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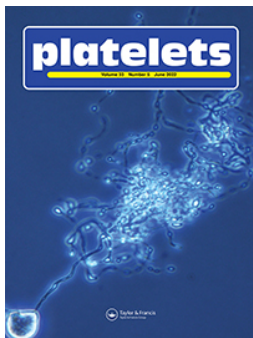
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Inhibition of Src but not Syk causes weak reversal of GPVI-mediated platelet aggregation measured by light transmission aggregometry

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Abstract

Src tyrosine kinases and spleen tyrosine kinase (Syk) have recently been shown to contribute to sustained platelet aggregation on collagen under arterial shear. In the present study, we have investigated whether Src and Syk are required for aggregation under minimal shear following activation of glycoprotein VI (GPVI) and have extended this to C-type lectin-like receptor-2 (CLEC-2) which signals through the same pathway. Aggregation was induced by the GPVI ligand collagen-related peptide (CRP) and the CLEC-2 ligand rhodocytin and monitored by light transmission aggregometry (LTA). Aggregation and tyrosine phosphorylation by both receptors were sustained for up to 50 min. The addition of inhibitors of Src, Syk or Bruton's tyrosine kinase (Btk) at 150 sec, by which time aggregation was maximal, induced rapid loss of tyrosine phosphorylation of their downstream proteins, but only Src kinase inhibition caused a weak (~10%) reversal in light transmission. A similar effect was observed when the inhibitors were combined with apyrase and indomethacin or glycoprotein IIb-IIIa (GPIIb-IIIa) antagonist, eptifibatide. On the other hand, activation of GPIIb-IIIa by GPVI in a diluted platelet suspension, as measured by binding of fluorescein isothiocyanate-labeled antibody specific for the activated GPIIb-IIIa (FITC-PAC1), was reversed on the addition of Src and Syk inhibitors showing that integrin activation is rapidly reversible in the absence of outside-in signals. The results demonstrate that Src but not Syk and Btk contribute to sustained aggregation as monitored by LTA, possibly as a result of inhibition of outside-in signaling from GPIIb-IIIa to the cytoskeleton through a Syk-independent pathway. This is in contrast to the role of Syk in supporting sustained aggregation on collagen under arterial shear.

Keywords

Aggregation, CLEC-2, Disaggregation, GPVI, Platelets, Tyrosine kinase

History

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Introduction

The immunoglobulin receptor glycoprotein VI (GPVI) and C-type lectin-like receptors 2 (CLEC-2) activate platelets through Src tyrosine kinases and spleen tyrosine kinase (Syk) via an immunoreceptor tyrosine-based activation motif (ITAM) and hemITAM, respectively. An ITAM has two repeats of the amino acid sequence YxxL separated by 6–12 amino acids, whereas a hemITAM has a single YxxL motif. Phosphorylation of the conserved tyrosine residues in these motifs leads to the binding of Syk through its tandem SH2 domains (in the case of CLEC-2, bridging two receptors) and activation of a signaling cascade that culminates in the activation of phospholipase C γ 2 (PLC γ 2) [1].

Recently, Ahmed et al. have shown that inhibition of GPVI signaling by a blocking Fab or with inhibitors of Src and Syk

kinases enhances disaggregation of platelets on a collagen surface at arterial shear in the presence of anticoagulation [2]. This extends an earlier observation from Andre et al. showing a role for Syk in the stabilization of aggregation under similar conditions [3]. Ahmed et al. proposed that stabilization is supported by the interaction of GPVI and fibrinogen [2], which has been identified as a novel activating ligand of the ITAM receptor [4], contributing to sustained activation of glycoprotein IIb-IIIa (GPIIb-IIIa). A critical role of GPIIb-IIIa in mediating sustained platelet aggregation under arterial shear on collagen has been previously shown using GPIIb-IIIa antagonists [5].

GPIIb-IIIa antagonists, including eptifibatide, have also been shown to reverse aggregation under minimal shear following stimulation by ADP as measured by light transmission aggregometry (LTA) [6–8]. Interestingly, it was also reported in the same study as data not shown that aggregation induced by a thrombin receptor activating peptide (TRAP) under the same conditions is not reversed by eptifibatide [8], although partial reverse of aggregation to TRAP by a combination of the GPIIb-IIIa antagonist, tirofiban, and prostacyclin has been described in a separate study [9]. Eptifibatide has also been shown to cause weak (< 20%) reversal of aggregation measured by LTA to collagen, albeit at a 100 times higher

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concentration than required to block aggregation [10]. In addition, Ahmed et al. have reported that the GPVI-blocking Fab, 9012, is unable to reverse aggregation as measured by LTA [2]. These results demonstrate that while aggregation measured by LTA is capable of being reversed, this is agonist-dependent and influenced by the experimental design.

There are several explanations for the failure of eptifibatide and Fab 9012 to fully reverse aggregation to collagen as measured by LTA, including poor penetration within the aggregate, the lack of flow, or masking by additional receptors. In the present study, we have asked whether small-molecule inhibitors of Src, Syk and Bruton's tyrosine kinase (Btk) that block signaling by GPVI and CLEC-2, and which have greater accessibility to the center of the aggregate, induce reversal of phosphorylation and aggregation.

