study, as GDI-platelets retain the capacity to be activated by other agonists or by higher agonist gradients. The concept of GDI could also help to explain the recent findings from in vivo experiments, showing the presence of a remarkably stable thrombus architecture encompassing a large shell of loosely attached, P-selectin negative platelets with little calcium mobilization surrounding a highly activated cluster of platelets in the thrombus core. GDI could be one mechanism responsible for maintaining low-grade activation in platelets forming the thrombus shell, despite the inevitable slow leakage of ADP, thromboxane A₂ and thrombin, eventually leading to agonist accumulation outside the core. In this context, it is interesting to note the different effects of GDI on paracrine signaling from ADP and thromboxane on the one hand and signaling from the thrombin receptors PAR1 and PAR4 on the other. Whereas GDI had incremental inhibitory effects on paracrine stimulation over a large range of gradients, resulting in progressively weaker platelet activation, a bimodal effect distribution was observed for PAR-mediated signaling, as platelet activation was effectively shut off when gradients decreased below a certain threshold. These differential effects of GDI could be instrumental for the formation of the core-shell thrombus architecture, as GDI could result in abolished thrombin signaling outside the thrombus core, whereas the gradual effects of GDI on paracrine stimulation would result in the intermediary platelet activation state found in the thrombus shell. The observation that the collagen activation pathway (represented by CRP-XL) was found to be unaffected by GDI in our study is noteworthy in this context, as collagen is not a diffusible agonist but remains attached to the damaged vessel wall upon injury. Thus, blood exposure to collagen is inherently restricted to the immediate vicinity of vessel damage, rendering GDI physiologically irrelevant as a regulatory mechanism.

While this study focused exclusively on the gradient-dependent effects of single agonists on platelet activation, platelets circulating near a vessel injury are exposed to multiple stimulatory gradients, primarily including the agonists ADP, thromboxane A₂ and thrombin. Adding another layer of complexity, these pro-hemostatic signaling pathways are counter-balanced by inhibitory signals released from intact endothelium such as PGI₂ and NO. Thrombin receptors are quite common in the human body, especially on cells in circulation. The observation that the collagen activation pathway (represented by CRP-XL) was found to be unaffected by GDI in our study is noteworthy in this context, as collagen is not a diffusible agonist but remains attached to the damaged vessel wall upon injury. Thus, blood exposure to collagen is inherently restricted to the immediate vicinity of vessel damage, rendering GDI physiologically irrelevant as a regulatory mechanism.
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Table 1: Summary of measures characterizing gradient-dependent inhibition of GPCR mediated platelet aggregation

<table>
<thead>
<tr>
<th>Agonist</th>
<th>$C_{agg}$ (µM)</th>
<th>$\Delta C_{nres}$ (s)</th>
<th>$t_{50}$ (s)</th>
<th>$C_{res}$ (µM)</th>
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<td>PAR1-AP</td>
<td>30</td>
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<td>+ inhibitors*</td>
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$C_{agg}$: The minimal agonist concentrations required to induce strong aggregation (> 65%) in all tested samples ($n \geq 5$).

$\Delta C_{nres}$: The highest agonist concentration gradient for which >75% of samples are unresponsive (maximal aggregation < 25%) to $C_{agg}$.

$t_{50}$: The minimal infusion time at which ≥ 50% of the samples ceased to aggregate.

$C_{res}$: Minimal agonist dose required to induce strong activation (>75% aggregation) when added at an infusion time of 2 sec in all samples, ($n > 5$) after rendering platelets unresponsive to $C_{agg}$ added with the gradient $\Delta C_{nres}$.

* For PAR1 and PAR4, $\Delta C_{nres}$ and $t_{50}$ were also determined in presence of P2Y1, P2Y12, and thromboxane synthesis inhibitors, shown here as ‘+ inhibitors’.

† $t_{50}$ was not determined since GDI was not observed for TPα in all experiments.

‡ The $C_{res}$ measurement for U46619 is calculated from experiments where gradient dependent inhibition was observed.

§ µg/mL.

