

Relative contribution of G-protein–coupled pathways to protease-activated receptor-mediated Akt phosphorylation in platelets

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Protease-activated receptors (PARs) activate G_q and $G_{12/13}$ pathways, as well as Akt (protein kinase B [PKB/Akt]) in platelets. However, the relative contribution of different G-protein pathways to Akt phosphorylation has not been elucidated. We investigated the contribution of G_q and $G_{12/13}$ to G_i/G_z -mediated Akt phosphorylation downstream of PAR activation. Selective $G_{12/13}$ activation failed to cause Akt phosphorylation in human and G_{α_q} -deficient mouse platelets. However, supplementing G_i/G_z signaling to $G_{12/13}$ caused significant increase in Akt phosphoryla-

tion, confirming that $G_{12/13}$ potentiates Akt phosphorylation. Inhibition of PAR-mediated Akt phosphorylation in the presence of the G_q -selective inhibitor YM-254890 was restored to the normal extent achieved by PAR agonists if supplemented with G_i signaling, indicating that G_q does not have any direct effect on Akt phosphorylation. Selective $G_{12/13}$ activation resulted in Src kinase activation, and Akt phosphorylation induced by costimulation of $G_{12/13}$ and G_i/G_z was inhibited by a Src kinase inhibitor but not by a Rho kinase inhibitor. These data demonstrate

that $G_{12/13}$, but not G_q , is essential for thrombin-induced Akt phosphorylation in platelets, whereas G_q indirectly contributes to Akt phosphorylation through G_i stimulation by secreted ADP. $G_{12/13}$ activation might mediate its potentiating effect through Src activation, and Src kinases play an important role in thrombin-mediated Akt phosphorylation. (Blood. 2006; 107:947-954)

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Introduction

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). It is known that PARs couple to G_q and $G_{12/13}$ pathways,¹ and we have demonstrated that thrombin and thrombin receptor–activating peptides cause G_i stimulation through P2Y₁₂ receptor activation by secreted ADP.² It has been shown that stimulation of platelets with thrombin results in Akt activation, and P2Y₁₂ ADP receptor is responsible for a significant proportion of Akt activation.³

Protein kinase B (PKB; also known as Akt) is a 57-kDa serine/threonine kinase⁴ that contains a pleckstrin homology (PH) domain adjacent to a centrally located catalytic domain that is connected to a short C-terminal tail.⁵ The catalytic domain of Akt contains a Thr³⁰⁸ phosphorylation site and a regulatory domain located in the C-terminal tail contains a Ser⁴⁷³ phosphorylation site.⁵ There are at least 3 different Akt isoforms identified in humans, which display more than 80% sequence homology and are named Akt1 (PKB α), Akt2 (PKB β), and Akt3 (PKB γ).⁶ It has been shown that the major Akt subtype in human platelets is Akt1 rather than Akt2.⁷ The Akt1 phosphorylation sites are Thr³⁰⁸/Ser⁴⁷³ and both translocation of Akt to cell membranes and phosphorylation of both Thr³⁰⁸ and Ser⁴⁷³ are required for full enzyme activity. Phosphatidylinositol (PI) 3-kinase has been shown to be implicated

in fibrinogen receptor activation,⁸ and Akt functions as one of several downstream effectors of PI 3-kinase.^{9,10} PI 3-kinase products PtdIns^{3,4}P₂ and PtdIns^{3,4,5}P₃ trigger the simultaneous phosphorylation of Akt by phosphatidylinositol-dependent kinase 1 (PDK1) and 2. Membrane attachment of Akt is a prerequisite for phosphorylation by PDK1 and PDK2, and PDK1 has been shown to phosphorylate Thr³⁰⁸ of Akt1 and the phosphorylation of Ser⁴⁷³ is independent of Thr³⁰⁸.¹¹

ADP-induced platelet aggregation requires coactivation of both the P2Y₁ and P2Y₁₂ receptors that couple to G_q and G_i , respectively, and concomitant signaling from G_q and G_i is sufficient and necessary for ADP-induced platelet aggregation.¹²⁻¹⁴ Epinephrine binds to the α_2A -adrenergic receptor and causes activation of the G_z pathway that leads to the pertussis toxin–insensitive inhibition of adenylyl cyclase.^{15,16} It has been shown that activated thrombin receptors in platelets couple to $G_{12/13}$ pathways,¹ which are involved in p160^{ROCK} activation and the subsequent shape change.^{17,18} Y-27632, a specific inhibitor of the Rho-associated protein kinase p160^{ROCK},¹⁹ blocks $G_{12/13}$ -induced platelet shape change that is independent of an increase of intracellular calcium, indicating that p160^{ROCK} plays a central role downstream of $G_{12/13}$ for mediating shape change.²⁰

Data from our own studies and others show that Akt is activated by various agonists including ADP, epinephrine, thrombin, and

