Journal of Thrombosis and Haemostasis, **8**: 2273–2282 DOI: 10.1111/j.1538-7836.2010.03992.x

ORIGINAL ARTICLE

Src family kinases are essential for primary aggregation by G_i-coupled receptors

C. A. NASH,* S. SÉVERIN,* B. B. DAWOOD,* M. MAKRIS,† A. MUMFORD,‡ J. WILDE,*§ Y. A. SENIS* and S. P. WATSON*

Centre for Cardiovascular Sciences, Institute of Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, Birmingham; †Department of Cardiovascular Science, University of Sheffield, Royal Hallamshire Hospital, Sheffield; ‡Bristol Heart Institute, University of Bristol, Bristol Royal Infirmary, Bristol; and §West Midlands Adult Haemophilia Centre, University Hospital Birmingham NHS Trust, Birmingham, UK

To cite this article: Nash CA, Séverin S, Dawood BB, Makris M, Mumford A, Wilde J, Senis YA, Watson SP. Src family kinases are essential for primary aggregation by G_i-coupled receptors. *J Thromb Haemost* 2010; **8**: 2273–82.

Summary. *Introduction and Background:* Adrenaline stimulates biphasic aggregation in plasma through the G_i-coupled α_{2A}-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 $P2Y_1$ and $P2Y_{12}$ 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 investigation of patients with suspected bleeding disorders. In a group of 90 unrelated patients with a clinically diagnosed platelet bleeding 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_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.

Correspondence: Craig Nash, Centre for Cardiovascular Sciences, Institute of Biomedical Research, College of Medical and Dental Sciences, University of Birmingham B15 2TT, UK.
Tel.: +44 121 4158679; fax: +44 121 4158817.
E-mail: can562@bham.ac.uk

Received 22 April 2010, accepted 7 July 2010

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 α_{2A}-adrenoreceptor knockout mouse, although it is unclear whether this is the case in humans [2].

The α_{2A}-adrenoreceptor and $P2Y_{12}$ ADP receptor signal via the G_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_i family of G proteins, namely G_z [4,5] and $G\alpha_{i2}$ [6], respectively. The α_{2A}-adrenoreceptor and $P2Y_{12}$ ADP receptor also couple to a second member of the G_i family of G proteins in mice, most likely $G\alpha_{i1}$ [7,8]. It is not known whether the $P2Y_{12}$ ADP receptor and the α_{2A}-adrenoreceptor couple to the same G_i proteins in human platelets.

The $P2Y_{12}$ ADP receptor and the α_{2A}-adrenoreceptor undergo synergy with G_q-regulated and $G_{12/13}$-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

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 PI 3-kinases is critical for G_i-mediated platelet activation. The structurally distinct inhibitors of PI 3-kinases, wortmannin and LY294002, inhibit activation of integrin $\alpha_{IIb}\beta_3$ by ADP in $P2Y_1$-deficient mice 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 $P2Y_{12}$ 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_i-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 α_{2A}-adrenoreceptor and $P2Y_{12}$ ADP receptor activation, demonstrating a critical role for Src family kinases in the proximal events in G_i 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 cell 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 (6×10^7 mL^{-1}) were stimulated under non-stirring conditions at 37 °C for 3 min with adrenaline, prostaglandin E_1 (PGE_1) 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 hemophilia 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),

