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Citation  

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Abstract

Platelet signalling responses to single agonists have been identified previously. However a model of the total platelet signalling network is still lacking. In order to gain insights into this network, we explored the effects of a range of platelet-function inhibitors in two independent assays of platelet function, namely fibrinogen binding and ADP secretion. In this study, we targeted the intracellular signalling molecules Syk and PI3K, the prostaglandin synthesis enzyme COX, surface receptors for TxA2 and ADP (P2Y1 and P2Y12) and the integrin cell adhesion molecule, αIIbβ3. We demonstrate that the platelet responses of fibrinogen binding and secretion can be differentially affected by the individual inhibitors permitting the generation of a model delineating novel regulatory links in the platelet signal network. Importantly, the model illustrates the interconnections among portions that are traditionally studied as separate modules, promoting a more integrated view of the platelet.

Abbreviations:
CRP: Collagen Related Peptide
GPVI: Glycoprotein VI
Syk: Spleen Tyrosine Kinase
PI3K: Phosphoinositide 3 kinase
COX: Cyclooxygenase
TxA$_2$R: Thromboxane A$_2$ Receptor,
TRAP: Thrombin Receptor Activating Peptide
PLA2: phospholipase A2

1. Introduction

Platelets play a critical role in haemostasis by monitoring the integrity of blood vessels. Following adhesion to exposed extracellular-matrix proteins, platelets activate, secrete, and aggregate on a damaged endothelium. Platelet activation proceeds through a poorly understood sequence of signalling events which culminates in the “inside-out” activation of the integrin $\alpha$IIb$\beta$3 cell adhesion molecule, regardless of the agonist. The heterodimeric $\alpha$IIb$\beta$3 cell adhesion molecule is the most abundant integrin on platelet surface, with about 80000 copies per cell and acts as a fibrinogen receptor. The common downstream effector of integrin activation is talin, upon which all the intracellular signalling routes converge irrespective of the agonist that initiated them. It is known that talin, once its auto-inhibition is relieved, inserts itself between the integrin cytoplasmic tails and binds to the $\beta$-tail, thereby causing the extracellular portions to undergo a conformational switch that increases the affinity for fibrinogen. Fibrinogen binding in turn elicits a signalling cascade (outside-in signalling) that is required for a full irreversible aggregation response and is therefore considered a key step in platelet activation.

Among post-activation events, ADP secretion and thromboxane (Tx)A$_2$ production are of undisputed importance since both provide short-distance signals for local amplification of the haemostatic response. ADP signalling in platelets is important for sustained aggregation,
and it is largely dependent on the G-protein coupled receptors, namely the P2Y1 receptor that is coupled to G\textsubscript{q}, and the P2Y12 receptor that is coupled to G\textsubscript{i11-12}.

\textit{TxA\textsubscript{2}} is a prostaglandin product of arachidonic acid and a potent platelet activator\textsuperscript{13}. The production of \textit{TxA\textsubscript{2}} begins with the release of arachidonic acid from membrane phospholipids or DAG that is activated by calcium-sensitive phospholipase A2 (PLA2). Arachidonic acid is then converted into prostaglandin H2 by cyclooxygenase (COX) and successively into \textit{TxA\textsubscript{2}} by thromboxane synthase. \textit{TxA\textsubscript{2}} is released by activated platelets and recruits more platelets to a site of vessel injury. Released ADP has a similar effect. Both ADP secretion and \textit{TxA\textsubscript{2}} release are therefore of great interest in the understanding of positive feedback mechanisms on clot development.

One of the most interesting platelets agonists is collagen as it not only binds to adhesive receptors on the platelet plasma membrane\textsuperscript{14}, it also initiates platelet activation through its signalling membrane-receptor glycoprotein (GP)\textsubscript{VI}\textsuperscript{15}. GPVI belongs to the immunoglobulin (Ig) superfamily\textsuperscript{16} and it is constitutively associated with FcR\gamma, a protein containing an immunoreceptor-tyrosine–based activation motif (ITAM)\textsuperscript{17}. The early events that follow GPVI engagement comprise the activation of Spleen Tyrosine Kinase (Syk)\textsuperscript{18} through phosphorylation or ubiquitination\textsuperscript{19-20}, and Phosphoinositide 3 kinase (PI3K)\textsuperscript{21}, possibly through its regulatory subunit\textsuperscript{22}. Recently, many studies investigating collagen signalling utilized collagen related peptide (CRP) instead of collagen because it specifically binds to GPVI therefore allowing for better molecular dissection of the signalling\textsuperscript{19,23}. \textit{αIIbβ3}, P2Y1, P2Y12, COX, \textit{TxA\textsubscript{2}}, Syk, and PI3K are all targets for antiplatelet therapy\textsuperscript{24} and are part of a signalling pathway that is relatively well characterized\textsuperscript{25}.