Materials and methods

Details on the source of materials and platelet preparation can be found in the *Online Supplementary Method*.

Light transmission aggregometry

Washed platelets at $2 \times 10^8/\text{ml}$ were stimulated with $10 \mu\text{g}/\text{mL}$ CRP, 100 nM rhodocytin or $15 \mu\text{M}$ TRAP-6 in a Chronolog model 700 aggregometer while stirring at 1200 rpm at 37°C . The following inhibitors were used: PP2 ($20 \mu\text{M}$), dasatinib ($10 \mu\text{M}$), PRT-060318 ($1 \mu\text{M}$), ibrutinib (200 nM), apyrase (2.5 U mL^{-1}) and indomethacin ($10 \mu\text{M}$), eptifibatide ($9 \mu\text{M}$) or DMSO as vehicle. Fibrinogen was not supplemented in the aggregation assay. The inhibitors were added at 150 sec after agonist stimulation, and the aggregation traces were monitored for 20 min . The degree of disaggregation (%) was calculated based on maximal aggregation minus the percentage of aggregation at 20 min after agonist stimulation.

Protein tyrosine phosphorylation

$5 \times$ SDS reducing sample buffer (10% SDS, 0.5 M DTT, 50% glycerol, 0.125 M Tris, $\text{pH } 6.8$) was added to washed platelets ($2 \times 10^8/\text{ml}$) stimulated with $10 \mu\text{g}/\text{mL}$ CRP or 100 nM rhodocytin in a Chronolog model 700 aggregometer. Where stated, washed platelets were pre-treated with $9 \mu\text{M}$ eptifibatide to block GPIIb-IIIa. Samples were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with blocking buffer (5% BSA in TBS-Tween), the membranes were incubated with primary antibody (diluted $1/1000$ in blocking buffer) overnight, washed, and then incubated with HRP-conjugated secondary antibody (diluted $1/10000$ in TBS-Tween) for 1 h at room temperature. Washed blots were visualized by ECL chemiluminescence and imaged with ECL autoradiography film.

Flow cytometry

Washed platelets ($2 \times 10^7/\text{ml}$) platelets were incubated with PAC-1-FITC for 20 min and then stimulated with $10 \mu\text{g}/\text{mL}$ CRP at 37°C . PP2 ($20 \mu\text{M}$), PRT-060318 ($1 \mu\text{M}$) or DMSO (as vehicle) were added 150 sec after stimulation and incubated for 20 min at 37°C . Platelets were identified and gated in an Accuri BD Flow Cytometer (BD Biosciences) according to the forward and side scatter signals. A total of $10,000$ platelet events were acquired per sample, and the mean of the fluorescence intensity (MFI) was analyzed.

Statistical analysis

Data are presented as mean \pm standard deviation of the mean with statistical significance taken as $p < .05$ unless otherwise stated. Statistical analysis was performed using Welch's t-test and one-way ANOVA multiple comparison, as stated. All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc. La Jolla, USA).

Results

Platelet aggregation induced by GPVI and CLEC-2 is minimally reversed by the addition of an inhibitor of Src but not Syk and Btk inhibitors

We first examined the role of Src, Syk and Btk kinases in the maintenance of platelet aggregation in LTA in response to stimulation by GPVI. Aggregation to a maximally effective concentration of the GPVI ligand, CRP ($10 \mu\text{g}/\text{mL}$), peaked within 120 sec and was maintained for up to 50 min (not shown). Kinase inhibitors, used at maximally effective concentrations, were given at 150 sec and aggregation was monitored for 20 min . The reversal in light transmission at this time was $1.5 \pm 2.8\%$ in the presence of vehicle and 3.0 ± 3.4 and $0.4 \pm 0.5\%$ in the presence of the Syk and Btk inhibitors, PRT-060318 and ibrutinib, respectively (Figure 1 Ai-ii). These values were not significantly different. In contrast, the Src inhibitor PP2 caused a small but significant reverse in light transmission which started after approximately 5 min and reached $11.4 \pm 4.0\%$ over 20 min ($P < .05$; Figure 1 Ai-ii), dasatinib also showed a tendency to reverse but this was not significant ($7.4 \pm 7.2\%$). We further investigated the effect of Syk inhibition on aggregation at earlier times. The addition of the Syk inhibitor PRT-060318 at 15 , 30 , and 45 sec after agonist stimulation blocked further aggregation but did not induce reversal over 20 min (not shown), consistent with the previous report from Zou et al [9].

The addition of inhibitors of Btk and Syk kinases at 150 sec post-agonist addition also had no significant effect on aggregation induced by rhodocytin over 20 min relative to the vehicle control (Figure 1 Bi-ii). However, as with GPVI signaling, the Src inhibitors PP2 and dasatinib caused a small but significant reversal of aggregation: the degree of reversal in the presence of vehicle over 20 min was $0.41 \pm 0.5\%$ compared with 10.8 ± 10.0 and $5.8 \pm 5.7\%$ in the presence of PP2 and dasatinib ($P < .05$), respectively (Figure 1 Bi-ii).