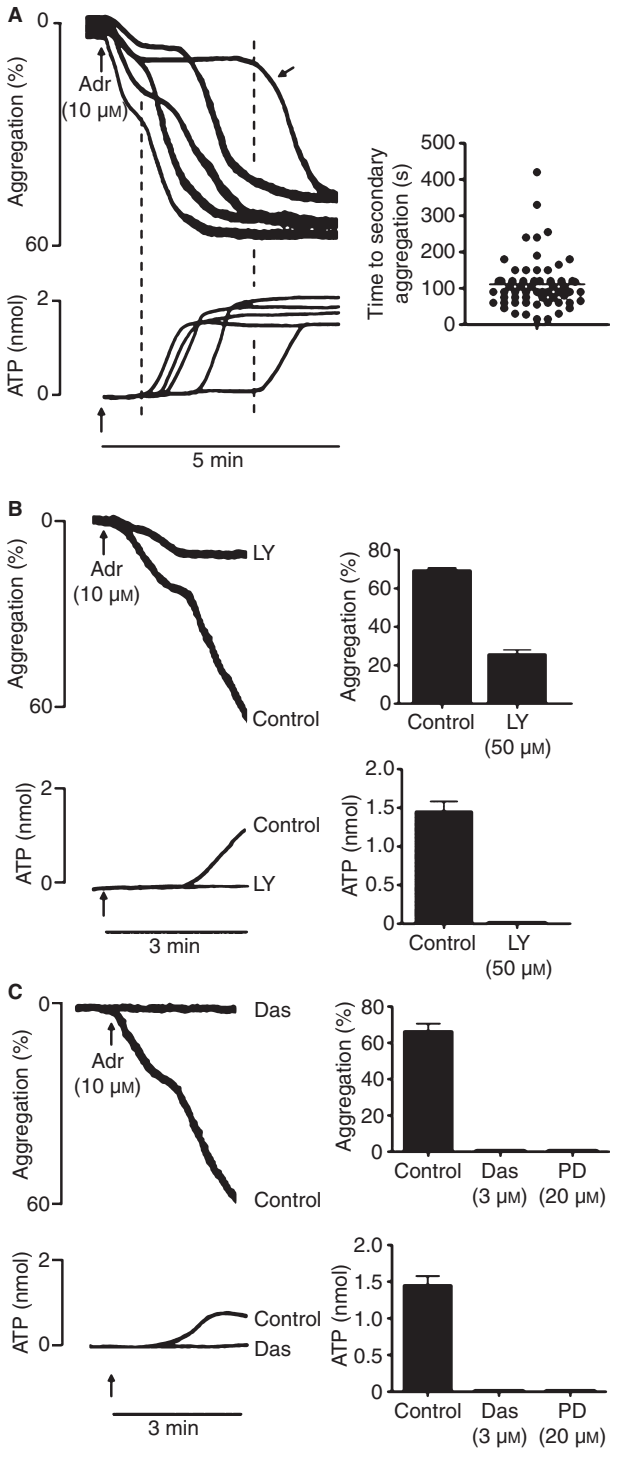

Fig. 1. Aggregation and secretion induced by adrenaline (Adr) is critically dependent on Src family kinases and partially dependent on phosphoinositide 3-kinases. (A) Overlaid sample traces for aggregation and dense granule secretion induced by adrenaline (10 μM) in platelet-rich plasma from 60 healthy control donors. Sample traces for aggregation and dense granule secretion for a control with extended time from primary to secondary aggregation, compared to a control with normal time to secondary aggregation, is indicated by the arrow and dashed line. The plot displays time to secondary aggregation in all 60 controls studied. (B) Aggregation and dense granule secretion induced by adrenaline (10 μM) in plasma in the presence or absence of LY294002 (LY) (50 μM). Traces are representative of three independent experiments, and histograms (3 min of stimulation) are means ± standard errors of the mean (SEMs). (C) Aggregation and dense granule secretion induced by adrenaline (10 μM) in plasma in the presence of either dasatinib (Das) (3 μM), PD0173952 (PD) (20 μM) or vehicle control. Representative traces are shown, and histograms represent means ± standard error of five independent experiments.

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_i 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_i-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

(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 $\alpha_{IIb}\beta_3$-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 $\alpha_{IIb}\beta_3$ [36,37]. On the other hand, the 5-hydroxytryptamine $(5\text{-HT})_{2A}$/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 $\alpha_{IIb}\beta_3$. 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

