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ORIGINAL ARTICLE

Repeated platelet activation and the potential of previously activated platelets to contribute to thrombus formation

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Abstract

Background: Especially in disease conditions, platelets can encounter activating agents in circulation.

Objectives: To investigate the extent to which previously activated platelets can be reactivated and whether in-and reactivation applies to different aspects of platelet activation and thrombus formation.

Methods: Short-and long-term effects of glycoprotein VI (GPVI) and G protein-coupled receptor (GPCR) stimulation on platelet activation and aggregation potential were compared via flow cytometry and plate-based aggregation. Using fluorescence and electron microscopy, we assessed platelet morphology and content, as well as thrombus formation.

Results: After 30 minutes of stimulation with thrombin receptor activator peptide 6 (TRAP6) or adenosine diphosphate (ADP), platelets secondarily decreased in PAC-1 binding and were less able to aggregate. The reversibility of platelets after thrombin stimulation was concentration dependent. Reactivation was possible via another receptor. In contrast, cross-linked collagen-related peptide (CRP-XL) or high thrombin stimulation evoked persistent effects in $\alpha_{IIb}\beta_3$ activation and platelet aggregation. However, after 60 minutes of CRP-XL or high thrombin stimulation, when $\alpha_{IIb}\beta_3$ activation slightly decreased, restimulation with ADP or CRP-XL, respectively, increased integrin activation again. Compatible with decreased integrin activation, platelet morphology was reversed. Interestingly, reactivation of reversed platelets again resulted in shape change and if not fully degranulated, additional secretion. Moreover, platelets that were previously activated with TRAP6 or ADP regained their potential to contribute to thrombus formation under flow. On the contrary, prior platelet triggering

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with CRP-XL was accompanied by prolonged platelet activity, leading to a decreased secondary platelet adhesion under flow.

Conclusion: This work emphasizes that prior platelet activation can be reversed, whereafter platelets can be reactivated through a different receptor. Reversed, previously activated platelets can contribute to thrombus formation.

KEYWORDS
receptors, G-protein-coupled, platelet activation, glycoprotein VI, thrombus formation, integrins

1 | INTRODUCTION

Blood platelets express a broad range of receptors, which support the roles of platelets in thrombus formation, such as shape change, granule release, and aggregation [1,2], among which the integrin $\alpha_{IIb}\beta_3$ receptor is one of the most highly expressed receptors on platelets, with an abundance of 80 000 surface copies [3]. In resting platelets, the affinity of integrin $\alpha_{IIb}\beta_3$ for its ligands is low [4]. Upon platelet activation, shape change occurs, integrins undergo an activating conformational change, and the granular content is secreted. During shape change, discoid platelets change into spheres that protrude filopodia [5]. The integrins are activated by extending or "opening" the extracellular regions of both integrin chains, which allows them to bind ligands, such as fibrinogen, with a higher affinity [6]. However, upon strong stimulation, integrin closure can occur when platelets reach a procoagulant state [7]. This process is unidirectional because the integrin β3 subunit is cleaved by calpain [7–9]. Alternatively, integrin inactivation can occur when continuous agonist-induced signaling is abrogated by inhibitors, resulting in the disassembly of thrombi [10,11]. It is still unclear when and to what extent the reversal of platelet activation processes occurs. Further, it is unknown whether platelets take up plasma components after secretion, to form granules again, as a mechanism to recycle. Wencel-Drake et al. [12] showed that thrombin receptor activator peptide 6 (TRAP6)-induced binding of fibrinogen to platelets decreased over time, thereby demonstrating increased integrin $\alpha_{IIb}\beta_3$ internalization and reduced aggregability. Interestingly, these platelets were still able to respond to adenosine diphosphate (ADP). The ability to reverse platelet activation by different agonists and its consequences for thrombus formation remains to be investigated.

Within a thrombus, platelets in contact with adhesive ligands, such as the glycoprotein VI (GPVI) ligand collagen, form the thrombus core, which is tightly packed and sustained by higher concentrations of thrombin. This core is surrounded by the thrombus shell, in which the platelets bind loosely and activation here is sustained by soluble agonists such as the G protein-coupled receptor (GPCR) ligands ADP and thrombin (at a lower concentration compared with the core). As the variability in the potency of stimuli, by type and concentration of agonist is crucial for thrombus organization [13], we set out to investigate to which extent platelet inactivation occurs upon agonist stimulation by comparing GPVI vs GPCR stimulation. Given that platelets can encounter activating agents during their lifetime (8-10 days), in pathophysiologic conditions [14], after flowing over an incipient thrombus or after being loosely incorporated in a thrombus shell, we aimed to determine whether previously activated platelets recycle and reset their capacity to respond to agonists. Additionally, we also studied the thrombogenic potential of previously activated platelets.

Essentials

- Given that platelets can encounter activating agents during their lifetime, we aimed to determine whether previously activated platelets can recycle and reset their capacity in response to agonists.
- Short-and long-term effects of glycoprotein VI (GPVI) and G protein-coupled receptor (GPCR) stimulation on platelet morphology, activation, and thrombus formation were evaluated using flow cytometry, plate-based aggregation, electron microscopy, and microfluidics.
- The reversal of platelet activation and reactivation by a second stimulus occurs when platelets are exposed to the agonists thrombin receptor activator peptide 6 (TRAP6), low thrombin, or adenosine diphosphate (ADP), but to a lesser extent in high thrombin- or cross-linked collagen-related peptide (CRP-XL)-stimulated platelets.
- Previously activated platelets returning to a resting state can contribute to thrombus formation.

2 | METHODS

2.1 | Materials

Cross-linked collagen-related peptide (CRP-XL) was obtained from Professor Richard Farndale (University of Cambridge, UK). TRAP6 (SFLLRN) was from Bio Connect. ADP and fibrinogen were purchased from Sigma-Aldrich, and Horm type I collagen was obtained from Nycomed Pharma. Fluorescein isothiocyanate (FITC)-conjugated PAC-1 antibody against active integrin $\alpha_{IIb}\beta_3$ and FITC-conjugated anti-CD61 antibody against β_3 were from BD Bioscience. Alexa Fluor (AF) 647-conjugated human fibrinogen and Annexin A5 AF647-conjugated

were from Invitrogen. AF647-labeled anti-human CD62P mAb was from Biolegend. AF488-conjugated anti-human CD42b (anti-GPIb) was from R&D systems.

2.1.1 | Blood collection

Blood was obtained by venepuncture from healthy volunteers, who had not received antiplatelet medication for at least 2 weeks. All volunteers gave full informed consent according to the Declaration of Helsinki. The study was approved by the Maastricht University Medical Ethics Committee (MET2017-0285). After discarding the first 2 mL of blood, to avoid contact activation, blood samples were collected into 3.2% trisodium citrate (Vacuette tubes, Greiner Bio-One). Platelet counts were within the reference range (150-450 $\times$ 10^9 platelets/L), as measured with a Sysmex XP-300 analyzer (Sysmex).

2.1.2 | Preparation of washed platelets and plasma

Platelet isolation was performed as described before [15]. In short, platelet-rich plasma (PRP) was obtained by centrifugation of citrated whole blood at 258 g for 15 minutes. To isolate platelets from PRP, PRP was centrifuged at 2200 g for 2 minutes, after adding 1:10 acid citrate dextrose (80 mM Trisodium citrate (.2H2O), 52 mM Citric acid, 183 mM D-(+)-glucose). Hereafter, the platelet pellet was resuspended in HEPES buffer pH 6.6 (10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose, and 0.1 % bovine serum albumin). After the addition of Apyrase (0.1 U/mL) and acid citrate dextrose (1:15), another centrifugation step (2200 g, 2 minutes) followed. Eventually, platelets were resuspended in HEPES buffer pH 7.45 (10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose, and 0.1 % bovine serum albumin). Platelet-poor plasma was obtained from citrated blood, by double centrifugation at 2500 g for 10 minutes.

2.1.3 | Plate-based aggregation

Measurements of aggregation were performed using 96-well plates (Greiner). PRP isolated from the citrated blood of healthy donors was incubated with 30 μM ADP, 3 μg/mL CRP-XL, or 15 μM TRAP6. Immediately after stimulation, after 30 and 60 minutes of incubation with the agonist at 37 °C, subsamples were loaded into plates and shaken at 1200 rpm for 5 minutes at 37 °C using a plate shaker (Quantifoil Instruments). Platelet-poor plasma was set as the control for maximal aggregation. Absorption at 405 nm was measured using a FlexStation 3 (Molecular Devices).

2.1.4 | Flow cytometric analysis

Washed platelets (50 $\times$ 10^9 plts/L) were incubated with low or high doses of agonist (5 and 50 μM ADP; 1 and 5 nM thrombin; 7.5 and 15 μM TRAP6; 0.5 and 5 μg/mL CRP-XL) in the presence of 2 mM $CaCl_2$, at 37 °C. Integrin activation and P-selectin exposure were measured in subsamples after 10, 30, or 60 minutes of stimulation using FITC PAC-1 (1.25 μg/mL) or AF647 anti-CD62P (2.5 μg/mL). PAC-1 and anti-CD62P antibodies were added during the last 10 minutes of agonist incubation. After 60 minutes of agonist incubation, samples were restimulated with the highest concentrations of ADP (50 μM), CRP-XL (5 μg/mL), TRAP6 (15 μM), or thrombin (5 nM) for 10 minutes and again integrin activation and P-selectin were measured. Flow cytometry was performed using an Accuri C6 flow cytometer and software (BD Bioscience).

2.1.5 | Scanning and transmission electron microscopy

Washed platelets in the presence of 2 mM $CaCl_2$, unstimulated or stimulated with 5 μg/mL CRP-XL, 15 μM TRAP6, 5 nM thrombin, or 50 μM ADP were subsampled after 10 and 60 minutes and fixed with 1.5% glutaraldehyde in 0.1 M phosphate buffered to pH 7.4. Subsamples were also taken after 60 minutes for restimulation with 50 μM ADP or 5 μg/mL CRP-XL, whereafter fixation followed.

For scanning electron microscopy (SEM), fixed samples were washed with 0.1 M cacodylate (pH 7.4), followed by incubation in 1% osmium tetroxide and 1.5% $K_4Fe(CN)_6$ in 0.1 M sodium cacodylate (pH 7.4) for 1 hour at 4 °C. Platelets were rinsed with Milli-Q water and dehydrated at room temperature in a graded ethanol series (from 70 up to 100%), whereafter they were washed twice in hexamethyldisilazane for 10 minutes. When dried, SEM samples were ready for imaging using an SEM Jeol JSM-IT200 InTouchScope (Jeol).

