Src family kinases are essential for primary aggregation by G\textsubscript{i}-coupled receptors

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Summary. Introduction and Background: Adrenaline stimulates biphasic aggregation in plasma through the G\textsubscript{i}-coupled \(\alpha_2\)A-adrenoreceptor. In the present study, we demonstrate that both primary and secondary wave aggregation induced by adrenaline in plasma is blocked by two structurally distinct inhibitors of Src family kinases, dasatinib and PD0173952. Methods and Results: In contrast, primary aggregation is partially inhibited or unaffected in the presence of inhibitors of cyclo-oxygenase, phosphoinositide (PI) 3-kinases, and P2Y\textsubscript{1} and P2Y\textsubscript{12} ADP receptors, although secondary aggregation is abolished. The ability of adrenaline to inhibit adenylyl cyclase and to synergize with platelet agonists in mediating platelet activation in plasma is retained in the presence of Src family kinase inhibition. Moreover, adrenaline does not activate Src family kinases, as determined by western blotting of their regulatory tyrosines, suggesting that constitutive signaling from Src family kinases may underlie their role in activation. Adrenaline is widely used in clinical laboratories for investigating patients with suspected bleeding disorders. In a group of 90 unrelated patients with a clinically diagnosed platelet disorder, we identified four who did not exhibit primary wave aggregation in response to adrenaline, although the catecholamine potentiated the response to other agonists, and five who failed to undergo secondary wave aggregation. In contrast, adrenaline stimulated biphasic aggregation in 60 controls. All of the patients with a defective response to adrenaline had impaired ADP-induced platelet activation. Conclusions: The present results indicate a previously unappreciated role for Src family kinases in mediating G\textsubscript{i} signaling in plasma, and demonstrate heterogeneity in response to adrenaline in patients with a clinically diagnosed platelet disorder.

Keywords: adrenaline, dasatinib, G-protein coupled receptor, Src family kinases.

Introduction

Blood platelets play a vital role in hemostasis and thrombosis. When there is a breach in the endothelial wall, platelets adhere to subendothelial matrix proteins and undergo activation, leading to inside-out activation of platelet integrins, notably \(\alpha_{IIb}\beta_3\), and stable adhesion. Platelet activation is reinforced by the release of the secondary agonists, ADP and thromboxane \(A_2\), and by the formation of thrombin, all of which signal through G protein-coupled receptors [1]. Adrenaline also plays a minor role in supporting hemostasis in mice, as shown with the use of an \(\alpha_2\)A-adrenoreceptor knockout mouse, although it is unclear whether this is the case in humans [2].

The \(\alpha_2\)A-adrenoreceptor and P2Y\textsubscript{12} ADP receptor signal via the G\textsubscript{i} family of G proteins, which inhibit adenylyl cyclase and activate phosphoinositide (PI) 3-kinases [3]. Studies in mutant mice have shown that the two receptors couple to distinct members of the G\textsubscript{i} family of G proteins, namely G\textsubscript{i1} [4,5] and G\textsubscript{i2} [6], respectively. The \(\alpha_2\)A-adrenoreceptor and P2Y\textsubscript{12} ADP receptor also couple to a second member of the G\textsubscript{i} family of G proteins in mice, most likely G\textsubscript{i4} [7,8]. It is not known whether the P2Y\textsubscript{12} ADP receptor and the \(\alpha_2\)A-adrenoreceptor couple to the same G\textsubscript{i} proteins in human platelets.

The P2Y\textsubscript{12} ADP receptor and the \(\alpha_2\)A-adrenoreceptor undergo synergy with G\textsubscript{i4}-regulated and G\textsubscript{i2/13}-regulated pathways, inducing powerful platelet activation [9,10]. On their own, however, they induce weak activation in plasma and have no effect in ‘washed’ platelets [11–13]. The molecular basis of activation in plasma and synergy with other agonists is not fully

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understood. It has been known since the 1970s that a reduction in cAMP is not sufficient to induce platelet aggregation, although it is unclear whether this plays a contributory role [8,14,15]. On the other hand, there is considerable evidence that activation of PI3-kinases is critical for G protein-mediated platelet activation. The structurally distinct inhibitors of PI3-kinases, wortmannin and LY294002, inhibit activation of integrin αIIβ3 by ADP in P2Y12-deficient mouse platelets [16], and mice deficient in PI 3-kinase-γ display a small reduction in ADP-induced aggregation [17]. On the other hand, a much greater reduction in response is seen in the presence of the PI 3-kinase-β inhibitor TGX221 [18,19], and in PI 3-kinase-β-deficient mouse platelets [20,21], revealing this to be the predominant isoform downstream of the P2Y12 ADP receptor.

