Differential requirements for calcium and Src-family kinases in platelet GPIIb/IIIa activation and thromboxane generation downstream of different G protein pathways

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Abstract

$G_{12/13}$ or $G_q$ signaling pathways activate platelet GPIIb/IIIa when combined with $G_i$ signaling. We tested whether combined $G_i$ and $G_z$ pathways also cause GPIIb/IIIa activation, and compared the signaling requirements of these events. Platelet aggregation occurred by combined stimulation of $G_i$ and $G_z$ pathways in human platelets, and in P2Y$_1$-deficient and $G_\alpha_q$-deficient mouse platelets, confirming that the combination of $G_i$ and $G_z$ signaling causes platelet aggregation. When $G_i$ stimulation was combined with $G_z$ stimulation, there was a small mobilization of intracellular calcium. Chelation of intracellular calcium decreased the extent of this platelet aggregation, whereas it abolished the $G_q$ plus $G_i$-mediated platelet aggregation. Co-stimulation of $G_i$ plus $G_z$ pathways also caused thromboxane generation that was dependent on outside-in signaling and was inhibited by PP2, a Src family tyrosine kinase inhibitor. Src family tyrosine kinase inhibitors also inhibited platelet aggregation and decreased the PAC-1 binding caused by co-stimulation of $G_i$ and $G_z$ signaling pathways in aspirin-treated platelets. However, Src-family kinase inhibitors did not affect $G_q$ plus $G_i$-mediated platelet aggregation. We conclude that the combination of $G_i$ plus $G_z$ pathways have different requirements than $G_q$ plus $G_i$ pathways for calcium and Src family kinases in GPIIb/IIIa activation and thromboxane production.
Introduction

Adenosine diphosphate (ADP) is contained in the dense granules of platelets and is released upon platelet activation by agonists such as collagen, thrombin, and thromboxane A$_2$. Released ADP plays a significant role in the potentiation of many platelet responses, including GPIIb/IIIa activation $^1$, dense granule secretion $^2$, and platelet procoagulant activity $^3$-$^5$, though ADP itself also causes platelet aggregation $^6$ and thromboxane A$_2$ generation $^7$. While the potentiating effects of ADP have been realized, the signaling mediators for ADP-induced platelet activation are still being identified.

ADP stimulates platelet aggregation by signaling through the P2Y$_1$ and P2Y$_{12}$ receptors $^1$-$^3$, $^8$-$^10$. Stimulation of the G$_q$-coupled P2Y$_1$ receptor leads to PLC$\beta$ activation and subsequent intracellular calcium mobilization $^{11}$. Increased intracellular calcium mobilization causes platelet shape change through a process of filopodial extension and actin reorganization $^{12,13}$. The P2Y$_{12}$ receptor couples to G$_i$ and leads to the inhibition of adenylyl cyclase $^{14}$-$^{17}$, but also activates PI-3 kinase $^{18}$, Rap1b $^{19,20}$, Akt $^{21}$-$^{24}$, and G protein-coupled inwardly rectifying potassium channels (GIRKS) $^{25}$. It is the signaling downstream of this receptor that is predominantly responsible for the potentiation of many platelet responses $^{26}$-$^{31}$.

Platelets contain members of the G$_q$, G$_i$, G$_s$, and G$_{12/13}$ families of heterotrimeric G proteins $^{10,32}$. Heterotrimeric G proteins contain $\alpha$, $\beta$, and $\gamma$ subunits, and are classified based on the $\alpha$ subunit $^{33}$. We have previously shown that co-stimulation of G$_q$ and G$_i$ signaling pathways, downstream of the P2Y$_1$ and P2Y$_{12}$ receptors, respectively, leads to platelet aggregation $^{34}$. Recently, we and others have shown that combined signaling of G$_{12/13}$ and G$_i$ pathways is also sufficient to cause platelet aggregation $^{35,36}$. 
Mice deficient in Gq, G12, or G13 proteins confirm that signaling through multiple G protein pathways are necessary for normal platelet aggregation. Gq-deficient mice have platelets that undergo shape change but no aggregation in response to thromboxane A2 or thrombin, while ADP causes neither shape change nor aggregation. Consistent with observations that G12/13 signaling is pro-aggregatory when combined with Gi signaling, mice deficient in alpha subunit of G13 have impaired platelet aggregation and granule secretion responses. Mice deficient in the alpha subunit of G2 have both decreased ADP-induced platelet aggregation and decreased inhibition of adenylyl cyclase, suggesting that the P2Y12 receptor primarily couples to G2. Mice that are deficient in the alpha subunit of Gz have an impaired response to epinephrine-mediated effects, suggesting that the α2A adrenergic receptor couples primarily to Gz. Both G12 and Gz belong to the G1 class of G proteins. Because the interactions between the multiple G protein pathways has been shown to elicit different effects than either pathway alone, we characterized the functional effects of the Gi and Gz pathways on platelet activation. We then compared the signaling requirements for Gi and Gz signaling with those of Gq and Gi signaling, two pathways simultaneously activated by ADP.

