Protease-activated receptors 1 and 4 do not stimulate G\textsubscript{i} signaling pathways in the absence of secreted ADP and cause human platelet aggregation independently of G\textsubscript{i} signaling

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Introduction

Platelet activation plays a major role in hemostasis and thrombosis. Several agonists, including adenosine diphosphate (ADP), thrombin, and thromboxane A\textsubscript{2}, can activate platelets.\textsuperscript{1} These agonists cause platelets to change their shape, to aggregate, and to release the contents of granules. Thrombin, generated at the site of vascular damage by extrinsic and intrinsic coagulation cascades, is an important agonist for platelet activation. Thrombin mediates its cellular effects primarily through a family of G protein–coupled protease-activated receptors (PARs). These receptors are activated by a unique mechanism in which the protease creates a new extracellular amino-terminus that functions as a tethered ligand, resulting in intramolecular activation.\textsuperscript{2,3} Three of the 4 known PARs, PAR1, PAR3, and PAR4, are activated by thrombin. PAR1 is detected in human platelets and has a major role in activation of human platelets by thrombin, but it plays no role in mouse platelets.\textsuperscript{4} PAR2 functions as a receptor for trypsin but not for thrombin.\textsuperscript{5} PAR3 is necessary for mouse platelets to be activated by low concentrations of thrombin.\textsuperscript{6} PAR3 functions as a cofactor for the activation of PAR4 by thrombin in mouse platelets.\textsuperscript{7} There is another receptor, PAR4, which appears to function in both mouse and human platelets.\textsuperscript{4,8} PAR4 also mediates platelet responses to the lower concentrations of \(\alpha\)-thrombin and AYPGKF but had no effect at higher concentrations. Similar results were obtained with platelets from mice deficient in the P2Y12. We conclude that (1) thrombin- and thrombin receptor-activating peptide–induced inhibition of adenylyl cyclase in platelets depends exclusively on secreted adenosine diphosphate that stimulates G\textsubscript{i} signaling pathways and (2) thrombin and thrombin receptor-activating peptides cause platelet aggregation independently of G\textsubscript{i} signaling. (Blood. 2002;99:3629-3636)

Thrombin is an important agonist for platelet activation and plays a major role in hemostasis and thrombosis. Thrombin activates platelets mainly through protease-activated receptor 1 (PAR1), PAR4, and glycoprotein Ib. Because adenosine diphosphate and thromboxane A\textsubscript{2} have been shown to cause platelet aggregation by concomitant signaling through G\textsubscript{q} and G\textsubscript{i} pathways, we investigated whether coactivation of G\textsubscript{q} and G\textsubscript{i} signaling pathways is the general mechanism by which PAR1 and PAR4 agonists also activate platelet fibrinogen receptor (\(\alpha IIb\beta 3\)). A PAR1-activating peptide, SFLLRN, and PAR4-activating peptides GYPGKF and AYPGKF, caused inhibition of stimulated adenylyl cyclase in human platelets but not in the presence of either Ro 31-8220, a protein kinase C selective inhibitor that abolishes secretion, or AR-C66096, a P2Y12 receptor–selective antagonist; \(\alpha\)-thrombin–induced inhibition of adenylyl cyclase was also blocked by Ro 31-8220 or AR-C66096. In platelets from a P2Y12 receptor–defective patient, \(\alpha\)-thrombin, SFLLRN, and GYPGKF also failed to inhibit adenylyl cyclase. In platelets from mice lacking the P2Y12 receptor, neither \(\alpha\)-thrombin nor AYPGKF caused inhibition of adenylyl cyclase. Furthermore, AR-C66096 caused a rightward shift of human platelet aggregation induced by the lower concentrations of \(\alpha\)-thrombin and AYPGKF but had no effect at higher concentrations. Similar results were obtained with platelets from mice deficient in the P2Y12. We conclude that (1) thrombin- and thrombin receptor-activating peptide–induced inhibition of adenylyl cyclase in platelets depends exclusively on secreted adenosine diphosphate that stimulates G\textsubscript{i} signaling pathways and (2) thrombin and thrombin receptor-activating peptides cause platelet aggregation independently of G\textsubscript{i} signaling. (Blood. 2002;99:3629-3636)