Studies in a variety of cells [22–24], including platelets [25–28], have provided evidence that Src family kinases contribute to signaling by G protein-coupled receptors, although their role in mediating platelet activation by G protein-coupled receptors is not known, owing to limited bioavailability of the first generation of Src family kinase inhibitors in plasma. The mixed Abl and Src family kinase inhibitor dasatinib, which is used in the treatment of imatinib-resistant chronic myeloid leukemia, is bioavailable in plasma and can be used to study Src family kinases, as platelets do not express Abl, as determined by SAGE library analysis and western blotting (unpublished). Dasatinib has recently been shown to inhibit platelet activation by the tyrosine kinase-linked collagen receptor glycoprotein (GP)VI [29]. A second, structurally distinct, Src family kinase inhibitor, PD0173952, is also available in plasma [30]. The use of two structurally distinct inhibitors provides a powerful way to investigate the role of Src family kinases, as they are unlikely to have other, non-specific, actions in common.

In the present study, we demonstrate that dasatinib and PD0173952 prevent primary wave aggregation of platelets induced by α2A-adrenoreceptor and P2Y12 ADP receptor activation, demonstrating a critical role for Src family kinases in the proximal events in G protein signaling. On the other hand, inhibition of cAMP formation and synergy with protease-activated receptor (PAR)1 are unaffected. We also show that patients with a clinically diagnosed bleeding disorder associated with loss of adrenaline-induced can be subdivided according to whether primary wave aggregation in response to the catecholamine is retained. These results demonstrate a critical role for Src family kinase activity in initiating platelet activation by adrenaline, and reveal a previously unrecognized heterogeneity in response in patients with impairment in adrenaline-induced platelet activation that will guide further studies on the identification of the underlying defect.

Materials and methods

Materials

Sources of all chemicals and reagents used in this study can be found in Data S1.

Preparation of human platelet-rich plasma (PRP) and washed platelets

Washed platelets [31] and platelet-rich plasma [32] were prepared as described previously, with minor modifications as described in Data S1.

Platelet aggregation and secretion studies

Platelet aggregation and ATP secretion were measured with a Chrono-Log lumi-aggregometer (Chrono-Log, Havertown, PA, USA), as previously described [32].

Platelet biochemistry

Washed human platelet whole lysates were prepared and western blotted as previously described [33]. Preparation of lysates from PRP was performed as described in Data S1.

cAMP assay

Washed platelet (6 × 10⁷ mL⁻¹) were stimulated under non-stirring conditions at 37°C for 3 min with adrenaline, prostaglandin E1 (PGE1) or both together. Samples were subsequently lysed and analyzed using the Parameter™ cAMP assay (R&D Systems, Abingdon, UK), according to the manufacturer’s instructions.

Patient studies

The study participants were identified by systematic analysis of subjects registered at UK haemophilia centers with mild platelet function disorders. This study has received UK Multicentre Research Ethics Committee approval, and informed consent was obtained in accordance with the Declaration of Helsinki.

Statistics and data analysis

Data were analyzed with GRAPHPAD PRISM software (La Jolla, CA, USA), and statistical analysis was carried out using Student’s t-test.