The platelet contains many serine/threonine and tyrosine kinases that are responsible for transducing intracellular signals upon agonist stimulation. Serine/threonine kinases such as protein kinase C (PKC) and Akt are activated by thrombin, collagen and thromboxane A2, and are important for platelet responses such as dense granule release and GPIIb/IIIa activation. ADP, though not dependent on PKC, does contribute to Akt activation through P2Y12 receptor signaling. Tyrosine kinases such as Syk and members of the Src family of tyrosine kinases are activated by
agonists such as thrombin and collagen, and also play a role in platelet aggregation. Syk-deficient platelets have impaired ability to aggregate in response to ADP and epinephrine, while Src family tyrosine kinase inhibitors decrease ADP-mediated effects as well as PAR1-mediated PAC-1 Ab binding. While these kinases are important to platelet function downstream of many agonists, the implications of their activity have not yet been elucidated.

Here, we demonstrate that combined stimulation of $G_i$ and $G_z$ pathways leads to fibrinogen receptor activation and thromboxane generation in platelets and depends on signaling pathways distinct from those required for $G_q$ and $G_i$ mediated platelet functional responses. PI-3 kinases and Src family kinases are important mediators of $G_i$ and $G_z$-mediated functional responses, whereas PI-3 kinase and calcium are important for $G_q$ and $G_i$-mediated platelet responses.

**Materials and Methods**

**Materials:**

ADP and epinephrine were purchased from Chrono-Log Corp. (Havertown, PA). Fluorescein isothiocyanate-conjugated monoclonal antibody PAC-1 was purchased from BD Pharmingen. Fura-2, AM was purchased from Molecular Probes (Eugene, OR). The acetoxymethyl ester of 5,5-dimethyl-bis-(o-aminophenoxy)ethane-$N’,N’,N’$ tetra-acetic acid (dimethyl BAPTA-AM), LY294002, PP1, PP2, PP3 and Ro 31-8220 were purchased from Biomol (Plymouth Meeting, PA). AR-C 69931MX was a gift from Astra-Zeneca Research Laboratories-Charnwood, (Loughborough, UK). MRS 2179, 2-MeSADP, apyrase grade VII, human fibrinogen, and acetylsalicylic acid were purchased...
from Sigma (St. Louis, MO). The phospho-Src (416) antibody was purchased from Cell Signaling (Beverly, MA).

**Animals** - Gαq-deficient mice were acquired from T. Kent Gartner, University of Memphis, with permission from Stefan Offermanns, University of Heidelberg, Germany. P2Y1-deficient mice were generated via subcontract with Lexicon Genetics Inc. (Woodlands, TX) and were described in our previous publications.

**Platelet preparation:**
Whole blood was drawn from healthy, consenting human volunteers into tubes containing 1/6 volume of ACD (2.5 g of sodium citrate, 1.5 g of citric acid, and 2 g of glucose in 100 mL of deionized water). Blood was centrifuged (Eppendorf 5810R centrifuge, Hamburg, Germany) at 230 rcf for 20 minutes at room temperature to obtain platelet rich plasma (PRP). PRP was incubated with 1 mM acetylsalicylic acid for 30 minutes at 37°C. The PRP was then centrifuged for 10 minutes at 980 rcf (room temperature) to pellet the platelets. Platelets were resuspended in Tyrode’s buffer (138 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 3 mM Na₂HPO₄, 5 mM glucose, 10 mM Hepes pH 7.4, 0.2% bovine serum albumin) containing 0.01 U/mL apyrase. Cells were counted using a Z1 Coulter Particle Counter. For flow cytometry studies, cells were adjusted to a concentration of 1.875 X10⁷ platelets/mL. Platelets were left at room temperature for 30 minutes after resuspension to obtain a quiescent state.
Aggregometry and ATP release:
Aggregation of 0.5 mL of washed platelets was analyzed using a P.I.C.A. lumiaggregometer (Chrono-log Corp., Havertown, PA). Aggregation was measured using light transmission under stirring conditions (900 rpm) at 37°C. Agonists were added simultaneously for platelet stimulation, however platelets were pre-incubated with each inhibitor (where noted) as follows: 10 µM Ro 31-8220 for 3 minutes at 37°C and 25 µM LY294002, 10 µM PP1, 10 µM PP2, 10 µM PP3, and 1 µM dimethyl BAPTA-AM for 10 minutes at 37°C. Each sample was allowed to aggregate for at least 3 minutes. The chart recorder (Kipp and Zonen, Bohemia, NY) was set for 0.2 mm/s. All samples contained exogeneously added human fibrinogen (1 mg/mL). For ATP release, 5 µL of 2 µM luciferin/luciferase was added to the washed platelets 1 minute prior to initiation of secretion. Aggregation and ATP release were measured simultaneously. Aggregation tracings are representative of results obtained from three separate experiments on three different donors.