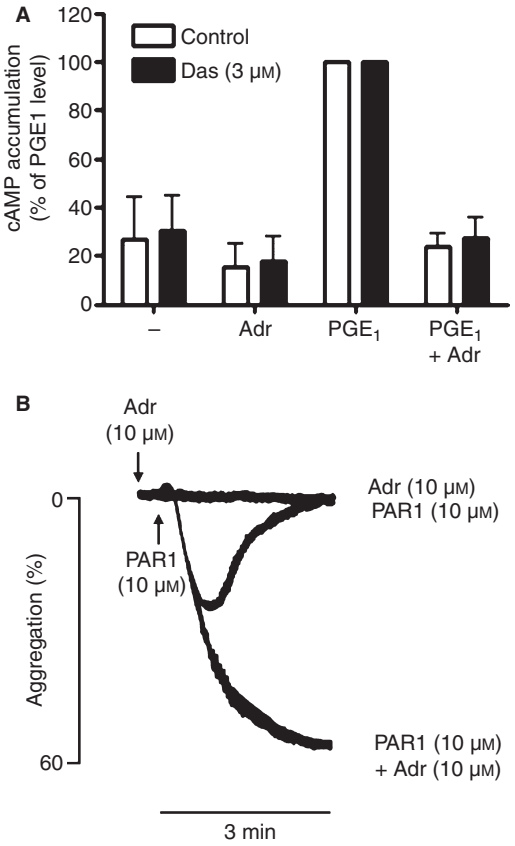

Fig. 2. Adrenaline (Adr)-mediated cAMP inhibition is not Src family kinase-dependent. (A) Washed platelets (6×10^7 mL^{-1}) were stimulated in the presence of prostaglandin E_1 (PGE_1) (20 μM), adrenaline (10 μM) or both together, and subsequently lysed. These experiments were performed following preincubation with either dasatinib (Das) (3 μM), PD0173952 (20 μM) or vehicle control. Samples were then analyzed with an enzyme-linked immunosorbent assay-based method, with the concentration of cAMP under PGE_1 stimulation being arbitrarily set to 100%. (B) Aggregation of platelet-rich plasma samples induced by adrenaline (10 μM), protease-activated receptor 1 (PAR1) peptide (10 μM) or both agonists in the presence of dasatinib (3 μM). The results shown are representative of those from three experiments. Aggregation was allowed to continue for 3 min.

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 dasatanib (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.