However the responses to pathway perturbations are not always intuitive, indicating that some of the regulatory circuits are still unknown and awaiting discovery. For this reason we have determined the sensitivity of fibrinogen binding and ADP secretion after pathway perturbation. By considering both the responses simultaneously and by complementing our
findings with the relevant literature, we were able to trace a potential wiring diagram that links the target proteins, ADP secretion and integrin activation. Such a model, although not definitive, facilitates the analysis of the network properties and sets the ground for a quantitative study.

2. Materials and methods

2.1. Materials:
Thrombin receptor activating peptide (TRAP) was obtained from Bachem (Merseyside, UK). Collagen–related peptide (CRP-X) used in these studies was obtained from the laboratory of Professor Richard Farndale (Cambridge, UK). All other reagents were obtained from Sigma-Aldridge except the following: Syk inhibitor II (Calbiochem Merk), SQ29548, MRS2395 & MRS2179 (Enzo Life Sciences, UK), and tirofiban (Merck Sharp & Dohme Ltd. Hertfordshire, UK). Chronolume was obtained from Chronolog (Labmedics Limited, UK) and Oregon Green labelled Fibrinogen was obtained from Molecular Probes via Biosciences Limited, Ireland.

2.2. Washed Platelet preparation
Washed platelets (WP) were prepared from donors who gave informed consent. Venous blood was drawn into 15% (v/v) of acid-citrate-dextrose (ACD) anticoagulant (38mM citric acid anhydrous, 75 mM sodium citrate, 124mM dextrose). Blood was centrifuged at 150 x g for 10 minutes and the platelet rich plasma (PRP) was collected. PRP was acidified with ACD at pH 6.5 and 1 μM prostaglandin E1 (PGE1) was added. The platelets were pelleted 720 x g for 10 minutes and resuspended in buffer (6 mM dextrose, 130 mM NaCl, 9 mM NaHCO₃, 10 mM sodium citrate, 10 mM Tris base, 3 mM KCl, 0.81 mM KH₂PO₄ and 0.9 mM MgCl₂6H₂O, pH 7.35) and the concentration adjusted to 3x10⁵ platelets/μl. WP were supplemented with 1.8 mM CaCl₂ immediately prior to use.

2.3. ADP release assay
Platelet ADP secretion was assessed as previously described\textsuperscript{26-27}. Inhibitors were dispensed in a final volume of 10 µl into different wells of a 96-well white plate (Sigma-Aldrich, Ireland) and 70 µl of WP were gently pipetted on top. The inhibitors used were Syk inhibitor II (from 1.37 to 1000 nM), wortmannin (from 0.34 to 250 nM), aspirin (from 1.37 to 100 µM), SQ29548 (from 2.194 to 1600 nM), MRS2179 (from 68.5 nM to 50 µM), MRS2395 (from 68.5 nM to 50 µM), and tirofiban (from 0.685 pM to 0.5 nM).

Platelets were incubated in the presence or absence of incremental doses of inhibitors for 15 minutes at 37°C. CRP (1 µg/ml; from Dr Richard Farndale, Cambridge, UK) or TRAP (10µM; Bachem, UK) was then added and allowed to activate platelets for 10 minutes at 37°C. Finally, 10 µl of the detection reagent Chronolume (Chronolog; Labmedics Limited, UK) were dispensed in the wells and the sample luminescence was detected using a Wallac 1420 Multilabel Counter (Perkin Elmer). For the inhibitor studies shown in figure 2, data was obtained from 6 independent donors. All inhibitors were tested on the same 6 donors in parallel. Data were expressed as a percentage of a maximal response, defined as the response to CRP or TRAP in the absence of any inhibitor. Values obtained in the presence of inhibitors were subtracted from this 100% response. Data presented represent mean ± sem for 6 independent donors.