For transmission electron microscopy (TEM), fixed samples were washed with 0.1 M cacodylate (pH 7.4) followed by incubation in 1% osmium tetroxide and 1.5% $K_4Fe(CN)_6$ in 0.1 M sodium cacodylate (pH 7.4) for 1 hour at 4 °C. Platelets were rinsed with Milli-Q water, dehydrated at room temperature in a graded ethanol series (from 70 up to 100%), and embedded in Epon. Epon was then polymerized for 48 hour at 60 °C. Around 50 nm sections were cut using a diamond knife (Diatome) on a Leica UC7 ultramicrotome and were transferred onto 50 Mesh copper grids covered with a formvar and carbon film, whereafter they were poststained with uranyl acetate and lead citrate. TEM samples were imaged using FEI Tecnai T12 microscopes (Thermo Fisher Scientific) at 120kV using an Eagle CCD camera.

2.1.6 | Thrombus formation with preactivated platelets

The ability of prior stimulated platelets to contribute to thrombus formation was investigated using the Maastricht flow chamber [16]. Therefore, washed platelets (500 $\times$ 10^9 plts/L) in the presence of 2 mM $CaCl_2$ were either left unstimulated (control) or were stimulated with ADP (50 μM), TRAP6 (15 μM) or CRP-XL (5 μg/mL) at 37 °C. A subsample of the (un)stimulated platelets was taken directly or after 30 minutes and was added (250 $\times$ 10^9 plts/L) to washed red blood

cells (40%) and fibrinogen (80 μg/mL), to expose the platelets to a blood-like environment. $DiOC_6$ (0.5 μg/mL, f.c.) was added to the 'reconstituted blood' to visualize the adhered platelets. Coverslips were coated with microspots of 100 μg/mL collagen type I or 100 μg/ml human fibrinogen + 12.5 μg/mL von Willebrand factor-Binding Peptide (VWF-BP), as described elsewhere [16].

After 1-minute plasma perfusion, platelets in the reconstituted environment were perfused through the Maastricht flow chamber, at a wall-shear rate of 1000 s^{-1}. After 3 minutes perfusion, tile scans and confocal fluorescence images were made of the whole microspot, using a Zeiss LSM7 Microscope equipped with a 63 × oil-immersion objective (Carl Zeiss).

2.1.7 | Fibrinogen uptake and secretion

Washed platelets (100 × 10^9/L), incubated with 30 μg/mL AF647-fibrinogen in the presence of 2 mM $CaCl_2$, were left unstimulated (control) or were stimulated with 5 μg/mL CRP-XL, 15 μM TRAP6 or 50 μM ADP. Furthermore, subsamples were stained with an anti-GPIbα AF488 conjugated antibody (1.3 μg/mL), 10 minutes before fixation. After 10 and 60 minutes of stimulation, subsamples were fixed with paraformaldehyde (1% f.c.) for 15 minutes. Hereafter, fixed samples were centrifuged at 2200 g for 2 minutes. The platelet pellet was resuspended in PBS and samples were subsequently spun down at 250 g for 10 minutes on poly-L-lysine coated coverslips (0.01%). Z-stacks were recorded, using a Leica DMI 4000 microscope (Leica Microsystems) to investigate the cellular localization of AF647 fibrinogen in platelets using Fiji software [17].

2.2 | Statistical analysis

The normality of data was assessed with the Shapiro-Wilk test and the ROUT method was used to detect outliers. Data are presented as mean ± SD in case of normality and homogeneity of variances. Mean values were compared using 1-way analysis of variance. If appropriate, data were presented as median ± IQR and the Kruskal-Wallis test was applied. Differences with p values of <.05 were considered statistically significant: $^*p$ <.05, $^{**}p$ <.01, and $^{***}p$ <.001. GraphPad Prism 8 software was used for statistical analysis.

3 | RESULTS

3.1 | Platelet activation and aggregation potential decreases over time following agonist stimulation

To investigate the capacity of platelets to aggregate after previous exposure to an agonist, PRP was incubated with CRP-XL (3 μg/mL), TRAP6 (15 μM), or ADP (30 μM) for different time intervals (5, 30, and 60 minutes) (Figure 1A). The aggregation response appeared to be the highest immediately after the stimulation. After 30 minutes of incubation with TRAP6, aggregation significantly decreased by 32% ± 10%. After 60 minutes of incubation, aggregation significantly reduced with all 3 agonists, although the reduction was higher after TRAP6 (48% ± 8%) or ADP (35% ± 18%) than after CRP-XL (15% ± 6%).

We hypothesized that there may be different reversibility of platelet activation in washed platelets compared with the platelets in plasma, for instance, because of the presence of exonuclease activity or higher levels of fibrinogen. To study the intrinsic potential of platelets to reverse, experiments were therefore performed using washed platelets. Agonist-induced integrin $\alpha_{IIb}\beta_3$ activation (FITC-PAC1 mAb), secretion (P-selectin expression, AF647-CD62P mAb), and PS exposure (AF647-Annexin A5) were measured by performing 2-color flow cytometric analysis using washed platelets (Figure 1B–C). Platelets were activated with low or high concentrations of CRP-XL, TRAP6, thrombin, or ADP for 10, 30, or 60 minutes. Both TRAP6 and thrombin were used as agonists to differentiate between the contribution of protease-activated receptor-1 (PAR1) alone and PAR1 plus PAR4 to (the reversal of) platelet activation.

After 10 minutes of agonist stimulation, there was a significant increase in PAC-1 positive platelets, compared with the unstimulated condition for all agonists (Figure 1B). After 30 and 60 minutes of stimulation with TRAP6 or ADP, PAC-1 binding decreased and did not significantly differ from the unstimulated condition anymore (Figure 1B). On the other hand, PAC1-binding after 30- and 60-minutes stimulation with 5 μg/mL CRP-XL or 5 nM thrombin was still significantly higher than PAC-1 binding of the unstimulated (control) platelets (Figure 1B). In contrast, after stimulation with 1 nM thrombin, PAC-1 binding decreased after 60 minutes (Figure 1B). To distinguish between the role of PAR1 and PAR4 in thrombin-induced platelet activation, experiments were performed in the presence of the PAR1 inhibitor Atopaxar (5 μM), which confirmed that activation with 1 nM thrombin was completely dependent on PAR1 signaling because integrin activation and P-selectin expression were fully inhibited. In contrast, Atopaxar did not suppress the platelet activation responses upon 5 nM thrombin, indicating that sustained signaling is mediated through PAR4 (Supplementary Figure 1).

Because our data suggest that the reversibility of platelets after thrombin stimulation depends on the thrombin concentration, the effect of different CRP-XL concentrations on the reversibility of GPVI-induced platelet activation was further investigated. For this purpose, a dose range of CRP-XL concentrations (5, 2.5, 1, and 0.5 μg/mL) was tested. After 10 minutes stimulation, there was an increase in PAC-1 and P-selectin positive platelets compared with the unstimulated condition, regardless of the CRP-XL concentrations used, which was still the case after 60 minutes. Hence, PAC-1 binding after CRP-XL stimulation remained relatively high over time independent of the concentration, in contrast to thrombin. (Supplementary Figure 2). The P-selectin expression after high dose CRP-XL, TRAP6, or thrombin stimulation remained high over time (80%-90%) (Figure 1C).

Platelet activation by the agonists did not result in significant phosphatidylserine exposure (Supplementary Figure 3A). Since PAC-1 binding following agonist stimulation decreased over time, we checked whether this was due to β3 cleavage or internalization by