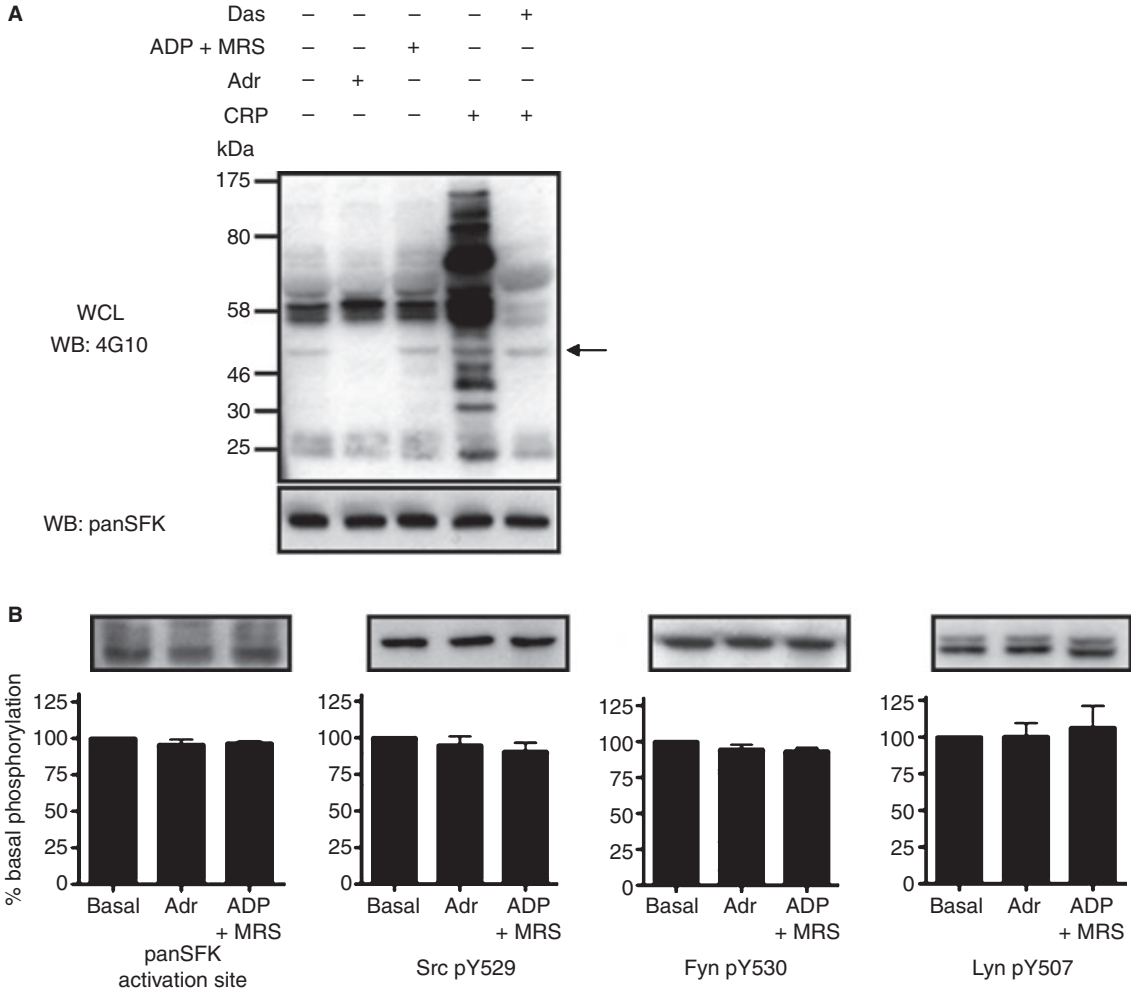

Fig. 3. Adrenaline (Adr) or $P2Y_{12}$ ADP receptor activation does not stimulate tyrosine phosphorylation in platelets. Human washed platelets were stimulated with adrenaline (1 mM), ADP (10 μM) in the presence of MRS2179 (MRS) (100 μM) or collagen-related peptide (CRP) (10 μg mL^{-1}) in the presence 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.

$P2Y_{12}$-mediated aggregation is dependent on Src family kinases

Experiments were performed to investigate whether activation of platelets in plasma by the G_i-coupled $P2Y_{12}$ ADP receptor is also dependent on Src family kinases. These studies were carried out in the presence of the $P2Y_1$ ADP receptor antagonist MRS2179, and were restricted to concentrations of ADP of up to 10 μM, as shape change was observed at higher concentrations, presumably because of partial rescue from the competitive blockade. These experiments were of particular interest, given both the physiologic significance of the $P2Y_{12}$ ADP receptor and the fact that it is coupled to $G\alpha_{i2}$ rather than G_z, which is the major G protein used by adrenaline [4–6].

ADP (10 μM) stimulated primary aggregation but not secretion in PRP in the presence of the $P2Y_1$ ADP receptor antagonist MRS2179 (Fig. 4). Aggregation was not preceded by shape change, as was the case for adrenaline. ADP-induced aggregation (10 μM) 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 $P2Y_1$ ADP receptor blockade, ADP (10 μM) 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, $P2Y_{12}$ 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_z, which is regulated by the α_{2A}-adrenoreceptor but not by the $P2Y_{12}$ ADP receptor.