We extended the experiments to the G protein-coupled receptor agonist TRAP-6. As with CRP and rhodocytin, we observed that the Src inhibitor PP2 ($9.9 \pm 3.1\%$, $P < .05$) caused a small but significant reverse in light transmission relative to vehicle ($1.0 \pm 0.1\%$); in contrast, however, there no significant reversal of aggregation in the presence of dasatinib ($6.0 \pm 5.0\%$) and PRT-060318 ($2.1 \pm 0.9\%$) (Figure S1). The lack of significance with dasatinib, despite the clear trend, may reflect variation between donors and the relatively small nature of the reversal.

The results show that aggregation induced by CRP, rhodocytin and TRAP-6 is maintained for up to 20 min in the presence of inhibitors of Syk and Btk whereas there was a small but significant reversal of aggregation to CRP in the presence of Src kinase inhibitors.

The effect of tyrosine kinase inhibitors on tyrosine phosphorylation

Experiments were performed to measure the reversal of tyrosine phosphorylation upon kinase addition. Tyrosine phosphorylation of Syk at Y525/526, LAT at Y200 and Btk at Y551 was increased over 20 -fold in the first 150 sec of stimulation by CRP ($10 \mu\text{g}/\text{mL}$)

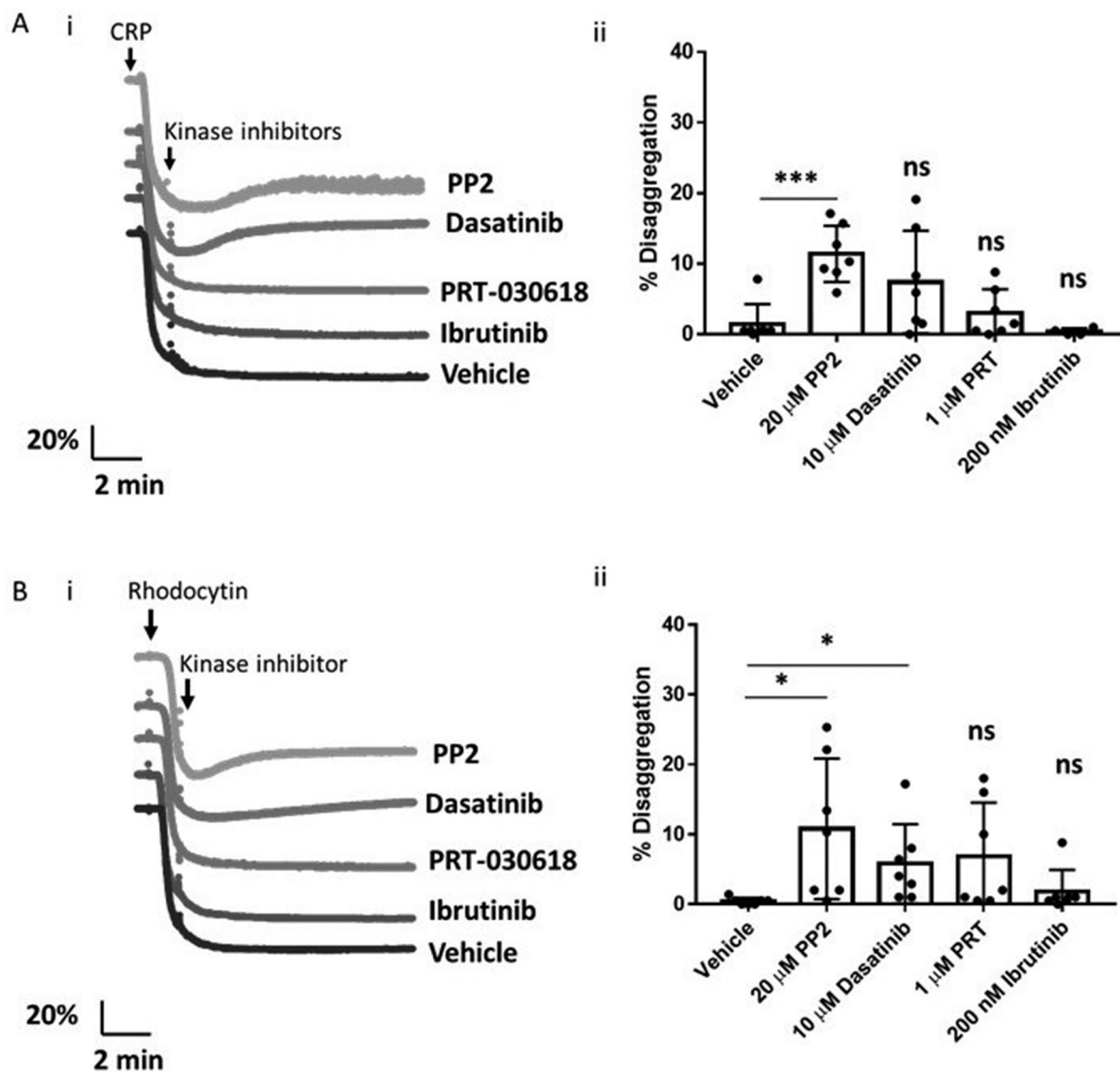


Figure 1. Src kinase inhibitors partially reversed GPVI and CLEC-2 mediated platelet aggregation. Washed platelets at 2×10^8 /ml were stimulated with (A) 10 μ g/mL CRP or (B) 100 nM rhodocytin and incubated with PP2 (20 μ M), dasatinib (10 μ M), PRT-060318 (1 μ M), ibrutinib (200 nM) or vehicle, after 150 sec of agonist stimulation. LTA monitored for 20 min. (i) Representative traces of seven identical aggregation experiments. (ii) Mean \pm SD % disaggregation after 20 min of agonist stimulation. *($P < .05$), **($P < .01$) and ***($P < .001$) calculated using Welch's t-test indicate statistically significant differences. ns, not significant. $N = 7$ separate donors.

