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

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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, i.e., determined not only by agonist concentrations *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 exhibits different kinetics, with platelet activation occurring at lower agonist gradients for protease-activated receptor 4 than for protease-activated receptor 1, but shares 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. Furthermore, 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 (GPCR) mediate activation in response to stimulation with multiple important soluble agonists, including thrombin, adenosine diphosphate (ADP) and thromboxane A_2.[1] These signaling events are critical for triggering platelet hemostatic activities such as adhesion,[2] 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 GPCR 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).

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Vascular damage is associated with a localized rapid increase in the concentrations of soluble agonists acting on platelet stimulatory GPCR. 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.[3] Recent studies of intra-thrombus architecture have shown that spatial differences in thrombus porosity result in distinct diffusion rates of solutes,[4] 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,[5] 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,[6,7] and can be predicted to enhance the information processing ability of cells in relation to changes in the ambient stimulation level.[8,9] 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. At the other end 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.[10] 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 (*Online Supplementary Figure S1*), enabling us to verify the presence of a mechanism for gradient-dependent inhibition (GDI) of platelet activation involving activation of cyclic adenosine monophosphate (cAMP)-dependent signaling mechanisms.

Methods

Blood collection and sample preparation

Whole blood from healthy adult volunteers was collected into tubes containing hirudin, sodium citrate or acid-citrate-dextrose as per the local Ethics Committee of Linköping University Hospital and platelet-rich plasma or washed platelets were prepared using standard procedures as described in the *Online Supplement*.

Light transmission aggregometry

Platelet aggregation was measured by light transmission aggregometry using a Chronolog Corporation model 490-X, Haverton, USA aggregometer. A pump controlled agonist infusion system (*Online Supplementary Figure 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 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 (*Online Supplementary Figure S1D*). Predefined algorithms were followed for the parameters in the aggregometry experiments (Figures 1A, 3A *and Online Supplementary Figure S2A*) to avoid the potential for bias associated with the *ad hoc* experimental design. Based on that, aggregation was measured after infusing the same volume and concentration of agonists for 2, 40, 80, 160, 320, 640 or 1,280 s. The details of the experimental conditions, including the use of various inhibitors and the stability of all the agonists used in the study under experimental conditions, are described in the *Online Supplement*.

Flow cytometry

The effect of agonist gradients on platelet α-granule release was assessed by taking aliquots from samples identical to those used in the aggregometry experiments except for the inclusion of a step in which samples were pre-incubated with 1 μM tirofiban for 10 min at room temperature to prevent aggregation. Samples were collected 1 min after completion of agonist infusion, labeled and analyzed by flow cytometry as described in the *Online Supplement*.

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 the *Online Supplement*.

Fluorescence microscopy

Resting platelets, platelets activated by PAR1 activating peptide (PAR1-AP) and platelets with induced GDI were visualized by fluorescence microscopy after staining F-actin according to the manufacturer's protocol, as described in the *Online Supplement*.

Electron microscopy

Transmission electron microscopy was used to visualize subcellular differences between resting, activated platelets and platelets with induced GDI, as described in the *Online Supplement*.

Results

A gradient-dependent mechanism modulates G protein-coupled receptor-mediated platelet activation

The minimal agonist concentration (C_{agg}) required to induce strong aggregation (>65%) in all samples (n≥5) with an infusion time of 2 s was determined (Table 1) using the algorithm shown in *Online Supplementary Figure S2A* (results in *Online Supplementary Figure S2B*). To verify the presence of GDI, we then sought to identify the highest concentration gradient (ΔC_{nres}) at which no significant aggregation (<25%) was observed in ≥75% of samples at a final agonist concentration of C_{agg} (algorithm in Figure

1A). Raw curves from the aggregometry experiments used to define ΔC_{nres} for the different agonists are shown in Figure 1B and a color map to aid visual interpretation is provided in Figure 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 PGE_1 (*Online Supplementary Figure S3*), even when using the longest infusion time of 1,280 s. For the TPα 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 cer-