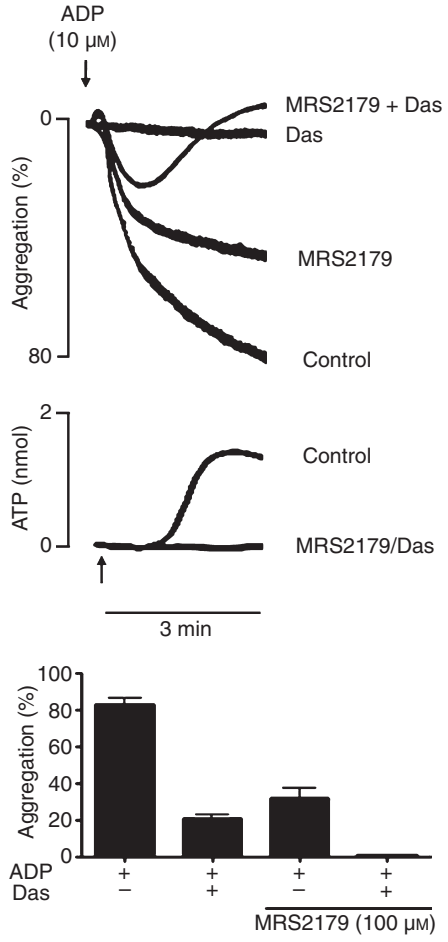

Fig. 4. Aggregation induced by $P2Y_{12}$ activation is critically dependent on Src family kinases. Platelet-rich plasma was stimulated with ADP (10 μM) in the presence of MRS2179 (100 μM). Samples were treated with dasatinib (Das) (3 μM), PD0173952 (20 μM) or vehicle control. Representative traces and 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-specificpeptides, ristocetin and U46619 (stable thromboxane A_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_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_1-elevated cAMP to a similar degree to controls (not shown). In contrast to the results for adrenaline, ADP (10 μM) 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 $P2Y_1$ and $P2Y_{12}$ 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

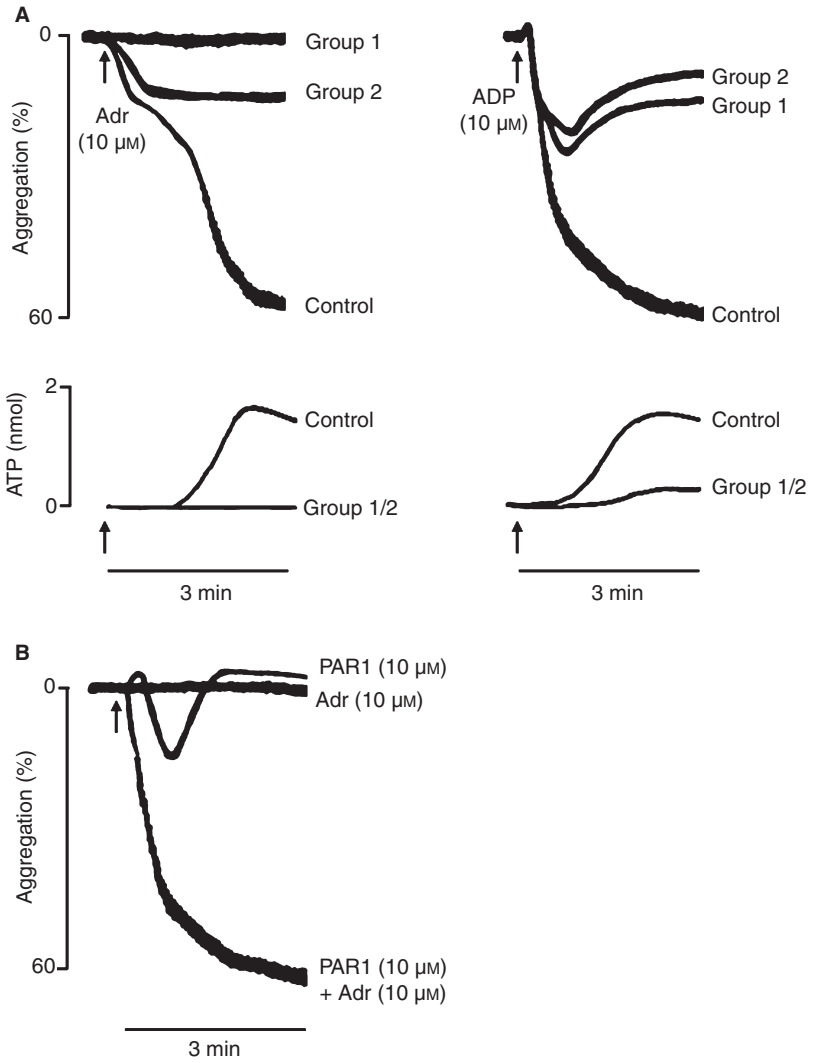

Fig. 5. Heterogeneity in responses to adrenaline (Adr) in patients with clinical bleeding disorders. (A) Platelet aggregation stimulated in PRP by adrenaline (10 μM) or ADP (10 μM) in patients relative to example control samples. Traces are representative of those seen following stimulation with adrenaline in four and five patients for group 1 and group 2, respectively, and with ADP. (B) Aggregation induced by adrenaline (10 μM), protease-activated receptor 1 (PAR1) peptide (10 μM) or both agonists together in a patient with a G_i-like defect. The results shown are representative of those seen in four patients. Aggregation was allowed to continue for 3 min.