2.4. Flow cytometry of fibrinogen binding

Washed platelet aliquots (20 µl) were dispensed in 15mm plastic tubes and pre-treated with increasing concentrations of the various inhibitors for 15 minutes in a final volume of 90 µl. Oregon Green labelled Fibrinogen (10 µl, to a final concentration of 0.15 mg/ml, Biosciences Limited, Ireland) and the agonist were then added and the reaction was allowed to proceed for 10 minutes. The assay was terminated by the addition of 1ml of cold buffer. Platelet-associated fluorescence was estimated using a FACSCalibur flow cytometer. Assays with inhibitors were performed in parallel with the ADP secretion assays (above) on the same 6 independent donors. Data were expressed as a percentage of a maximal response, defined as
the response to CRP or TRAP in the absence of any inhibitor. Values obtained in the presence of inhibitors were subtracted from this 100% response.

2.5. Statistical Analysis

Standard errors were determined using Instat, the statistical package associated with GraphPad Prism 4 for Windows. Statistical changes in tirofiban-treated platelets were determined performing paired t-tests for each dose, with R software for statistical computing and graphics. R is a computer language and environment for data handling and analysis and it was used here in order to calculate the level of significance of the observations regarding the tirofiban.

3. Results

3.1 Characterization of Assay parameters in human Platelets

Collagen related peptide (CRP) was chosen as an agonist for this study as it binds to the collagen receptor glycoprotein (GP) VI, but not to the integrin α2β1. To identify a useful dose of CRP peptide to use in our studies, we examined the effects of increasing doses of CRP peptide on platelet ADP secretion and fibrinogen binding assays. CRP induces a dose-dependent response that is saturated at concentrations in excess of 2 µg/ml in both assays. A dose of 1µg/ml CRP was chosen for all future studies as it consistently caused a submaximal response (Figure 1). A dose of agonist that induced an 80% maximal response was considered optimal for these studies as it would permit the identification of both inhibitory and activating agents in our assays. Moreover, this dose of CRP is capable of causing a response that is equivalent to 62% of the response to a maximal dose (8µM) of TRAP peptide in ADP-secretion assays and 81% of the maximal TRAP induced fibrinogen-binding response (8µM; Figure 1D). Thus, although the dose of CRP used in our study might appear low, we establish that this batch of CRP-X peptide is highly potent and that the dose chosen for our study causes a substantial and equivalent activation of platelets in both assays used in our study. This dose of CRP is capable of inducing platelet aggregation (data not shown) as expected.
However, because platelet aggregation responses is not a sensitive measure of platelet response \(^{28}\) and because we wished to examine small increments in response associated with specific inhibitors, we opted to examine the related index of fibrinogen binding in our assays.

3.2 Effect of pharmacological inhibitors upon CRP-induced fibrinogen binding and ADP release.

To determine the role of intracellular pathways in the regulation of CRP-induced integrin activation and ADP secretion we investigated the effect of the selective inhibitors Syk inhibitor II\(^{29}\), wortmannin\(^{30}\), aspirin\(^{31}\), SQ29548\(^{32}\), MRS2179\(^{33}\), MRS2395\(^{34}\). It is known that Syk is a central component of the intracellular signalling cascade elicited by GPVI engagement\(^{19}\). Syk inhibitor-II retains its selectivity for Syk at doses up to 1 µM (doses higher than 1 µM were not investigated due to the documented lack of selectivity\(^{29}\)). Increasing doses of Syk inhibitor-II produce similar inhibition profiles in ADP secretion and integrin activation, consistent with a role of Syk as a crucial check-valve in the control of the two activation endpoints under study (Fig. 2A).

