Reciprocal Cross-Talk Between P2Y₁ and P2Y₁₂ Receptors At The Level Of Calcium Signaling In Human Platelets

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Running title: Reciprocal regulation of P2Y₁ and P2Y₁₂ signaling

Financial support: ARH is supported by an A.J. Clark Studentship from the British Pharmacological Society. SJM is a British Heart Foundation Research Fellow. The work was supported by project grants from the Wellcome Trust (grant nos. 064785 & 069572) and the British Heart Foundation (grant no. PG/2000087).

Word count: 4981 (Abstract: 200)

Scientific heading: Hemostasis, Thrombosis and Vascular Biology

Keywords: purine receptor, G-protein-coupled receptors, c-src kinase, ADP, PI 3-kinase
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**ABSTRACT**

ADP, an important platelet agonist, acts through two G-protein-coupled receptors (GPCRs) P2Y₁ and P2Y₁₂, which signal through Gq and Gi respectively. There is increasing evidence for cross-talk between signalling pathways downstream of GPCRs and here we demonstrate cross-talk between these two ADP receptors in human platelets. We show that P2Y₁₂ contributes to platelet signalling by potentiating the P2Y₁-induced calcium response. This potentiation is mediated by two mechanisms; inhibition of adenylate cyclase and activation of PI 3-kinase. Furthermore, the Src family kinase inhibitor PP1 selectively potentiates the contribution to the calcium response by P2Y₁₂, although inhibition of adenylate cyclase by P2Y₁₂ is unaffected. Using PP1 in combination with the inhibitor of PI 3-kinase LY294002, we show that Src negatively regulates the PI 3-kinase-mediated component of the P2Y₁₂ calcium response. Finally we were able to show that Src kinase is activated through P2Y₁ but not P2Y₁₂. Taken together we present evidence for a complex signalling interplay between P2Y₁ and P2Y₁₂, where P2Y₁₂ is able to positively regulate P2Y₁ action and P2Y₁ negatively regulates this action of P2Y₁₂. It is likely that this interplay between receptors plays an important role in maintaining the delicate balance between platelet activation and inhibition during normal haemostasis.
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INTRODUCTION

Platelets form an integral part of the physiological response to vascular injury, where together with the products of the clotting cascade, they form a haemostatic plug that serves to stem the flow of blood from the injured vessel. Under normal conditions there is a fine balance between platelet activation and inhibition and any abnormal perturbation of this balance can result in serious disease states such as unstable angina, stroke and heart attack, or bleeding disorders. ADP is an important platelet activator and has been shown to be critical for a full platelet response at sites of vascular injury 1-5. ADP activates platelets through two purinergic G-protein coupled receptors (GPCRs): P2Y₁ and P2Y₁₂. P2Y₁ couples to Gαq and subsequent activation of phospholipase C and increase in cytosolic calcium 2,6-8. P2Y₁ has also been shown to couple to activation of p160 Rho kinase, and this, together with the increase in cytosolic calcium, is responsible for platelet shape change in response to ADP 7,9-14. It is accepted, on the basis of P2Y₁ knockout studies and pharmacological blockade, that this receptor is absolutely required for both the ADP-induced rise in intracellular calcium and shape change responses, as selective inhibition of P2Y₁ results in abolition of these responses 2,7,8.

The molecular identity of P2Y₁₂ remained elusive until recently 5 and was originally termed P2YAC, P2T or P2CYC. Pharmacological studies demonstrated that P2Y₁ alone was unable to account for all of the effects of ADP on platelet activation 2,9,15 and these additional activities were attributed to the P2Y₁₂ receptor, later shown to be coupled to inhibition of adenylate cyclase through Gαi 5,9. P2Y₁₂ was shown to play an important role in the reversible aggregation response to ADP, as well as potentiating aggregation responses to other agonists 16-19. More recently it has been questioned whether P2Y₁₂-mediated inhibition of adenylate cyclase can account for all of the observed events downstream of P2Y₁₂ 20-22, and it has been
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subsequently demonstrated that P2Y\textsubscript{12} can also couple to activation of PI3-kinase (PI3K)\textsuperscript{19,23-25}. This has been shown to be important in mediating the pro-aggregatory properties of P2Y\textsubscript{12}, although the precise functional roles of PI3K remain to be elucidated\textsuperscript{23,24}. One possible role may involve modulation of the P2Y\textsubscript{1}-dependent calcium response by P2Y\textsubscript{12}. Although pharmacological blockade or genetic removal of P2Y\textsubscript{1} can ablate the calcium response to ADP, there is conflicting evidence regarding the role of P2Y\textsubscript{12} in ADP-mediated calcium responses. Sage et al. (2000)\textsuperscript{26} and Fox et al. (2004)\textsuperscript{27} have shown that P2Y\textsubscript{12} may be involved in the contribution to a cytosolic calcium rise by ADP, whereas Daniel et al. (1998)\textsuperscript{22} provide evidence that P2Y\textsubscript{12} is not involved in this process.