Intracellular calcium mobilization:
Calcium mobilization was measured in platelets that were loaded with 2 µM fura-2, AM in PRP for 45 minutes at 37°C, and washed platelets were isolated as noted above and brought to a final concentration of 2 X 10^8 platelets/mL in Tyrode’s buffer. Samples of Fura-2 AM-loaded platelets (0.5 mL) were placed in a quartz cuvette with a magnetic stirbar, and incubated for 1 minute at 37°C in a temperature-controlled chamber. An Aminco Bowman Series 2 Luminescence Spectrometer was used for measurement of intracellular calcium mobilization. Two wavelengths (340 and 380 nm) were used for
excitation and the emitted light was measured at 510 nm. Samples were stimulated after 1 minute incubation at 37°C. F_min was obtained by addition of 20 mM Tris and 4 mM EGTA, and F_max was determined by adding 0.25% Triton and saturating levels of CaCl_2. Calculation of the calcium mobilization was performed as outlined previously 45.

**Sample Preparation and Western Blotting**

Platelets were resuspended in Tyrode’s buffer at a concentration of 2 x 10^8 platelets/mL as noted above in “Platelet Preparation.” Platelets were stimulated in the presence of 10µM SC57101A, a GPIIb/IIIa antagonist, to eliminate outside-in signaling. Platelets were stimulated with 10µM ADP + 10µM MRS2179 for selective G_i signaling, 10µM epinephrine for G_z signaling, or a combination of both for G_i and G_z signaling. The reaction was stopped with 3X Sample Buffer, boiled for 10 minutes, and platelet lysates were subjected to sodium dodecyl sulfate - polyacrilamide gel electrophoresis (SDS-PAGE) and Western blotting according to a procedure that has been described previously 23. Membranes were probed with a primary antibody that recognizes Src Tyr416 (1:1000 titer) (Cell Signaling, Beverly, MA, USA) as an indication of Src activity, then probed with an anti-mouse secondary Ab (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland) that is conjugated to alkaline phosphatase. Chemi-luminescence was measured using a Fujifilm LAS-3000 Luminescent Image Analyzer.

**Analysis of PAC-1 binding:**

Activation of GPIIb/IIIa was measured by PAC-1 mAb binding to washed platelets and subsequent analysis by flow cytometry. Aspirin-treated platelets were isolated by
centrifugation as noted, then counted, and brought to a concentration of $1.7 \times 10^7$ platelets/mL. PAC-1-FITC mAb (5 µL) was present in each sample. The samples were stimulated for a period of 10 minutes in the dark, and then diluted with 450 µL of Tyrode’s buffer. Each sample was transferred to a 12 X 75 mm cuvette (Fisher Scientific, Pittsburgh, PA) and analyzed by flow cytometry, using FACSCAN (BD Biosciences, San Jose, CA), to measure an increase in fluorescence that indicates an increase in GPIIb/IIIa receptor activation. The experiment was performed three times and data are presented as mean +/- S.E.

**Mouse platelet preparation:**

C57BL mice were sedated and anesthetized using 0.05 cc ketamine injected intramuscularly into the hind quarter. After induction, blood was drawn from the right ventricle into a 1cc syringe containing 0.1 cc of 3.8% sodium citrate. After drawing the blood, the needle of the syringe was removed (to eliminate shearing of the cells) and the blood was ejected into an Eppendorf tube containing 200 µL of 3.8% sodium citrate. Blood was centrifuged at 100 rcf for 10 minutes to obtain platelet rich plasma. 3.8% sodium citrate (200 µL) was added to the remaining cells to extract more PRP, and spun at 100 rcf for 10 minutes. PGE1 (1 µM) was added to the PRP, and was centrifuged at 500rcf for 10 minutes at room temperature to pellet platelets. Platelets were resuspended in 1 mL of Tyrodes containing 0.01 U/mL apyrase.

**Measurement of Thromboxane A2 generation:**

Washed, aspirin-treated human platelets were prepared as noted above, and brought to a
concentration of $2 \times 10^8$ platelets/mL. Stimulations were performed in a platelet
aggregometer under stirring conditions (900 rpm) at 37°C. All antagonists were added 1
minute prior to the stimulation, and all agonists were added simultaneously for
stimulation. All samples contained exogeneously added human fibrinogen (1mg/ml).
Stimulations occurred for 3.5 minutes and were stopped by snap freezing. The samples
were stored at –80°C until thromboxane analysis was performed. Levels of thromboxane
generation were determined in duplicate using a Correlate-EIA Thromboxane B$_2$ Enzyme
Immunoassay Kit (Assay Designs, Inc, Ann Arbor, Michigan), according to
manufacturer’s instructions. Data represents the average of three days data +/-standard
error.

**Results**

*Co-stimulation of $G_i$ and $G_z$ signaling causes platelet aggregation*: Our laboratory is
interested in the G protein signaling pathways that lead to the activation of GPIIb/IIIa and
platelet aggregation $^{34,35}$. We began by measuring the aggregation of washed human
platelets to determine if a combination of $G_i$ signaling through the P2Y$_{12}$ receptor and $G_z$
signaling through the $\alpha_{2A}$ adrenergic receptor could cause platelet aggregation. We used
a washed aspirin-treated platelet preparation devoid of any plasma proteins to eliminate
the positive feedback from generated thromboxane A$_2$ and thrombin.