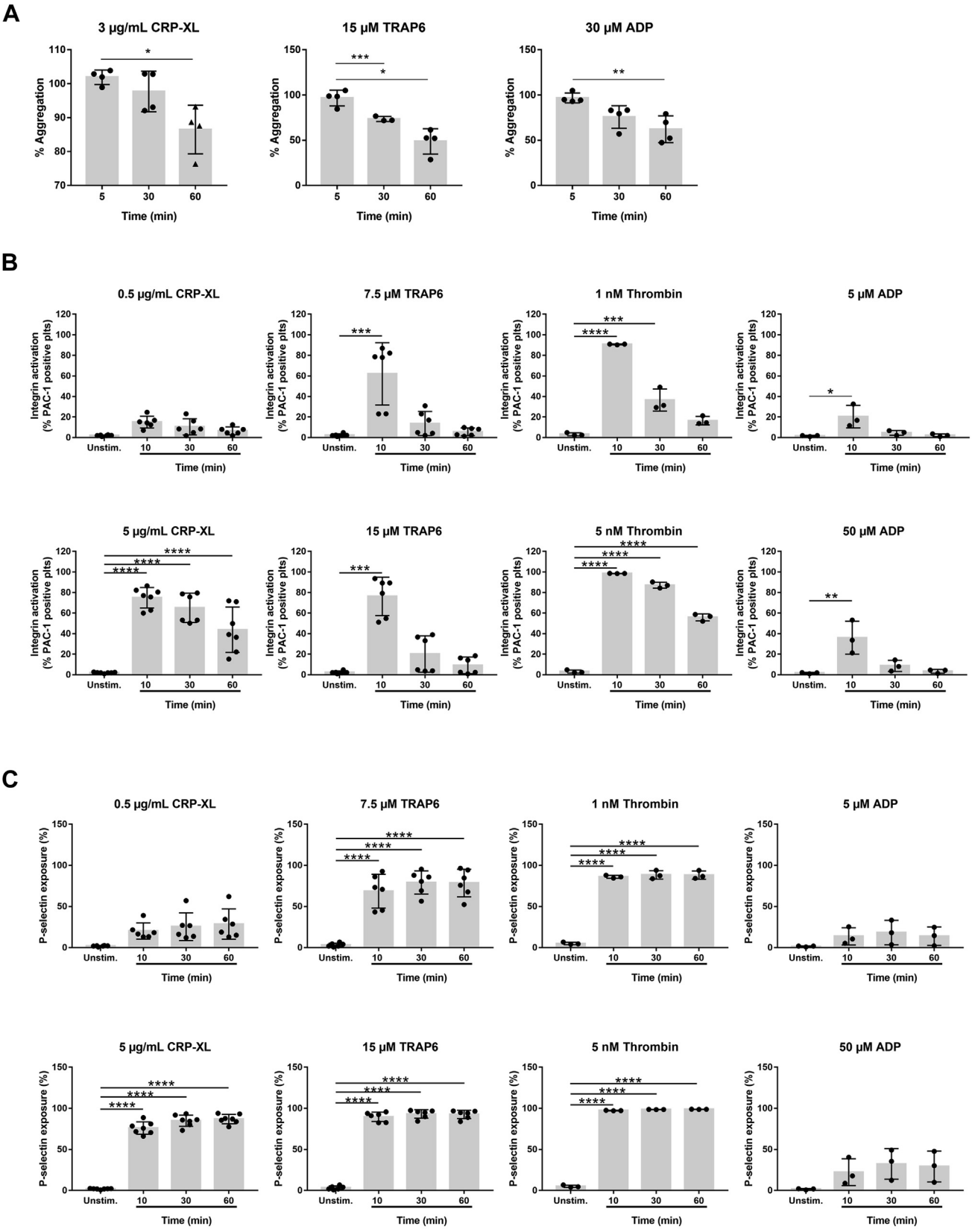

FIGURE 1 Agonist-induced platelet integrin $\alpha_{IIb}\beta_3$ activation and aggregation decrease over time. **A**. Platelet-rich plasma preincubated with 3 µg/mL CRP-XL, 15 µM TRAP6, and 30 µM ADP, for 5, 30, or 60 minutes, respectively. Platelet aggregation was assessed by well-plate-based light transmission changes. **B, C**. Washed platelets were activated with low and high concentrations of agonist (0.5 and 5 µg/mL CRP-XL; 7.5 and 15 µM TRAP6; 1 and 5 nM Thrombin; 5 and 50 µM ADP), in the presence of 2 mM $CaCl_2$. After 10, 30, and 60 minutes of activation, integrin $\alpha_{IIb}\beta_3$ activation **(B)** and P-selectin expression **(C)** were measured by flow cytometry. Mean ± SD, n = 3-7; 1-way ANOVA, $^*p$ <.05, $^{**}p$ <.01, $^{***}p$ <.001, $^{****}p$ <.0001 or Median ±IQR, n = 6; Kruskal-Wallis $^*p$ <.05, $^{**}p$ <.01, $^{***}p$ <.001, $^{****}p$ <.0001. ADP, adenosine diphosphate; CRP-XL, cross-linked collagen-related peptide; TRAP6, thrombin receptor activator peptide 6.

measuring CD61 expression (Supplementary Figure 3B). No alterations in levels of β3 could be detected over time. Together, this suggests that at least a part of the activated integrins on activated platelets can be transformed into a 'low affinity' state.

3.2 | Previously activated platelets can be reactivated by agonists

Next, we investigated whether previously stimulated platelets can be reactivated by a second stimulus, once integrin activation was reversed (60 minutes after stimulation). To this end, previously stimulated platelets were reactivated with a high agonist dose (5 μg/mL CRP-XL, 15 μM TRAP6, 5 nM thrombin, or 50 μM ADP). Interestingly, integrin activation of platelets previously incubated with CRP-XL, only increased significantly after restimulation with ADP (from 5% ± 3% to 39% ± 18% for low CRP-XL and from 29% ± 11% to 57% ± 17% for high CRP-XL); or after restimulation with TRAP6 if previously stimulated with low CRP-XL (from 5% ± 3% to 31% ± 4%). Restimulation with a second CRP-XL dose did not increase the integrin activation (Figure 2A). Further, platelets that were prior activated with TRAP6, only showed elevated integrin activation after restimulation with CRP-XL, up to 44% ± 13% if previously stimulated with low TRAP6 or up to 51% ± 9% after high TRAP6 stimulation. A restimulation with CRP-XL, up to 68% ± 20%, was also possible after prior activation with low thrombin (Figure 2A). Markedly, platelets previously stimulated with ADP showed integrin reactivation induced by either TRAP6 or CRP-XL (Figure 2A). TRAP6 increased the fraction of PAC1-positive platelets from 2.3% ± 1.3% to 29% ± 7.3% (low dose ADP) and 32% ± 11% (high dose ADP), while CRP-XL resulted in 56% ± 3.7% (low dose ADP) and 55% ± 7.0% (high dose ADP) PAC-1 positive platelets (Figure 2A).

Under conditions whereupon the initial activation P-selectin expression was maximal, as observed with high TRAP6, thrombin, or CRP-XL (Figure 2B), P-selectin expression remained maximal over time, regardless of restimulation. However, as expected, ADP induced only limited P-selectin expression, which then significantly increased upon restimulation with TRAP6 or CRP-XL (Figure 2B). Taken together, in platelets, there is a time-dependent pattern of granular secretion, integrin inactivation, and reactivation through heterologous receptors.

3.3 | Platelet fibrinogen uptake and secretion

We questioned whether the reversal of platelet activation also entails the reuptake of secreted proteins or plasma proteins, such as fibrinogen, which can be secreted again on activation by a second stimulus. Therefore, washed unstimulated platelets or platelets stimulated with CRP-XL, TRAP6, or ADP were exposed to AF647-labeled fibrinogen and after 10-and 60-minutes incubation, fibrinogen binding and uptake were assessed by flow cytometry and confocal microscopy. Flow cytometric analysis revealed that all stimulated platelets were fibrinogen-positive after 10 minutes, but also after 60 minutes (Figure 3A). Because the location of AF647-fibrinogen, on the top or in the middle of the platelets, could not be distinguished by flow cytometry, we applied confocal microscopy imaging. As observed from Z-stack images, after incubation in the absence of an agonist, AF647-fibrinogen was not bound to platelets or taken up (Figure 3B, C upper panels). After 10 minutes of CRP-XL, TRAP6 or ADP stimulation, AF647-fibrinogen was localized in the middle and on the top of the platelets, which was still the same after 60 minutes of CRP-XL incubation, while with TRAP6 or ADP, AF647 fibrinogen was completely in the middle (Figure 3B, C lower panels) and no fibrinogen signal could be detected at the plasma membrane. In case fibrinogen was observed in the middle of the platelets, it was unclear whether fibrinogen was partly or completely internalized or bound to platelets in the open canalicular system (OCS). Eventually, upon (re)stimulation of the platelets with CRP-XL, AF647-fibrinogen was again bound to the middle and the top of the platelets (Figure 3D), as fibrinogen was observed in Z-stack images from the surface and the middle of the platelets.

3.4 | Platelet shape and content change on activation and inactivation

To assess whether platelets that underwent shape change and formed filopodia can return to the smooth, discoid morphology, which is typical for resting platelets, we performed SEM on platelets stimulated with 5 μg/mL CRP-XL, 15 μM TRAP6, 5 nM thrombin or 50 μM ADP for 10 or 60 minutes. The SEM images were quantified by counting the number of platelets that were discoid, protruded filopodia, or had an intermediate phenotype (filopodia ±) and relating this to the total number of platelets per visual field. Upon stimulation with any of the agonists, there was a significant increase in platelets protruding filopodia, while the unstimulated platelets retained their smooth and discoid shape (Figure 4A, B). Interestingly, after 60 minutes of TRAP6 or ADP stimulation, there was a major reduction (of ±80%) in platelets with the filopodia phenotype and a significant increase (of 67% and 57%, respectively) in platelets with the intermediate "filopodia ±" phenotype. This shift in platelet phenotype indicates that not only integrin activation is reversible, but morphologic change is as well. Under conditions in which integrin activation was more persistent, as upon thrombin or CRP-XL activation, the activated platelet morphology remained almost unchanged, with a minor shift (7%) from filopodia to "filopodia ±" phenotype after 60 minutes thrombin stimulation, and no significant changes upon CRP-XL over time. Additionally, no major change could be observed when thrombin- or CRP-XL-stimulated platelets were restimulated with CRP-XL or ADP, respectively. Remarkably, when TRAP6- or ADP-stimulated platelets were restimulated with CRP-XL, platelets were able to form filopodia again.

Further, we investigated the platelet content in resting and activated conditions using TEM (Figure 4C). As expected, resting washed platelets were filled with granules, both after 10 and 60 minutes. The OCS, which is a network of intracellular membrane channels where granules accumulate before secretion [18], was visible in resting platelets, but upon TRAP6 activation, it expanded, as observed after