Two emerging themes in G protein-coupled receptor (GPCR) signalling and regulation are (i) that GPCRs from different classes may regulate each other’s activities through cross-talk mechanisms and (ii) that GPCRs can couple to signalling pathways distinct from the activation of G-proteins, specifically those involving tyrosine kinases. Here we address the question of interplay between these important ADP receptors, P2Y\textsubscript{1} and P2Y\textsubscript{12}, in human platelets using a variety of pharmacological approaches. We were able to show directly that P2Y\textsubscript{12} modulates the P2Y\textsubscript{1}-mediated calcium response through two of its signalling pathways; activation of PI3 kinase and inhibition of adenylate cyclase. In turn, we show that P2Y\textsubscript{1} feeds back negatively upon the PI3 kinase component of this response, and that this feedback is mediated through a novel activation of Src kinase. We hypothesise that this reciprocal receptor regulation may provide a means by which the delicate balance between resting and activated platelet states is maintained.
METHODS

Materials
Monoclonal anti-Src antibody was from Upstate Biotechnology Ltd (Milton Keynes, UK). Polyclonal rabbit anti-Src antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-specific antibodies anti-Src (Tyr416) and anti-PKB (Thr308) were from Cell Signaling Technologies (New England Biolabs, UK). Src family kinase inhibitor PP1 was from Alexis Corp (Nottingham, UK). The broad-spectrum phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), inactive PP1 analogue PP3 and Raytide peptide were from Calbiochem (La Jolla, CA, USA). Fura 2-AM, A3P5P, apyrase, forskolin, SQ22536 and BAPTA-AM were from Sigma (Poole, Dorset, U.K.). The P2Y12 antagonist AR-C69931MX was a generous gift from AstraZeneca. 2-methylthio-AMP (2MeSAMP) was a kind gift from Dr Pamela Conley (Portola Pharmaceuticals Inc., CA, USA). γ-[32P]-ATP was from Amersham (Amersham, UK).

Preparation and stimulation of human platelets
Human blood was drawn from healthy, drug-free volunteers on the day of the experiment, under ethical approval from the Local Research Ethics Committee, United Bristol Healthcare Trust. Acid citrate dextrose (ACD: 120 mM sodium citrate, 110 mM glucose, 80 mM citric acid, used at 1:7 vol/vol) was used as anticoagulant. Platelet rich plasma (PRP) was prepared by centrifugation at 200g, for 17 min and platelets were then isolated by centrifugation for 10 min at 400g, in the presence of 0.02 u/ml apyrase and prostaglandin E1 (PGE1; 140 nM) for all assays other than measurement of intracellular cyclic AMP (cAMP), or where otherwise indicated, where PGE1 was omitted. The pellet was resuspended to a density of 4x10^8 platelets/ml in a modified Tyrodes-HEPES buffer (145 mM NaCl, 2.9 mM KCl, 10 mM
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HEPES, 1 mM MgCl2, 5 mM glucose, pH 7.3). To this platelet suspension, 10 µM indomethacin was added as well as 0.02 units/ml apyrase to remove any leaked ADP, and a 30 min resting period was allowed before stimulation. Stimulation of platelets was performed either in a fluorimeter for calcium measurements or in an aggregometer at 37°C, with continuous stirring at 800 rpm. For measurements of cAMP and Src activity, platelet responses were assessed in the presence of EGTA (1 mM) to inhibit aggregation responses.

Generation of 1321N1 astrocytoma cells stably expressing P2Y1

1321N1 human astrocytoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units ml⁻¹ penicillin G, and 100 µg ml⁻¹ streptomycin sulfate at 37 °C in a humidified atmosphere of 95% air, 5% CO₂. pCMVneo containing P2Y1 was linearized before being mixed with Lipofectamine 2000 and incubated with plated cells according to the manufacturer's instructions. Briefly, 20 µg of DNA was incubated with 20 units of Xho and Xba. Linearized DNA was mixed with DMEM and Lipofectamine before addition to cells in monolayer and left overnight. After 24 hrs, medium was replaced with normal complete medium; after an additional day, the medium was supplemented with 400 µg/ml geneticin. Surviving colonies were expanded and cell lines expressing HA-tagged P2Y1 receptor isolated by immunofluorescent microscopy as previously described (28, data not shown). Receptor expression was assessed using a primary anti-HA-monoclonal antibody (HA-11) and a goat anti-mouse fluorescein-conjugated secondary antibody.

Measurement of cytosolic calcium

Measurement of cytosolic calcium was performed as previously described 29. Briefly, 3 µM Fura-2-AM was added to platelet rich plasma, and incubated at 30 °C for 45 min in the
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presence of 10 µM indomethacin. Platelets were washed and re-suspended in modified Tyrodes and stimulated at room temperature in the absence of EGTA. For experiments with 1321N1-P2Y\textsubscript{1} cells, cells were grown on poly-L-lysine coated plates to ~ 60% confluence. Cells were washed with Locke’s solution (154 mM NaCl, 5.6 mM KCl, 1.2mM MgCl\textsubscript{2}, 2.2 mM CaCl\textsubscript{2}, 5 mM HEPES, 10 mM glucose, pH 7.4) and loaded with Fura-2-AM (3 µM) at 37 °C for 60 mins. Cells were washed, removed by trypsinisation and resuspended in PBS in the presence of 0.02u/ml apyrase. Cells were stimulated in suspension at room temperature. Fluorescence excitation was made at 340 nm and 380 nm and emission at 510 nm was measured using a Hitachi F-4500 spectrofluorimeter. Data are presented as the excitation fluorescence ratio (340:380 nm).