The agonist 2-MeSADP, which stimulates both the P2Y$_1$ and P2Y$_{12}$ platelet
receptors, caused the aggregation of washed, aspirin-treated human platelets. Using 2-
MeSADP in the presence of a P2Y\textsubscript{1} receptor antagonist causes signaling selectively through the G\textsubscript{i}-coupled P2Y\textsubscript{12} receptor. The platelet aggregation was blocked with the P2Y\textsubscript{1} receptor antagonist MRS2179, confirming that 2-MeSADP requires signaling through both P2Y\textsubscript{1} and P2Y\textsubscript{12} receptors to cause platelet aggregation\textsuperscript{34}. Epinephrine-mediated signaling through the G\textsubscript{z} pathway also did not cause aggregation, however the combination of G\textsubscript{i} stimulation (2-MeSADP plus MRS2179) with G\textsubscript{z} signaling (epinephrine) caused aggregation in human platelets (Figure 1A). The aggregation was slower than 2-MeSADP-induced aggregation and was irreversible. This platelet aggregation was inhibited by antagonism of either the P2Y\textsubscript{12} receptor with AR-C69931MX or the \( \alpha_{2A} \) adrenergic receptor using yohimbine, confirming that the aggregation is mediated by the combination of these two receptors (Figure 1A). Platelet aggregation caused by P2Y\textsubscript{12}-selective stimulation plus epinephrine also occurred in P2Y\textsubscript{1}-deficient or G\textsubscript{q}-deficient mouse platelets (Figure 1B and Figure 1C); therefore, signaling through the G\textsubscript{i} and G\textsubscript{z} pathways is sufficient to cause aggregation in both human and mouse platelets.

**Intracellular signaling events resulting from combined G\textsubscript{i} and G\textsubscript{z} signaling:** Because our previous work showed that combined G\textsubscript{12/13} and G\textsubscript{i} signaling mobilizes small amounts of intracellular calcium\textsuperscript{35}, we tested the effects of G\textsubscript{i} and G\textsubscript{z} signaling on intracellular calcium mobilization. Washed, aspirin-treated platelets were loaded with 2 µM Fura-2 and stimulated through either G\textsubscript{i} or G\textsubscript{z} signaling alone, then in combination, to measure whether increases in intracellular calcium occurred with the combination of G\textsubscript{i} and G\textsubscript{z} signaling pathways. Interestingly, though G\textsubscript{z} or G\textsubscript{i} signaling alone did not cause any calcium mobilization, the combination of the two signaling pathways produced a small
increase in fluorescence indicating that mobilization of intracellular calcium occurs upon simultaneous stimulation of $G_i$ and $G_z$ pathways (Figure 2).

$G$ protein-mediated activation of GPIIb/IIIa involves many intracellular signaling mediators, including: calcium, protein kinase C, and PI-3 kinase $^{10,32}$. We tested the effects of inhibiting these molecules on the aggregation caused by combined $G_i$ plus $G_z$-mediated or $G_q$ plus $G_i$-mediated signaling in aspirin-treated platelets by using the PKC inhibitor Ro 31-8220, the intracellular calcium chelator dimethyl BAPTA-AM, and the PI-3 kinase inhibitor LY 294002. PKC inhibition, using $10 \mu M$ Ro 31-8220, had no effect on the aggregation caused by either co-stimulation of $G_i$ and $G_z$ signaling pathways, or on $G_q$ and $G_i$-mediated platelet aggregation (Figure 3). Pre-incubation with $1 \mu M$ dimethyl BAPTA-AM slowed and decreased the extent of platelet aggregation for $G_i$ and $G_z$ signaling by $35\%$ (S.D. ± 5.29), and completely inhibited the aggregation caused by $G_q$ and $G_i$ signaling (Figure 3). PI-3 kinase inhibition with $25 \mu M$ LY294002 completely blocked platelet aggregation that was caused by $G_i$ and $G_z$, and also dramatically blocked the aggregation that was caused by $G_q$ and $G_i$ signaling (Figure 3).

The Src family tyrosine kinase inhibitor, PP2, dose-dependently decreased platelet aggregation caused by combined $G_i$ and $G_z$ stimulation (Figure 4A). Platelet aggregation was drastically decreased at the maximal concentration of $10 \mu M$ PP2. To confirm the inhibitory effect of Src family tyrosine kinase inhibitors on platelet aggregation, we also tested PP1, a Src-family tyrosine kinase inhibitor, and PP3, a control compound that does not inhibit tyrosine kinase activity. Whereas PP1 decreased the extent of aggregation caused by combined $G_i$ and $G_z$ signaling, the control compound PP3 had no effect (Figure 4B). On the other hand, PP1, PP2, or PP3 did not have a
significant inhibitory effect on the aggregation caused by Gq plus Gi-induced aggregation (Figure 4B). We next measured if the agonist-induced inside-out signaling causes Src activity. After stimulating the platelets, we subjected the platelet lysates to SDS-PAGE and Western blotting in the presence of 10 µM SC57101A, a GPIIb/IIIa antagonist. We probed the membrane for the presence of the phosphorylation of Src tyrosine 416, which is an indicator of Src activity. Compared with control, both Gi and Gz signaling were able to cause an increase in Src (416) phosphorylation (Figure 4C).