NA, not applicable; ND, not determined.
**Figure legends:**

**Fig. 1: Gradient-dependent inhibition of GPCR-mediated platelet aggregation**
To experimentally verify the presence of a gradient dependent mechanism modulating platelet aggregation, light transmission aggregometry was conducted according to the experimental algorithm in (A), where the identification of an agonist gradient (ΔCnres) at which no significant aggregation (<25 %) occurred despite reaching an agonist concentration sufficient to elicit strong aggregation when using a 2 s agonist infusion time, was interpreted as proof for the presence of gradient-dependent inhibition. (B) Aggregation raw curves obtained using the algorithm in A for different agonists and gradients. n≥5. (C) Heat map showing mean max aggregation for all experiments in B. (D) Logistic regression was performed to calculate the infusion time at which >50 % of samples could be expected to give <25 % aggregation for the respective agonists (t50). Dots represent the outcome of individual experiments where data has been dichotomized so that >25 % aggregation is denoted as “aggregation” and < 25 % aggregation is denoted as “no aggregation”. Confidence bands represent 95% confidence intervals.

**Fig. 2: Characterization of gradient-dependent inhibition using thrombin, ADP, paracrine signaling inhibition and alpha granule secretion**
(A-B) To quantify the effects of GDI on platelet aggregation induced by thrombin, LTA was performed on PRP pre-incubated with 4mM GPRP to prevent fibrin polymerization. 1 U/ml thrombin was added with different infusion times as indicated, n≥3. (C) Platelet aggregation induced by ADP added with different infusion times as indicated. (D-F). The role of paracrine stimulation in GDI of PAR signaling was quantified by performing LTA on PRP in presence of P2Y1, P2Y12 and thromboxane synthesis inhibitors (MRS2179, cangrelor and ASA, respectively), using agonists PAR1-AP (30µM) and PAR4-AP (300µM), n≥3 (G). The effects of GDI on alpha granule release was analyzed by measuring the percentage of platelets positive for CD62P (P-selectin) using flow cytometry at different infusion rates, (n ≥5). Data represent mean ± s.d. * P<0.05, **P<0.01 and ***P<0.001

**Fig. 3: Agonist-specific effects of gradient-dependent inhibition**
(A) Algorithm for defining C_res, the minimal concentration required to induce aggregation in GDI-platelets. (B) GDI was induced by exposing platelets to C_agg with ΔC_res for agonists ADP, PAR1-AP and PAR4-AP. Platelets were then challenged with the same agonist by adding multiples of C_agg with an infusion time of 2s. To investigate whether the determinant of the aggregation induced by adding PAR1-AP or PAR4-AP at the concentration C_res (as shown above in (B)), was the increased agonist gradient or the final agonist concentration, subsequent infusions of C_res using either the 2s high gradient ⓞ or the GDI gradient ΔC_res ☉ were performed (C).

**Fig. 4. Mechanistic characterization of gradient dependent inhibition**
Western blotting was performed on resting platelets (RST: resting- treated with 320s infusion of saline), activated platelets (ACT: activated- treated with 2s infusion of 30 µM PAR1-AP) or GDI-platelets (GDI: treated with 320s infusion of 30 µM PAR1-AP) with staining for
VASP phosphorylation at Ser157 or total VASP (A). Experiments were performed with or without pre-treatment with a PKA inhibitor (H89) or PGI2. The quantitation of the signal intensity where, PGI2 mediated phosphorylation signal was set to 100%, is given in (B), n=3. Effects of the PKA inhibitor H89 on platelet aggregation (C) induced by 30 µM PAR1-AP added at 2s or 320s infusion time. (D) Levels of total and phosphorylated AKT (Ser 473) in resting, activated or GDI platelets determined by western blotting. (E) Effect of pre-incubation with PGI2 (0.01, 0.1 and 1 nM) on aggregation induced by 30 µM PAR1-AP added with different infusion times. (F) Effect of the phosphodiesterase-3 inhibitor milrinone (Mil; 3µM) on maximal platelet aggregation induced by Cagg (30 µM PAR1-AP, 300 µM PAR4-AP, 5 µM ADP, 2µM U46619, 0.16 µg/ml CRP-XL) added with different infusion times compared to control (Ctr). (G-H) Effects of epinephrine (Epi; 0.1, 1 and 10 µM) on GDI for agonists PAR1-AP (G) or thrombin (H) at different infusion times presented as log scale on x-axis (GDI calculated as % inhibition of maximal aggregation compared to 2 s infusion time). For all the experiments, data represent mean ± s.d., n≥3 and * P<0.05, **P<0.01 and ***P<0.001.

Fig. 5: Actin rearrangement and subcellular differences in GDI platelets
Morphological and cytoskeletal differences in resting, activated and GDI platelets (for 30µM PAR1-AP) were visualized by fluorescence microscopy (A) using AF546-Phalloidin staining of F-actin (scale bar is 3 µm) and by electron microscopy (B). In electron micrographs, glycogen bodies (GB), alpha granules (AG) and dense granules (DG) are shown with arrows and peripheral microtubular loops in GDI platelets are shown with arrowheads with a higher magnification in GDI-II. The scale bar in the images is 1 µm except in GDI-II where it is 0.5 µm.