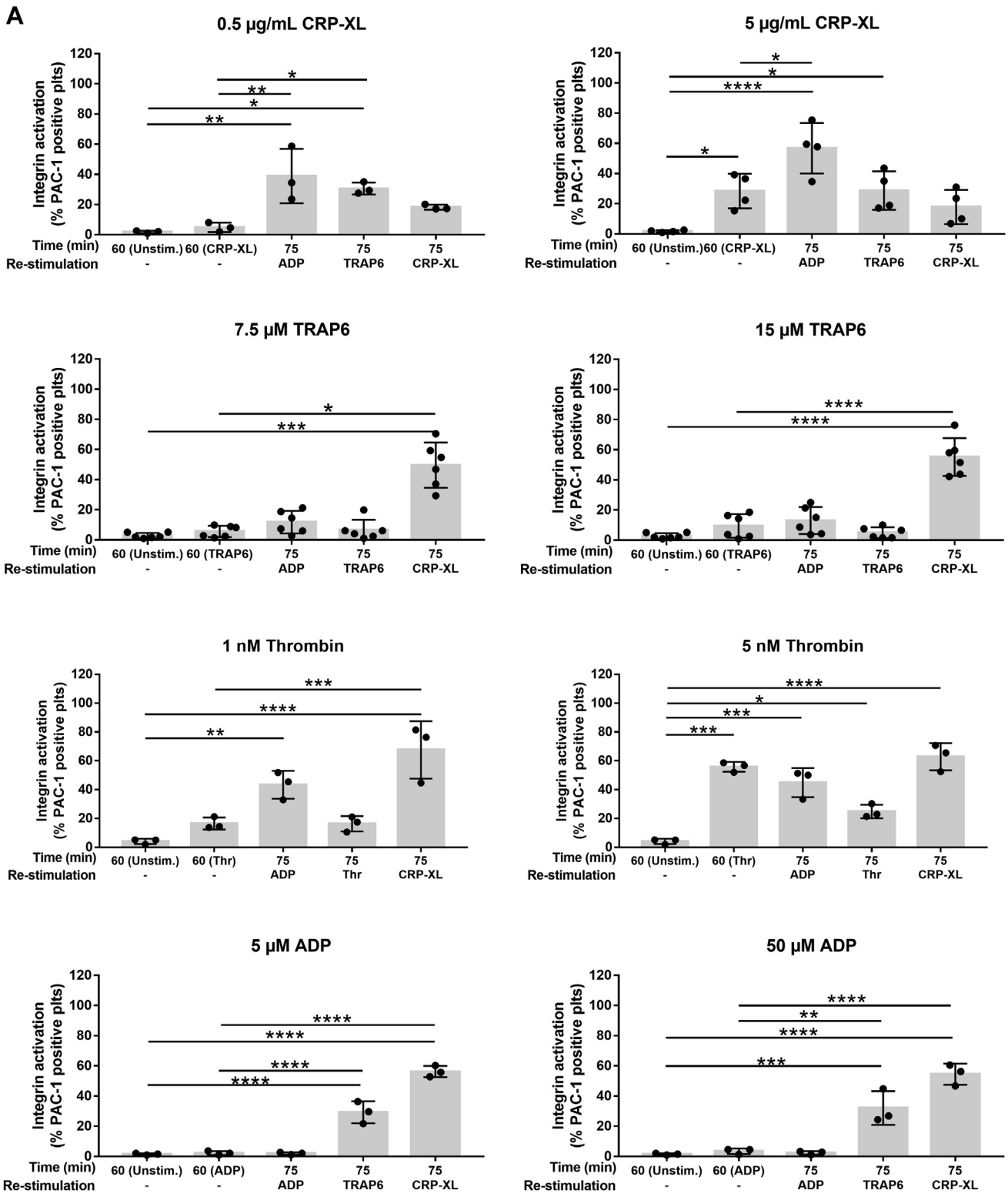

FIGURE 2 When platelet integrin $\alpha_{IIb}\beta_3$ reverses, platelets can be reactivated again. Washed platelets were activated with low and high concentrations of agonist (0.5 and 5 µg/mL CRP-XL; 7.5 and 15 µM TRAP6; 1 and 5 nM Thrombin; 5 and 50 µM ADP), in the presence of 2 mM $CaCl_2$. After 60 minutes of activation, platelets were restimulated with high agonist concentrations (50 µM ADP; 7.5 µM TRAP6 or 5 nM thrombin; 5 µg/mL CRP-XL), integrin $\alpha_{IIb}\beta_3$ activation **(A),** and P-selectin expression **(B)** were measured by flow cytometry, using PAC-1 and anti-P-selectin antibodies, respectively. Mean ± SD, n = 3-6; 1-way ANOVA, *p <.05, **p <.01, ***p <.001, ****p <.0001 or Median ±IQR, n = 6; Kruskal-Wallis *p <.05, **p <.01, ***p <.001, ****p <.0001. ADP, adenosine diphosphate; CRP-XL, cross-linked collagen-related peptide; TRAP6, thrombin receptor activator peptide 6.

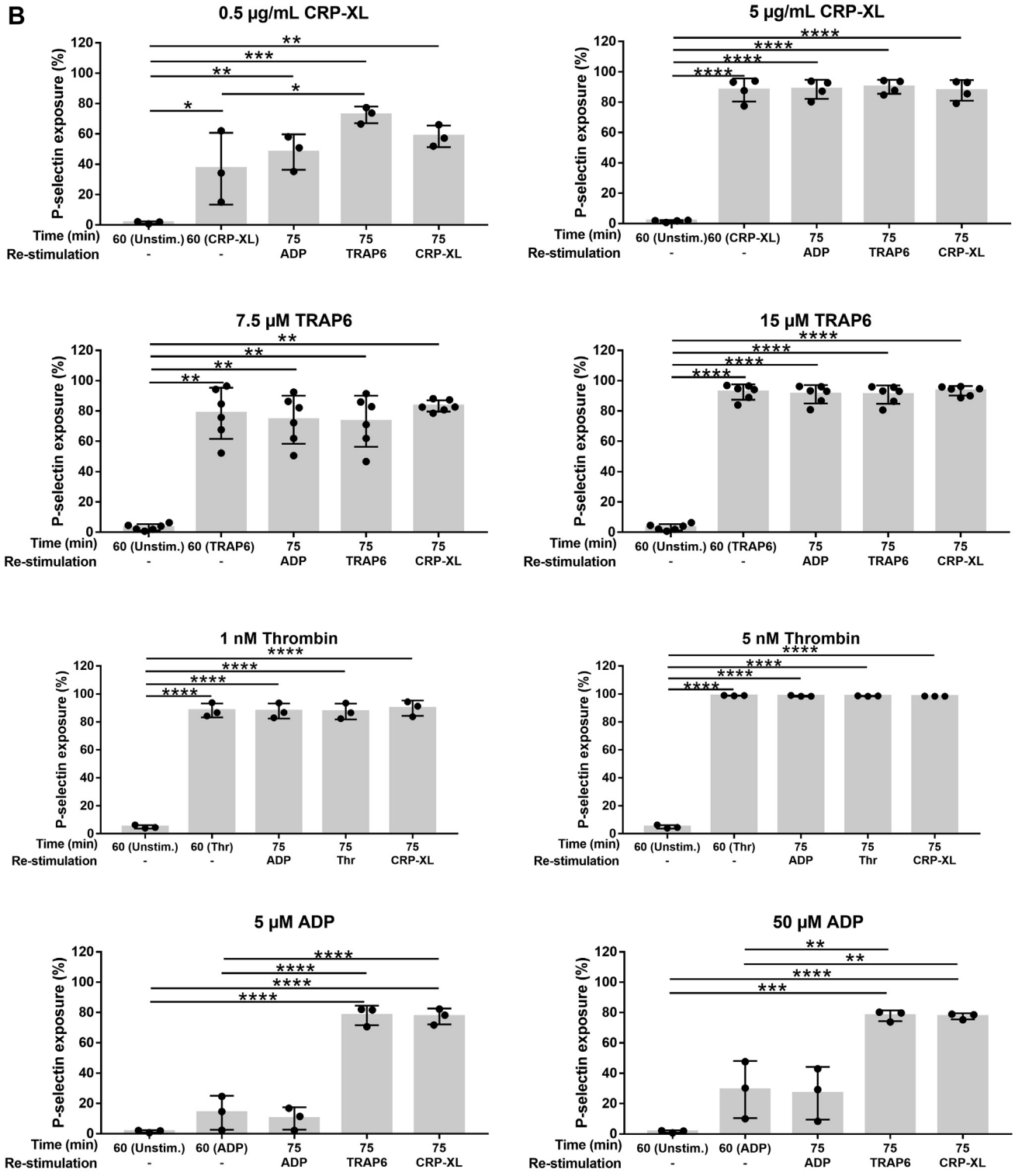

FIGURE 2 (continued)

10 and 60 minutes of incubation. On the other hand, the OCS seemed to become less pronounced after stimulation with CRP-XL, possibly because of fusion with the platelet plasma membrane and platelet fragmentation. Since the stimulation with TRAP6, thrombin, or CRP-XL for 10 and 60 minutes resulted in secretion, no α or dense granules could be observed. Although SEM images showed that TRAP6-stimulated platelets reversed (return to discoid shape and decreased filopodia length) after 60 minutes, such reversal was not detectable in the TEM images of TRAP6-stimulated platelets over time. No differences were noticed intracellularly upon restimulation after 60 minutes of TRAP6- or CRP-XL-stimulated conditions. Stimulation with the weak agonist ADP did not result in granule-depleted platelets, as