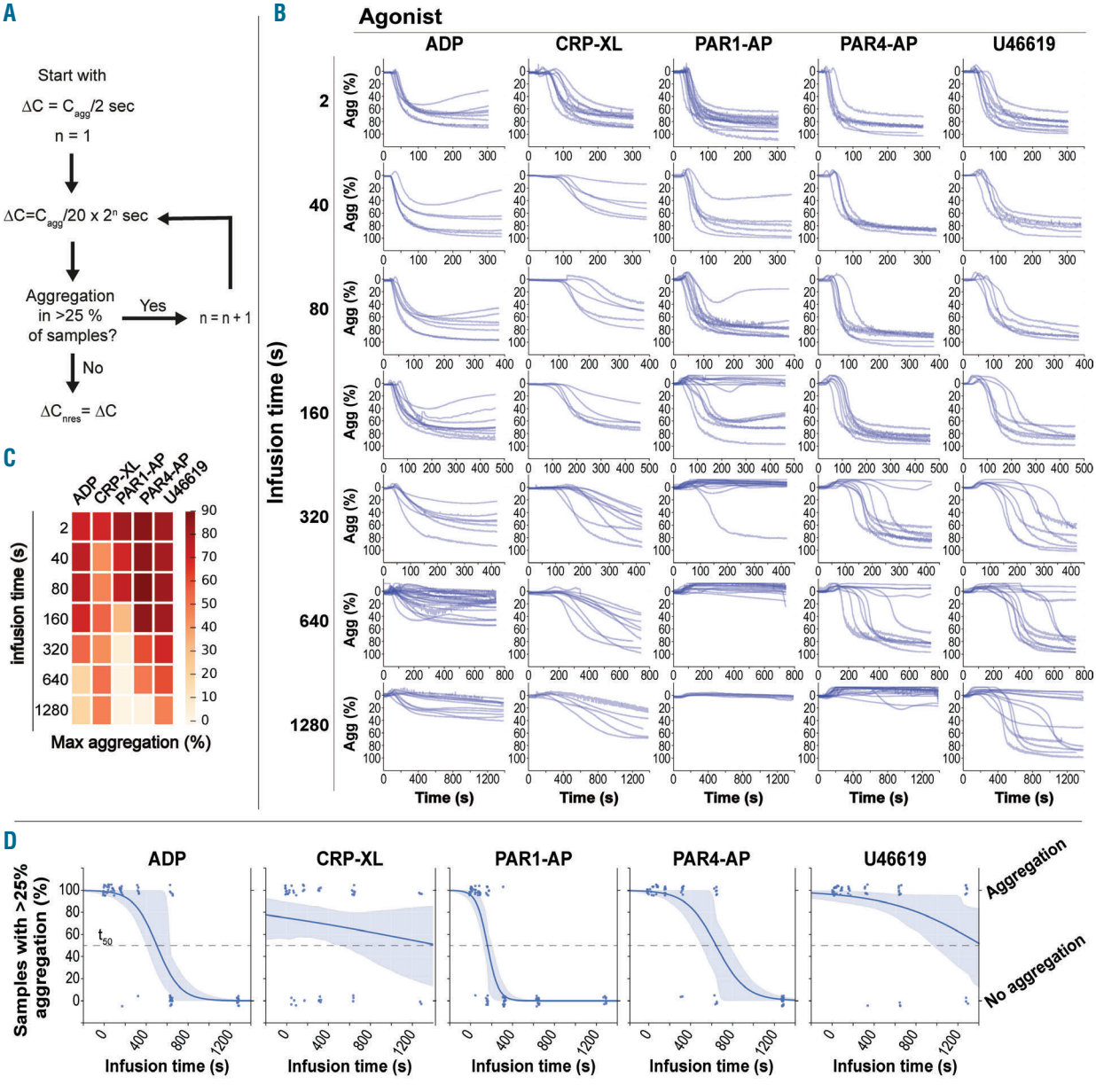

Figure 1. Gradient-dependent inhibition of G protein-coupled receptor-mediated platelet aggregation. To experimentally verify the presence of a gradient-dependent mechanism modulating platelet aggregation, light transmission aggregometry was conducted according to the experimental algorithm in (A), where the identification of an agonist gradient (ΔC_{nres}) 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 of 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 maximum 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 (t_{50}). Dots represent the outcome of individual experiments for which data have been dichotomized so that >25% aggregation is denoted as "aggregation" and <25% aggregation is denoted as "no aggregation". Confidence bands represent 95% confidence intervals.

tain agonist and individual-specific threshold resulting in a complete inhibition of aggregation (Figure 1B,C). For example, in the case of PAR4-AP, the maximum aggregation observed was either below 20% or above 60%, but not in between; 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 to discriminate between "aggregation" and "no aggregation" (Figure 1D, Table 1). In this model, we defined the measure t_{50} as the minimal infusion time at which ≥50% of the samples ceased to aggregate. Aggregation induced by PAR1-AP was most sensitive to GDI, with a t_{50} of 151 s, whereas the effects of GDI on the aggregatory response to stimulation with PAR4-AP required significantly longer infusion times, with a t_{50} of 607 s.

To investigate the impact of GDI on thrombin-induced platelet activation, we performed infusion experiments with thrombin (final concentration 1 U/mL) in citrated platelet-rich plasma treated with Gly-Pro-Arg-Pro (GPRP) to prevent fibrin polymerization (Figure 2A,B). Our results show that GDI has dramatic effects on the platelet aggregatory response to thrombin stimulation even at short infusion times, with almost complete inhibition at an infusion time of 80 s. The difference in the effect distribution of GDI between PAR and ADP receptors is illustrated by a comparison of the representative curves for thrombin (Figure 2A) with those for ADP (Figure 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-