Wortmannin inhibits mammalian class 1a, class 1b and class 3 PI 3-kinases (PI3Ks) at low-nanomolar concentrations by covalently binding to a catalytically relevant lysine residue in the p110 subunit\(^{30}\). At concentrations greater than 100 nM, wortmannin will inhibit some isoforms of PI 4-kinase\(^{35}\) and phospholipase A\(_2^{36}\); therefore, only one dose greater than 100 nM was used in our studies in order to maintain the specificity for PI3K. As illustrated in Fig. 2B, the CRP-induced fibrinogen binding is only partially inhibited by the inhibition of PI3K activity, whereas the secretion of ADP is completely abolished at concentrations of 100 nM. In both of these assays, it is clear that the PI3K inhibitor has reached its maximum effect, as the dose-inhibition curves reach a plateau. Moreover, the shape of these dose-inhibition curves conforms to classical inhibition criteria. Thus, the range of drug doses required to induce an inhibition of the response of between 10 and 90% was approximately 80 fold and Hill co-efficients were close to 1.0. Aspirin (Fig. 2C) and SQ29548 (Fig. 2D) were used in order to evaluate the contribution of thromboxane signalling in CRP-stimulated activation of
platelets. The first inhibits the intracellular production of thromboxane by inhibition of COX\textsuperscript{31}, whereas the latter blocks the thromboxane receptor on the cell membrane\textsuperscript{32}. Both inhibitors showed only a mild inhibition of fibrinogen-binding to CRP-activated platelets. Due to the high variability observed in SQ29548-treated platelets (Fig. 2D), it is difficult to draw reliable conclusions. The inhibition profile of aspirin-treated platelets, on the other hand, with its low variability (Fig. 2C), allows us to affirm that thromboxane signalling is likely to be responsible for 20\% of the fibrinogen binding response and for the 40\% of ADP secretion.

Furthermore, we observed that the antagonism of purinergic P2Y1 and P2Y12 receptors, with MRS2179 and MRS2395 respectively, has little effect at any dose except the highest dose tested (Fig. 2E and 2F), suggesting that GPVI-induced activation is mostly independent of single ADP receptors. It seems noteworthy that the pharmacological inhibition of both the receptors at higher doses has a more prominent effect on the secretory response than on the fibrinogen binding response. However, at lower doses, P2Y12 inhibitor appears to slightly promote secretion.

3.3. Tirofiban increases the secretion of ADP at low doses

Tirofiban is an RGD-mimetic\textsuperscript{37} that binds integrin α\textsubscript{IIb}β\textsubscript{3}\textsuperscript{38} and hinders the binding of fibrinogen. When washed platelets were incubated with this integrin inhibitor, we observed that doses that were able to almost ablate the binding of fibrinogen were in fact enhancing the secretion of ADP in response to CRP-activation (Fig. 3). We also used thrombin receptor activating peptide (TRAP; 8μM) as an agonist and observed a similar phenomenon (Fig. 3B). The significance was verified by Student t-test.

4. Discussion

In this present study, our data demonstrate a differential effect of a range of clinically-relevant inhibitors on collagen-induced ADP secretion and fibrinogen binding in human platelets. Thus, fibrinogen binding is inhibited by the α\textsubscript{IIb}β\textsubscript{3}-inhibitor, tirofiban and by Syk
Inhibitor II, but is only partially inhibited by wortmannin or aspirin and is unaffected by the ADP receptor antagonists MRS2179 and MRS2395. In contrast, ADP secretion is fully inhibited by Syk inhibitor II and by wortmannin, is partially inhibited by aspirin and only affected by high concentrations of the ADP-receptor antagonists. Surprisingly, ADP secretion was substantially and significantly enhanced by the αIIbβ3-antagonist, tirofiban suggesting the presence of a biochemical feedback link between integrin activation and platelet granule secretion. We attempted to construct a novel model of platelet signaling pathways that incorporates this new information.

Several studies contributed to the development of current models of the early events in collagen signalling (as schematized on the left of Fig. 4, black links)\textsuperscript{25}. It is known that, upon GPVI engagement, the signal propagates from FcRγ to Fyn/Lyn, Syk, PI3K and finally PLCγ2. The membrane proximal steps of ADP signalling and the release of TxA\textsubscript{2} are also known, at least in some aspects. Likewise, the inside-out integrin signalling was studied extensively and a model was developed that accounts for a number of observations\textsuperscript{8}. However, it was not known, to date, how these signalling modules integrated to produce the overall responses observed in activated platelets. The model hereby presented (Fig. 4), while partial, accomplishes the purpose of demonstrating the platelet signalling in an integrative manner, which we believe will prove valuable to the advancement of platelet systems biology. Extensive literature mining was performed in order to contextualize our results though some of the important nodes were not analyzed due to space constraints. Of note, the contribution of the various PKC isoforms and the details of cytoskeletal regulation, although of undeniable importance, were omitted.