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 α_{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_q-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 $P2Y_1$ but not the $P2Y_{12}$ ADP receptor in washed platelets.

The nature of the Src family kinases that underlie the action of the α_{2A}-adrenoreceptor and $P2Y_{12}$ 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 $P2Y_{12}$-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_i 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_i 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_i-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 $\alpha_{IIb}\beta_3$, 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_i-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 $P2Y_1$ and $P2Y_{12}$ 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_i 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_i-based signaling rather than abrogation of Src family kinase-mediated activation.

Clinical implications for investigation of patients with suspected G_i defects

The present results emphasize the critical role of Src family kinases in initiating platelet activation by G_i-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_i 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_q-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.

Acknowledgements

This work was supported by the following grants from the British Heart Foundation: FS/08/023/25089, PG/06/129, PG/06/038 and RG/09/007). C. A. Nash is supported by a BHF

studentship, Y. A. Senis is a BHF Intermediate Fellow, and S. P. Watson holds a BHF Chair.

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.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

1 Varga-Szabo D, Pleines I, Nieswandt B. Cell adhesion mechanisms in platelets. *Arterioscler Thromb Vasc Biol* 2008; **28**: 403–12.
2 Pozgajova M, Sachs UJ, Hein L, Nieswandt B. Reduced thrombus stability in mice lacking the alpha2A-adrenergic receptor. *Blood* 2006; **108**: 510–14.
3 Pierce KL, Premont RT, Lefkowitz RJ. Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* 2002; **3**: 639–50.
4 Kelleher KL, Matthaei KI, Hendry IA. Targeted disruption of the mouse Gz-alpha gene: a role for Gz in platelet function? *Thromb Haemost* 2001; **85**: 529–32.
5 Yang J, Wu J, Kowalska MA, Dalvi A, Prevost N, O'Brien PJ, Manning D, Poncz M, Lucki I, Blendy JA, Brass LF. Loss of signaling through the G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. *Proc Natl Acad Sci USA* 2000; **97**: 9984–9.
6 Jantzen HM, Milstone DS, Gousset L, Conley PB, Mortensen RM. Impaired activation of murine platelets lacking G alpha(i2). *J Clin Invest* 2001; **108**: 477–83.
7 Patel YM, Patel K, Rahman S, Smith MP, Spooner G, Sumathipala R, Mitchell M, Flynn G, Aitken A, Savidge G. Evidence for a role for Galphai1 in mediating weak agonist-induced platelet aggregation in human platelets: reduced Galphai1 expression and defective Gi signaling in the platelets of a patient with a chronic bleeding disorder. *Blood* 2003; **101**: 4828–35.
8 Yang J, Wu J, Jiang J, Mortensen R, Austin S, Manning DR, Woulfe D, Brass LF. Signaling through Gi family members in platelets. Redundancy and specificity in the regulation of adenylyl cyclase and other effectors. *J Biol Chem* 2002; **277**: 46035–42.
9 Nieswandt B, Schulte V, Zywietz A, Gratacap MP, Offermanns S. Costimulation of Gi- and G12/G13-mediated signaling pathways induces integrin alpha IIbbeta 3 activation in platelets. *J Biol Chem* 2002; **277**: 39493–8.
10 Dorsam RT, Kim S, Jin J, Kunapuli SP. Coordinated signaling through both G12/13 and G(i) pathways is sufficient to activate GPIIb/IIIa in human platelets. *J Biol Chem* 2002; **277**: 47588–95.