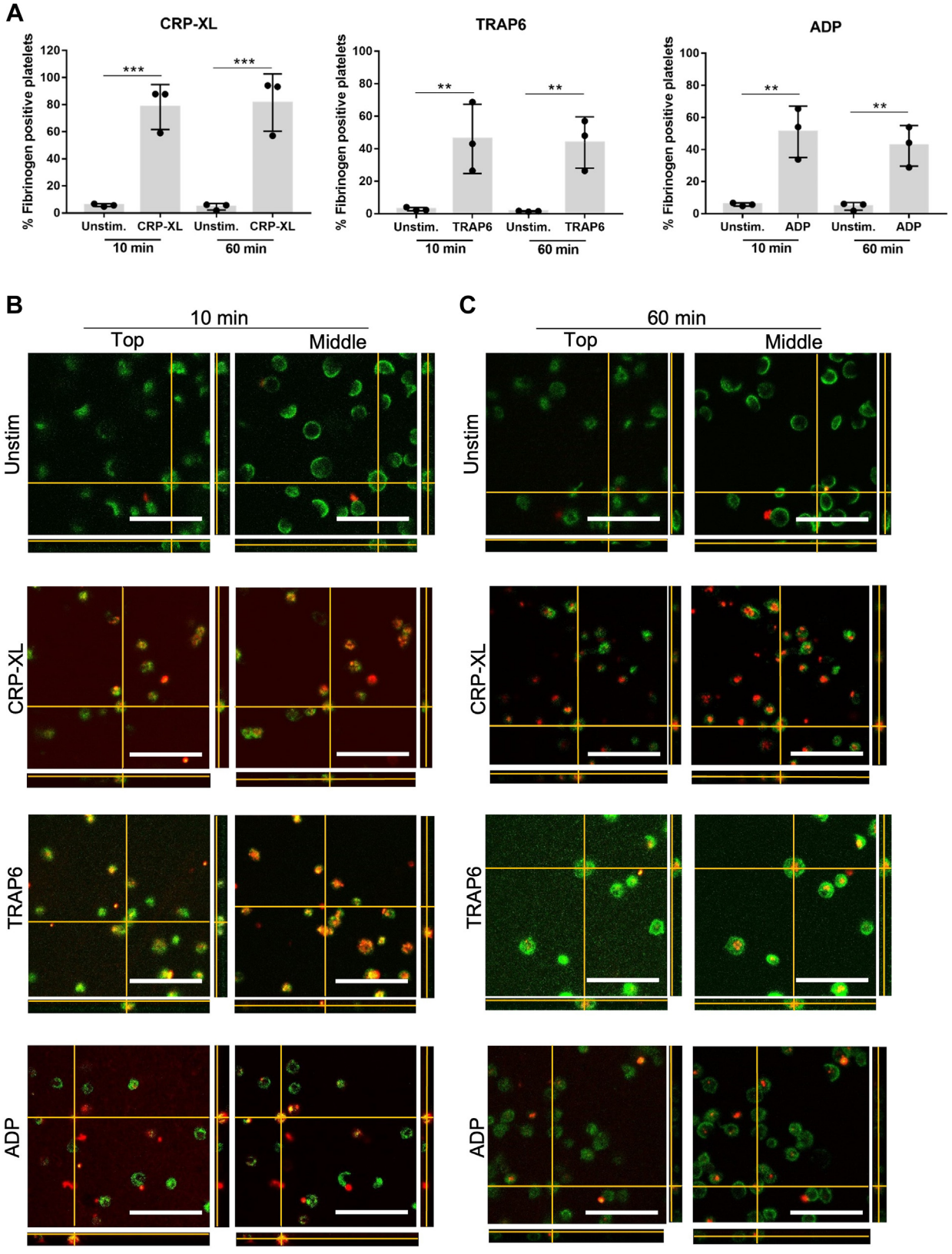

FIGURE 3 Activated platelets internalize fibrinogen. Washed platelets in the presence of AF647 Fibrinogen and 2 mM $CaCl_2$, unstimulated or stimulated with 5 μg/mL CRP-XL,15 μM TRAP6, or 50 μM ADP for 10 or 60 minutes. **A,** Fibrinogen binding was measured by flow cytometry. **B-D,** Washed platelets in the presence of AF647 Fibrinogen and 2 mM $CaCl_2$, unstimulated or stimulated with 5 μg/mL CRP, 15 μM TRAP6 or 50 μM ADP for 10 minutes **(B)** or 60 minutes **(C)**, and restimulated with ADP or CRP-XL **(D)**, were fixed and stained for GPIb (anti-GPIbα AF488 conjugated antibody). Shown are representative microscopy overlays, extracted from Z-stacks. Scale bar = 40 μm. **E,** Quantification of platelets in which fibrinogen binding was localized to the plasma membrane (top of the Z-stack, colocalized with GPIbα) and platelets in which the fibrinogen was detected intracellularly/within the open canicular system (OCS) (middle of the Z-stack). Mean ± SD, n = 3; 1-way ANOVA, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$. ADP, adenosine diphosphate; CRP-XL, cross-linked collagen-related peptide; TRAP6, thrombin receptor activator peptide 6.

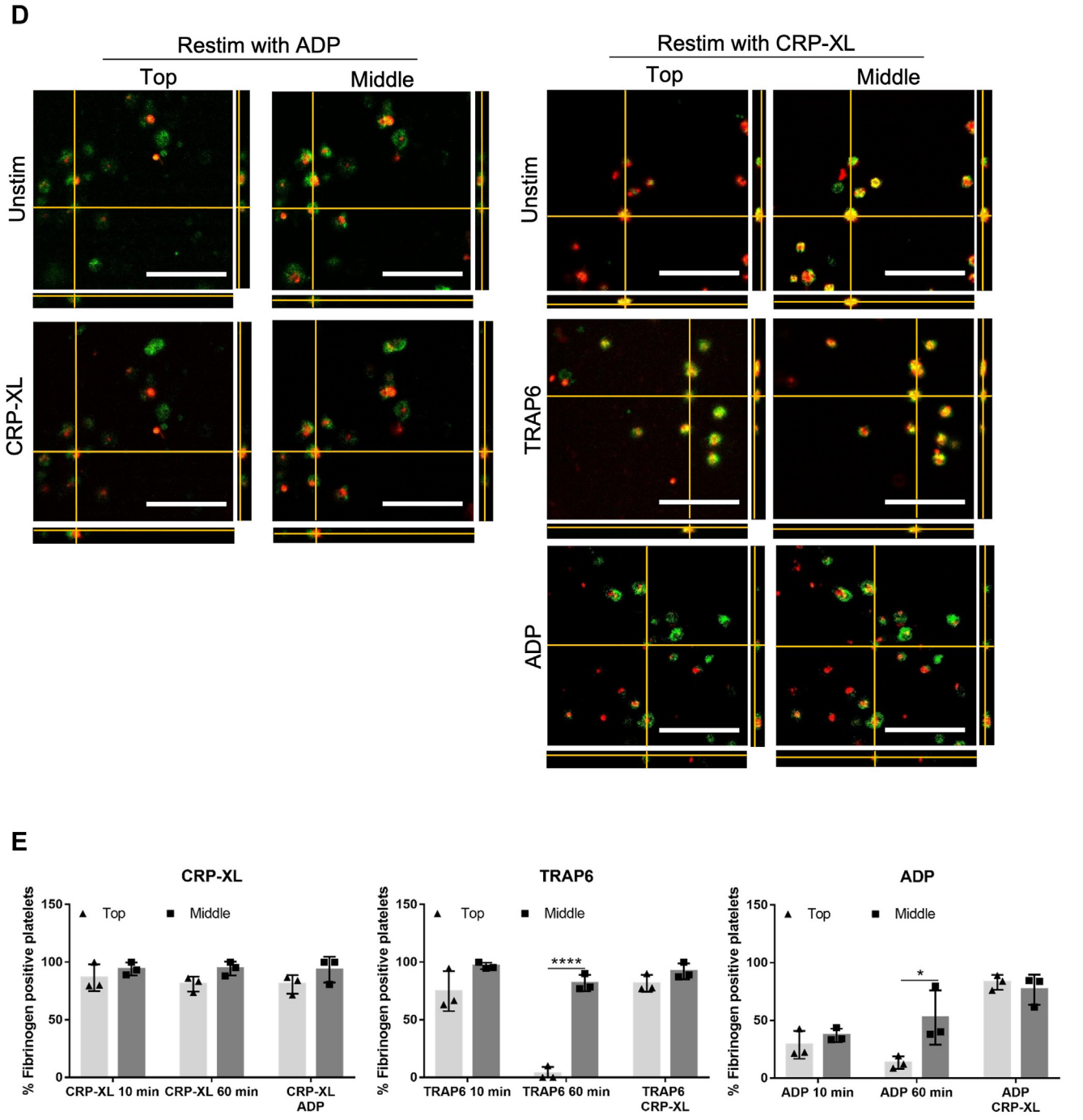

FIGURE 3 (continued)

α-granules were still visible after stimulation. The α-granules, however, started to accumulate in the middle of the platelet, after ADP stimulation, as described before [19]. After CRP-XL restimulation, previously ADP-stimulated platelets further secreted their granules. The outer morphology of ADP-stimulated platelets showed filopodia, as observed from the SEM samples. Overall, the SEM images showed that platelet morphology upon natural inactivation is reversible from filopodia-forming platelets to disc-shaped platelets, whereas the TEM images revealed that the initial signs of intracellular reversibility were not detectable yet.

3.5 | Thrombus formation of previously activated platelets on collagen and fibrinogen surfaces

Because we observed that platelets could be reactivated after a prior stimulation, we hypothesized that previously stimulated platelets could still contribute to thrombus formation in a blood-like environment under flow. Therefore, washed platelets were incubated with CRP-XL, TRAP6, or ADP and reconstituted with fibrinogen and red blood cells, immediately or after long-term incubation of 30 minutes. Then, the reconstituted blood was perfused over microspots coated