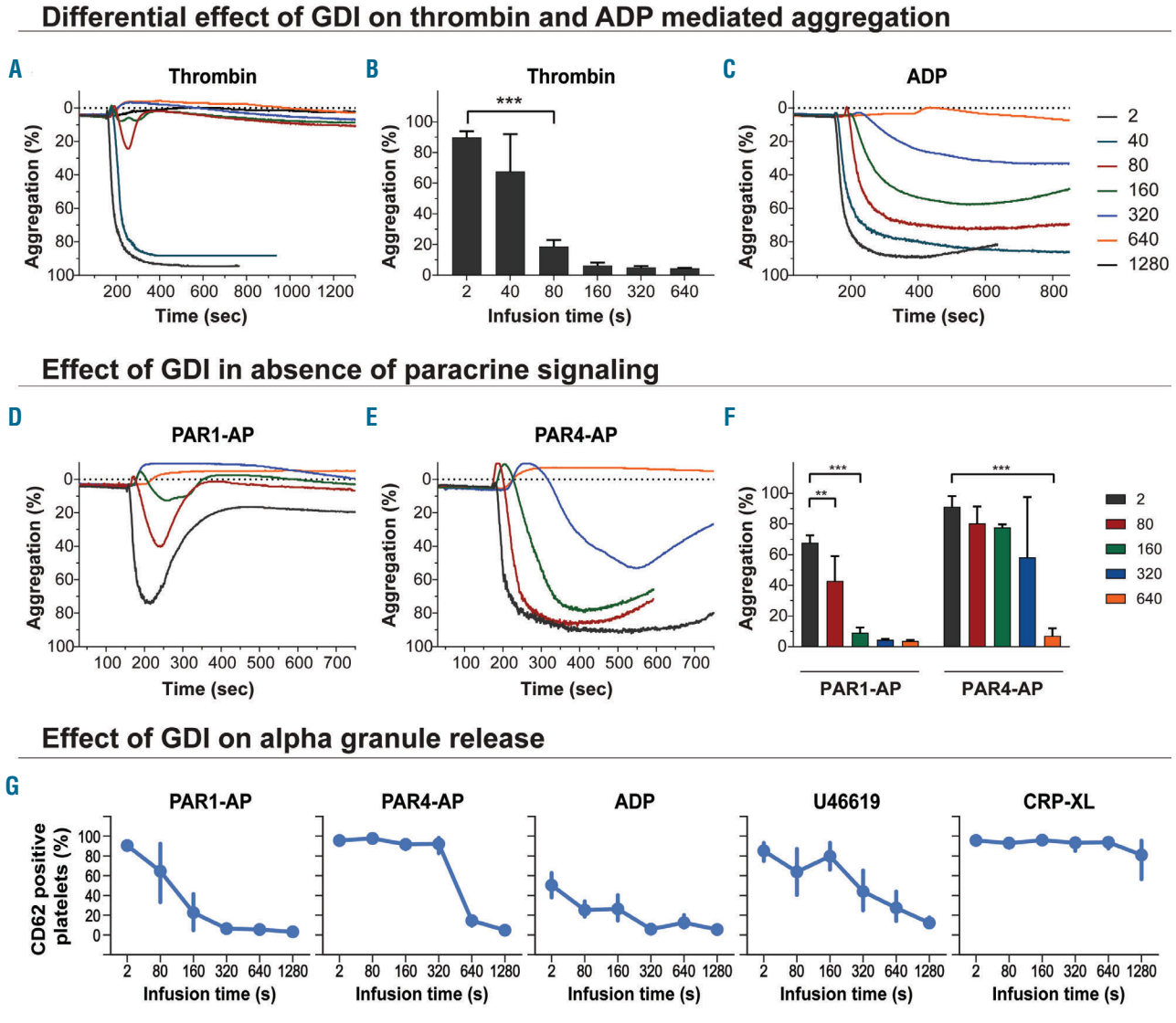

Figure 2. Characterization of gradient-dependent inhibition using thrombin, ADP, paracrine signaling inhibition and α-granule secretion. (A,B) To quantify the effects of gradient-dependent inhibition (GDI) on platelet aggregation induced by thrombin, light transmission aggregometry was performed on platelet-rich plasma pre-incubated with 4 mM GPRP to prevent fibrin polymerization. Thrombin (1 U/mL) 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 light transmission aggregometry on platelet-rich plasma in the presence of $P2Y_1$, $P2Y_{12}$ and thromboxane synthesis inhibitors (MRS2179, cangrelor and ASA), using the agonists PAR1-AP (30 μM) and PAR4-AP (300 μM), n≥3 (G). The effects of GDI on α-granule release were analyzed by measuring the percentage of platelets positive for CD62P (P-selectin), using flow cytometry, at different infusion rates, n ≥5. Data represent mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001

AP to mimic the composite stimulus of PAR obtained with thrombin (*Online Supplementary Figure S4*).

The secondary mediators ADP and thromboxane A_2 play an important role in amplifying stimulatory signaling from PAR receptors and have previously been shown to be susceptible to desensitization.[11-13] To assess how ADP and thromboxane A_2 affect GDI of PAR receptor-mediated platelet activation, we performed additional experiments in which PAR-APs were infused in the presence of inhibitors of $P2Y_1$, $P2Y_{12}$, 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_{50} was reduced from 151 s to 77 s and from 607 to 320 s for PAR1 and PAR4, respectively (Figure 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 α-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 by other groups showing that stimulation with ADP is not sufficient to evoke a robust paracrine response in platelets.[14-16] 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 the gradient was lowered 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 (Figure 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 1,280 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 α-granule release were evident at longer infusion times, i.e., 640 s and 1,280 s.