11 Baurand A, Raboisson P, Freund M, Leon C, Cazenave JP, Bourguignon JJ, Gachet C. Inhibition of platelet function by administration of MRS2179, a P2Y1 receptor antagonist. *Eur J Pharmacol* 2001; **412**: 213–21.
12 Fabre JE, Nguyen M, Latour A, Keifer JA, Audoly LP, Coffman TM, Koller BH. Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. *Nat Med* 1999; **5**: 1199–202.
13 Leon C, Hechler B, Freund M, Eckly A, Vial C, Ohlmann P, Dierich A, LeMeur M, Cazenave JP, Gachet C. Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. *J Clin Invest* 1999; **104**: 1731–7.
14 Haslam RJ. Interactions of the pharmacological receptors of blood platelets with adenylate cyclase. *Ser Haematol* 1973; **6**: 333–50.
15 Daniel JL, Dangelmaier C, Jin J, Kim YB, Kunapuli SP. Role of intracellular signaling events in ADP-induced platelet aggregation. *Thromb Haemost* 1999; **82**: 1322–6.
16 Kauffenstein G, Bergmeier W, Eckly A, Ohlmann P, Leon C, Cazenave JP, Nieswandt B, Gachet C. The P2Y(12) receptor induces platelet aggregation through weak activation of the alpha(IIb)beta(3) integrin – a phosphoinositide 3-kinase-dependent mechanism. *FEBS Lett* 2001; **505**: 281–90.
17 Hirsch E, Bosco O, Tropel P, Laffargue M, Calvez R, Altruda F, Wymann M, Montrucchio G. Resistance to thromboembolism in PI3Kgamma-deficient mice. *FASEB J* 2001; **15**: 2019–21.
18 Jackson SP, Schoenwaelder SM, Goncalves I, Nesbitt WS, Yap CL, Wright CE, Kenche V, Anderson KE, Dopheide SM, Yuan Y, Sturgeon SA, Prabaharan H, Thompson PE, Smith GD, Shepherd PR, Daniele N, Kulkarni S, Abbott B, Saylik D, Jones C, *et al*. PI 3-kinase p110beta: a new target for antithrombotic therapy. *Nat Med* 2005; **11**: 507–14.
19 van der Meijden PE, Schoenwaelder SM, Feijge MA, Cosemans JM, Munnix IC, Wetzker R, Heller R, Jackson SP, Heemskerk JW. Dual P2Y 12 receptor signaling in thrombin-stimulated platelets – involvement of phosphoinositide 3-kinase beta but not gamma isoform in $Ca2^+$ mobilization and procoagulant activity. *FEBS J* 2008; **275**: 371–85.
20 Canobbio I, Stefanini L, Cipolla L, Ciraolo E, Gruppi C, Balduini C, Hirsch E, Torti M. Genetic evidence for a predominant role of PI3Kbeta catalytic activity in ITAM- and integrin-mediated signaling in platelets. *Blood* 2009; **114**: 2193–6.
21 Martin V, Guillermet-Guibert J, Chicanne G, Cabou C, Jandrot-Perrus M, Plantavid M, Vanhaesebroeck M, Payrastre B, Gratacap MP. Deletion of the p110beta isoform of phosphoinositide 3-kinase in platelets reveals its central role in Akt activation and thrombus formation in vitro and in vivo. *Blood* 2000; **115**: 2008–13.
22 Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ. Beta-arrestin-dependent formation of beta2 adrenergic receptor–Src protein kinase complexes. *Science* 1999; **283**: 655–61.
23 Ma YC, Huang J, Ali S, Lowry W, Huang XY. Src tyrosine kinase is a novel direct effector of G proteins. *Cell* 2000; **102**: 635–46.
24 Sun Y, McGarrigle D, Huang XY. When a G protein-coupled receptor does not couple to a G protein. *Mol Biosyst* 2007; **3**: 849–54.
25 Dorsam RT, Kim S, Murugappan S, Rachoor S, Shankar H, Jin J, Kunapuli SP. Differential requirements for calcium and Src family kinases in platelet GPIIb/IIIa activation and thromboxane generation downstream of different G-protein pathways. *Blood* 2005; **105**: 2749–56.
26 Hardy AR, Jones ML, Mundell SJ, Poole AW. Reciprocal cross-talk between P2Y1 and P2Y12 receptors at the level of calcium signaling in human platelets. *Blood* 2004; **104**: 1745–52.
27 Shankar H, Murugappan S, Kim S, Jin J, Ding Z, Wickman K, Kunapuli SP. Role of G protein-gated inwardly rectifying potassium channels in P2Y12 receptor-mediated platelet functional responses. *Blood* 2004; **104**: 1335–43.
28 Torti M, Crouch MF, Lapetina EG. Epinephrine induces association of pp60src with Gi alpha in human platelets. *Biochem Biophys Res Commun* 1992; **186**: 440–7.