Our results suggest the existence of three novel functional links depicted in green, red and blue in Fig. 4. The green link represents a functional interaction that connects Syk to integrin activation without passing through the kinase activity of PI3K. Fig. 2A and 2B indicate that Syk is necessary for both responses, as previously described\textsuperscript{18}, and that the catalytic function
of PI3K has a non-redundant role in ADP secretion while being only partially responsible for integrin activation. Composing these two observations, we hypothesized that the activation signal brought about by phosphorylated Syk is not fully absorbed by the catalytic activity of PI3K, thus implying a branching of the signalling route upstream of PI3K. PI3Ks are heterodimers comprised of a catalytic (p110) and a regulatory subunit (p85) that were both reported to be involved in collagen signalling. Wortmannin only inhibits kinase activity therefore a role for p85 cannot be excluded. The residual fibrinogen binding function observed in wortmannin-treated platelets is to be considered independent from the kinase activity of PI3K and from the signalling downstream of ADP receptors as ADP secretion is abolished. Since the nature of Syk-to-αIibβ3 link was not investigated, a black-box was interposed which will be empty in case the link is direct. This observation agrees, to a large extent, with data obtained from p85α deficient mice which used platelet aggregation rather than fibrinogen binding to illustrate an inhibition of platelet function. Platelets treated with wortmannin may be expected to recapitulate the phenotype of the p85α-/- platelets as these platelets where also shown to down-regulate the expression of class AI p110, the catalytic subunits of PI3K. The authors showed an approximate 40% to 60% reduction of platelet aggregation in response to high-dose CRP (2-5 µg/ml) but the complete absence of aggregation in response to low-dose CRP (1µg) (38). However, batch to batch variability in CRP peptides necessitates bioassay of each separate batch to establish potency (Prof. R. Farndale; personal communication). Our data demonstrate that the batch of CRP that we use is highly potent (Fig 1A&B) and that a dose of 1µg/ml causes an 80% maximal response. Our results therefore are consistent with the reduction observed in p85α-/- platelets stimulated with higher doses of CRP (2-5 µg/ml), rather than with the complete ablation of response in platelets stimulated with low-dose CRP, likely because our batch was very potent (Fig.1). In any case, the parallel between the phenotype of p85α knockout mice platelets to that of wortmannin-inhibited human platelets should not be over-estimated, as the two systems are very different, not only for species-dependent issues, but also because the altered expression profiles of p110 subunits confounds the interpretation of the p85α-depleted phenotype.
Thus, we can state that a PI3K-independent pathway contributes to integrin activation and fibrinogen binding in human platelets.

The red link is suggested by Fig. 1F, where high doses of MRS2395 cause an inhibition of ADP secretion. This would imply that platelet-released ADP augments dense granule secretion in a positive feedback loop. In contrast, low doses of MR2395 seem to slightly, but significantly (P<0.01) augment ADP secretion. This might suggest that a concentration-sensing mechanism in place that facilitates secretion when extracellular ADP levels are perceived to be low, but which inhibits excessive secretion when plasma ADP levels are high. Thus, such mechanism results in positive signalling only when a certain concentration threshold is exceeded. Such a mechanism would guarantee a controlled release of ADP.