We used the FITC-labelled PAC-1 monoclonal antibody, which recognizes the active conformation of GPIIb/IIIa, to measure if the combination of Gi and Gz signaling pathways is mediating platelet aggregation through activation of GPIIb/IIIa. While there was no increase in the fluorescence of samples stimulated with either Gi or Gz signaling alone, the combination of both agonists produced an increase in fluorescence indicating that the aggregation is mediated by GPIIb/IIIa activation (Figure 5). Pre-treating the aspirin-treated platelets with the Src family tyrosine kinase inhibitor PP2 caused a decrease in the mean fluorescence of platelets that were stimulated with Gi and Gz signaling pathways (Figure 5).

**Effect of co-stimulation of Gi and Gz signaling pathways on thromboxane generation:**

There was a marked difference in the extent of platelet aggregation of non-aspirin-treated platelets versus aspirin-treated platelets when treated with combined Gi and Gz signaling (Figure 6). ADP alone caused platelet aggregation in both aspirin-treated and non-aspirin-treated platelets, though selective Gi or Gz stimulation alone failed to cause platelet aggregation. The aggregation stimulated via Gi and Gz signaling was significantly more robust in non-aspirin-treated human platelets compared to aspirin-
treated platelets (Figure 6). Whereas ADP caused dense granule release in non-aspirin-treated human platelets, either Gi or Gz stimulation alone did not lead to dense granule release. Thromboxane from combined Gi and Gz stimulation resulted in dense granule release in the non-aspirin-treated platelets, but not in the aspirin-treated human platelets (data not shown).

Because the difference in platelet aggregation was dependent on aspirin, we continued by measuring thromboxane generation after combined Gi and Gz signaling. A competitive ELISA assay was used to quantitate the amount of thromboxane A2 generation that occurred after 3.5 minutes of stimulation. Because of the short half-life of generated thromboxane A2, samples were probed for their thromboxane B2 content, a more stable product that reflects thromboxane A2 production. Either Gi or Gz stimulation alone generated similar levels of thromboxane compared with unstimulated human platelets (Figure 7A). Combined Gi and Gz stimulation caused a sizeable increase in thromboxane A2 generation compared with the thromboxane levels generated by 100 nM 2-MeSADP (Figure 7A).

Src-family tyrosine kinase inhibition decreased the aggregation caused by combined Gi and Gz stimulation (Figure 4). We, therefore, tested the effect of PP2 on thromboxane generation from combined stimulation of these pathways. Src family tyrosine kinase inhibition completely inhibited thromboxane production in human platelets from combined Gi and Gz signaling (Figure 7B). To confirm that there was no contribution from P2Y1 receptor signaling, we performed the same experiment in P2Y1-/-mice. Similar to human platelets, thromboxane was generated in P2Y1-deficient mouse platelets only when Gi and Gz signaling were combined (Figure 7C). This confirms that
co-stimulation of the P2Y12 and α2A adrenergic receptors is also sufficient to cause thromboxane generation in mouse platelets. Similar to human platelets, pre-treating the mouse platelets with 10 µM PP2 inhibited the thromboxane production that resulted from combined G\textsubscript{i} and G\textsubscript{z} stimulation (Figure 7C).

It has previously been reported that fibrinogen must be present for ADP to cause thromboxane generation in platelets\textsuperscript{46}. We noted that fibrinogen was also necessary for thromboxane production via combined G\textsubscript{i} and G\textsubscript{z} signaling(Figure 7D). In the absence of fibrinogen, thromboxane levels were comparable to unstimulated samples. We also used a GPIIb/IIIa antagonist, SC57101, in the presence of fibrinogen, to test if the effect of fibrinogen was GPIIb/IIIa-specific. The GPIIb/IIIa antagonist blocked thromboxane production when fibrinogen was present, suggesting that fibrinogen interactions with GPIIb/IIIa are necessary for thromboxane generation via combined G\textsubscript{i} and G\textsubscript{z} signaling (Figure 7D).

**Discussion**

The G\textsubscript{i} signaling pathway is central to platelet activation, primarily because the Gi pathway potentiates alpha- and dense granule release, GPIIb/IIIa activation, and platelet procoagulant activity. Whereas the G\textsubscript{i} pathway alone does not cause aggregation, G\textsubscript{i} signaling can interact with other G protein signaling pathways to cause platelet aggregation. Previous work has shown that G\textsubscript{i} signaling causes platelet aggregation when it is combined with either G\textsubscript{q} or G12/13 signaling\textsuperscript{9,34-36}. Hence, we examined the functional responses that occur when G\textsubscript{i} is combined with G\textsubscript{z} signaling, another member of the G\textsubscript{i} family of G proteins, to further characterize the complexities of the interactions between the G\textsubscript{i} and other G protein pathways.
Platelet aggregation triggered by co-stimulation of the P2Y\textsubscript{12} and \(\alpha_{2A}\) adrenergic receptor results in signaling through multiple members of the G\(_i\) family because each receptor signals primarily through different members of the G\(_i\) family \(^{17}\). Though the G\(_i\) and G\(_z\) pathways couple to many of the same effectors, there still remains the possibility that these pathways signal through distinct signaling pathways. However, there is no published evidence for this.