29 Gratacap MP, Martin V, Valera MC, Allart S, Garcia C, Sie P, Recher C, Payrastre B. The new tyrosine-kinase inhibitor and anticancer drug dasatinib reversibly affects platelet activation in vitro and in vivo. *Blood* 2009; **114**: 1884–92.
30 Auger JM, Kuijpers MJ, Senis YA, Watson SP, Heemskerk JW. Adhesion of human and mouse platelets to collagen under shear: a unifying model. *FASEB J* 2005; **19**: 825–7.
31 Fuller GL, Williams JA, Tomlinson MG, Eble JA, Hanna SL, Pohlmann S, Suzuki-Inoue K, Ozaki Y, Watson SP, Pearce AC. The C-type lectin receptors CLEC-2 and Dectin-1, but not DC-SIGN, signal via a novel YXXL-dependent signaling cascade. *J Biol Chem* 2007; **282**: 12397–409.
32 Dawood BB, Wilde J, Watson SP. Reference curves for aggregation and ATP secretion to aid diagnosis of platelet-based bleeding disorders: effect of inhibition of ADP and thromboxane A(2) pathways. *Platelets* 2007; **18**: 329–45.
33 Pearce AC, Senis YA, Billadeau DD, Turner M, Watson SP, Vigorito E. Vav1 and vav3 have critical but redundant roles in mediating platelet activation by collagen. *J Biol Chem* 2004; **279**: 53955–62.
34 Weiss HJ, Lages B. The response of platelets to epinephrine in storage pool deficiency – evidence pertaining to the role of adenosine diphosphate in mediating primary and secondary aggregation. *Blood* 1988; **72**: 1717–25.
35 Haslam RJ, Davidson MM, Davies T, Lynham JA, McClenaghan MD. Regulation of blood platelet function by cyclic nucleotides. *Adv Cyclic Nucleotide Res* 1978; **9**: 533–52.
36 Jin J, Quinton TM, Zhang J, Rittenhouse SE, Kunapuli SP. Adenosine diphosphate (ADP)-induced thromboxane A(2) generation in human platelets requires coordinated signaling through integrin alpha(IIb)beta(3) and ADP receptors. *Blood* 2002; **99**: 193–8.
37 Cattaneo M, Gachet C, Cazenave JP, Packham MA. Adenosine diphosphate (ADP) does not induce thromboxane A2 generation in human platelets. *Blood* 2002; **99**: 3868–9.
38 Pawson T. Protein modules and signalling networks. *Nature* 1995; **373**: 573–80.
39 Senis YA, Tomlinson MG, Ellison S, Mazharian A, Lim J, Zhao Y, Kornerup KN, Auger JM, Thomas SG, Dhanjal T, Kalia N, Zhu JW, Weiss A, Watson SP. The tyrosine phosphatase CD148 is an essential positive regulator of platelet activation and thrombosis. *Blood* 2009; **113**: 4942–54.
40 Campus F, Lova P, Bertoni A, Sinigaglia F, Balduini C, Torti M. Thrombopoietin A complements G(i)- but not G(q)-dependent pathways for integrin {alpha}(IIb){beta}(3) activation and platelet aggregation. *J Biol Chem* 2005; **280**: 24386–95.
41 Kim S, Garcia A, Jackson SP, Kunapuli SP. Insulin-like growth factor-1 regulates platelet activation through PI3-Kalpha isoform. *Blood* 2007; **110**: 4206–13.
42 Mori J, Pearce AC, Spalton JC, Grygielska B, Eble JA, Tomlinson MG, Senis YA, Watson SP. G6b-B inhibits constitutive and agonist-induced signaling by glycoprotein VI and CLEC-2. *J Biol Chem* 2008; **283**: 35419–27.
43 Anderson KE, Jackson SP. Class I phosphoinositide 3-kinases. *Int J Biochem Cell Biol* 2003; **35**: 1028–33.
44 Vanhaesebroeck B, Leevers SJ, Panayotou G, Waterfield MD. Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem Sci* 1997; **22**: 267–72.