Electrophoresis of proteins and western blotting

Following stimulation, platelets were lysed directly into an equal volume of 2X Laemmli sample solvent and boiled for 5 min. Proteins were resolved by electrophoresis in 10% gradient SDS-PAGE gels. Samples were then transferred to PVDF membranes, using a Bio-Rad Trans-Blot SD semi-dry transfer cell, blocked with 10% bovine serum albumin and incubated for 1 hour at room temperature with appropriate primary antibody (1 µg/ml). Membranes were then washed before incubation with appropriate secondary antibody followed by thorough washing. Bound peroxidase activity was detected using enhanced chemiluminescence (ECL, Pharmacia-Amersham, UK).

Src in vitro kinase assay

Src activity was assayed on kinase immunoprecipitated from basal or activated platelets. Reactions were stopped, after 3 min stimulation with agonist, by lysis of platelets with an equal volume of 2X NP-40 extraction buffer (2% Nonidet P40, 300 mM NaCl, 20 mM Tris, 1
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mM phenylmethylsulphonyl fluoride, 10 mM EDTA, 2 mM Na₃VO₄, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 µg/ml pepstatin, pH 7.3). Lysates were incubated with protein-A sepharose and 3 µg polyclonal rabbit anti-Src antibody overnight at 4°C. Beads were then washed and resuspended in 10 µl of kinase assay (KA) buffer (5 mM MgCl₂, 5 mM MnCl₂, 100 mM NaCl, 10 µM ATP, 2 mM Na₃VO₄, 20 mM HEPES pH 7.2) and 10 µg Raytide peptide added to each sample. The reaction was started by addition of 10 µl ATP buffer (0.15mM ATP, 30mM MgCl₂ and 200µCi/ml γ-[³²P]-ATP in KA buffer). After incubation at 30°C for 30 min the reaction was terminated by addition of 10% phosphoric acid. Samples were applied to 3x3 cm squares of P81 ion exchange chromatography paper, extensively washed in 0.5% phosphoric acid followed by a wash in acetone. Papers were then dried and labeled Raytide quantified by liquid scintillation counting.

Whole cell cyclic AMP accumulation

Platelets suspensions at 37°C, prepared as described above, were pre-treated for 5 min with a mixture of the phosphodiesterase inhibitor IBMX (1 mM) ± forskolin (1 µM) in the absence or presence of ADP (10 µM). Cyclic AMP accumulation was terminated by addition of ice cold 100 % trichloroacetic acid (TCA) and samples were left to lyse on ice for 1-2 hrs. The resulting samples were spun at 4000g for 5 min and the cAMP-containing supernatant neutralized with 1 M NaOH and TE buffer (50mM Tris-HCl, 4 mM EDTA, pH 7.4). Cyclic AMP levels were subsequently determined in each sample using a binding assay as previously described.³⁰
RESULTS

P2Y_{12} contributes to the ADP-induced calcium response

It is well established that the P2Y_{1} receptor expressed on human platelets couples to Go_{q} and is required for the calcium response to ADP \(^{2,7}\). It is also becoming established that there is a significant degree of cross-talk and interplay between G protein-coupled receptors and their signalling pathways, and we were therefore interested to investigate the possibility that P2Y_{12} may play a role in contributing to the ADP-induced calcium response. Fig. 1(i) shows a representative calcium response to ADP (10 \(\mu\)M), and confirms the dependency of this response upon activation of P2Y_{1} as the selective antagonist of this receptor, A3P5P, completely abrogates the response. Interestingly however, the selective P2Y_{12} antagonist AR-C69931MX is also able to partially block the response. This effect of AR-C69931MX could be replicated with another selective antagonist of P2Y_{12}, 2-methylthio-AMP (2MeSAMP) (Fig. 1(ii)). In order to demonstrate that these two antagonists had no effect upon P2Y_{1} receptors, 1321N1 astrocytoma cells, which express no endogenous P2 receptors \(^{31-34}\), were stably transfected with P2Y_{1}. Calcium responses in these cells were monitored in response to ADP, and there was no change in the peak response in the presence of either P2Y_{12} antagonist (Fig. 1(iii)).