Our work, and work by others, has shown that partial irreversible platelet aggregation occurs with the co-activation of the G\(_{12/13}\) and G\(_i\) pathways \(^{35,36}\). Though 100 \(\mu\)M ADP has been reported to cause platelet aggregation in the absence of any calcium mobilization in mouse platelets \(^47\), we have found that a combination of low-level P2Y\(_{12}\) receptor and \(\alpha_{2A}\) adrenergic receptor stimulation results in a small increase in calcium in human platelets, which is similar to our previous work with combined G\(_{12/13}\) and G\(_i\) stimulation \(^35\). The mechanism of this increase of intracellular calcium is presently unclear; however \(\beta\gamma\)-mediated activation of PLC or calcium leakage resulting from inhibition of adenyl cyclase are two possibilities. Chelation of intracellular calcium moderately decreased the extent of aggregation for G\(_i\) plus G\(_z\) signaling, while ADP-mediated signaling through G\(_q\) and G\(_i\) was completely inhibited by intracellular calcium chelation.

Calcium is known to play a significant role in thromboxane A\(_2\) generation in platelets \(^48\). The thromboxane A\(_2\) generated by 2-MeSADP was consistently less than the levels generated with combined G\(_i\) and G\(_z\) stimulation in human platelets. This is unexpected primarily because ADP mobilizes higher levels of intracellular calcium through the G\(_q\)-coupled P2Y\(_1\) receptor, than the combined signaling through G\(_i\) and G\(_z\)
pathways. Thus, combined G\textsubscript{i} and G\textsubscript{z}-mediated thromboxane generation may activate phospholipase A\textsubscript{2} (PLA\textsubscript{2}) more readily than ADP-mediated signaling, leading us to speculate that this robust activation of PLA\textsubscript{2} is not completely dependent on intracellular calcium mobilization.

A Src family tyrosine kinase inhibitor, PP2, decreased the platelet aggregation caused by P2Y\textsubscript{12} receptor and \(\alpha_{2A}\) adrenergic receptor stimulation. Others have used pharmacologic approaches to suggest that Src-family tyrosine kinase activity affects platelet aggregation caused by other agonists. Two Src-family tyrosine kinase inhibitors, PP2 and PD173956, inhibited PAR1-stimulated PAC-1 binding by 35\% \textsuperscript{43}. ADP and epinephrine-induced fibrinogen binding was decreased by piceatannol, an inhibitor of Syk and Src \textsuperscript{41}. This suggests that multiple agonists stimulate GPIIb/IIIa activity that is partially regulated by tyrosine kinase activity. The Src family tyrosine kinase inhibitor PP1 also inhibited the aggregation caused by combined G\textsubscript{i} and G\textsubscript{z} signaling, while the control compound PP3 had no effect on this aggregation (Figure 4B). PAC-1 binding, which is indicative of GPIIb/IIIa activation \textsuperscript{49}, resulted from combined stimulation of G\textsubscript{i} and G\textsubscript{z} signaling. Src tyrosine kinase inhibition led to a decrease in PAC-1 binding, suggesting that tyrosine kinases have a role in the modulation of GPIIb/IIIa activity downstream of these two pathways. Notably, PP2 did not completely inhibit the platelet aggregation or GPIIb/IIIa activation that is caused by co-stimulation of G\textsubscript{i} and G\textsubscript{z} signaling. Therefore, while Src-family tyrosine kinases may increase the extent of GPIIb/IIIa activation, they are not absolutely necessary for GPIIb/IIIa activation.

Recent work has measured Src activity that occurs downstream of ADP-mediated stimulation\textsuperscript{50}. This work showed that ADP-mediated Src activation is activated primarily
by the P2Y<sub>1</sub> receptor, but did not consider that ADP-mediated Src activation is still increased above baseline when ADP is signaling solely through the P2Y<sub>12</sub> receptor<sup>50</sup>. Thus, it appears that both the P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors are able to cause Src activation through G<sub>q</sub> and G<sub>i</sub> signaling respectively, albeit to different levels. Our data illustrates that Src is activated downstream of the P2Y<sub>12</sub> receptor and the α<sub>2A</sub> adrenergic receptor. Thus, either G<sub>i</sub> or G<sub>z</sub> signaling is sufficient to activate Src, and this Src activity plays a role in the level of GPIIb/IIIa activation.

Multiple enzymes have been implicated in the regulation of PLA<sub>2</sub> including protein kinase C<sup>51</sup>, MAP kinases<sup>52</sup>, and calcium<sup>53</sup>, though the candidates that phosphorylate and activate PLA<sub>2</sub> remain the subject of controversy. Lyn-deficient mice have impaired thromboxane generation in response to γ-thrombin when compared to wild type mice<sup>42</sup>. Thus, Src family tyrosine kinases play a role in the generation of thromboxane A<sub>2</sub> in platelets that are activated by G<sub>i</sub> and G<sub>z</sub> signaling pathways, though it is presently unclear how proximal this family of kinases is to PLA<sub>2</sub> activation.