Fig. 6. Gradient-triggered activation of the cAMP-dependent pathway controls signaling from stimulatory GPCRs.
Signaling pathways with a net stimulatory effect on platelet activation are colored red and signaling pathways with a net inhibitory effect are colored blue. (A) In the presence of high agonist concentration gradients, strong activation of multiple stimulatory pathways will produce Akt phosphorylation, which in itself results in inhibition of the cAMP dependent pathway by stimulation of PDE3 activity39. In the presence of low agonist concentration gradients (B), gradient-dependent activation of the cAMP-dependent pathway will counteract stimulatory signaling from GPCRs, resulting in a refractory state characterized by an absence of Akt phosphorylation but prominent VASP phosphorylation and non-responsiveness to high concentrations of agonists. As proof for the involvement of the cAMP-dependent pathway in GDI, we found that GDI was effectively shut off by inhibition of adenylyl cyclase with epinephrine, and partially reversed by inhibition of PKA with H89, while GDI was potentiated by stimulation of the cAMP-dependent pathway with PGI2 or milrinone (B).

Fig. 7. Proposed role of GDI in creating and maintaining the core-shell thrombus architecture
As GDI potently inhibits thrombin-induced platelet activation over a narrow range of temporal concentration gradients, we propose that stimulatory signaling via the PAR receptors will effectively be shut off outside the thrombus core (A). In contrast, as GDI exhibits a gradual increase over a larger range of gradients for ADP and thromboxane A₂, paracrine signaling from these agonists will only be partially inhibited, resulting in an intermediary state of platelet activation in the thrombus shell (B).
A) Start with
\( \Delta C = C_{agg}/2 \) sec
\( n = 1 \)
\( \Delta C = C_{agg}/20 \times 2^n \) sec
Aggregation in >25% of samples?
Yes \( n = n + 1 \)
No \( \Delta C_{res} = \Delta C \)

B) Agonist
- ADP
- CRP-XL
- PAR1-AP
- PAR4-AP
- U46619

C) Infusion time (s)
Max aggregation (%)

D) Samples with >25% aggregation (%)
Differential effect of GDI on thrombin and ADP mediated aggregation

Effect of GDI in absence of paracrine signaling

Effect of GDI on alpha granule release
Find concentration $C_{res}$ at which GDI-platelets aggregate when added at 2 sec infusion time.

Start with

$\Delta C = C_{nres}$

$n = 1$

Add $C = C_{agg} \times 2^{n-1}$

$\Delta C = C/2$ sec

Aggregation? No $n = n + 1$

Yes $C_{res} = C$

---

B)

Max aggregation (%)

ADP ($\mu$M) | PAR1-AP ($\mu$M)
---|---
5 | 30 | 60 | 300
10 | 20 | 60 | 300
20 | 10 | 60 | 300
25 | 5 | 60 | 300

---

C)

Gradient profile

PAR1-AP ($\mu$M)
---
0 | 50 | 100 | 1500
50 | 100 | 1500
100 | 1500

Aggregation response

Time (sec)
---
0 | 500 | 1000 | 1500
500 | 1000 | 1500
1000 | 1500

---

Gradient profile

PAR4-AP ($\mu$M)
---
0 | 200 | 400 | 2000
200 | 400 | 2000
400 | 2000

Aggregation response

Time (sec)
---
0 | 1000 | 2000 | 3000
1000 | 2000 | 3000
2000 | 3000
Effect of GDI on PKA, VASP and AKT

Enhancement of GDI

Inhibition of GDI
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, PGI2, 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 SärI, 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, Immuno-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 (PGI2) 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 MgSO4.7H2O, 8.47 mM Na2HPO4.2H2O, 10 mM glucose and 1.73 mM KH2PO4, PH 7.3) supplemented with PGI2, 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 Cagg 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), PGI2 (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 with PRP 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 MgCl₂, 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 (PGI₂) 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 PGI₂ 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, PGI₂ is not very stable in aqueous solutions², its stable analog PGE₁ was used instead to stimulate the IP receptor. PGE₁ 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 PGE₁ in saline during the time course of the experiment, PGE₁ 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 A₂ 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 ΔC₅₀ 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.

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**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.

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References