In order to determine the maximal extent of inhibition of the calcium response by the P2Y_{12} blocker AR-C69931MX, a concentration-inhibition curve was determined. Increasing concentrations of the antagonist inhibited the ADP-induced calcium rise, which was maximally inhibited by 1 \(\mu\)M, although suppression of the calcium response was not complete, being reduced to between 40 and 50% of the maximal value (Fig. 2(i)). This demonstrated that P2Y_{12} partially contributed to the calcium response elicited by ADP. This contribution was also demonstrated to be a potentiation of the maximal response to ADP,
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rather than a synergistic parallel shift in the dose-response curve, since Fig. 2(ii) shows a drop in the maximal effect of ADP in the presence of AR-C69931MX (1 µM), with no significant change in EC\textsubscript{50} to ADP (2.5±1.0x 10\textsuperscript{-7} in the absence of AR-C69931MX compared to 6.1±2.0 x 10\textsuperscript{-7} in the presence of AR-C69931MX).

*Sub-maximally effective A3P5P reveals a calcium response dependent upon P2Y\textsubscript{12}*

Figure 3 shows a concentration-response relationship for the P2Y\textsubscript{1}-specific antagonist A3P5P. At a sub-maximal concentration of A3P5P (200 µM), where the calcium response to ADP was reduced to approximately 25% of the control response, addition of AR-C69931MX (1 µM) was able to abolish completely the calcium response. Thus, at 200 µM A3P5P, the calcium response is dependent upon potentiation by P2Y\textsubscript{12} and this sub-maximal concentration of A3P5P therefore provides a convenient means of studying the P2Y\textsubscript{12}-mediated contribution to the calcium response to ADP.

*P2Y\textsubscript{12} contributes to the calcium response to ADP through 2 mechanisms: activation of PI3-kinase and reduction in cellular cAMP*

Previous reports have shown that P2Y\textsubscript{12} couples to PI3-kinase (PI3K) and that this is important for ADP-dependent platelet aggregation\textsuperscript{23,24}. We have shown that both ADP and thrombin are able to induce phosphorylation of the kinase PKB on T308 using a phosphopeptide specific antibody and that the PI3K antagonist LY294002 was able to completely abolish phosphorylation of PKB induced by ADP (Fig. 4(i)). Threonine 308 is phosphorylated by the PI3K-dependent enzyme PDK1, demonstrating that both agonists were able to induce PI3K activation in agreement with previous reports\textsuperscript{23,24}. The mechanism by which the P2Y\textsubscript{12}-mediated activation of PI3K contributes to other ADP-mediated responses has not been clearly elucidated. Using the sub-maximal A3P5P approach described above,
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we studied the P2Y<sub>12</sub>-dependent calcium response and noted that it was partially inhibited by LY294002, a PI3K-specific antagonist (Fig. 4(ii) & (iii)). The calcium rise induced by ADP when LY294002 was added in conjunction with maximal AR-C69931MX however was no different than with AR-C69931MX alone (Fig. 4(iii)). These findings indicate that PI3K plays no role in mediating the P2Y<sub>1</sub>-induced calcium response, but provides a significant component of the P2Y<sub>12</sub>-mediated contribution to the ADP-induced calcium rise.

We were interested to investigate the nature of the remaining component of the P2Y<sub>12</sub>-dependent calcium response, as the response was only partially attenuated by inhibition of PI3K. In the presence of a maximal concentration of AR-C69931MX, where activation of P2Y<sub>12</sub> is fully blocked, ADP is not able to induce a reduction in cAMP. Fig. 4(iv) shows that in the presence of this inhibitor, the calcium response to ADP is inhibited by approximately 50% and that whilst the adenylate cyclase inhibitor SQ22536 added in addition to AR-C69931MX is able to partially restore the calcium response, it is unable to completely recover the response from inhibition of P2Y<sub>12</sub>. This demonstrates that the P2Y<sub>12</sub> dependent calcium response involves inhibition of cAMP as well as an additional mechanism, which we show is mediated by PI3K.

*P2Y<sub>12</sub> contribution to the calcium response is poteniated by the Src kinase inhibitor PP1*

G protein-coupled receptors have been shown to couple to activation of tyrosine kinases<sup>35-37</sup>, and indeed the ADP-induced aggregation response has been shown to partially depend upon Src kinase activity<sup>38</sup>. It was therefore important to address whether Src kinases played a role in the ADP-induced calcium response. Figure 5(i) shows that inhibition of Src by the selective inhibitor PP1 has no effect upon the calcium to response to ADP alone. In the presence of sub-maximal A3P5P however, where the response depends upon activation of P2Y<sub>12</sub>, the response is potentiated by PP1, but not the inactive analogue PP3 (Figs 5(i) & (ii)).
Figure 5(ii) shows that PP1 has no effect upon a P2Y₁-mediated calcium signal (i.e. in the presence of a maximal concentration of AR-C69931MX), and the effect is dependent upon PI3K activity as it is not observable in the presence of LY294002. These data suggest that Src kinases specifically inhibit the PI3K-mediated P2Y₁₂-dependent signalling events, but not P2Y₁-mediated signalling.