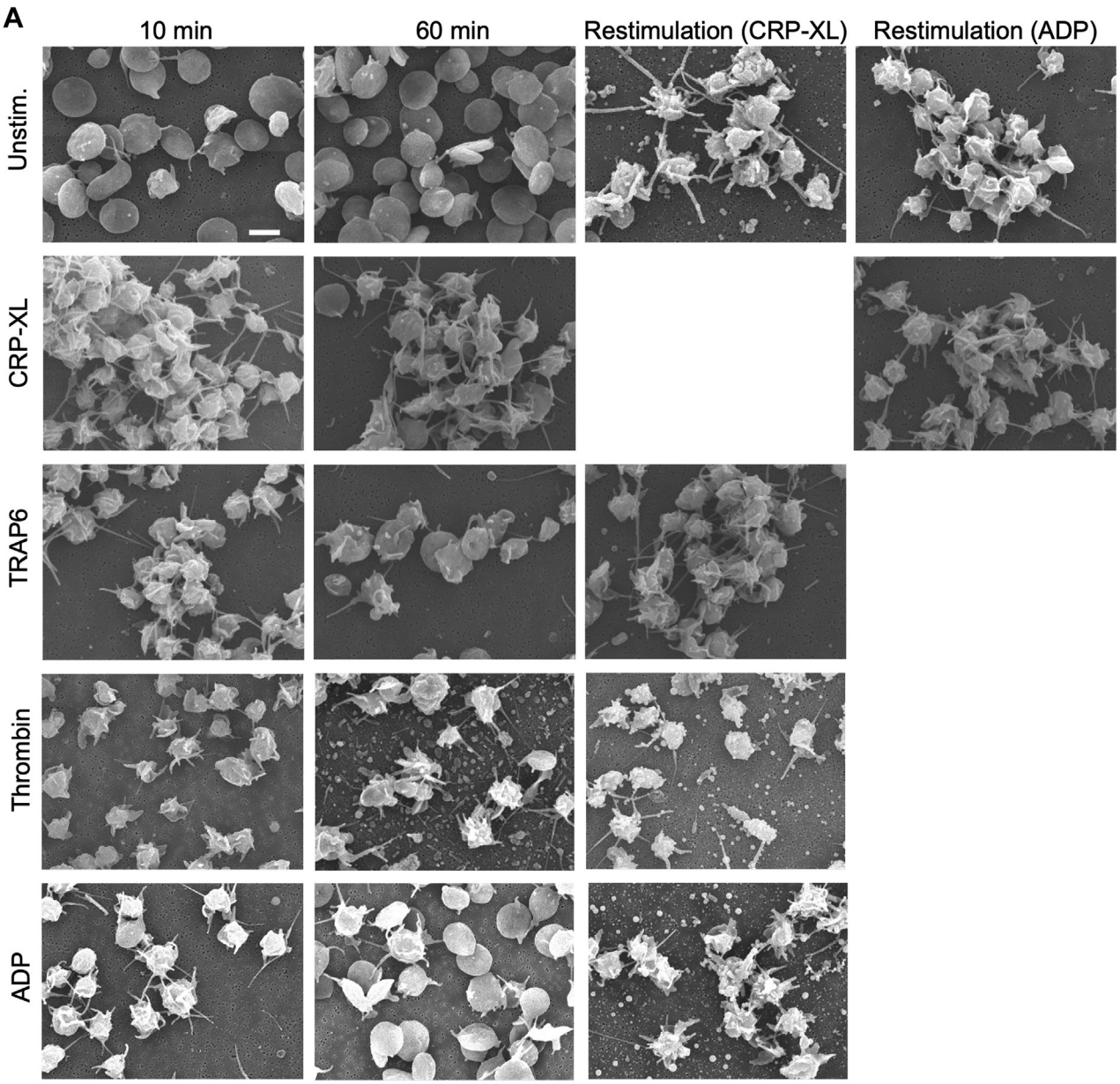

FIGURE 4 Upon reversal of platelet activation, platelets return to their initial discoid morphology, but intracellular reversibility was not detected. Washed platelets were unstimulated or stimulated with 5 μg/mL CRP-XL, 15 μM TRAP6, 5 nM thrombin, or 50 μM ADP for 10 or 60 minutes, in the presence of 2 mM $CaCl_2$. After 10 or 60 minutes of stimulation, or after restimulation, platelet morphology was imaged using scanning electron microscopy, scale bar = 2μm **(A)**. Quantification of platelets being disc-shaped ('Discoid'), having an intermediate phenotype ("filopodia ±"), or forming filopodia ("filopodia") **(B)**. Mean ± SD, n = 3; 2-way ANOVA, $^{*}p$ <.05, $^{**}p$ <.01, $^{***}p$ <.001, $^{****}p$ <.0001, compared with 10 minutes and $^{\#}p$ <.05, $^{\#\#}p$ <.01, $^{\#\#\#}p$ <.001, $^{\#\#\#\#}p$ <.0001, compared with 60 minutes. The platelet content was imaged using transmission electron microscopy, scale bar = 500 nm **(C)**. ADP, adenosine diphosphate; CRP-XL, cross-linked collagen-related peptide; TRAP6, thrombin receptor activator peptide 6.

with collagen or fibrinogen, which were previously rinsed with plasma for von Willebrand factor binding. Unstimulated (control) platelets, reconstituted in a blood-like environment, formed thrombi homogeneously spread over the collagen and fibrinogen surfaces (Figure 5A), with similar coverage of the area per microspot after 0 or 30 minutes (Figure 5E). Platelets prestimulated with CRP-XL formed large thrombi, which were heterogeneously distributed over the microspots resulting in an overall decrease in surface coverage, both when the platelets were added directly after stimulation (0 minute) and after 30 minutes of stimulation (Figure 5B, E). When platelets were pretreated with ADP or TRAP6, again large thrombi were formed shortly after stimulation, however, after the 30 minutes stimulation the thrombi formed on the collagen and fibrinogen surfaces again resembled those formed by unstimulated platelets which homogeneously covered the spot (Figure 5C, D). The percentage of surface area coverage on collagen I spots was significantly lower for platelets immediately after

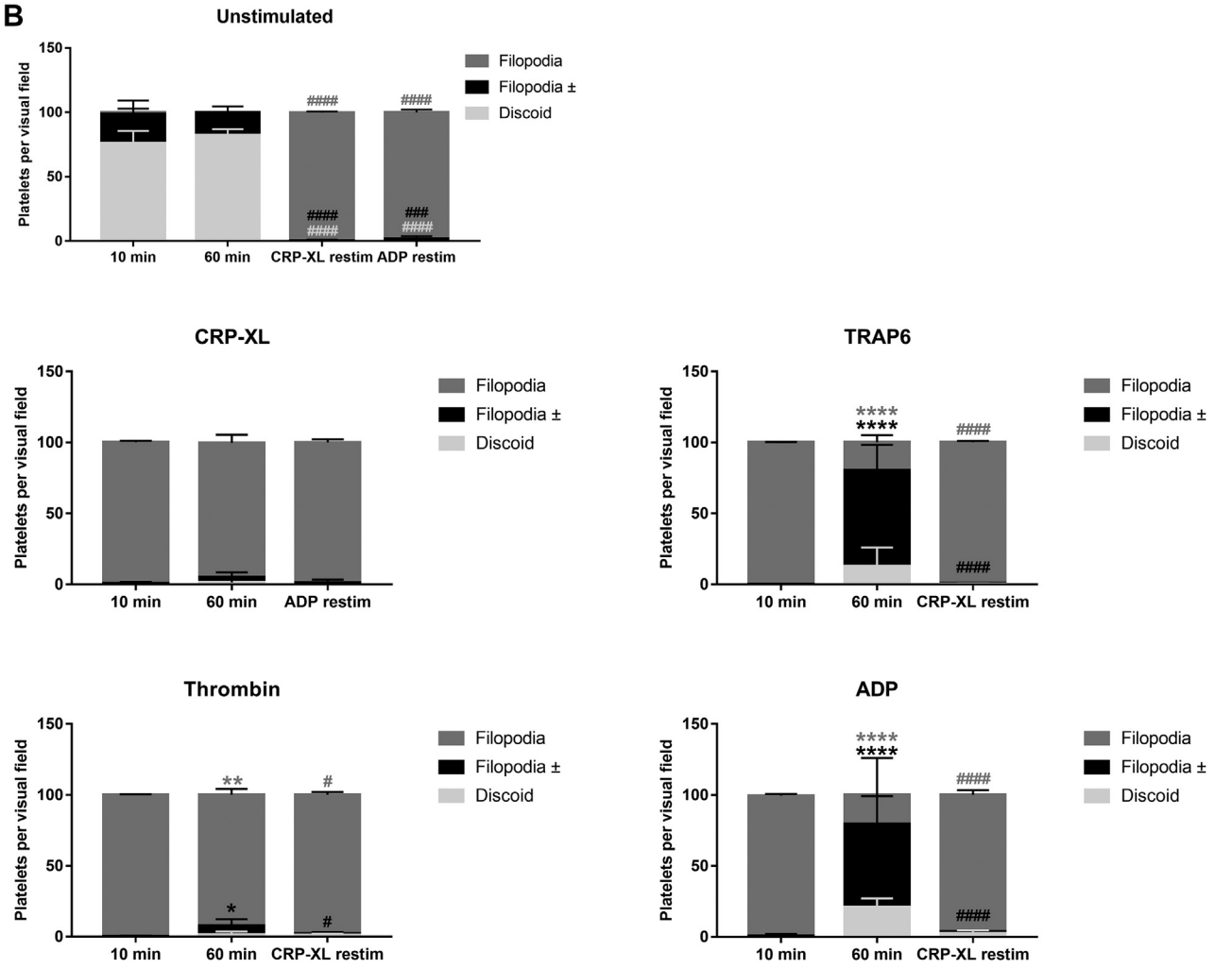

FIGURE 4 (continued)

stimulation with ADP or TRAP6, compared to after 30 minutes of stimulation, in which case the thrombi resembled those of unstimulated (control) platelets (Figure 5E). Taken together, after 30 minutes, the effects induced by stimulation through GPCR receptors were abolished, whereafter the platelets previously stimulated with GPCR agonists could again contribute to thrombus formation, with a potential comparable with unstimulated platelets. On the other hand, the effects of stimulation through GPVI were sustained, leading to a decreased potential of GPVI-stimulated platelets to contribute to secondary thrombus formation under flow.

4 | DISCUSSION

In the present article, we show that reversal of platelet activation and reactivation by a second stimulus occurs when platelets are exposed to the agonists TRAP6, low thrombin, or ADP, but to a lesser extent in high thrombin- or CRP-XL-stimulated platelets. Integrin $\alpha_{IIb}\beta_3$ activation and aggregation decreased over time, especially in TRAP6- or ADP-activated platelets. Remarkably, upon reversal of integrin $\alpha_{IIb}\beta_3$ activation, platelets started to return to their initial smooth, discoid morphology. These platelets could be reactivated again with specific agonists, depending on the initial trigger, resulting in the reformation of filopodia and residual granule content secretion. Interestingly, we showed for the first time that platelets previously activated with the GPCR agonists TRAP6 or ADP regained their potential to contribute to thrombus formation under flow. In contrast, prior platelet triggering with the GPVI agonist CRP-XL was accompanied by more prolonged platelet activity, leading to a decreased secondary platelet adhesion under flow. The observation that platelets previously stimulated through GPCRs regained their potential to contribute to thrombus formation under flow supports all previous reports which described that GPCRs recycle after desensitization [20,21]. The finding that integrin $\alpha_{IIb}\beta_3$ activation after GPCR (PAR1) stimulation is transient, and rather persistent upon GPVI stimulation, is in agreement with the recent findings by Zou et al. [11] showing that there is a maintained Ca^{2+} signal with CRP-XL, in comparison with