While the effects of GDI on GPCR signaling were prominent also when measuring platelet intracellular calcium concentrations (*Online Supplementary Figure S5*), a comparative quantitative analysis of GDI was not feasible because of 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 $[Ca^{2+}]_{max}$ for PAR4-AP and U46619. To examine whether this phenomenon was a unique feature of platelets or whether 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 those observed in platelets, with diminishing calcium mobilization with increasing infusion times (*Online Supplementary Figure S6*).

Gradient-dependent inhibition of G protein-coupled receptor-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 ΔC_{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 Figure 3A). As shown in Figure 3B and Table 1, GDI-platelets could be activated by immediate addition of C_{agg} x 2 for PAR1-AP and C_{agg} x 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 x C_{agg}, a result consistent with previous findings.[11,12] Additional experiments shown in *Online Supplementary Figure 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 whether adding C_{res} with the gradient ΔC_{nres} to GDI-platelets could elicit the same aggregation response as adding C_{res} instantaneously (Figure 3C). In these experiments, GDI-platelets were exposed to either instantaneous or prolonged gradient infusion to reach the final concentration C_{res}. In contrast to the 2 s infusion, no aggregation was observed when adding C_{res} with the ΔC_{nres} gradient using the agonists for which ΔC_{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 a 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 and ADP (*Online Supplementary Figure 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 GPCR,[17-20] we compared platelet PAR1 receptor density in resting platelets 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 (*Online Supplementary Figure S9*). A role for clathrin-mediated receptor endocytosis was also excluded, as GDI was unaffected by pre-treatment with the dynamin inhibitor Dynasore[21] (*Online Supplementary Figure S10*). Western blotting revealed a prominent phosphorylation of VASP at serine 157 in GDI-platelets, which was not observed in resting or activated platelets (Figure 4A,B). 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 prostacyclin (PGI_2) (*Online Supplementary Figure S3B*), did not in itself cause aggregation of platelets, nor did it affect aggregation induced by rapid (2 s) addition of 30 μM PAR1-AP. However, the effects of GDI on PAR1-induced platelet aggregation were partially reversed (Figure 4C). This effect was also reflected in a decreased level of VASP phosphorylation in PKA-inhibited GDI-platelets treated with PAR1-AP (Figure 4A,B). Furthermore, western blotting revealed markedly decreased AKT phosphorylation in GDI-platelets in comparison with that in activated platelets (Figure 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 PGI_2 (concentrations 0.01, 0.1 and 1 nM) to increase adenylyl cyclase activity did not affect aggregation induced by a 2 s infusion of 30 μM PAR1-AP, but significantly and dose-dependently enhanced GDI at the 80 s and 160 s infusion times (Figure 4E). Similarly, pre-incubation of platelets with the phosphodiesterase-3 inhibitor milrinone (3 μM) to inhibit cAMP degradation had no effect on aggregation at the 2s infusion time, and did not affect platelet aggregation induced by CRP-XL, at either the 2 s or the 1,280 s infusion time (Figure 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 s 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 (Figure 4G,H). This effect was most prominent for thrombin, as 1 μM epinephrine was sufficient to block GDI completely for all tested infusion times.

The role of the VASP/PKA pathway in cytoskeleton remodeling has been described previously.[23] Also, VASP has been shown to interact with F-actin and regulation of F-actin rearrangement is modulated by differential phosphorylation of VASP.[24] we, therefore, assessed morphological and cytoskeletal changes in platelets induced by GDI 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 (Figure 5A). To confirm this finding and obtain more insights into the structural characteristics unique to GDI-