and maintained for up to 50 min (Figure 2 Ai-ii). Tyrosine phosphorylation of Btk at Y223 and PLC γ 2 at Y1217 showed a slower and lower rate of increase of up to 5-fold over the first 150 sec, and this was also sustained over 50 min. The Src kinase inhibitors, PP2 and dasatinib, added at 150 sec, induced rapid dephosphorylation of Syk Y525/526, LAT Y200, Btk Y223, Btk Y551 and PLC γ 2 Y1217, with phosphorylation declining to basal levels by 20 min (Figure 3 Ai-ii). One notable difference between the two kinase inhibitors is the loss of phosphorylation of a band at 50–60 kDa in the presence of dasatinib which corresponds to Src family kinases. This may be due to inhibition of Csk which phosphorylates Src kinases at their inhibitory tyrosine residue [11]. The Syk inhibitor, PRT-060318, also caused rapid inhibition of tyrosine phosphorylation of proteins that lie downstream of Syk, namely LAT Y200, Btk Y223, Btk Y551 and PLC γ 2 Y1217, but only partially reduced phosphorylation at Y525/526, which is known to be phosphorylated by Src kinases and Syk itself (Figure 3 Ai-ii). Similarly, ibrutinib only inhibited tyrosine phosphorylation of proteins that lie downstream of Btk, namely at its

site of autophosphorylation, Y223, and of PLC γ 2 at Y1217 (Figure 2 Bi-ii).

A similar set of results were observed for the CLEC-2 ligand rhodocytin, with phosphorylation sustained for up to 50 min (Figure 2 Bi-ii), and rapid reversal of downstream substrates in the presence of inhibitors of Src, Syk and Btk kinases (Figure 3 Bi-ii). There were however several differences to those with GPVI, namely that PP2 did not inhibit LAT Y200 phosphorylation, and there was no significant difference on phosphorylation of Syk Y525/526 or PLC γ 2 Y1217, when treated with PRT-060318 and ibrutinib, respectively. The explanation for these differences is not known but may be due to a small overall increase and the sensitivity of detection.

These results show that CRP and rhodocytin stimulate sustained tyrosine phosphorylation with rapid inhibition of their downstream substrates upon addition of kinase inhibitors. This indicates that the failure of the kinase inhibitors to induce a complete reversal of aggregation is not due to a lack of inhibition of tyrosine phosphorylation.