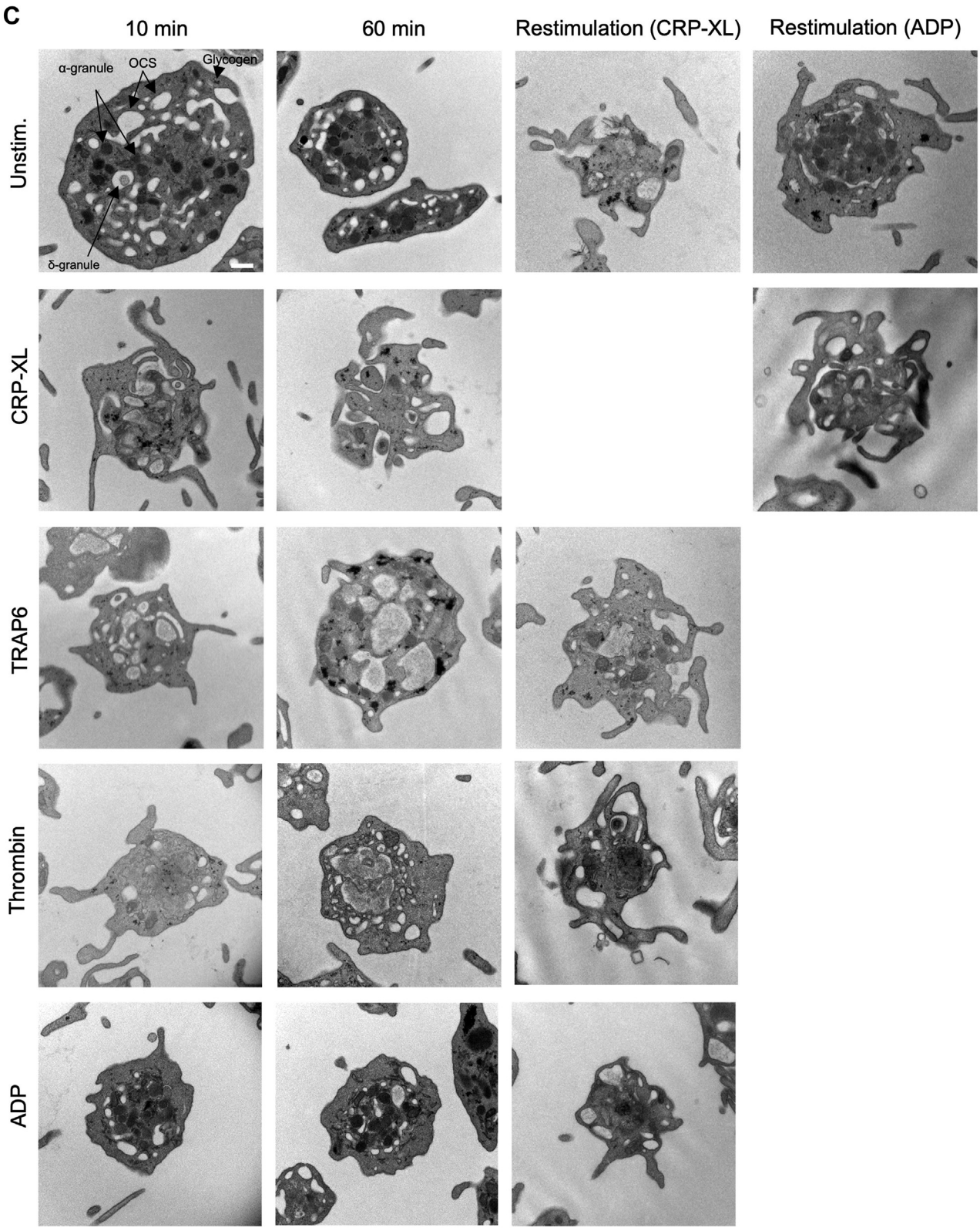

FIGURE 4 (continued)

TRAP6, which points to a longer-term activation state of the platelets. Comparable to the transient activation after TRAP6 stimulation, our results pointed to higher reversibility after platelet stimulation with a lower thrombin concentration. This finding can be explained by the high dependency on PAR1 after low thrombin stimulation, as we showed that PAR1 inhibition abolished the activation responses with

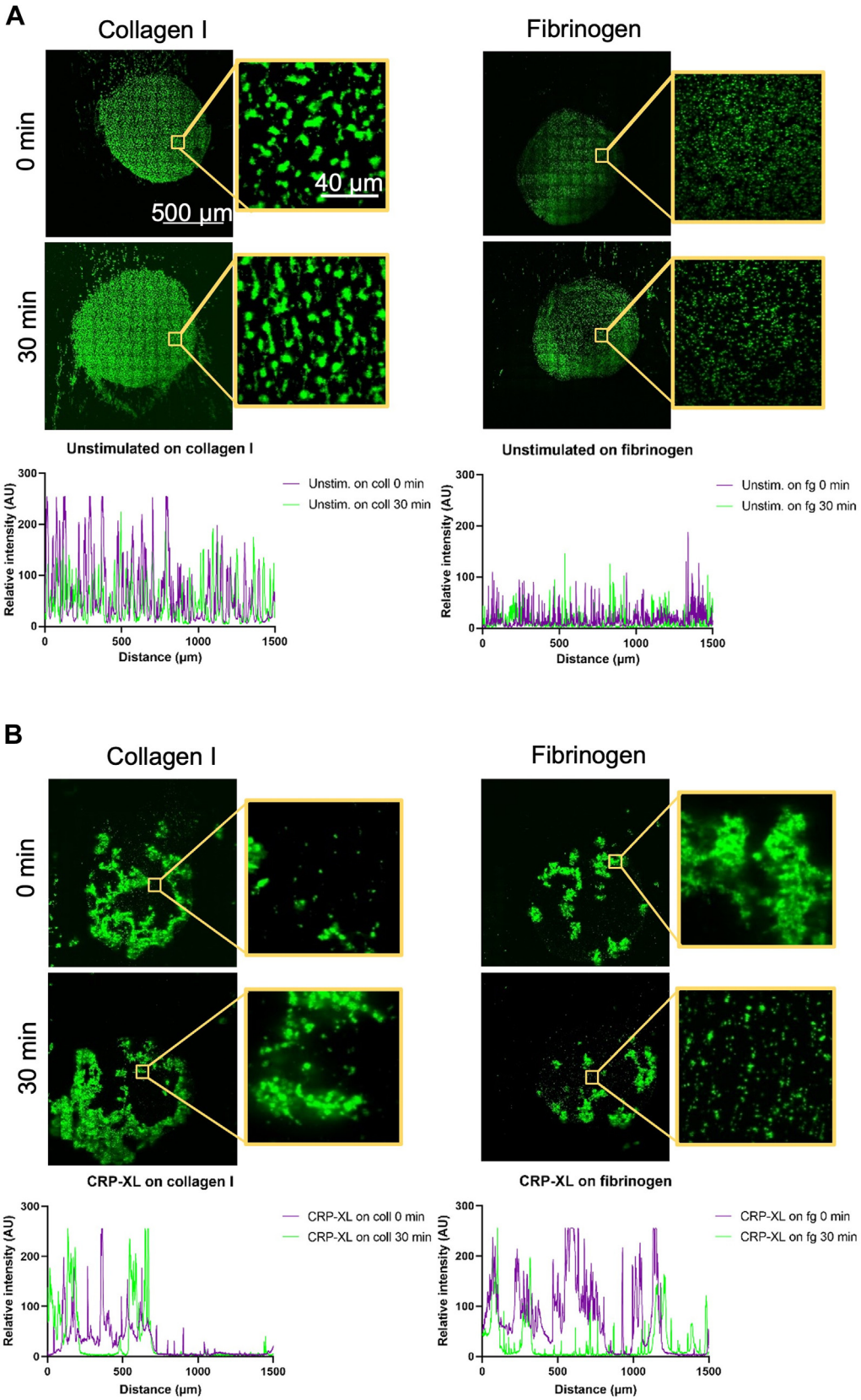

FIGURE 5 GPCR-stimulated platelets regain their aggregation potential under flow, whereas platelet stimulation through GPVI is accompanied by more prolonged platelet activity, leading to a decreased secondary platelet adhesion under flow. Washed platelets, in the presence of 2 mM $CaCl_2$, unstimulated **(A)**, stimulated with 5 µg/mL CRP-XL **(B)**, 15 µM TRAP6 **(C),** or 50 µM ADP **(D)**, stained with DiOC6, reconstituted in a "blood-like" environment, perfused over the microspots collagen I and fibrinogen, at a wall-shear rate of 1000 s^{-1}. After 3 minutes of perfusion, tile scans were made of the whole microspot. Representative images and cross-sectional intensity profile plots of surfaces are shown. **E,** Percentages of fluorescence surface area coverage (%SAC). Mean ± SD, n = 3-5; 1-way ANOVA, $^{*}p$ <.05, $^{**}p$ <.01, $^{***}p$ <.001, $^{****}p$ <.0001. ADP, adenosine diphosphate; CRP-XL, cross-linked collagen-related peptide; TRAP6, thrombin receptor activator peptide 6.

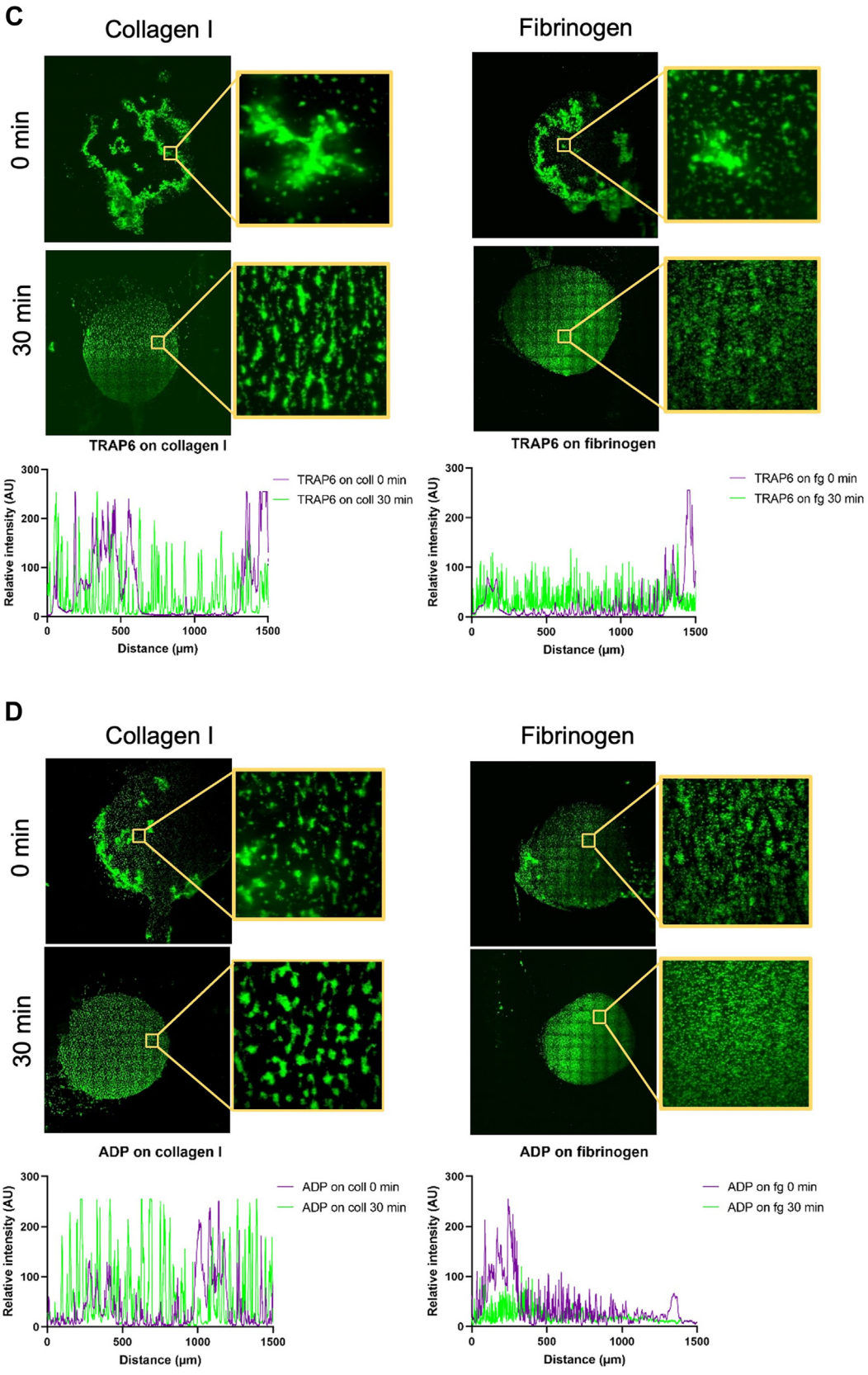

FIGURE 5 (continued)