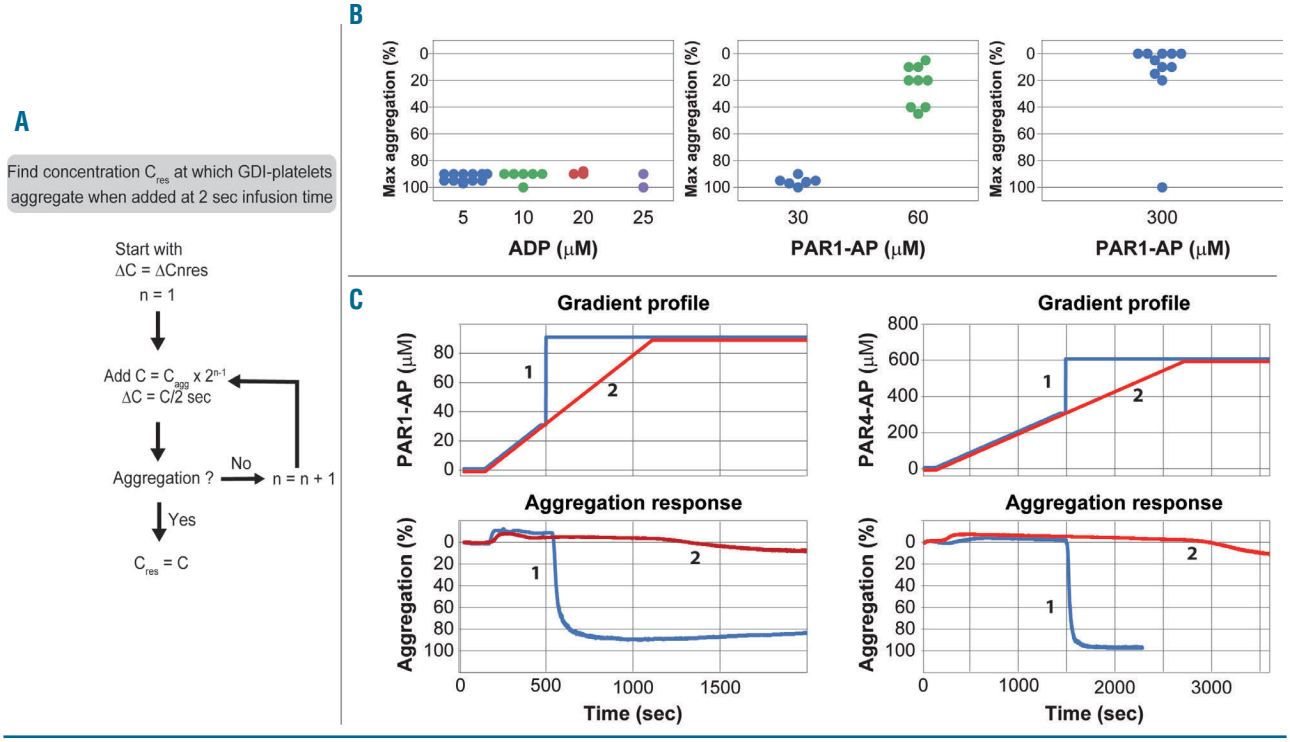

Figure 3. Agonist-specific effects of gradient-dependent inhibition. (A) Algorithm for defining C_{res}, the minimal concentration required to induce aggregation in platelets showing gradient-dependent inhibition (GDI). (B) GDI was induced by exposing platelets to C_{agg} with ΔC_{nres} for the agonists 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 2 s. (C) 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 2 s high gradient (1) or the GDI gradient ΔC_{nres} (2), were performed.