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the activating peptide GYPGKF is more effective in inducing human platelet aggregation than is GYPGQV.12 Recently, AYPGKF was shown to be a selective and more potent PAR4AP.17 Although these receptors have been identified, the molecular events leading to PAR agonist–induced platelet aggregation are unknown and the signaling events triggered by the activating peptides remain to be elucidated.

ADP-induced platelet aggregation requires coactivation of both the P2Y1 receptor (platelet ADP receptor coupled to stimulation of phospholipase C) and P2Y12 receptor (platelet ADP receptor coupled to inhibition of adenyl cyclase) that couple to \(G_q\) (heterotrimeric guanosine triphosphate–binding protein that stimulates phospholipase C) and \(G_i\) (heterotrimeric guanosine triphosphate–binding protein that inhibits adenyl cyclase), respectively, and concomitant signaling from \(G_q\) and \(G_i\) is sufficient for ADP-induced platelet aggregation.18–20 Thromboxane \(A_2\) also causes platelet aggregation by coactivation of \(G_q\) and \(G_i\) pathways.21 Thrombin and thrombin receptor–activating peptides have been shown to activate both \(G_q\) and \(G_i\) pathways.22–28 The signal transduction mechanisms of thrombin-induced platelet aggregation are less clear, and it has not yet been defined whether coactivation of \(G_q\) and \(G_i\) signaling pathways is the general mechanism by which all agonists activate platelet fibrinogen receptor.

Characterization of the signal transduction pathways that mediate thrombin’s action on platelets is necessary for understanding hemostasis and thrombosis. To determine the mechanisms of PAR1 and PAR4 in human platelet activation, we performed multiple approaches in human platelets, including analysis of a P2Y12 receptor–defective patient’s platelets, utilizing selective P2 receptor antagonists, blocking granule secretion, and the use of P2Y12 receptor–deficient mouse platelets. We report here that thrombin and thrombin receptor–activating peptides cause \(G_i\) stimulation through P2Y12 receptor activation by secreted ADP, and that they cause platelet aggregation independently of \(G_q\) stimulation.

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**Materials and methods**

**Reagents**

Apyrase (type V), fibrinogen (type I), and bovine serum albumin (fraction V) were purchased from Sigma (St Louis, MO). Hexapeptides SFLLRN, GYPGKF, and AYPGKF were custom synthesized at Research Genetics (Huntsville, AL). Luciferin-luciferase reagent was purchased from Chrono-Log (Havertown, PA). Imipramine was from ICN (Costa Mesa, CA); Prostaglandin E (PGE)\(_2\) and Ro 31-8220 (bisindolylmaleimide) were from Biomol Research Laboratories (Plymouth Meeting, PA); AR-C66096 and AR-C69931MX were gifts from AstraZeneca (Loughborough, United Kingdom). Yohimbine was from Research Biologicals International (Natick, MA). All other reagents were reagent grade, and deionized water was used throughout.

**Preparation of human platelets**

Human blood was collected from a pool of healthy volunteers or a patient, after informed consent, in a one-sixth volume of acid citrate dextrose (ACD: 2.5 g sodium citrate, 1.5 g citric acid, and 2.0 g glucose in 100 mL \(H_2O\)). Platelet-rich plasma (PRP) was prepared by centrifugation of citrated blood at 230g for 20 minutes at room temperature. Acetylsalicylic acid was added to PRP to a final concentration of 1 mM, and the preparation was incubated for 1 hour at 37°C followed by centrifugation at 1000g for 10 minutes at room temperature. The platelet pellet was resuspended in Tyrode buffer (pH 7.4) containing 138 mM NaCl, 2.7 mM KCl, 2 mM MgCl\(_2\), 0.42 mM NaH\(_2\)PO\(_4\), 5 mM glucose, 10 mM HEPES, 0.2% bovine serum albumin, and 0.05 U/mL apyrase. This low concentration of apyrase is not enough to block responses to ADP, but will prolong the responsiveness of platelets to ADP by preventing desensitization of the P2Y receptors. These conditions have been standardized in the laboratory. The platelet count was adjusted to 2 × 10\(^{10}\) cells per milliliter.