ADP requires signaling through the P2Y<sub>1</sub> receptor, P2Y<sub>12</sub> receptor, and outside-in signaling through GPIIb/IIIa to cause thromboxane A<sub>2</sub> generation<sup>46</sup>. Similarly, we have found that combined P2Y<sub>12</sub> receptor and α<sub>2A</sub> adrenergic receptor stimulation causes thromboxane A<sub>2</sub> generation with a requirement for outside-in signaling through GPIIb/IIIa. An alternative explanation is that post-aggregatory events may be necessary for thromboxane generation, and that fibrinogen is facilitating platelet-to-platelet contact for the post-aggregatory response. We cannot presently differentiate between the two because of limited knowledge regarding post-aggregatory events.
While $G_{12/13}$, $G_q$, or $G_z$ can interact with P2Y$_{12}$-mediated $G_i$ signaling to cause platelet aggregation, the signaling intermediates for these pathways differ (illustrated in Figure 7E). PI-3 kinases and Src family kinases are pro-aggregatory signals for $G_i$ plus $G_z$-mediated platelet aggregation, while calcium and PI-3 kinase are important for $G_q$ plus $G_i$-mediated platelet aggregation. Though calcium chelation decreased the extent of platelet aggregation caused by $G_{12/13}$ plus $G_i^{35}$, the pro-aggregatory signals downstream of $G_{12/13}$ pathway have not yet been identified. Based on the recent findings of Hardy et al. $^{50}$, we speculate that Src family kinases are upstream of the PI-3 kinases. The diversity in the signaling of these somewhat non-redundant pathways may serve as a protection from deficiencies, but also allow multiple pro-aggregatory signals to contribute to this highly complex, fine-tuned system.
References

31. van Gestel MA, Heemskerk JW, Slaaf DW, Heijnen VV, Reneman RS, oude Egbrink MG. In vivo blockade of platelet ADP receptor P2Y12 reduces embolus and
Figure Legends

Figure 1. Platelet aggregation induced by simultaneous stimulation of the Gi and Gz pathways. A) Aspirin-treated, washed human platelets were stimulated with 100 nM 2-MeSADP, 10 µM epinephrine or a combination of both agonists, as noted. MRS2179 (100 µM), 100 nM AR-C69931MX, or 10 µM yohimbine, antagonizing P2Y1, P2Y12, and α2A adrenergic receptor antagonists, respectively, were added 1 minute prior to platelet stimulation. All aggregation tracings were performed in the presence of 1 mg/mL fibrinogen. Tracings are representative of results obtained from three donors and three separate experiments. B and C) Aspirin-treated, washed platelets were isolated from P2Y1- or Gq-deficient mice as noted in material methods. Samples were stimulated with either 3 µM ADP or 10 µM Epinephrine at 37°C under stirring conditions (900 rpm). In the case where both ADP and epinephrine were added, the additions of the agonists were made simultaneously. All tracings are representative of data obtained from three separate experiments.

Figure 2. Intracellular calcium mobilization in response to simultaneous stimulation of the Gi and Gz signaling. Fura-loaded, aspirinated, washed human platelets were stimulated as noted under stirring conditions at 37°C. Where noted, the antagonist 200 µM MRS2179 was added one minute prior to the addition of other agonists. Arrows indicate addition of agonist. Tracings are representative of results obtained from three different donors.
Figure 3. The effect of kinase inhibitors on platelet aggregation caused by the simultaneous stimulation of $G_i$ and $G_z$ or $G_q$ and $G_i$ pathways. Platelets were pre-incubated with kinase inhibitors as follows: 10 minutes at 37°C with DMSO (vehicle), 3 minutes at 37°C with 10 µM Ro 31-8220, 10 minutes pre-incubation at 37°C with 1 µM dimethyl BAPTA-AM, or 10 minutes pre-incubation with 25 µM LY294002. Platelets were stimulated with 100 nM 2-MeSADP, 10 µM epinephrine or a combination of both agonists, as noted, for $G_i$ and $G_z$ stimulation. MRS2179 (100 µM) was added 1 minute prior to platelet stimulation. 10 µM ADP was used as an agonist for stimulation of $G_q$ and $G_i$ pathways. All aggregation tracings were performed in the presence of 1 mg/mL fibrinogen. Tracings are representative of results obtained from three donors and three separate experiments.