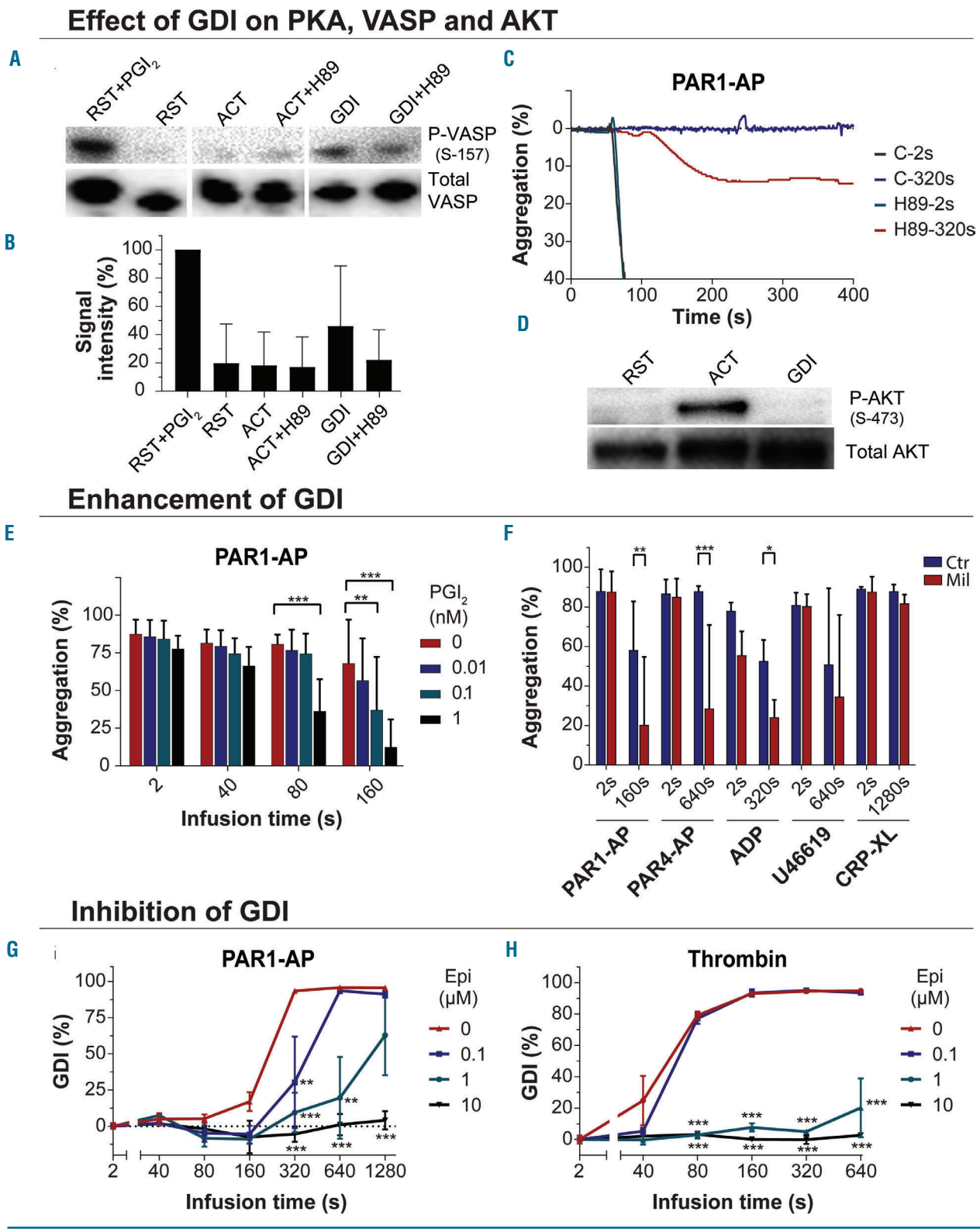

Figure 4. Mechanistic characterization of gradient-dependent inhibition. (A) Western blotting was performed on resting platelets (RST: treated with a 320 s infusion of saline), activated platelets (ACT: treated with a 2 s infusion of 30 μM PAR1-AP) or platelets showing gradient-dependent inhibition (GDI: treated with a 320 s infusion of 30 μM PAR1-AP) with staining for VASP phosphorylation at Ser157 or total VASP. Experiments were performed with or without pre-treatment with a PKA inhibitor (H89) or PGI_2. (B) Quantitation of the signal intensity, with the PGI_2-mediated phosphorylation signal set at 100%, n=3. (C) Effects of the PKA inhibitor H89 on platelet aggregation induced by 30 μM PAR1-AP added at a 2 s or 320 s 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 PGI_2 (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; 3 μM) on maximal platelet aggregation induced by C_{agg} (30 μM PAR1-AP, 300 μM PAR4-AP, 5 μM ADP, 2 μM 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 the agonists PAR1-AP (G) and thrombin (H) at different infusion times presented as a log scale on the x-axis (GDI calculated as % inhibition of maximal aggregation compared to that with the 2 s infusion time). For all the experiments, data represent mean ± standard deviation, n≥3. **P*<0.05, ***P*<0.01 and ****P*<0.001.

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 in GDI-platelets than in resting platelets in which they were mostly present in clusters (Figure 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 characterizing 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 A_2 (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 downregulation of signal transduction after prolonged or repeated stimulation of a receptor with an agonist.[25] These definitions imply that desensitization induces a state of