Results

Adrenaline stimulates biphasic platelet aggregation

Adrenaline (> 3 μM) stimulated biphasic, irreversible aggregation and dense granule secretion in plasma, with the onset of secretion occurring concomitantly with the second phase of aggregation, as illustrated in Fig. 1A. Adrenaline-induced aggregation was not preceded by shape change. The mean onset of second wave aggregation and secretion in a population of 60 healthy controls was at 110 ± 9 s. Interestingly, in five of the controls, none of whom has a known bleeding disorder, there was a significant delay in secondary aggregation and secretion of more than 200 s, as illustrated in Fig. 1A (arrow),
where the second phase of aggregation can be seen to start at approximately 300 s, even though the primary phase began within 10 s of agonist addition. Furthermore, the delay in the second phase was not decreased by increasing the concentration of adrenaline (not shown). The delay in aggregation was observed on up to five occasions over a period of 2 years in the five affected controls. Thus, these results demonstrate that adrenaline always induces biphasic aggregation and secretion in healthy controls, although, in less than 5% of cases, second wave aggregation and secretion are delayed, and this may explain why earlier studies have reported that a proportion of controls fail to undergo adrenaline-induced second wave aggregation [34]. The observation that sustained, biphasic adrenaline-induced aggregation is always present is of relevance for the study of patients with clinically diagnosed bleeding disorders who fail to exhibit adrenaline-induced secondary wave aggregation (see later).

Adrenaline-induced primary aggregation is dependent on Src family kinases but not PI 3-kinases

The signaling events that underlie adrenaline-induced primary wave aggregation are not fully understood. As PI 3-kinases are known to be involved in G<sub>i</sub> signaling in platelets (see Introduction), we investigated the effect of the pan-PI 3-kinase inhibitor LY294002 on platelet activation by adrenaline in PRP. LY294002 (50 µM) partially inhibited primary aggregation and abolished secondary aggregation and dense granule secretion induced by adrenaline (Fig. 1B). A three-fold higher concentration of LY294002 had no further effect on the response to the catecholamine (not shown), suggesting that maximal blockade of PI 3-kinases had been achieved. These results demonstrate a partial role for PI 3-kinases in adrenaline-induced primary wave aggregation, and that the lipid kinase is also essential for continuation to secondary wave aggregation and dense granule secretion.

The observation that primary aggregation is reduced but not abolished in the presence of LY294002 and that inhibition of cAMP production is not sufficient to induce platelet activation by G<sub>i</sub>-coupled receptors [35] suggests the presence of one or more further signaling pathways. In light of this, we investigated the role of Src family kinases in platelet activation by adrenaline in PRP, as they have been reported to contribute to signaling by G protein-coupled receptors in a variety of cells [22–24], including platelets [25–28]. To achieve this, we used two structurally distinct inhibitors of Src family kinases, dasatinib and PD0173952 [29,30], which are both bioavailable in plasma. The inhibitors were used at concentrations that were just sufficient to prevent activation of platelets by collagen

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(not shown), which induces activation through a Src family kinase-driven pathway. At this concentration, the two inhibitors blocked primary and secondary wave aggregation and dense granule secretion induced by adrenaline (Fig. 1C). As the two inhibitors are structurally distinct, and therefore unlikely to have similar non-specific actions, this provides a powerful argument for a role of Src family kinases in initiating platelet activation by adrenaline.

Experiments were also designed to investigate the role of a variety of surface receptors in initiating platelet activation by adrenaline in plasma. The contribution of thromboxane A_2 generation and the P2Y_1 and P2Y_12 ADP receptors was investigated with the cyclo-oxygenase inhibitor indomethacin and the receptor antagonists MRS2179 and cangrelor, respectively. Secondary wave aggregation and dense granule secretion were abolished in the presence of indomethacin, although primary aggregation was not altered (Fig. S1A). In contrast, MRS2179 and cangrelor only partially inhibited the second wave of aggregation and dense granule secretion, and had no effect on primary wave aggregation (Fig. S1A). These observations are in accord with previously published findings of ourselves and other groups [32]. Dense granule secretion and, as expected, both phases of aggregation were inhibited in the presence of the α_hβ_3-blocking peptide integrilin (Fig. S1B), consistent with a model in which G_i stimulation of dense granule secretion occurs through synergy with outside-in signals from α_hβ_3 [36,37]. On the other hand, the 5-hydroxytryptamine (5-HT)_2A/5-HT_2C receptor antagonist ketanserin or chelation of thrombopoietin (TPO) with a specific antibody had no effect on platelet activation in plasma by adrenaline (not shown).