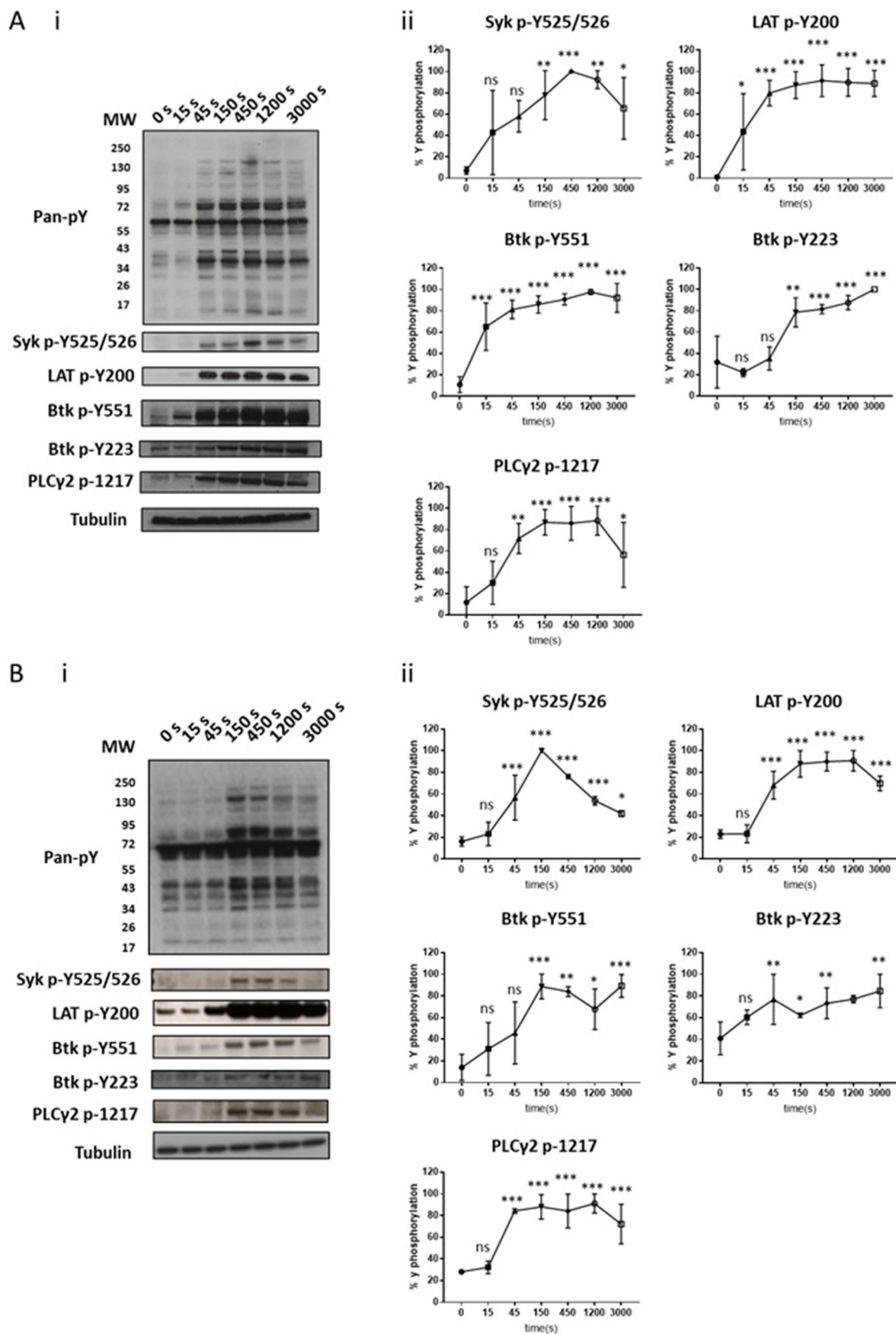


Figure 2. Tyrosine phosphorylation is sustained for 50 min in GPVI and CLEC-2 mediated protein phosphorylation. Washed platelets at 4×10^8 /ml were stimulated with (A) 10 μ g/mL CRP or (B) 100 nM rhodocytin in the presence of 9 μ M epifibatide. Platelets were lysed with 5x reducing sample buffer at stated time after addition of agonist. Whole cell lysates were probed for whole cell phosphorylation or kinase phosphorylation with the stated antibodies. (i) Representative blot and (ii) mean \pm SD % of tyrosine phosphorylation from 3 experiments. * ($P < .05$), ** ($P < .01$) and *** ($P < .001$) assessed by one-way ANOVA multiple comparison analysis indicate statistically significant differences. ns, not significant. N = 3 separate donors.