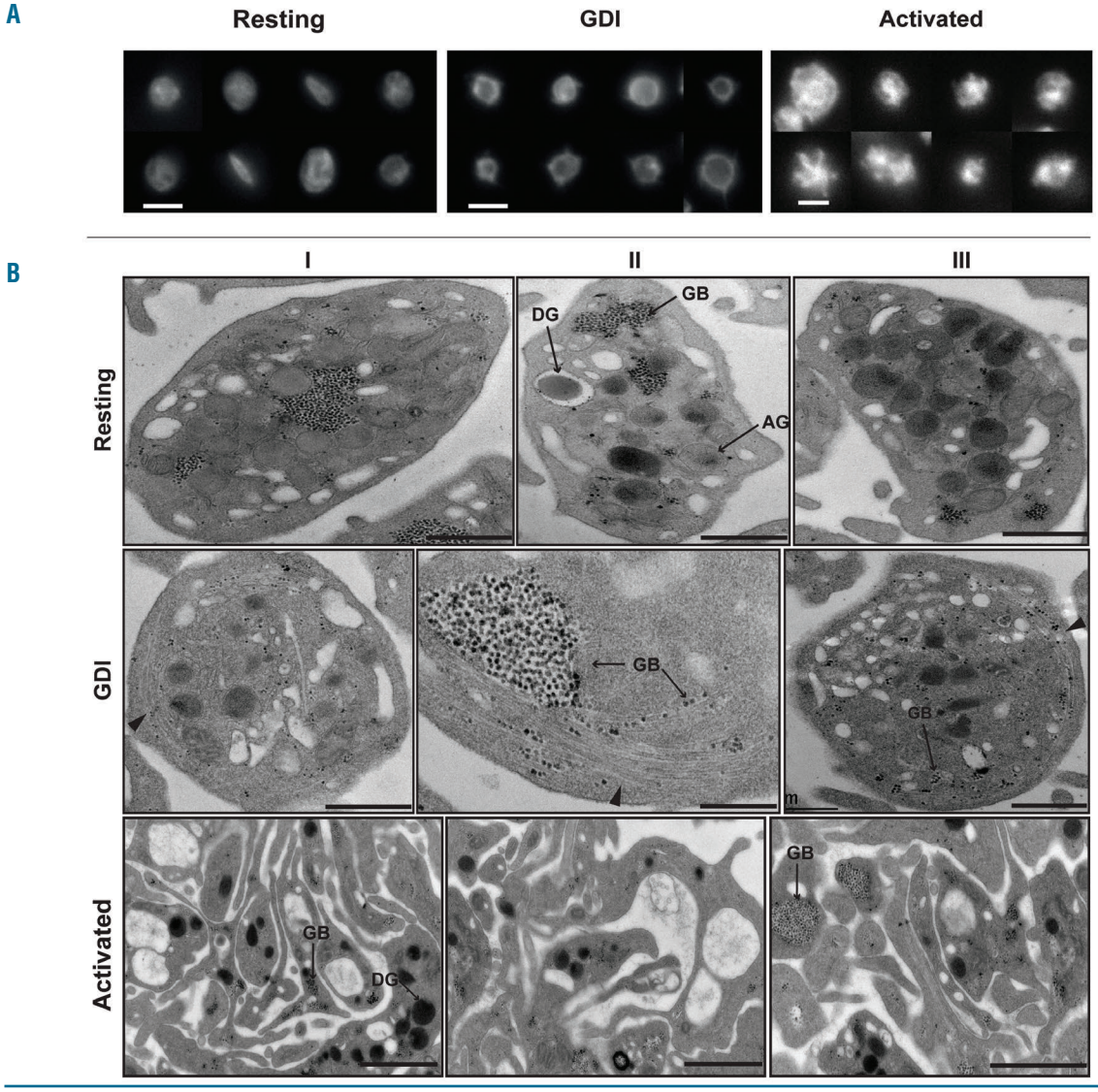

Figure 5. Actin rearrangement and subcellular differences in resting platelets, activated platelets and platelets showing gradient-dependent inhibition. (A,B) Morphological and cytoskeletal differences in resting platelets, platelets activated by 30 μM PAR1-AP, and platelets showing gradient dependent inhibition (GDI) for 30 μM PAR1-AP were visualized by fluorescence microscopy (A) using AF546-Phalloidin staining of F-actin (scale bar represents 3 μm) and by electron microscopy (B). In electron micrographs, glycogen bodies (GB), α-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 represents 1 μm except in GDI-II where it represents 0.5 μm.

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 $P2Y_{12}$ receptors occurs rapidly enough to affect ADP signaling during the timescales relevant for this study.[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.[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 such as PAR1, requiring only subnanomolar concentrations of thrombin to effect significant receptor cleavage over time.[17,27,28]

Whereas GPCR desensitization typically involves slow cellular processes such as altered protein translation and receptor internalization,[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,[26] PAR1 receptor density was found to be unaffected in GDI-platelets (*Online Supplementary Figure S9*). Secondly, inhibiting dynamin had no effect on GDI (*Online Supplementary Figure 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 (Figure 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

Table 1. Summary of measures characterizing gradient-dependent inhibition of G protein-coupled receptor-mediated platelet aggregation.

Agonist	C_{agg} (μM)	ΔC_{nres} (s)	t_{50} (s)	C_{res} (μM)
PAR1-AP	30	320	151	60
+ inhibitors*	30	160	77	ND
PAR4-AP	300	1280	607	300
+ inhibitors*	300	640	322	ND
ADP		640	485	NA
U46619†	2	1280	ND	2‡
CRP-XL	0.16§	NA	NA	NA

C_{agg}: the minimal agonist concentrations required to induce strong aggregation (>65%) in all tested samples (n≥5); ΔC_{nres}, the highest agonist concentration gradient for which >75% of samples are unresponsive (maximal aggregation <25 %) to C_{agg}; t_{50}: the shortest infusion time at which ≥50% of the samples ceased to aggregate; C_{res}: the lowest agonist dose required to induce strong activation (>65% aggregation) when added at an infusion time of 2 s in all samples (n>5) after rendering platelets unresponsive to C_{agg} added with the gradient ΔC_{nres}. *For PAR1 and PAR4, ΔC_{nres} and t_{50} were also determined in the presence of $P2Y_1$, $P2Y_{12}$, 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 was calculated from experiments in which gradient dependent inhibition was observed. §μg/mL. NA: not applicable; ND: not determined.