It is well established that adrenaline does not activate washed platelets prepared either by centrifugation or gel filtration. One possible explanation for this is the removal by washing of agonists in plasma that synergize with adrenaline. However, we have been unable to reconstitute the response to adrenaline by supplementing the modified Tyrodes buffer with candidate ligands, including fibrinogen, TPO and subthreshold concentrations of G protein-coupled receptor agonists (not shown), such as 5-HT.

Taken together, these results demonstrate that the second phase of aggregation and dense granule secretion induced by adrenaline are mediated through a synergistic interaction of the P2Y_1 and P2Y_12 ADP receptors and through generation of thromboxane A_2, which activates the thromboxane receptor. Dense granule secretion is also dependent on outside-in signaling through α_hβ_3. We have, however, been unable to find evidence for the role of additional platelet receptors in supporting activation by adrenaline in plasma, including 5-HT_2A and the TPO receptor c-Mpl.

**Src family kinases are not required for inhibition of cAMP or for synergy with PAR1**

In view of the critical role of Src family kinases in initiating adrenaline-induced platelet activation, we investigated whether Src family kinases are required for other platelet responses to adrenaline. In contrast to the above, however, dasatinib and PD0173952 had no effect on the ability of adrenaline to inhibit the elevation of cAMP induced by PGE_1 in washed platelets (Fig. 2A and not shown), consistent with the fact that Src family kinases are not required for activation of the G_i family of G proteins. Moreover, the ability of adrenaline to synergize with a low concentration of the G protein-coupled PAR1 agonist SFLLRN in PRP was also unaffected in the presence of dasatinib and PD0173952 (Fig. 2B), demonstrating that the synergy is independent of Src family kinases.

**Adrenaline does not stimulate tyrosine phosphorylation in platelets**

In view of the critical role of Src family kinases in mediating platelet activation by adrenaline, the effect of the catecholamine...
on tyrosine phosphorylation in washed platelets and in plasma was investigated. The studies in plasma required rapid cooling of the samples so as to enable their subsequent separation from the very high levels of albumin and other plasma proteins, which cause an unacceptably high level of non-specific binding in western blotting studies. We were unable to observe a detectable alteration in tyrosine phosphorylation by adrenaline in either washed platelets (Fig. 3A) or in plasma (Fig. S2A), as monitored by western blotting using the monoclonal antibody 4G10. This is in contrast to the dramatic increase in tyrosine phosphorylation induced by the GPVI-specific collagen-related peptide (CRP), which is abrogated by dasatinib (Fig. 3A) or by PD0173952 [30]. On the other hand, adrenaline induced dephosphorylation of an unidentified protein of 50 kDa in washed platelets (see arrow in Fig. 3A), indicating either inhibition of a constitutively active tyrosine kinase or activation of a protein tyrosine phosphatase.

Src family kinase activity is regulated by phosphorylation of inhibitory and activatory tyrosyl residues [38], and phosphorylation/dephosphorylation of either of these can alter activity. For example, the protein phosphatase CD148 has been shown to play a critical role in regulating Src family kinases in platelets activated through the collagen receptor GPVI [39]. Phosphorylation of activation loop tyrosines in all Src family kinases can be monitored by western blotting with a pan-phosphospecific antibody, and phosphorylation of the inhibitory tyrosines can be measured with individual phosphospecific antibodies: Fyn (Tyr530), Src (Tyr529) and Lyn (Tyr509) [39]. Adrenaline did not induce a detectable change in tyrosine phosphorylation of the activation tyrosines or the inhibitory tyrosines in Fyn, Lyn and Src, however, either in washed platelets (Fig. 3C) or in PRP (Fig. S2), indicating that the catecholamine does not induce activation of Src family kinases, or that, if this does occur, it falls below the level of detection.