Finally, the blue link refers to the finding that, strikingly, doses of tirofiban that were able to almost fully ablate the binding of fibrinogen were observed to significantly enhance ADP secretion (Fig. 3). The present model is informed by the finding that tirofiban can behave as a partial agonist\(^{43-44}\). Nevertheless, the exact mechanism underlying the phenomenon remains elusive. In particular the question is raised whether the clinical adverse effects of RGD-mimetics could be ascribed to this non-intuitive enhancement of platelet secretion responses. Bassler and colleagues\(^ {45}\) have demonstrated the capacity for platelet-released ADP to ‘prestimulate’ platelets in patients being treated with integrin antagonists. Our data confirm the capacity for one such RGD-mimetic, Tirofiban, to simultaneously inhibit fibrinogen binding while enhancing the platelet secretory response. Our data provides evidence suggesting an important role for this previously unknown (or unappreciated) component of the platelet signal pathway. In support of this a recent paper demonstrates the capacity of RGD and its mimetics, lotrafiban and tirofiban, to enhance dense-granule secretion in platelets\(^ {46}\). These authors similarly argued that this response may assist in understanding why integrin antagonists failed in clinical trials\(^ {47}\). This paradoxical activation of platelet secretion will exert both specific and non-specific effects on platelet function through specific
competitive platelet activation via ADP and non-specific effects exerted by other agents released simultaneously from platelet granules, respectively.

Thus, there appears to be a feedback loop between the integrin ADP-secretion which is perturbed by pre-treatment of platelets with ligand mimetic αIIbβ3 inhibitors. Understanding this pathway will be essential for the design of future anti-platelet therapies for the treatment and prevention of thrombosis.

Furthermore, some concluding remarks can be formulated about the results and the model. One general consideration that our results suggest is that the secretion of ADP appears to be more sensitive to inhibition as compared to the fibrinogen binding (Fig. 2). This is not surprising considering that, unlike adhesion, ADP secretion is an amplification response hence the necessity for a tighter control. Secondly, from our literature survey, especially regarding ADP and TxA2 receptors\textsuperscript{48-50}, it clearly emerged that coincidence detection is a design principle largely employed in this platelet signalling network. Lastly, the diagram in Fig. 4 displays numerous feed-forward loops and a relative paucity of negative regulation mechanisms. This might be an artifact caused by the scarcity of studies about negative regulation due to the focus on discovery of small molecule inhibitors of platelet aggregation. We anticipate that the full wiring diagram will have more negative regulators and that further investigation will aim at determining the weight of the links.

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7. Figure Legends

**Fig. 1. Effect of different doses of CRP on ADP release and fibrinogen binding.** A and B: Platelets were stimulated with increasing doses of CRP for 3 minutes and representative dose-response profiles are shown for ADP release (A) and for Oregon-Green Fibrinogen binding (B) (data represents one donor and error bars refer to the 3 replicates). C and D: Activation caused by CRP (1 µg/ml) is compared to activation caused by TRAP 8 µM and the results shown refer to 4 donors (error bars represent S.E.M). E, A representative image showing increased fibrinogen binding in resting platelets (Blue) or platelets activated for 3 minutes with TRAP (8 µM; Green) or CRP (1µg/ml;Pink) as determined by FACS analysis.

**Fig 2. Effect of Syk, PI3K, COX, TXA₂R, P2Y1 and P2Y12 inhibition upon fibrinogen binding and ADP release in CRP-stimulated human platelets.** Platelets were pre-treated with the indicated concentration of inhibitors. Following stimulation with CRP (1µg/ml), platelets were assayed for fibrinogen binding (■) and for ADP secretion (▲). Results were normalized for each donor relative to the response seen in unstimulated platelets (0%) and the maximal response seen in CRP-stimulated platelets (100%) in the absence of any inhibitors. Data points represent the means ± S.E.M. of six independent experiments.

**Fig.3. Effect of tirofiban on fibrinogen binding and ADP release.** Platelets were pre-treated with the indicated concentration of tirofiban and then stimulated with 1µg/ml CRP (panel A) or 8µM TRAP (panel B). Results were normalized for each donor as explained in
figure legend for Fig. 2. Data points represent the means ± S.E.M. of six (panel A) or four (panel B) independent experiments where * P < 0.001.

**Fig. 4. Platelet signalling.** The diagram schematizes some of the functional interactions within the GPVI signalling network. The star (*) indicates that the protein is in its active state. The red inhibition symbols correspond to the proteins that were inhibited. The circles indicate that the link has a positive effect, either of stimulation or catalysis, on the transition (grey arrow) that it refers too. The black boxes represent a set of functional interactions that are unknown or only partially known and they can be empty in case the interaction is direct. Black lines: interactions inferred from previous literature and this study. Green, red and blue lines: inferred from this study.
Figure 1
Figure 2.
Figure 3
References