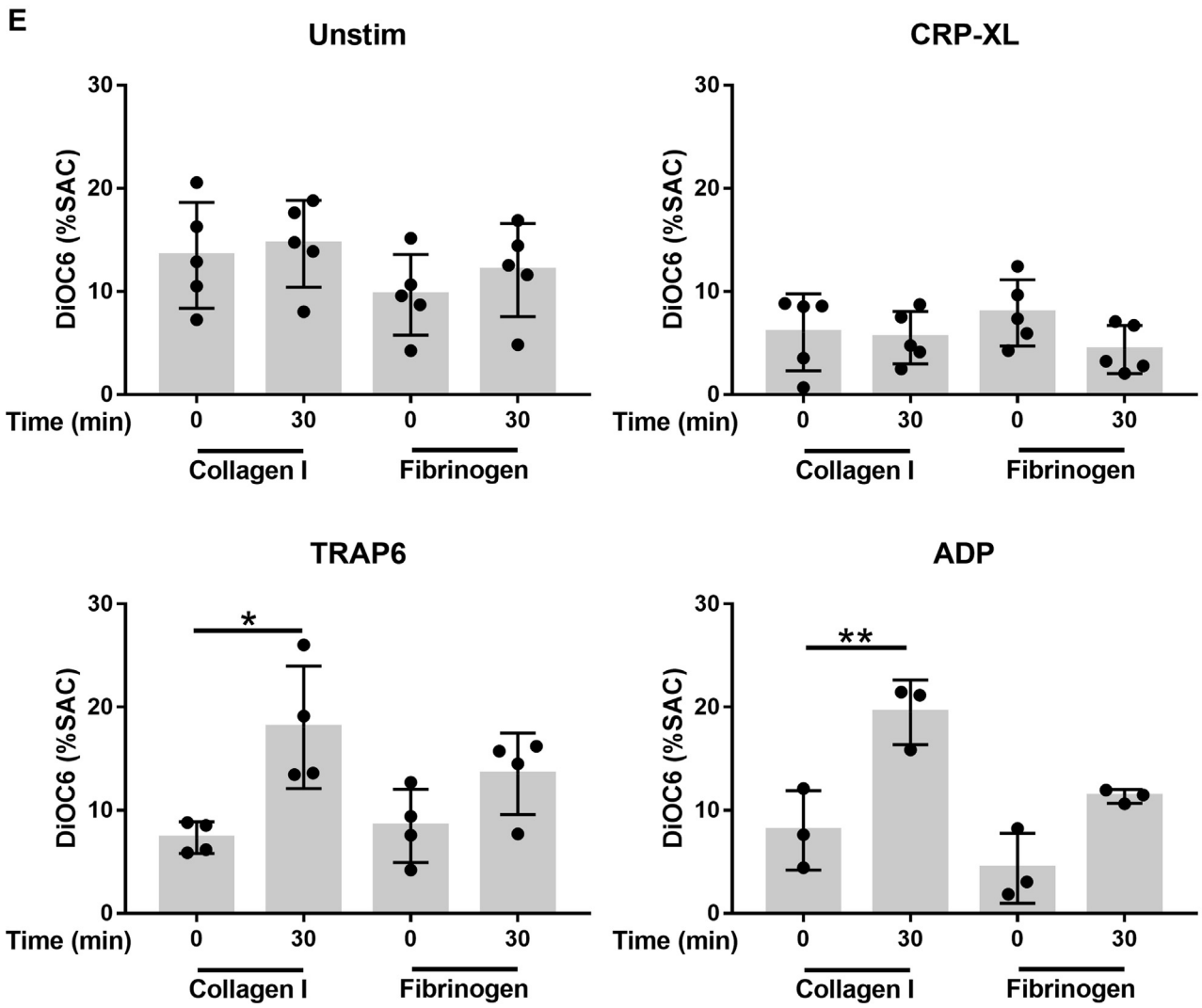

FIGURE 5 (continued)

low but not high thrombin, indicating that at higher thrombin concentrations platelet responses are sustained through PAR4, as described earlier [22].

With the agonist concentrations used in our assay, we showed that platelets did not increase in phosphatidylserine exposure, thus stating that the observed decreased integrin activation is not due to a procoagulant state. In other reports, the reversibility of platelet integrin activation was studied [10,11], however, the reversibility was evoked with a $P2Y_{12}$ receptor inhibitor. In this article, we investigated natural integrin inactivation over time, thus without the use of inhibitors. Whether the inhibitors enhance the process of natural integrin inactivation is so far unclear. Also, to which extent the plasma affects natural integrin inactivation and contributes to intrinsic platelet reversal, should be further investigated in the future. Furthermore, we observed that on receptor stimulation followed by natural integrin inactivation, the reactivation of platelets was possible through other receptors than through which the activation was initially evoked, indicating that the platelets were desensitized for previously stimulated receptors, but not completely exhausted. After long-term CRP-XL stimulation, platelets also showed slightly less integrin activation compared with immediately after stimulation. However, our data showed that the natural integrin inactivation on CRP-XL, in contrast to thrombin, is less dependent on the agonist concentration, suggesting that at low concentrations, the reversibility of platelets is less likely. A striking observation was that integrin activation in prior CRP-XL-stimulated platelets was induced by a second ADP stimulation, however, the underlying mechanism for this remains unclear. The present data hence point to a regulated mechanism of receptor switch-off. We speculate that our observations of integrin inactivation after stimulation through the GPCR receptors PAR1 and $P2Y_{1/12}$ over time are because of receptor desensitization, a mechanism that is dependent on the type of receptors, they are internalized and can be degraded or recycled later [20,23–26]. The concept of receptor desensitization is compatible with our data and suggests that on low thrombin concentrations, PAR1 is activated, and thereafter rapidly desensitized, whereafter platelets reverse. Instead, on high thrombin concentrations, the initial PAR1 activation is enforced by

PAR4, which retards platelet reversal to resting states. In agreement with our data, a previous article that compared the internalization and switching off of PAR1 and PAR4 reported that these processes occur slower for PAR4 than for PAR1 [24]. Integrin inactivation is probably observed to a lower extent following long-term GPVI stimulation because the GPVI receptor is not desensitized. However, ADAM-mediated shedding of the GPVI receptor after ligand exposure [27], might explain the unresponsiveness to restimulation of these platelets.

Our flow cytometry data showed that when platelets were stimulated with weak agonists (such as ADP) or low concentrations of agonist (e.g., low CRP-XL), further secretion was possible upon restimulation. It is likely that upon weak stimulation, only a part of the individual granules fuses with the platelet surface, while upon potent triggers, there is first granule-to-granule fusion followed by full degranulation [19]. Our data suggest that further secretion is only possible if platelets do not fully secrete. We found that stimulated platelets were able to take up fibrinogen from the environment, but whether the fibrinogen is internalized or accumulated in the OCS, is still unclear. The available literature suggests that fibrinogen after uptake concentrates in the OCS [28], and that the major determinant of platelet fibrinogen uptake is integrin $\alpha_{IIb}\beta_3$ since platelet fibrinogen was reduced in samples of Glanzmann patients [29], and that fibrinogen internalization modulates the return of stimulated platelets to a resting state [12].

Typically, we found that reversed platelets after PAR1 or $P2Y_{1/12}$ stimulation, in contrast to GPVI stimulation, could fully participate again in thrombus formation on coated surfaces. Translated to physiology, the difference between GPVI and GPCR (PAR1 or $P2Y_{1/12}$) receptor stimulation indicates that the activation of platelets by extracellular matrix ligands is more persistent, whereas the activation of circulating platelets by soluble agonists is more transient. With respect to thrombin, the platelets encountering low concentrations will be more likely to reverse than platelets subjected to higher thrombin concentrations, because prolonged intracellular signaling is mediated by PAR4. *In vivo*, platelets are exposed to a mixture of agonists, but depending on the platelet's location in a thrombus, the influence of certain agonists will be more prominent [13]. The formation of fibrinogen bridges is described to be a multistep process, in which initial contact between platelets and fibrinogen is reversible, followed by irreversible binding, whereafter it is probably not possible for platelets to disaggregate again [30,31]. It thus remains to be shown whether platelets passing by an incipient thrombus without being incorporated, but being exposed to localized high concentrations of several soluble agonists, as well as platelets upon initial reversible contact with fibrinogen, can return to their resting state before being removed from the circulation and as such can still contribute to hemostasis. Future studies are needed to unravel whether platelets can be recycled after exposure to multiple agonists, as this will more likely be the case *in vivo*, during thrombotic events. On another note, based on our data showing that platelets can be reactivated, it is questioned whether exhausted platelets, characterized by desensitized receptors, inactive integrins, and previous secretion [32], are truly exhausted and if this definition needs to be reconsidered.

In conclusion, our data show that platelet triggering through GPVI induces prolonged responses on platelets, while stimulation through the GPCR receptors PAR1 or $P2Y_{1/12}$ induces rather transient platelet effects, whereafter platelets return to a state in which they can be restimulated and might still contribute to vascular repair.

AUTHOR CONTRIBUTIONS

P.E.J.vd.M., C.I.J., J.W.M.H., H.T.C., and J.M.G. conceptualized the study. I.D.S. and C.C.F.M.J.B. devised the methodology and carried out investigations. I.D.S. undertook the data analysis and curation. P.E.J.vd.M., C.I.J., H.T.C., J.M.G. and J.W.M.H. collected the resources. I.D.S. prepared the original draft. P.E.J.vd.M., C.I.J., C.C.F.M.J.B., J.W.M.H., H.T.C., and J.M.G. reviewed and edited the manuscript. I.D.S. and C.C.F.M.J.B. visualized the study. P.E.J.vd.M., C.I.J., J.W.M.H., J.M.G., and H.T.C. acquired the funding. All authors read and approved the final version of the paper.

DECLARATION OF COMPETING INTERESTS

The authors report no competing interest associated with the work reported in the manuscript.

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SUPPLEMENTARY MATERIAL

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