Src kinase does not regulate P2Y₁₂-mediated inhibition of adenylate cyclase

In order to further clarify the inhibition of P2Y₁₂ by Src Kinase we decided to study a P2Y₁₂-specific signalling event – inhibition of adenylate cyclase. Figure 6 shows that as expected, the P2Y₁-specific antagonist A3P5P had no effect on ADP-mediated inhibition of a forskolin-stimulated rise in cAMP, confirming the lack of role for P2Y₁ in this event. We further showed the absence of a role for Src in this signalling pathway using PP1 and PP3, neither having any effect either alone or in conjunction with each of the receptor antagonists.

P2Y₁ but not P2Y₁₂ couples to activation of Src kinase

Although we showed that inhibition of Src kinases resulted in the selective potentiation of the PI3K component of the P2Y₁₂-dependent contribution to the calcium response, it was important to demonstrate that Src is directly activated by ADP and to determine the receptor to which it is coupled. We chose to assess activation of Src in two ways: (i) identification of phosphorylation of Y416, which correlates with activity and (ii) direct assay of Src activity in vitro. Figure 7(i) shows that Src is activated directly by ADP, as phosphorylation of Y416 is unaffected by pre-treatment of platelets with either EGTA (1 mM) to block αΙΙbβ₃ or indomethacin (10 μM) to block thromboxane A₂ generation, or both in combination where dense granule secretion is prevented. Collagen is used as a positive control in this assay, and shows that the level of ADP-induced activation is equivalent to that induced by collagen.
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By *in vitro* kinase assay we were able to show that activation of Src lies selectively downstream of P2Y<sub>1</sub>, as it is prevented by pretreatment of platelets with the P2Y<sub>1</sub>-selective antagonist A3P5P but not by the P2Y<sub>12</sub> antagonist, AR-C69931MX (Fig. 7(ii)). Although the activity of Src stimulated by ADP in the presence of both P2Y<sub>1</sub> and P2Y<sub>12</sub> blockade (51.4 ± 5%) is slightly above basal (43.4 ± 5%), the difference is non-significant (Students t-test, \( p>0.05 \)).
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DISCUSSION

Here we present evidence for cross-talk between the two platelet ADP receptors where P2Y₁₂ receptor activation positively modulates the P2Y₁-dependent calcium response, whilst P2Y₁ negatively modulates P2Y₁₂ through Src kinase activation. We show that modulation of P2Y₁ by P2Y₁₂ is mediated by both PI3K and inhibition of adenylate cyclase. In turn a negative feedback pathway from P2Y₁, mediated by Src tyrosine kinase, inhibits the PI3K-dependent signalling component. These findings are summarised in Fig. 7(iii). Calcium signalling therefore represents a point of cross-talk between these two ADP receptors, and a means of subtly modulating the response of platelets to this important agonist.

Although it has been shown that P2Y₁₂ is able to potentiate the platelet aggregation response to activation of a variety of platelet receptors, including the Gαq-coupled P2Y₁ and PAR-1, and the collagen receptor GP VI, the signalling mechanisms underlying this potentiation are not clearly elucidated and it is also not clear whether P2Y₁₂ is able to potentiate platelet responses such as cytosolic calcium rise. In Fig. 1 we have shown that whereas the calcium response to ADP is absolutely dependent upon activation of P2Y₁, there is also a component that depends upon P2Y₁₂. This effect is achieved by a reduction in the maximum calcium response (see Fig. 2(ii)), suggesting that P2Y₁₂ is able to potentiate the P2Y₁-mediated calcium response. We developed an experimental protocol in Fig. 3 that allowed us to examine the component of the ADP-induced calcium response that was dependent upon P2Y₁₂. The hypothesis underlying this protocol was that as the P2Y₁-dependent calcium response is potentiated by P2Y₁₂ receptor activation, under conditions where P2Y₁ receptors were substantially but not completely inhibited by using a submaximal concentration of A3P5P, the calcium response observed may be completely dependent upon contribution by P2Y₁₂ and that this would provide a useful means to study a calcium response for which
P2Y\textsubscript{12} was predominantly responsible. This is indeed the case, and at a concentration of 200 \(\mu\text{M}\) A3P5P, the remaining calcium response to ADP was found to be fully inhibited by the P2Y\textsubscript{12} antagonist AR-C69931MX. This protocol therefore provided a useful approach to dissecting the signalling mechanisms involved in mediating the P2Y\textsubscript{12}-dependent component of the calcium response.