**Preparation of washed mouse platelets**

Studies using P2Y12-null mice were performed under protocols that were approved by the Schering-Plough Animal Use and Care Committee. P2Y12-null mice were generated using standard techniques.29 In-house bred 129Sv × C57BL/6 F2 mice derived from the same 129 Sv parent ES cell line as the P2Y12-null mice that were used as controls. These wild-type mice do not differ genetically from P2Y12-null mice except at the P2Y12-targeted locus. Blood was collected from the vena cava of anesthetized mice into syringes containing ACD as anticoagulant. Red blood cells were removed by centrifugation at 250g for 15 minutes. PRP was recovered, and platelets were pelleted at 1500g for 5 minutes.

**Human platelet aggregation**

Agonist-induced platelet aggregation was measured using a lumaggregometer (Chrono-Log) at 37°C with stirring (900 rpm). A 0.5-mL sample of aspirin-treated washed platelets was stimulated with agonist, and change in light transmission was measured. In some experiments, Ro 31-8220 (10 \(\mu\)M), a protein kinase C (PKC) inhibitor, was added and incubated for 5 minutes at 37°C with stirring before agonist stimulation to block secretion. The extent of platelet aggregation was measured 210 seconds after the addition of the agonist, and the maximum extent of aggregation was taken as 100%.

**Mouse platelet aggregation**

The mouse platelet aggregations were performed using washed platelets from the P2Y12 receptor–deficient mice and the control wild-type mice. The aggregations were performed in 96-well plates at room temperature by the procedure described by Bednar et al.30 Platelets were resuspended in modified Tyrode buffer at 2 × 10\(^{10}\) platelets per milliliter. Aggregation was initiated by adding agonist, and the plates were rotated at setting 6 on a microtiter plate shaker (Lab-Line Instruments, Melrose, IL). Optical density readings were obtained at 30-second to 1-minute intervals at 405 nm using a microtiter plate reader ( Molecular Devices, Sunnyvale, CA).

**Platelet secretion**

Platelet secretion was determined by measuring the release of \([^{3}H]\)5-HT. PRP was incubated with \([^{3}H]\)5-HT (1 \(\mu\)Ci/mL [37 kBq/mL]) and 1 mM acetylsalicylic acid for 1 hour at 37°C. The PRP was centrifuged, and the platelet pellet was resuspended in HEPES-buffered Tyrode solution containing imipramine at a final concentration of 1 \(\mu\)M to prevent reuptake of secreted \([^{3}H]\)5-HT. Platelet secretion was performed in the lumiggregometer at 37°C with stirring. The activation of labeled \([^{3}H]\)5-HT platelets was stopped 2 minutes after adding agonist with the addition of stopping solution containing formaldehyde and ethylenediaminetetraacetic acid according to the method previously described.31 Samples were collected and centrifuged at 5000g for 1 minute, and the supernatant was collected to measure the radioactivity using a Wallac 1409 liquid scintillation counter (Gaithersburg, MD).

Platelet secretion was also independently determined by measuring the release of adenosine triphosphate (ATP) by adding luciferin-luciferase reagent to the platelet sample after aggregation is completed. Platelet secretion was performed in the lumiggregometer at 37°C with stirring, and step change in the luminescence record indicated the amount of ATP released during aggregation.