Fig. 3. Adrenaline (Adr) or P2Y12 ADP receptor activation does not stimulate tyrosine phosphorylation in platelets. Human washed platelets were stimulated with adrenaline (1 mM), ADP (10 μM) in the presence of MRS2179 (MRS) (100 μM) or collagen-related peptide (CRP) (10 μg mL⁻¹) in the presence or absence of dasatinib (Das) (1 μM), and subsequently lysed. Samples were immunoblotted with pan-pTyr monoclonal antibody 4G10 or phosphospecific antibodies, as shown. Mean data are representative of three independent experiments ± standard errors of the mean. SFK, Src family kinase; WB, western blot; WCL, whole cell lysate.
P2Y12-mediated aggregation is dependent on Src family kinases

Experiments were performed to investigate whether activation of platelets in plasma by the G\textsubscript{i}-coupled P2Y\textsubscript{12} ADP receptor is also dependent on Src family kinases. These studies were carried out in the presence of the P2Y\textsubscript{1} ADP receptor antagonist MRS2179, and were restricted to concentrations of ADP of up to 10 \(\mu\text{M}\), as shape change was observed at higher concentrations, presumptively because of partial rescue from the competitive blockade. These experiments were of particular interest, given both the physiologic significance of the P2Y\textsubscript{12} ADP receptor and the fact that it is coupled to G\textsubscript{\alpha_{12}} rather than G\textsubscript{\alpha_{i2}}, which is the major G protein used by adrenaline [4–6].

ADP (10 \(\mu\text{M}\)) stimulated primary aggregation but not secretion in PRP in the presence of the P2Y\textsubscript{1} ADP receptor antagonist MRS2179 (Fig. 4). Aggregation was not preceded by shape change, as was the case for adrenaline. ADP-induced aggregation (10 \(\mu\text{M}\)) under these conditions (i.e. presence of MRS2179) was completely inhibited by the same concentrations of dasatinib and PD0173952 (Fig. 4) that blocked adrenaline-induced aggregation (Fig. 1C). On the other hand, in the absence of P2Y\textsubscript{1} ADP receptor blockade, ADP (10 \(\mu\text{M}\)) stimulated sustained aggregation in plasma, which was converted to a reduced and transient response in the presence of dasatinib (Fig. 4A) or PD0173952 (not shown). In washed platelets, P2Y\textsubscript{12} ADP receptor activation by ADP did not alter the level of tyrosine phosphorylation of whole cell lysates, including that of Src family kinases (Fig. 3A,C). Interestingly, the absence of dephosphorylation of the 50-kDa protein suggests that this is mediated through G\textsubscript{\alpha_{12}}, which is regulated by the \(\alpha_{2A}\)-adrenoreceptor but not by the P2Y\textsubscript{12} ADP receptor.

**Fig. 4.** Aggregation induced by P2Y\textsubscript{12} activation is critically dependent on Src family kinases. Platelet-rich plasma was stimulated with ADP (10 \(\mu\text{M}\)) in the presence of MRS2179 (100 \(\mu\text{M}\)). Samples were treated with dasatinib (Das) (3 \(\mu\text{M}\)), PD0173952 (20 \(\mu\text{M}\)) or vehicle control. Representative traces and mean data ± standard errors of the mean from three independent experiments are shown. Maximal aggregation was measured in the histogram.

Heterogeneous defects in response to adrenaline in patients with clinically diagnosed platelet disorders

Adrenaline is routinely used in clinical laboratories for monitoring platelet activation in patients with a suspected platelet disorder. We also use adrenaline, along with eight other stimuli [ADP, arachidonic acid, collagen, CRP, PAR1-specific and PAR4-specific peptides, ristocetin and U46619 (stable thromboxane A\textsubscript{2} analog)], in our investigations on patients with clinically diagnosed platelet bleeding disorders [32]. Over the course of investigations on 90 unrelated patients, we identified nine with a defect in both adrenaline-induced and ADP-induced aggregation and secretion, which could not be explained solely by loss of response to ADP on the basis of reference to previously determined control curves [32]. Aggregation induced by low concentrations of the other platelet agonists was reduced to a level consistent with the loss of the feedback action of ADP [32], suggesting that the primary defect in these patients was an impairment in G\textsubscript{i} signaling by ADP and adrenaline.