Figure 4. The effect of Src-family tyrosine kinase inhibition on platelet aggregation caused by the simultaneous stimulation $G_i$ and $G_z$ pathways. A) Platelets were pre-incubated at 37°C with DMSO (vehicle) or varying doses of PP2 for 10 minutes prior to the stimulation of the platelets. Platelets were stimulated with 100 nM 2-MeSADP plus 100 µM MRS 2179 ($G_i$ signaling), 10 µM epinephrine ($G_z$ signaling), or a combination of both agonists, as noted. MRS2179 (100 µM) was added 1 minute prior to platelet stimulation. B) Washed human platelets were pre-incubated at 37°C with DMSO (vehicle) or with 10 µM PP1, 10 µM PP2, or 10 µM PP3 for 10 minutes prior to the stimulation of the platelets. Platelets were stimulated with 100 nM 2-MeSADP, 10 µM epinephrine or a combination of both agonists, as noted. MRS2179 (100 µM) was added 1 minute prior to platelet stimulation. 10 µM ADP was added for simultaneous stimulation of $G_q$ and $G_i$ pathways. All aggregation tracings were performed in the
presence of 1 mg/mL fibrinogen. Tracings are representative of results obtained from three donors and three separate experiments. C) Washed, aspirin-treated platelets (2x10^8 platelets/mL) were stimulated as noted in Materials and Methods. Chemiluminescence was measured on membranes that were probed with an anti-phospho-Src(416) antibody for measurement of Src kinase activity. The intensity of the bands was first calculated as a ratio of phosphorylated to total Src in each lane. Then the ratios from three independent experiments were normalized to the control, taken as 1, and expressed as fold increase in other lanes (mean ± SEM). The blot shown is representative of three independent experiments.

Figure 5. The effect of Src-family tyrosine kinase inhibition on PAC-1 binding that is induced by combined G_i and G_z stimulation. Aspirin-treated washed human platelets were stimulated for 10 minutes with the noted agonists in the presence of FITC-labeled PAC-1 mAb. Samples were diluted with Tyrode’s buffer and analyzed on a flow cytometer for increases in fluorescence that are correlated with GPIIb/IIIa activation. Data were calculated as mean fluorescence of the total platelet population. Each bar is the average of three experiments ± S.E. from three donors. Single asterisk (*) denotes p<0.05 compared to unstimulated. Double asterisk (**) denotes p<0.05 when compared to single asterisk samples.

Figure 6. The effect of aspirin on platelet aggregation caused by simultaneous stimulation of the G_i and G_z pathways. Upper panel contains aggregation tracings of non-aspirin-treated washed human platelets, while the lower panel contains aspirin-
treated washed human platelets. Where noted, the antagonist 100 µM MRS2179 was added one minute prior to the addition of other agonists. The agonists 100 nM 2-MeSADP and 10 µM epinephrine were added simultaneously where noted. The arrows indicate addition of agonists. All samples are performed in the presence of 1 mg/mL fibrinogen. Tracings are representative of data obtained from three different donors.

**Figure 7. The effect of combined G\textsubscript{i} and G\textsubscript{z} signaling on thromboxane generation in human and mouse platelets.**

A) Thromboxane B\textsubscript{2} was generated from washed human platelets in the presence of 1 mg/mL fibrinogen and stopped after 3.5 minutes of stimulation. Levels of TxB\textsubscript{2} were obtained using a 96-well competitive ELISA kit as noted in the Materials and Methods section. B) Samples were pre-incubated with varying doses of PP2 for 10 minutes at 37°C, and then stimulated with combined G\textsubscript{i} and G\textsubscript{z} signaling. C) The effect of combined G\textsubscript{i} and G\textsubscript{z} stimulation on thromboxane generation in P2Y\textsubscript{1}-deficient mouse platelets. Where noted, samples were pre-incubated with 10 µM PP2 for 10 minutes at 37°C. D) The effect of GPIIb/IIa antagonism on thromboxane generation induced by combined P2Y\textsubscript{12} and \(\alpha\textsubscript{2A}\) adrenergic stimulation. The GPIIb/IIa antagonist, 10 µM SC57101A, was added 1 minute prior to the addition of agonists (where noted). Similarly, 1 mg/mL fibrinogen was added to samples 1 minute prior to stimulation (where noted). In all bar graphs, the bars are representative of the average thromboxane A\textsubscript{2} generated (+/- standard error) from three different donors on three days of experiments. E) Final model depicting G\textsubscript{i} and G\textsubscript{z}-induced platelet aggregation – The agonists 2-MeSADP and ADP both activate the P2Y\textsubscript{1} and P2Y\textsubscript{12} receptors. MRS2179 and AR-C 69931MX antagonize the P2Y\textsubscript{1} and P2Y\textsubscript{12} receptors, respectively (noted by
hatch marks). Epinephrine is an agonist for the \( \alpha_{2A} \) adrenergic receptor and is blocked by the antagonist yohimbine. The combined signaling through the P2Y\(_{12} \) and \( \alpha_{2A} \) adrenergic receptors mediates Src family tyrosine kinase activity, which modulates integrin activation.
Figure 1A  
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Figure 1B&C
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Figure 2
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Figure 3
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Figure 4A
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Figure 4B
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Fig. 4C
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Figure 5
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Figure 6
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Figure 7A
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Figure 7B
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Figure 7C
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Figure 7D
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GPIIb/IIIa Activation

P2Y1 receptor

P2Y12 receptor

Gi

Gq

Gz

ADP

2-MeSADP

MRS 2179

AR-C 69931MX

Epinephrine

Yohimbine

α2A adrenergic receptor

Calcium

PI 3-Kinase

(+ ) Src

Calcium

PI 3-Kinase

GPIIb/IIIa Activation

Figure 7E
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Differential requirements for calcium and Src-family kinases in platelet GPIIb/IIIa activation and thromboxane generation downstream of different G protein pathways

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