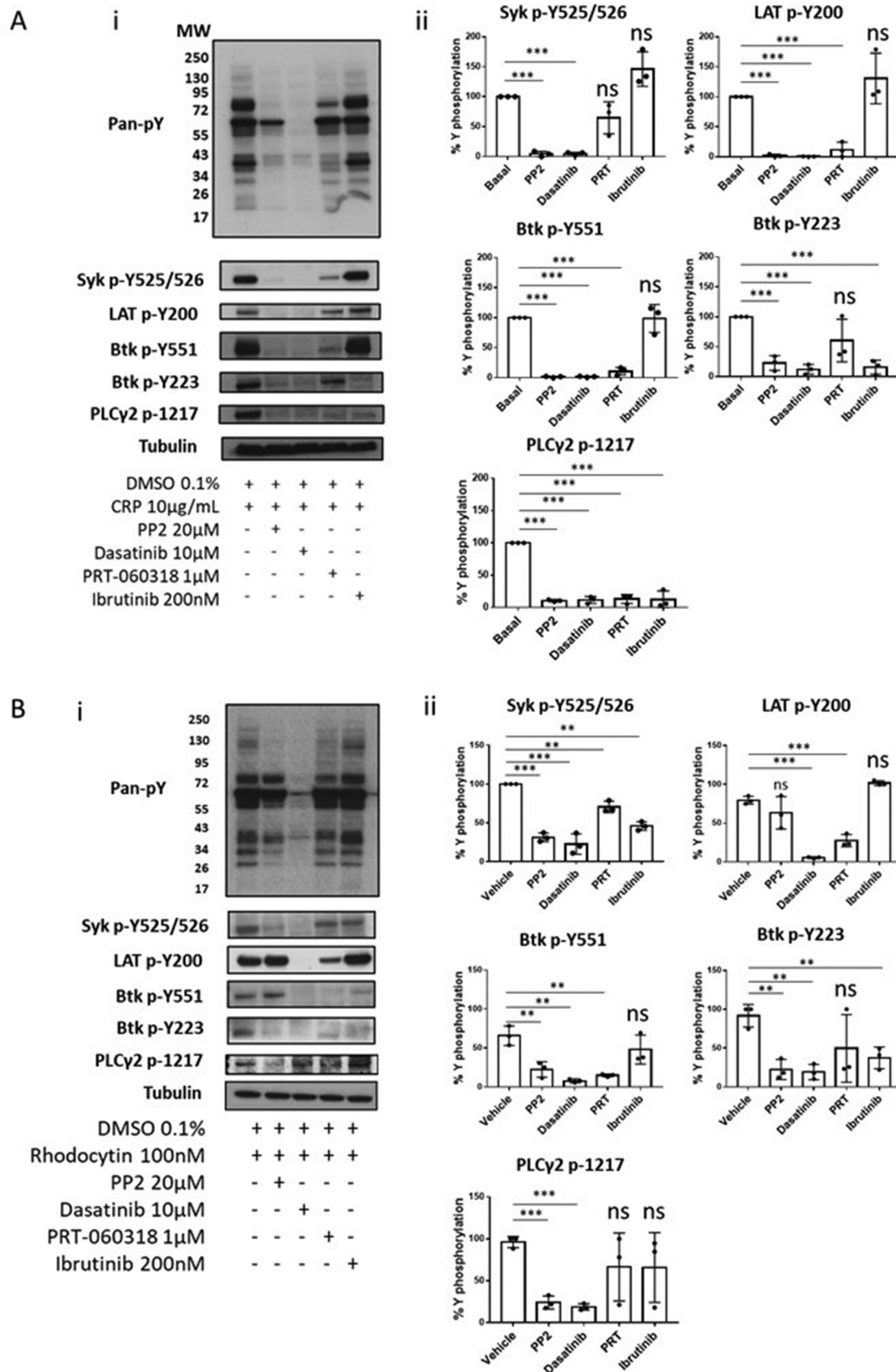


Figure 3. Kinase inhibitors reverse GPVI and CLEC-2 mediated protein phosphorylation. Washed platelets at 4×10^8 /ml were stimulated with (A) 10 μg/mL CRP or (B) 100 nM rhodocytin CRP in the presence of 9 μM epifibatide. Platelets were incubated with PP2 (20 μM), dasatinib (10 μM), PRT-060318 (1 μM), ibrutinib (200 nM) or vehicle after 150 sec of agonist stimulation. Platelets were then lysed with 5X reducing sample buffer 20 min after addition of agonist. Whole cell lysates were probed for whole cell phosphorylation or kinase phosphorylation with the stated antibodies. (i) Representative blot and (ii) mean \pm SD % of tyrosine phosphorylation from 3 experiments. * ($P < .05$), ** ($P < .01$) and *** ($P < .001$) calculated using Welch's t-test indicate statistically significant differences. ns, not significant. N = 3 separate donors.