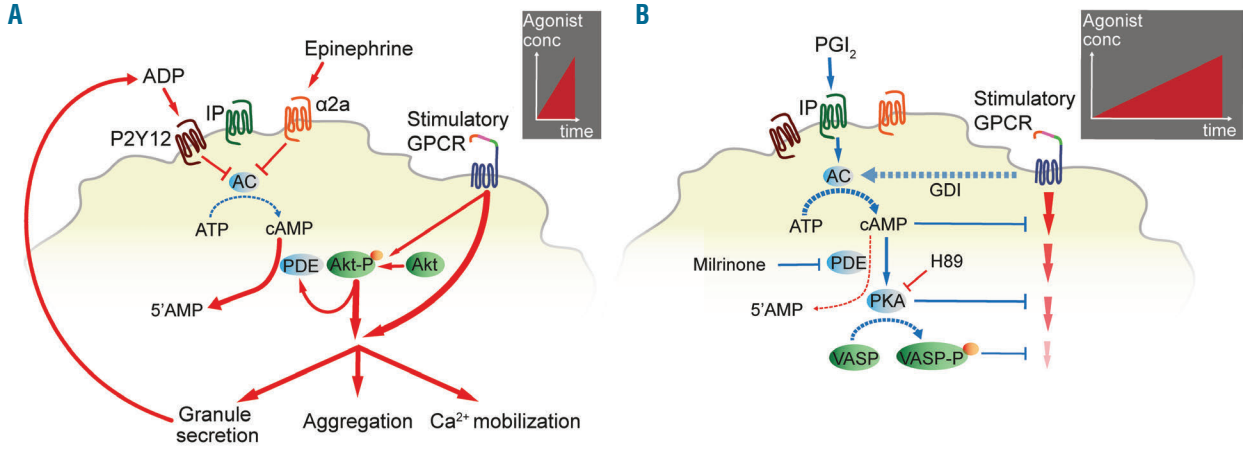

Figure 6. Gradient-triggered activation of the cAMP-dependent pathway controls signaling from stimulatory G protein-coupled receptors. Signaling pathways with a net stimulatory effect on platelet activation are colored red and signaling pathways with a net inhibitory effect are colored blue. (A) In the 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 activity.[39] (B) In the presence of low agonist concentration gradients, gradient-dependent activation of the cAMP-dependent pathway will counteract stimulatory signaling from G protein-coupled receptors (GPCR), 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 of the involvement of the cAMP-dependent pathway in gradient-dependent inhibition (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 PGI_2 or milrinone.

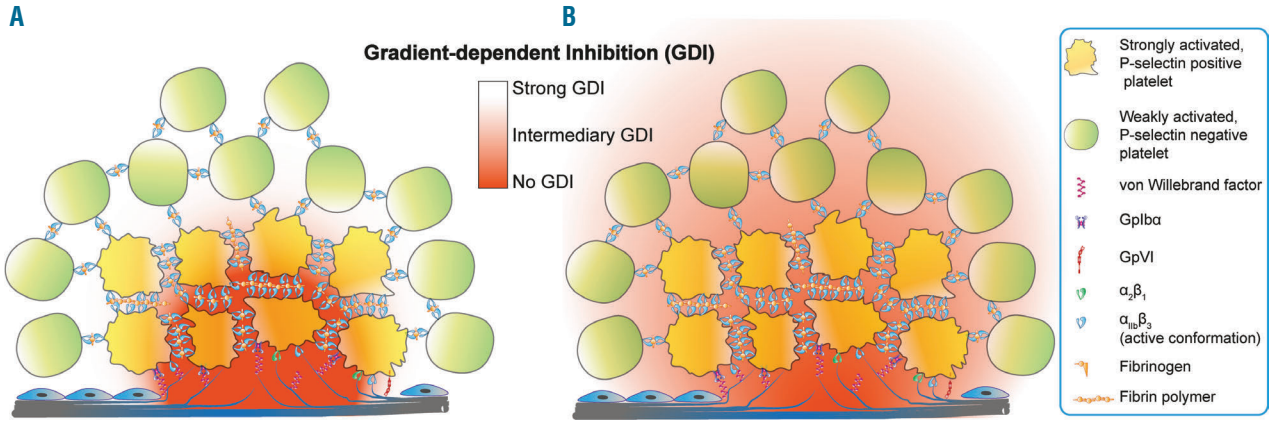

Figure 7. Proposed role of gradient-dependent inhibition in creating and maintaining the core-shell thrombus architecture. (A) As gradient-dependent inhibition (GDI) potently inhibits thrombin-induced platelet activation over a narrow range of temporal concentration gradients, we propose that stimulatory signaling via the PAR receptors will effectively be shut off outside the thrombus core. (B) In contrast, as GDI exhibits a gradual increase over a larger range of gradients for ADP and thromboxane A_2, paracrine signaling from these agonists will only be partially inhibited, resulting in an intermediary state of platelet activation in the thrombus shell.

cAMP-dependent pathway, as ADP is known to decrease cAMP levels via inhibition of adenylyl cyclase.[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 situations characterized by excessive diffusion of soluble platelet agonists, for example in cancer,[31-34] sepsis,[34] and intensive care.[35-37] 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 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 core of the thrombus.[4,10] 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 A_2 and thrombin,[38] eventually leading to agonist accumulation outside the core (Figure 7A,B). 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 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 A_2 and thrombin. Adding another layer of complexity, these pro-hemostatic signaling pathways are counter-balanced by inhibitory signals released from intact endothelium such as PGI_2 and nitric oxide. Thrombin receptors are quite common in the human body, especially on cells in circulation. The presence of GDI in epithelial cells suggests that this phenomenon may be exhibited by other cell types.

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