There is conflicting evidence in the literature regarding the possible role of P2Y\textsubscript{12} in mediating a calcium rise. Sage \textit{et al}. (2000) and recently Fox \textit{et al}. (2004) have shown that P2Y\textsubscript{12} may contribute to a cytosolic calcium rise to ADP \textsuperscript{26,27}, whereas it has been accepted largely on the basis of work by Daniel \textit{et al}. (1998) that P2Y\textsubscript{12} is not involved in this response \textsuperscript{22}. The discrepancy between these results is most likely due to the different platelet preparation conditions used. Daniel \textit{et al}. (1998) \textsuperscript{22} prepared platelets without the use of either PGE\textsubscript{1} or prostacyclin, whereas in contrast, both Sage \textit{et al}. (2000) \textsuperscript{26} and Fox \textit{et al}. (2004) \textsuperscript{27} deliberately used PGE\textsubscript{1} in order to assess the role of cAMP in the inhibition of the calcium response, and were therefore able to reveal a G\textsubscript{i}-mediated potentiation of the calcium response by P2Y\textsubscript{12}. We too have prepared platelets in the presence of PGE\textsubscript{1} and it was therefore likely that blockade of the calcium response by AR-C69931MX may be partially due to inhibition of the P2Y\textsubscript{12}-mediated G\textsubscript{i} response on adenylate cyclase. We sought to confirm this using the adenylate cyclase inhibitor SQ22536. In Fig. 4 we show that SQ22536 could partially rescue inhibition of the calcium rise by AR-C69931MX, and conclude that part of the P2Y\textsubscript{12}-dependent calcium response is therefore mediated by modulation of platelet cAMP levels. The inhibition of adenylate cyclase is physiologically relevant because platelets are normally continuously exposed to endothelially-derived prostacyclin, and therefore \textit{in vivo} calcium levels are likely to be modulated by P2Y\textsubscript{12} inhibition of adenylate cyclase. There is substantial evidence demonstrating an inhibitory role for cAMP, through
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activation of PKA, in the control of IP3-mediated calcium release from stores, and also for activation of plasma membrane calcium ATPases extruding calcium from the cytosol⁴²-⁴⁵.

However, the rescue of the response by SQ22536 was not complete, suggesting that P2Y₁₂ may use an additional signalling mechanism to mediate its calcium response. It is clear that P2Y₁₂ may couple to activation of PI3K and we therefore hypothesised that this pathway may contribute also to the calcium response seen. In the presence of LY294002, the selective PI3K inhibitor, the calcium response to ADP was indeed reduced, suggesting a contribution to the calcium signal through PI3K. Several possible mechanisms may operate, including activation of PLCγ isoforms and Btk, which are known to be PIP₃-dependent mechanisms leading to a rise in cell calcium⁴⁶-⁴⁸. The response proved to be mediated by P2Y₁₂ and not P2Y₁, because in the presence of full P2Y₁₂ blockade, where only P2Y₁ would be acting to induce a calcium rise, the response was no longer attenuated by LY294002. In contrast, using the sub-maximal A3P5P protocol, it is clear that the P2Y₁₂-dependent component is partially suppressed by LY294002. This is consistent with data from PI3-kinase-γ knock out mice where it has been shown that P2Y₁₂ but not P2Y₁ couples to activation of this lipid kinase ²³, contributing to the platelet aggregation response. These data indicate that there are two signalling pathways that mediate the P2Y₁₂-dependent calcium response; PI3K and inhibition of cAMP levels through Gᵢ. Interestingly SQ22536 was able to recover the P2Y₁₂-inhibited calcium response to a level comparable to the response when PI3K was inhibited, suggesting that these two pathways may be the only signalling mechanisms required by P2Y₁₂ to mediate its calcium response.

Having demonstrated a mechanism by which P2Y₁₂ signalling may modulate responses to P2Y₁, we were interested to investigate whether any reciprocal cross-talk may occur whereby
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P2Y1 signalling feeds back to influence P2Y12-mediated events. There is increasing evidence that GPCRs can initiate signalling events involving non receptor tyrosine kinases such as Src kinase. For example, Luttrell et al. demonstrated that the stimulation of β2-adrenoceptors leads to the activation of Src through the binding of arrestins, and that this activates the MAP kinase cascade. Recently Jin et al. (2002) have shown that the ADP-induced aggregation response relies partially upon activation of Src kinases. Platelets express multiple members of the Src tyrosine kinase family, including Src itself, which is heavily expressed and is critically involved in signalling downstream of adhesion receptor activation. In Fig. 5 we were able to demonstrate that inhibition of Src by PP1 causes potentiation of the P2Y12-dependent calcium response component, although there was no effect upon the calcium response mediated by P2Y1 alone. PP1 did not alter the response to ADP alone either, but potentiated the calcium response only under conditions of sub-maximal inhibition of P2Y1 by A3P5P. The reason for this is likely to be because when pathways are maximally activated in the absence of receptor antagonists, there is no room for potentiation of the calcium response by PP1. When the calcium response has been diminished partially by application of a sub-maximal concentration of A3P5P however, a potentiation of the response by the Src inhibitor PP1 is revealed. The response is likely to occur under physiological conditions because the potency of ADP for P2Y1 and P2Y12 is not greatly different, being 1-2 µM, and therefore ADP will normally activate P2Y1 and P2Y12 simultaneously. This indicated that Src kinases may negatively regulate P2Y12 signalling, and it was therefore important to address two questions: (i) which component of P2Y12 signalling was negatively regulated by Src? and (ii) which receptor (P2Y1 or P2Y12) couples to activation of Src? We addressed the first question in two ways. In the first instance, Fig. 6 demonstrates that Src does not regulate the inhibition of adenylate cyclase mediated by P2Y12, as PP1 has no effect upon this response. On the other hand, in Fig. 5(ii) we hypothesised that if the PI3K
component of the P2Y12 calcium response was potentiated by PP1, then this potentiation should be abolished in the presence of the PI3K inhibitor LY294002. This was indeed the case, where potentiation of the signal induced PP1 was ablated in the presence of LY294002, indicating that Src kinases may negatively regulate the PI3K component of the signal from P2Y12. We had shown in Fig. 4 (i) that ADP induces a weak phosphorylation of PKB on T308, confirming activation of PI3 kinase. We were not however able to demonstrate reliably whether PP1 could potentiate this response, due to the weak nature of the response to ADP. We cannot therefore conclude whether Src may directly affect PI3 kinase activity. The precise nature of the step regulated by Src kinases is therefore not clear, but taking the evidence from Figs 5 and 6 together, it is likely not to directly regulate the P2Y12 receptor, but may target a step on the pathway from G\betaγ, through PI3K to modulation of calcium release or entry. The nature of this modulation step is currently the focus of ongoing investigation in our laboratory.