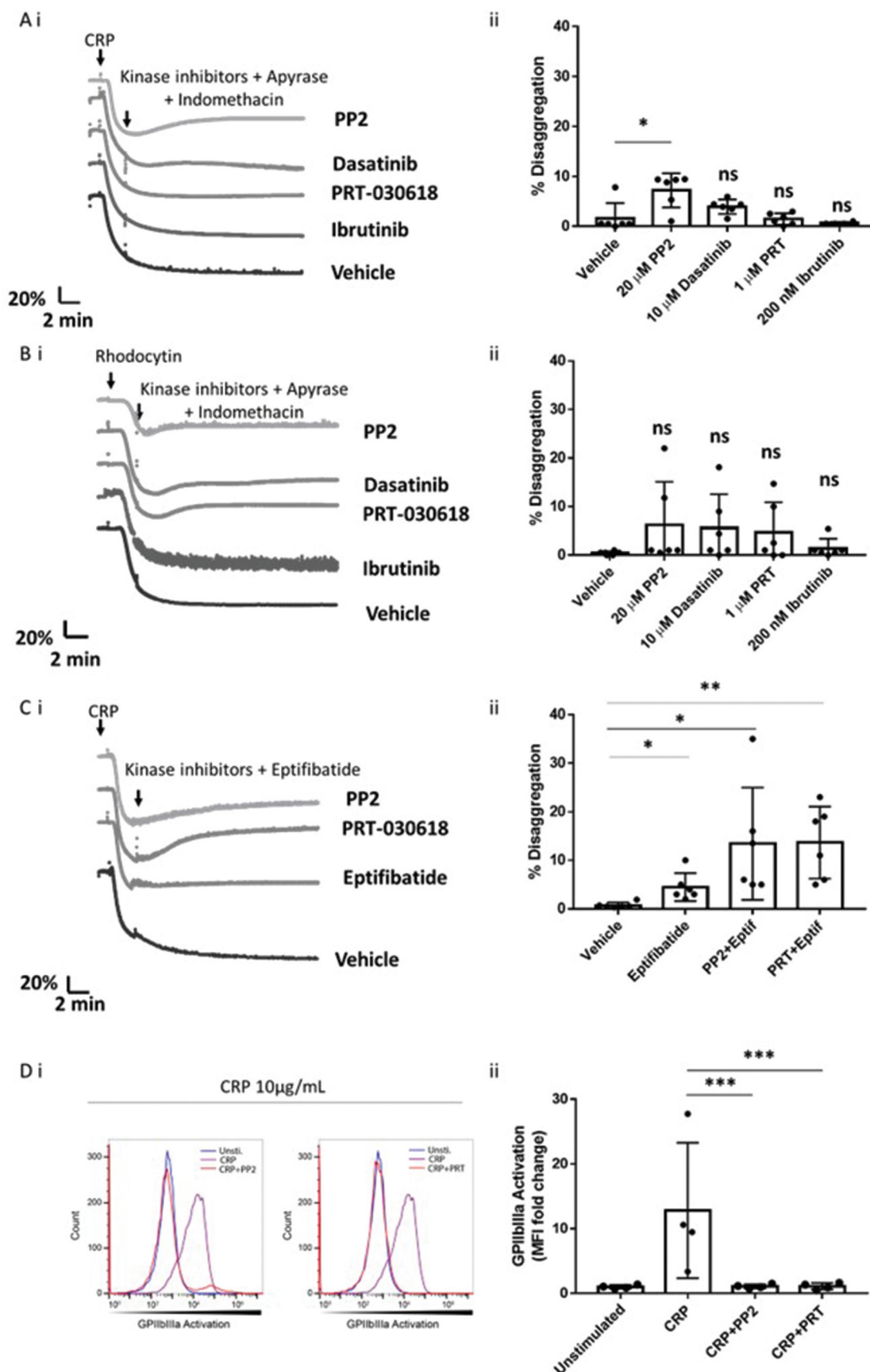


Figure 4. Kinase inhibitors together with secondary mediators or GPIIb-IIIa antagonist partially reversed GPVI and CLEC-2 mediated platelet aggregation and reverse GPIIb-IIIa activation. Washed platelets at 2×10^8 /ml were stimulated with (A) 10 μ g/mL CRP or (B) 100 nM rhodocytin and incubated with PP2 (20 μ M), dasatinib (10 μ M), PRT-060318 (1 μ M), ibrutinib (200 nM) or vehicle together with 10 μ M indomethacin and 2.5 U/mL apyrase, after 150 sec of agonist stimulation and monitored by LTA for 20 min. (C) Washed platelets (2×10^8 /ml) were stimulated with 10 μ g/mL CRP and then treated with eptifibatide 9 μ M alone or together with PP2 (20 μ M) or PRT-060318 (1 μ M), after 150 sec of agonist stimulation and monitored by LTA for 20 min. (i) Representative traces of six identical aggregation experiments. (ii) Mean \pm SD % of disaggregation after 20 min

Platelet aggregation is sustained in the presence of tyrosine kinase inhibitors when combined with apyrase and indomethacin

The observation that aggregation is maintained in the presence of inhibitors of Src, Syk and Btk shows that this is independent of GPVI or CLEC-2 signaling, which contrasts with the role of GPVI in supporting thrombus stabilization under flow. One potential explanation for this is the masking of the role of GPVI by the release of feedback messengers ADP and TxA₂. To investigate the contribution of the secondary mediators in the maintenance of the aggregates, we used the ADP/ATP scavenger apyrase and the cyclooxygenase inhibitor indomethacin in the presence of inhibitors of tyrosine kinases. Aggregation to CRP was maintained in the presence of apyrase and indomethacin when given on their own or in combination with the kinase inhibitors. The degree of recovery over 20 min in the presence of the combination of apyrase, indomethacin and the kinase inhibitors was as follows: vehicle: $1.6 \pm 3.0\%$, PP2: $7.2 \pm 3.4\%$; dasatinib: $3.9 \pm 1.4\%$; PRT-060318: $1.5 \pm 1.2\%$; and ibrutinib: $0.6 \pm 0.3\%$ (Figure 4 Ai-ii). Among these inhibitors, only PP2 led to a significant reversal compared to vehicle ($P < .05$), although there was also a trend with dasatinib. A similar set of results were observed in rhodocytin-stimulated platelets. The maximal reversal in the presence of apyrase and indomethacin and vehicle, PP2, dasatinib, PRT-060318 or ibrutinib was: 0.4 ± 0.4 , 6.2 ± 8.9 , 5.6 ± 7.0 , 4.7 ± 6.2 and $1.4 \pm 2.0\%$, respectively (Figure 4 Bi-ii).

Platelet aggregation is sustained in the presence of GPIIb-IIIa inhibition

The above experiments were repeated in the presence of GPIIb-IIIa antagonist eptifibatide to investigate the role of GPIIb-IIIa in GPVI-mediated aggregation. The maximal reversal in the presence of eptifibatide alone or in combination with Src and Syk inhibitors was 4.5 ± 2.9 , 13.4 ± 11.6 and $13.7 \pm 7.4\%$, respectively (Figure 4 Ci-ii), which were significant ($P < .05$) compared to vehicle ($0.7 \pm 0.6\%$).

Platelet GPIIb-IIIa activation is reversed by tyrosine kinase inhibitors

Experiments were designed to investigate whether activation of GPIIb-IIIa in the absence of aggregation is reversible. Activation of GPIIb-IIIa was measured using PAC-1-FITC by flow cytometry in a dilute suspension of platelets to prevent aggregation. PP2 and PRT-060318 were added 150 sec after stimulation by CRP. Both inhibitors induced full reversal of GPIIb-IIIa activation in 20 min (1.1 ± 0.3 and 1.1 ± 0.5 fold change in MFI) compared to sustained activation in the presence of vehicle (12.8 ± 10.5 fold) (Figure 4 Di-ii). This demonstrates that activation of GPIIb-IIIa by GPVI is a reversible process in the absence of aggregation.