**Measurement of cAMP in human platelets**

PRP from normal human blood was incubated with 2 \(\mu\)Ci/mL [74 kBq/mL] \([^{3}H]\)adrenaline and aspirin (1 \(\mu\)M) for 1 hour at 37°C followed by centrifugation at 1000 × g for 10 minutes at room temperature. A 0.5-mL aliquot of washed platelets was stimulated with the following reagents: PGE\(_1\), AR-C66096, yohimbine, or Ro 31-8220 and with SFLLRN or GYPGKF. Three and one-half minutes later, the reaction was stopped by addition of 1
M HCl, and 4000 dpm (disintegrations per minute) [\(^{14}\)C]cyclic adenosine monophosphate (cAMP) was added as the recovery standard. The level of cAMP was determined as described previously. The results were expressed as a percentage of inhibition, taking PGE\(_1\)-induced cAMP levels as 100%. In the study involving a P2Y12-defective patient, cAMP levels were determined by radioimmunoassay kit from Amersham (Piscataway, NJ).

Measurement of mouse platelet adenylyl cyclase activity

Production of cAMP in platelets was quantitated using the Adenylyl Cyclase Activation FlashPlate Assay Kit (NEN-Life Sciences, Boston, MA) following the manufacturer’s instructions. The 100- \mu\text{L} reaction mix contained 50 \mu\text{L} washed platelets (1 \times 10^6/mL) in Stimulation Buffer (NEN-Life Sciences) containing the phosphodiesterase inhibitor isobutyl-methylxanthine (Sigma). Receptor agonists and PGE\(_1\) were dissolved in Dulbecco phosphate-buffered saline (Gibco, Gaithersburg, MD) supplemented with 0.2% bovine serum albumin, 1 g/L glucose, and 10 mM MgCl\(_2\); and were added at the final concentrations indicated in the text and figures. We observed no significant differences in adenylyl cyclase responses between male and female mice.

Results
Evaluation of G\(_i\), signaling by PAR1AP and PAR4AP

PAR1 and PAR4, similar to thromboxane A\(_2\), can cause release of dense granule contents, and the ADP thus released could activate the G\(_i\) pathways through activation of the P2Y12 receptor. To determine whether PAR1 and PAR4 receptors can couple to G\(_i\) signaling pathways independently of secreted ADP, we utilized multiple complimentary approaches. The first approach was to block secretion, thereby eliminating the contribution of secreted ADP to G\(_i\) stimulation. PKC has been shown to play a major role in the induction of platelet secretion. To assess the role of secreted ADP on cAMP levels upon stimulation of platelets with SFLLRN and GYPGKF, we used Ro 31-8220, a selective inhibitor of PKC isoforms. The ability of Ro 31-8220 to block agonist-induced dense granule secretion was confirmed by \([\text{H}]\text{5-HT}\) release and by the measurement of ATP release using luciferin-luciferase reagent. SFLLRN and GYPGKF caused a decrease in the PGE\(_1\)-stimulated cAMP levels in the absence of Ro 31-8220 (Figure 1). However, in the presence of Ro 31-8220, neither SFLLRN nor GYPGKF caused inhibition of stimulated adenylyl cyclase (Figure 1). This indicated that the stimulation of PAR1 by SFLLRN and stimulation of PAR4 by GYPGKF does not activate G\(_i\) signaling pathways in the absence of secretion.

Platelet dense granules contain ADP, which inhibits adenylyl cyclase and reduces levels of cAMP following activation of P2Y12. Hence, the second approach utilized AR-C66096, a selective antagonist at the G\(_i\)-coupled P2Y12 receptor, to eliminate the contribution of signaling from this G\(_i\)-coupled receptor to PAR-mediated inhibition of adenylyl cyclase. SFLLRN- and GYPGKF-induced inhibition of PGE\(_1\)-stimulated adenylyl cyclase inhibition, which was blocked by AR-C66096 (Figure 1), suggesting that reversal of cAMP formation is due to the platelet dense granule. It should be noted that a granules can release small quantities of chemokines that can activate G\(_i\) pathways and the P2Y12 receptor antagonist would not affect this phenomenon. The residual inhibition of adenylyl cyclase seen in Figure 1 could be due to G\(_i\) stimulation by secreted chemokines.