The second of these questions was addressed by two approaches to assay Src activation state: (i) western blot analysis of phosphorylation of Y416, which lies in the activation loop of Src, and (ii) a direct in vitro approach to assay activity. Using the first approach we demonstrated that ADP was able to directly activate Src, with no requirement for αIIbβ3 activation or thromboxane A2, or secreted mediators. It was important however to use a more direct and quantifiable assay of Src activity to assess the role of P2Y1 or P2Y12 in activating Src. For these reasons we chose to assay Src activity directly in vitro using standard approaches, as we have used for other platelet tyrosine kinases29,52. Fig. 7(ii) shows that the P2Y12 receptor antagonist AR-C69931MX has no effect upon Src activation by ADP, whereas P2Y1 blockade by A3P5P reduces Src activity substantially. We conclude therefore that ADP activates Src kinase downstream of P2Y1 but not P2Y12. This suggested that this negative signalling role of Src regulating the PI3K component of the P2Y12 calcium response represents a point of
reciprocal cross-talk between P2Y\textsubscript{1} and P2Y\textsubscript{12} receptors. There are a number of ways in which P2Y\textsubscript{1} may activate Src although these are not investigated here. It has been shown that calcium-dependent tyrosine kinase (CADTK or Pyk2) can activate Src downstream of an increase in cytosolic calcium\textsuperscript{53,54} and Src has been shown to be linked to GPCR activation through binding to arrestins\textsuperscript{35}.

In conclusion, Fig. 7(iii) summarises the findings that P2Y\textsubscript{12} may regulate the P2Y\textsubscript{1}-activated calcium response to ADP through activation of PI3K and inhibition of adenylate cyclase. A reciprocal feedback pathway also operates whereby P2Y\textsubscript{1} inhibits P2Y\textsubscript{12} signalling through Src kinase. This negative signalling specifically affects the PI3K arm of the pathway from P2Y\textsubscript{12} to its calcium response. Details of these mechanisms are currently under investigation as it will be important to ascertain how P2Y\textsubscript{1} couples to Src activation and how this may negatively regulate the P2Y\textsubscript{12} signal.
ACKNOWLEDGEMENTS

The authors would like to thank AstraZeneca for the generous supply of AR-C69931MX. We are grateful to Dr Pamela Conley, Portola Pharmaceuticals Inc., for generously supplying P2Y\textsubscript{1} cDNA and 2MeSAMP.
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FIGURES

Figure 1. P2Y₁₂ potentiates the P2Y₁-dependent calcium response.

Fura 2-loaded platelets (i & ii) or 1321N1 cells (iii) were pre-incubated for 5 min with either AR-C69931MX (1 µM), 2MeSAMP (10 µM), A3P5P (1 mM) or vehicle as control as indicated. Cells were then stimulated with ADP (10 µM) and 340:380 fluorescence ratio followed as shown (i & ii). Traces shown are representative of at least three separate experiments. For (iii), mean peak responses are represented relative to the control response to 10 µM ADP in the absence of P2Y₁₂ receptor antagonists. Data shown are mean ± S.E.M. (n=3).
Figure 2. Concentration-dependent inhibition of calcium response by blockade of P2Y<sub>12</sub>

Fura 2-loaded platelets were incubated (i) in the presence of various concentrations of AR-C69931MX for 5 min or (ii) with or without 1 μM AR-C69931MX for 5 min. Platelets were then stimulated with ADP (10 μM) (for (i)) or various concentrations of ADP (for (ii)), as shown. The peak rise in cytosolic calcium concentration was measured and plotted against log concentration of antagonist (for (i)) or agonist (for (ii)). Data shown are mean ± S.E.M. (n=3).
Figure 3. Addition of a sub-maximal concentration (200 μM) of A3P5P reveals a calcium response dependent upon P2Y₁₂ activation