Examination of the aggregation response to adrenaline revealed heterogeneity in the response of the nine affected patients to adrenaline, with primary wave aggregation being absent in four patients (group 1) and reduced in five patients (group 2). In all cases, secondary wave aggregation and dense granule secretion were abolished. Sample traces illustrating this heterogeneity are shown in Fig. 5A. Interestingly, in patients with abolished primary wave aggregation, adrenaline was still able to inhibit PGE\textsubscript{1}-elevated cAMP to a similar degree to controls (not shown). In contrast to the results for adrenaline, ADP (10 \(\mu\text{M}\)) stimulated transient aggregation in all nine of the affected patients, but on the other hand, this was also different to the response in over 60 controls, where it induced sustained aggregation, as shown in Fig. 5B. The response of the nine patients to ADP could not be differentiated into two distinct groups, as was the case for adrenaline, most likely because the response is mediated through a synergy between P2Y\textsubscript{1} and P2Y\textsubscript{12} receptors.

The marked reduction in adrenaline-induced and ADP-induced aggregation in the nine patients, and the relatively mild effect on the response to other platelet agonists, suggests that...
the primary defect is one of impaired $G_i$ signaling. The possibility that the loss of response to adrenaline could resulted from the presence of an additional mutation in the receptor itself seems unlikely, as adrenaline potentiated aggregation to a threshold concentration of a PAR1-specific peptide in the four patients who did not show a primary aggregation response to the catecholamine (see Fig. 5C for a sample trace). Thus, these results suggest that patients with a defective response to ADP and adrenaline can be subdivided on the basis of whether adrenaline is able to mediate primary aggregation. Further studies will be required to establish the molecular basis of the defect in the nine patients.

**Discussion**

The present results demonstrate that adrenaline-induced primary wave aggregation is dependent on Src family kinases, whereas it is partially or fully preserved following inhibition of thromboxane $A_2$ formation, antagonism of the $P2Y_1$ and $P2Y_{12}$ ADP receptors, or blockade of PI 3-kinases. Primary wave aggregation induced by the $P2Y_{12}$ ADP receptor (monitored in the presence of MRS2179) is also dependent on Src family kinases, even though the $P2Y_{12}$ ADP receptor and $a_2A$-adrenoreceptor couple to distinct members of the $G_i$ family of G proteins [4–6]. Src family kinases are not required for adrenaline-mediated inhibition of adenylyl cyclase or for synergy with PAR1. Together, these results therefore demonstrate a previously unappreciated role for Src family kinases in initiating platelet activation by $G_i$-coupled receptors in plasma, and are in agreement with previous studies that have reported a role for Src family kinases in the synergy between $G_i$-coupled and $G_{ai}$-coupled receptors in washed platelets, although, under these conditions, $G_i$ activation is not sufficient to mediate activation [25,27]. In contrast, however, Hardy et al. [26]
reported a role for Src family kinases downstream of the P2Y<sub>1</sub> but not the P2Y<sub>12</sub> ADP receptor in washed platelets.

The nature of the Src family kinases that underlie the action of the 5-HT<sub>2A</sub>-adrenoreceptor and P2Y<sub>12</sub> ADP receptor is not known. Furthermore, this cannot be investigated with the use of mouse platelets, as adrenaline does not induce aggregation in mouse PRP [2] and P2Y<sub>12</sub>-mediated aggregation of mouse platelets is Src family kinase-independent [16], thereby demonstrating a marked species difference. Also, there are no selective inhibitors of individual Src family kinase isoforms. There is also a possibility that the inhibitory action of dasatinib and PD0173952 is mediated through a mechanism other than Src family kinase inhibition, although the distinct structures of the two inhibitors and the fact that it is well established that Src family kinases are regulated by G protein-coupled receptors make this extremely unlikely.