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thrombin receptor–activating peptides.^{3,7,21} It has been shown that Akt phosphorylation stimulated by ADP was preserved in the platelets from G_q knockout mice, whereas thrombin-induced Akt phosphorylation was abolished.^{3,22} However, it is controversial whether thrombin-induced Akt phosphorylation is dependent on G_q or G_i signaling pathways. Woulfe et al²² reported that the activation of Akt by thrombin occurs in a G_q-dependent manner in which secreted ADP can only amplify Akt activation. We have suggested that thrombin-mediated Akt phosphorylation is largely dependent on G_i stimulation through P2Y₁₂ receptor activation by secreted ADP. Even though thrombin-induced Akt phosphorylation depends on secretion, Akt phosphorylation mediated by ADP and epinephrine is much weaker than that caused by thrombin. These findings raised the possibility that there is a potentiating contribution of other G-protein signaling to Akt phosphorylation mediated by G_i or G_z signaling pathways. The relative contribution of G_q or G_{12/13} pathways to Akt phosphorylation and the downstream signaling events from G_{12/13} activation have not been fully understood. Therefore, we sought to define the contribution of G_q or G_{12/13} pathways to Akt phosphorylation mediated by G_i/G_z pathways in platelets.

In this study, we show that selective activation of G_{12/13} alone is not sufficient to cause Akt phosphorylation in platelets, but activation of G_{12/13} in combination with selective activation of G_i/G_z signaling in the absence of G_q can restore the Akt phosphorylation to the level achieved by PAR agonists in normal platelets, thus indicating the essential role of G_{12/13} pathways in potentiation of Akt phosphorylation downstream of PAR receptor activation. There has been an unresolved question of whether coupling of PAR1 to G_i is direct or indirect.²³ We have previously demonstrated that PAR1 activation does not lead to G_i stimulation in platelets using cAMP levels as an assay.² In this study, we confirm this important finding using Akt phosphorylation as the readout. The results also show that G_q-dependent pathways do not have any direct effect on PAR-mediated Akt phosphorylation and confirm our previous study that thrombin requires secreted ADP to induce Akt phosphorylation. We also show for the first time that G_{12/13} activation downstream of PARs leads to Src family kinase activation, and G_{12/13} might mediate its potentiating effect on Akt phosphorylation through Src family kinase.

Materials and methods

Approval for this study was obtained from the Institutional Review Board of Temple University (Philadelphia, PA). Informed consent was provided according to the Declaration of Helsinki.

Materials

Thrombin, 2-MeSADP, epinephrine, MRS-2179, apyrase (type V), and bovine serum albumin (fraction V) were purchased from Sigma (St Louis, MO). YFLLRNP was custom synthesized by New England Biolabs (Beverly, MA), and SFLLRN and AYPGKF were custom synthesized by Invitrogen (Carlsbad, CA). Anti-phospho-Akt (Ser⁴⁷³ and Thr³⁰⁸) and anti-phospho-Src (Tyr⁴¹⁶) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Alkaline phosphatase (AP)-labeled secondary antibody was from Kirkegaard & Perry Laboratories (Gaithersburg, MD). CDP-Star chemiluminescent substrates were purchased from Applied Biosystems (Foster City, CA). Y-27632, LY294002, and PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-D]pyrimidine) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). PP3 (4-amino-7-phenylpyrazolo[3,4-D]pyrimidine) was purchased from Calbiochem (San Diego, CA). AR-C69931MX was a gift from AstraZeneca (Loughborough, United Kingdom). YM-254890 was a generous gift from Yamanouchi

Pharmaceutical (Ibaraki, Japan). All other reagents were reagent grade, and deionized water was used throughout.

Animals

Gα_q-deficient mice were obtained from T. Kent Gartner,²⁴ with permission from Stefan Offermanns (University of Heidelberg, Heidelberg, Germany).

Preparation of human platelets

Whole blood was obtained from a pool of healthy volunteers in a one-sixth volume of ACD (2.5 g sodium citrate, 1.5 g citric acid, and 2.0 g glucose in 100 mL of H₂O). Platelet-rich plasma (PRP) was prepared by centrifugation of citrated blood at 230 rcf (*g*) for 20 minutes at room temperature (RT). Acetylsalicylic acid was added to PRP to a final concentration of 1 mM, and the preparation was incubated for 45 minutes at 37°C followed by centrifugation at 980 rcf (*g*) for 10 minutes at RT. The platelet pellet was resuspended in Tyrode buffer (138 mM NaCl; 2.7 mM KCl; 2 mM MgCl₂; 0.42 mM NaH₂PO₄; 5 mM glucose; 10 mM HEPES, pH 7.4; and 0.2% bovine serum albumin) containing 0.05 unit/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 × 10⁸ cells/mL.