Fura 2-loaded platelets were pre-incubated with various concentrations of A3P5P with or without addition of AR-C69931MX (1 μM). Platelets were then stimulated with ADP (10 μM) and the peak rise in cytosolic calcium concentration was measured and plotted against log concentration of A3P5P as shown. Data shown are mean +/- S.E.M. (n=3).
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Figure 4. P2Y12 component of the calcium response is mediated through PI3 kinase and inhibition of cAMP

For (i), platelets were pre-incubated with LY294002 (10 µM) or vehicle as control for 25 min and stimulated with ADP (10 µM) or thrombin (0.1 units/ml) for 3 min as indicated. Reactions were stopped by addition of an equal volume of Laemmli sample solvent, and proteins separated by SDS-PAGE and blotted using the phospho-peptide specific anti-pT308
PKB antibody. For (ii) and (iii) Fura 2-loaded platelets were incubated for 25 min with or without LY294002 (10 μM), followed by incubation for 5 min with a submaximal concentration of A3P5P (200 μM; i & ii) or AR-C69931MX (1 μM; ii) as indicated. Platelets were then stimulated with ADP (10 μM) and 340/380 nm fluorescence ratios plotted (ii) or peak rise in cytosolic calcium calculated represented as a bar graph (iii). For (ii) data are representative of 3 separate experiments and for (iii) data shown are mean +/- S.E.M. (n=3). For (iv), platelets were pre-treated for 5 minutes with AR-C69931MX and various concentrations of the adenylate cyclase inhibitor SQ22536 for 25 minutes. The peak rise in cytosolic calcium in response to ADP (10 μM) is plotted against log concentration of SQ22536. Data shown are mean ± S.E.M. (n=3).
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Figure 5. Src kinase inhibitor PP1 potentiates the P2Y₁₂ component of the ADP-induced calcium response.

(i & ii) Washed platelets previously loaded for 45 minutes with Fura 2-AM (3 μM), were incubated for 25 min with either PP1 (20 μM) or PP3 (20 μM), and/or 5 min with AR-C69931MX (1 μM) or A3P5P (200 μM) as indicated. For (i), a time-course of response is shown, representative of 3 separate experiments. For (ii), the peak rise in intracellular calcium elicited by ADP (10 μM) was measured and plotted for each condition as indicated. Data shown are mean +/- S.E.M. (n>3).
Figure 6. Src does not regulate P2Y_{12}-mediated inhibition of cAMP, but negatively feeds back on the PI3K component of the P2Y_{12}-mediated contribution to the calcium response.

(i) Washed platelets were incubated with forskolin (10 µM) and IBMX (10 µM) for 60 min. Platelets were then incubated for 1 min with EGTA (1 mM) and stimulated for 3 min with ADP (10 µM) where indicated. Some platelets were incubated prior to stimulation with either PP1 (20 µM) or PP3 (20 µM) for 25 min, and/or A3P5P (1 mM) or AR-C69931MX (1 µM) for 5 min as indicated. Reactions were stopped by addition of 1/10 volume 100% Trichloroacetic Acid and cAMP assayed as described in Methods. cAMP accumulation is expressed as a percentage of the accumulation for samples treated with forskolin/IBMX alone, and is mean +/- S.E.M. (n=3).
Figure 7. P2Y₁, but not P2Y₁₂, activates Src kinase.

For (i) platelets were prepared with or without indomethacin pre-treatment for 10 min as indicated. Platelets were then pre-incubated for 1 min with or without EGTA (1 mM) and stimulated for a further 3 min with 10 µM ADP. Proteins were separated by SDS-PAGE and blotted with phospho-peptide specific anti-Y416 Src antibody. For (ii) Src was immunoprecipitated from basal platelets or platelets incubated for 1 min with EGTA (1 mM)
and stimulated for a further 3 min with 10 µM ADP. Some platelets had been preincubated for 5 min with A3P5P (1 mM) or AR-C69931MX (1 µM) or both inhibitors as indicated. Immunoprecipitates were incubated with the exogenous tyrosine kinase substrate, Raytide, and incorporation of $^{32}$P into Raytide was measured by liquid scintillation counting. Data shown are mean ± S.E.M. (n=3).

(iii) This diagram summarises the data presented. Although P2Y$_1$ is absolutely required for induction of a calcium response, P2Y$_{12}$ is able to contribute to this response through two mechanisms; activation of PI3K and lowering of cellular cAMP levels. P2Y$_{12}$ thus feeds forward on the P2Y$_1$-mediated calcium response. On the other hand, P2Y$_1$ activates Src kinase which negatively regulates the PI3K component of the P2Y$_{12}$-mediated calcium response. P2Y$_1$ thus negatively feeds back on this part of the P2Y$_{12}$-mediated response, although not upon the cAMP component of the P2Y$_{12}$ signalling pathway. This demonstrates an intricate level of feedback cross-talk between these two receptors at the level of calcium signalling in platelets.
Reciprocal Cross-Talk Between P2Y₁ and P2Y₁₂ Receptors At The Level Of Calcium Signaling In Human Platelets

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