A patient was described with abnormal responses to ADP due to defective signaling from the P2Y12 receptor. In this patient, ADP-induced inhibition of adenylyl cyclase is abolished. To confirm our results obtained with the inhibitors and receptor antagonists, an independent study was performed using the platelets from this P2Y12 receptor-defective patient.
In addition, thrombin-induced inhibition of adenylyl cyclase was almost completely abolished in mouse platelets lacking the P2Y12 receptor (Figure 4B). Thrombin-induced inhibition of adenylyl cyclase in mouse platelets was recently shown to be blocked by a P2Y12 receptor antagonist.41 Because platelets express PAR1, PAR3, PAR4, and GPIb-V-IX complex, these data indicate that thrombin activation of Gi signaling through these thrombin receptors depends primarily on stimulation of the P2Y12 receptor by secreted ADP.

Concentration dependence of thrombin-, SFLLRN-, or PAR4AP-induced platelet aggregation and secretion

To determine the relationship between platelet aggregation and secretion in response to thrombin, SFLLRN, AYPGKF, and GYPGKF, we exposed platelets to different concentrations of the agonists. The extent of aggregation at 210 seconds after the addition of agonist was measured, and the maximum extent was taken as 100%. Maximal aggregation was observed at concentrations above 2 μM SFLLRN (Figure 5A), 200 μM AYPGKF (Figure 5B), 500 μM GYPGKF (not shown), or 0.08 U/mL thrombin (Figure 5C). The dense granule secretion was measured by [3H]5-HT release, and the maximum secretion was taken as 100%. Maximal secretion was observed at concentrations above 3 μM SFLLRN (Figure 5A), 1000 μM AYPGKF (Figure 5B), 700 μM GYPGKF (not shown), or 0.3 U/mL thrombin (Figure 5C).

Effect of receptor-selective antagonists on thrombin-, SFLLRN-, or AYPGKF-induced platelet aggregation

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caused concentration-dependent platelet aggregation. In the P2Y12-deficient mouse platelets, aggregation by lower concentrations of AYPGKF was dramatically inhibited (Figure 7). However, aggregation induced by higher concentrations of AYPGKF in the P2Y12-deficient mouse platelets appeared similar to the aggregation in wild-type littermates. Similarly, the concentration-response curve in the P2Y12 receptor–deficient mouse platelets was shifted to the right at the lower concentrations of thrombin but was similar to wild-type littermates with higher thrombin concentrations. These results confirm that PAR4-mediated platelet aggregation does not depend on the ability of secreted ADP to activate the P2Y12 receptor.