G protein-coupled receptors have been reported to activate Src family kinases through direct receptor activation [23,24], via a G<sub>i</sub> protein [28] and through β-arrestin [22]. However, we have been unable to demonstrate altered tyrosine phosphorylation of the major Src family kinase isoforms in platelets, raising the possibility that activation is a consequence of synergy between constitutively active Src family kinases and other components of G<sub>i</sub> signaling (the nature of which is not known). Thus, plasma may induce a basal level of Src family kinase activation that, although insufficient for activation, undergoes synergy with G<sub>i</sub>-coupled receptors to enable aggregation to occur. For example, subthreshold activation of Src family kinases could be achieved by agonists such as TPO or insulin-like growth factor, which potentiate platelet activation but do not induce activation on their own [40,41], by the presence of plasma fibrinogen, which generates outside-in signals through binding to constitutively active 2<sub>1A</sub>β<sub>3</sub>, or by subthreshold levels of G protein-coupled receptor agonists, including 5-HT, thrombin and ADP. We have not been able, however, to reconstitute adrenaline-mediated activation of washed platelets by addition of TPO, fibrinogen or subthreshold concentrations of various G protein-coupled receptor agonists, such as 5-HT (not shown), although it may be that several agonists in combination are required to mediate this effect. Constitutive activation of Src family kinases has also been reported in washed platelets [42], demonstrating that this may be agonist-independent, although it is noteworthy that this is not sufficient to reconstitute adrenaline-induced aggregation. The present results further demonstrate that PI 3-kinases play a partial (but not absolute) role in adrenaline-induced primary aggregation and are required for this to proceed to secondary aggregation and secretion. It is possible that Src family kinase-dependent signals mediate the activation of PI 3-kinases by G<sub>i</sub>-coupled receptors [16-20], given that activation of PI 3-kinase is dependent on binding of its SH2 domain to phosphotyrosine [43,44].

During the study of 90 unrelated patients with clinically diagnosed platelet-based bleeding disorders, nine patients were found with defects in both adrenaline-induced and ADP-induced aggregation. Among these, a subset of four patients did not exhibit adrenaline-induced primary wave aggregation, and five were unable to generate secondary wave aggregation. In contrast, ADP induced transient rather than sustained aggregation in all nine patients, reflecting costimulation of P2Y<sub>1</sub> and P2Y<sub>12</sub> ADP receptors. Secretion induced by adrenaline and ADP was completely blocked in the nine patients. The impairment in response to both adrenaline and ADP suggests that the defect occurs at the level of G<sub>i</sub> signaling rather than at the level of the receptor. The observation that adrenaline-induced primary aggregation was blocked in four of these patients raises the possibility that this is mediated by a defect at the level of Src family kinases, although further studies are required to investigate this, especially as the decrease in response to collagen is consistent with loss of G<sub>i</sub>-based signaling rather than abrogation of Src family kinase-mediated activation.

**Clinical implications for investigation of patients with suspected G<sub>i</sub> defects**

The present results emphasize the critical role of Src family kinases in initiating platelet activation by G<sub>i</sub>-coupled receptors, and demonstrate heterogeneity in the loss of response to adrenaline in patients with clinically diagnosed platelet bleeding disorders, as revealed by the presence or absence of primary wave aggregation.

Adrenaline is one of the main platelet agonists used in the clinical setting for testing patients with suspected platelet defects. Prior to this study, partial or complete loss of response to adrenaline was attributed to a defect at the level of the receptor or its G<sub>i</sub> signaling pathway. The present results reveal a third explanation for the loss of response in patients with complete rather than partial loss of adrenaline-induced aggregation, namely a defect in Src family kinase regulation. Furthermore, the demonstration that Src family kinase inhibition does not block cAMP inhibition or synergy with G<sub>q</sub>-coupled receptors means that these relatively straightforward assays can be used to further establish the basis of the defect in response to adrenaline and ultimately the nature of the clinical disorder.

**Addendum**

C. A. Nash designed and performed experiments, and wrote the article. S. Séverin designed experiments and wrote the article. B. B. Dawood performed all of the patient studies and reviewed the article. A. Mumford, M. Makris and J. Wilde supplied patients and reviewed the article. Y. A. Senis co-supervised the project and reviewed the article. S. P. Watson designed experiments and wrote the article.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Aggregation and secretion induced by adrenaline is dependent on \( \alpha_{IIb}\beta_3 \) integrin and secondary mediator release.

Fig. S2. Adrenaline does not stimulate tyrosine phosphorylation in PRP.

Data S1. Materials and methods.

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