Discussion

The present study shows that signaling through GPVI or CLEC-2 and the feedback agonists ADP and TxA₂ are not required for sustained aggregation in washed platelets as measured by LTA,

and that reversal is also not seen with the GPIIb-IIIa inhibitor, eptifibatide. On the other hand, a small decrease in aggregation is observed in the presence of two structurally distinct inhibitors of Src kinases dasatinib and PP2, but not in the presence of inhibitors of Syk and Btk. This result is also seen in response to aggregation induced by TRAP-6. This is likely to be due to loss of outside-in signaling by GPIIb-IIIa which serves to consolidate the aggregate through a Src kinase-dependent process. This was previously shown by Auger et al. who reported that maintenance of stable and compact platelet aggregates on collagen at arteriolar rates of flow is a dynamic process mediated by Src family kinases and the actin cytoskeleton [12]. The broad-spectrum nature and off-target effect of PP2 and dasatinib may also be a factor in the observation of partial aggregation reversal [13].

In contrast to the sustained aggregation, Src and Syk inhibitors rapidly induce reversal of activation of GPIIb-IIIa when measured in a dilute platelet suspension which does not support outside-in signaling by GPIIb-IIIa and platelet aggregation due to the absence of added fibrinogen and low platelet density. This shows that inside-out activation of integrin GPIIb-IIIa is a reversible process in contrast to the sustained aggregation monitored by LTA. This may reflect the absence of binding fibrinogen to activated GPIIb-IIIa, and subsequent outside-in signaling and that, once formed, the maintenance of platelet aggregation is also regulated by additional interactions between membrane proteins [14]. Unlike GPVI, CLEC-2 and PAR-1 do not interact with fibrinogen, but they stimulate the binding of fibrinogen to GPIIb-IIIa, and this may increase the interaction of fibrinogen with GPVI.

In contrast to the present findings, an increased rate of disaggregation has been reported at arteriolar rates of shear in thrombi formed on a collagen surface upon addition of a GPVI-blocking Fab or inhibitors of Src and Syk kinase [2]. This is a very different situation as the aggregate is exposed to high shear and collagen is only present at the site of thrombus initiation with the thrombus formed by the release of secondary mediators. The role of Src and Syk kinases in aggregate growth and stability under shear is therefore mediated by the coordinated signals from the binding of fibrinogen to integrin GPIIb-IIIa and GPVI [15]. It is therefore also likely that the presence of a lower concentration of secondary agonists on the outermost parts of the aggregate has contributed to increased susceptibility to disaggregation. In support of this, the initiation of disaggregation is a slow process that begins with loss of single platelets and is only associated with loss of aggregate structure at later time points [15]. The shedding of GPVI under shear may also contribute to the loss of thrombus stability [16].

The observation that inhibitors of GPVI and Src and Syk tyrosine kinase can promote de-aggregation under flow may have clinical significance including in the phase 2 trial of the GPVI-blocking Fab glenzocimab in combination with best treatment care in patients who have had a thrombotic stroke [17].

These results provide an important extension of a recent finding that CRP-stimulated platelets can maintain aggregation for a prolonged time [9] but contrast with the observation of the reversal of ADP-induced aggregation in the presence of a GPIIb-IIIa blocker, as monitored by LTA [6–8]. This difference may

of agonist stimulation. (N = 6) (D) Reversal of GPIIb-IIIa activation by tyrosine kinase inhibitors. Washed platelets ($2 \times 10^7/\text{ml}$) were incubated with PAC-1-FITC, stimulated with CRP ($10 \mu\text{g}/\text{mL}$) and then treated with vehicle, PP2 ($20 \mu\text{M}$) or PRT-060318 ($1 \mu\text{M}$) 150s after agonist stimulation. Flow cytometry measurements were done at 20 min after agonist stimulation. (i) Flow cytometry histograms depict activation of GPIIb-IIIa in platelets that were unstimulated (blue), treated with CRP ($10 \mu\text{g}/\text{mL}$) and followed with vehicle (purple), PP2 or PRT-060318 (red). (ii) MFI fold change \pm SD of four independent and identical experiments (N = 4). *($P < .05$), **($P < .01$) and ***($P < .001$) calculated using Welch's t-test indicate statistically significant differences. ns, not significant.

reflect the transient and weaker nature of ADP-induced aggregation brought about by the rapid desensitization of the P2Y₁ receptor [18]. The stronger level of activation may explain why Speich et al. (2009 and 2013) did not observe marked disaggregation in response to TRAP or collagen in the presence of the GPIIb-IIIa blocker eptifibatide [8,10].

In conclusion, the present study shows that strong aggregation appears to be an irreversible process when measured by LTA following activation by tyrosine kinase-linked receptors. Platelet aggregates are maintained despite inhibition of tyrosine kinase and G protein-coupled receptor signaling pathways, or blockade of integrin GPIIb-IIIa, and contrast to results observed on a collagen surface under the more physiological condition of arterial flow. The small reduction in aggregation measured by LTA in the presence of Src kinase inhibitors is likely to reflect loss of contraction as a result of inhibition of outside-in signaling from GPIIb-IIIa.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Supplementary material

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