Discussion

Thrombin is the most powerful activator of platelets, and characterization of the platelet receptors for thrombin is important for understanding thrombosis and hemostasis. Although it is known that cellular response to thrombin in platelets is mediated by PARs, the molecular mechanisms involved in PAR agonist–induced
platelet aggregation are yet to be clearly elucidated. It may be possible that platelet activation induced by PAR agonists, similar to ADP and thromboxane A2, requires coactivation of Gq and Gi signaling pathways.2,23,26,28 Previous studies suggested that thrombin and thrombin receptor–activating peptides cause both phosphoinositide hydrolysis and inhibition of adenylyl cyclase via at least 2 G proteins, Gq and Gi.22-28 Benka et al43 demonstrated that thrombin receptor is coupled to a member of the Gq family by using anti-Gq antibodies, which inhibited thrombin receptor activation peptide-induced platelet membrane guanosine triphosphatase activation. It has also been shown that platelets from Gq or Gi knock-out mice fail to aggregate or to release their granule contents in response to thrombin.44 On the other hand, thrombin has been shown to inhibit the adenylyl cyclase activity, which was inhibited by pertussis toxin.22,25,26 Giesberts et al26 also suggested that complete inhibition of cAMP requires activation of both PKC and Gi. It has also been shown that thrombin can couple to Gi in cells stably expressing the PAR1 receptor but not the PAR4 receptor.37 Even though previous studies have provided the evidence of involvement of each pathway, they have not identified the role of each pathway in thrombin-induced platelet activation. We utilized several complementary approaches to characterize the molecular mechanism of PAR1- and PAR4-induced platelet activation. In particular, we focused on the role of secretion and Gi pathways in platelet fibrinogen receptor activation. First, we blocked granule secretion using a specific PKC inhibitor. Second, we used receptor subtype–selective antagonists to eliminate the positive feedback from secreted ADP and epinephrine. Third, we used platelets from a P2Y12 receptor–defective patient. Finally, we utilized the P2Y12 knock-out mouse platelets to evaluate the role of the P2Y12 receptor in thrombin-induced platelet activation. In the last 3 approaches, wherein ADP secretion is preserved but the signaling through the P2Y12 receptor is blocked, activation of the P2Y12 receptor by secreted ADP would still contribute to the Gq-mediated functional responses.

Several groups have demonstrated that PAR1, upon activation by its activating peptide, couples to Gq, leading to inhibition of adenylyl cyclase.26,28,42 To determine whether PAR1– and PAR4–activating peptides can couple to Gi signaling pathways independently of secretion, we have used Ro 31-8220 and receptor-selective antagonists. PKC is essential for platelet secretion, and the PKC inhibitor Ro 31-8220 blocks the secretion of ADP in response to PAR1 and PAR4 agonists.34,36 In our hands, Ro 31-8220 completely blocks secretion (not shown). SFLLRN- or AYPGKF-induced adenylyl cyclase inhibition was dramatically blocked by Ro 31-8220, suggesting that these agonists depend on secreted ADP in Gi stimulation. Previously, Giesberts et al26 showed that a low concentration of thrombin depends on PKC and Gi to cause adenylyl cyclase inhibition. It is conceivable that, in their study, inhibition of PKC blocks secreted ADP, which can stimulate Gi through the P2Y12 receptor.

Blockade of PAR1AP- and PAR4AP-induced adenylyl cyclase inhibition by the P2Y12 receptor–selective antagonist AR-C66096 indicates that PAR receptor–activating peptides cause Gi stimulation through the secreted ADP. Thus, PAR1 and PAR4 fail to directly couple to Gi and inhibit cAMP formation in intact platelets (Figures 1 and 2) when activated by receptor-activating peptides. Using a complementary approach involving platelets from a P2Y12 receptor–defective patient,38 we also show that PAR1AP and PAR4AP failed to inhibit adenylyl cyclase. Finally, AYPGKF also failed to cause Gi stimulation in P2Y12 receptor–defective mouse platelets. Hence, our studies through complementary approaches demonstrate that SFLLRN, through activation of PAR1, and AYPGKF and GYPGKF, through activation of PAR4, cause Gi stimulation in platelets through secreted ADP.

Similar to PAR-activating peptides, thrombin-induced inhibition of adenylyl cyclase in human platelets was blocked when secretion was blocked by Ro 31-8220 or in the presence of AR-C66096 (Figure 4A). In the platelets from the patient lacking the P2Y12 receptor, thrombin failed to cause significant inhibition of adenylyl cyclase. Thus, thrombin cannot cause Gi stimulation in human platelets independently of secreted ADP. Because human platelets express PAR1, PAR3, PAR4, and GPIb-V-IX complex, it can be concluded that thrombin stimulation of these receptors does not activate the Gi pathway. These data contradict previous studies demonstrating Gi stimulation by thrombin in human platelet membranes wherein secretion of ADP does not occur.22,23,46 Even though thrombin has been shown to inhibit cAMP formation in HEL cell or platelet membrane preparations where secretion would not be involved, thrombin increases cAMP levels in intact HEL cells.25 These studies represent a difference of thrombin responses between intact platelets and membrane preparations and suggest that, in the membrane preparations, thrombin receptors can couple to other G proteins that are not stimulated in the native cell. Our data are also consistent with the studies of Selheim et al,47 who demonstrated that thrombin-induced phosphatidylinositol-3 kinase product formation in platelets depends on secreted ADP. In wild-type mouse platelets, thrombin causes inhibition of adenylyl cyclase but fails to do so in the platelets from P2Y12 receptor–defective mice (Figure 4B). Recent studies indicated that thrombin-induced inhibition of adenylyl cyclase in mouse platelets could be blocked by a P2Y12 receptor antagonist.41 Mouse platelets do not express PAR1 receptor, and thrombin effects are predominantly mediated by PAR4 receptor in these cells. Consistent with our observations, Faruqi et al17 demonstrated that PAR4 stably expressed in fibroblast cells does not inhibit adenylyl cyclase upon stimulation with thrombin.

Because thrombin, SFLLRN, and PAR4APs depend on secreted ADP for Gi stimulation, and costimulation of Gq and Gi is required for ADP- and U46619-induced platelet aggregation, we investigated the role of secreted ADP in thrombin-, SFLLRN- or
PAR4AP-induced platelet aggregation by 2 complementary pharmacological approaches. The first approach utilized AR-C66096. Platelet aggregation induced by PAR4APs and thrombin was diminished in the presence of AR-C66096 at lower agonist concentrations. However, AR-C66096 has no effect on platelet aggregation induced by higher agonist concentrations of PAR4APs and thrombin, thereby confirming a lack of dependence on secreted ADP. SFLLRN-induced platelet aggregation was rightward-shifted by AR-C66096, indicating a potentiating effect of secreted ADP. In the second approach, the P2Y12 knock-out mouse platelets aggregated normally to high concentrations of AYPGKF, although aggregation was diminished when lower agonist concentrations were used. Similarly, thrombin also has been shown to cause platelet aggregation in P2Y12-deficient mouse platelets.29 These findings correlate with the first approach and provide further evidence that PAR4-mediated platelet aggregation does not depend on Gq stimulation. The primary platelet aggregation mediated by PARs could result from Gq signaling alone or through coactivation of an unidentified G protein–coupled pathway in addition to Gi signaling. Even though it has been shown that both Gq and Gi pathways are necessary for ADP-induced platelet aggregation,18,20 it is conceivable that thrombin-, SFLLRN-, and PAR4AP-induced platelet aggregation do not require these 2 pathways. PAR1 and PAR4 also couple to G12/13 pathways. As result of this coupling, a PAR1AP, YFLLRN, causes human platelet shape change without intracellular calcium mobilization.40 In Gi-deficient mouse platelets, where PAR1 is not expressed, thrombin causes platelet shape change through activation of PAR4.44 However, activation of the G12/13 pathway does not result in platelet aggregation.40,44

In conclusion, we demonstrate that, in human platelets, thrombin- and SFLLRN depend on secreted ADP to stimulate Gq subsequent to activation of PAR1. In mouse platelets, upon stimulation by thrombin, its receptors GP Ib-V-IX, PAR3, and PAR4 fail to couple to Gi in the absence of P2Y12 receptor. Our results also demonstrate that thrombin-, PAR4AP-, or SFLLRN-induced platelet aggregation occurs independently of the Gq-coupled pathway, and concomitant signaling from the Gq and Gi signaling pathways is not required for these agonist-induced primary platelet aggregations.

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References
Protease-activated receptors 1 and 4 do not stimulate $G_i$ signaling pathways in the absence of secreted ADP and cause human platelet aggregation independently of $G_i$-signaling

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