Preparation of mouse platelets

Blood was collected from the vena cava of anesthetized mice into syringes containing one-tenth blood volume of 3.8% sodium citrate as anticoagulant. Red blood cells were removed by centrifugation at 100 rcf (*g*) for 10 minutes at RT. PRP was recovered and platelets were pelleted at 400 rcf (*g*) for 10 minutes at RT. The platelet pellet was resuspended in Tyrode buffer (pH 7.4) containing 0.05 unit/mL apyrase.

Western blotting

Platelets were stimulated with agonists under nonstirring conditions for the appropriate time, and the reaction was stopped by the addition of 3 × sodium dodecyl sulfate (SDS) sample buffer. In some experiments, Y-27632 (10 μM), a p160^{ROCK} inhibitor, and PP2 (10 μM), a Src family kinase inhibitor, were added and incubated for 5 minutes at 37°C without stirring before agonist stimulation. Levels of phospho-Akt and phospho-Src were determined after immunoblotting with anti-phospho-Akt (Ser⁴⁷³ and Thr³⁰⁸) and anti-phospho-Src (Tyr⁴¹⁶) antibodies. Platelet samples were boiled for 5 minutes and proteins were separated on 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride membrane. Nonspecific binding sites were blocked by incubation in Tris-buffered saline/Tween (TBST; 20 mM Tris, 140 mM NaCl, 0.1% [v/v] Tween 20) containing 0.5% (w/v) milk protein and 3% (w/v) bovine serum albumin (BSA) for 30 minutes at RT, and membranes were incubated overnight at 4°C with primary antibody (1:1000 in TBST/2% BSA) with gentle agitation. After 3 washes for 5 minutes each with TBST, the membranes were probed with AP-labeled goat anti-rabbit IgG (1:5000 in TBST/2% BSA) for 1 hour at RT. After additional washing steps, membranes were then incubated with CDP-Star chemiluminescent substrates for 10 minutes at RT and immunoreactivity was detected using Fujifilm Luminescent Image Analyzer (model LAS-3000 CH; Tokyo, Japan).

Human platelet aggregation

Agonist-induced platelet aggregation was measured using a lumi-aggregometer (Chrono-Log, Havertown, PA) 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. Agonists were added simultaneously for platelet stimulation, and platelets were preincubated with 10 μM Y-27632 or 50 nM YM-254890 for 5 minutes at 37°C before agonist stimulation in some experiments. Each sample was allowed to aggregate for at least 5 minutes. The chart recorder (Kipp and Zonen, Bohemia, NY) was set for 0.2 mm/s.

Results

Characterization of YFLLRNP-mediated Akt phosphorylation in human platelets

It has been shown that activated thrombin receptors in platelets couple to G_{12/13} pathways¹ that are involved in Rho kinase p160^{ROCK} activation and the subsequent shape change.^{17,18,25} Low concentrations of YFLLRNP, a heptapeptide binding to PAR1, causes shape change without calcium mobilization in platelets.²⁶ This shape change is mediated by the activation of G_{12/13} pathways without activation of the G_q pathways. In human platelets, we used this selective agonist of G_{12/13} pathways in combination with selective activation of G_i/G_z pathways to demonstrate the contribution of G_{12/13} signaling to Akt phosphorylation. To determine the kinetics of Akt phosphorylation, Ser⁴⁷³ phosphorylation was monitored over a time range of 0.5 to 20 minutes. Figure 1A shows that an increase in Ser⁴⁷³ phosphorylation in response to YFLLRNP (500 μM) was detectable within 1 minute of stimulation, and the level of phosphorylation peaked at around 5 minutes of stimulation. We exposed platelets to different concentrations of YFLLRNP ranging from 40 to 500 μM, and Ser⁴⁷³ phosphorylation was measured at 5 minutes after stimulation. Figure 1B illustrates that YFLLRNP induced a concentration-dependent increase in Akt phosphorylation. An increase in Ser⁴⁷³ phosphorylation was detectable at concentrations above 200 μM YFLLRNP, suggesting that higher concentrations of YFLLRNP activate both G_q and G_{12/13} signaling. However, when human platelets were stimulated with low concentrations of YFLLRNP that activate only the G_{12/13} signaling cascade, YFLLRNP failed to cause Akt phosphorylation, suggesting that G_{12/13} signaling alone is not capable of causing Akt phosphorylation in human platelets.

Contribution of G_{12/13} signaling to Akt phosphorylation in human platelets

Activation of G_{12/13} pathways results in platelet shape change that involves Rho kinase.^{18,25} As shown in Figure 2A, YFLLRNP-induced (60 μM) platelet shape change was completely inhibited in the presence of 10 μM Y-27632,²⁵ Rho kinase p160^{ROCK} inhibitor, suggesting that a low dose of YFLLRNP causes selective activation of G_{12/13} pathways. Immunoblot analysis revealed that YFLLRNP (60 μM) failed to phosphorylate Akt at Ser⁴⁷³ in human platelets, whereas stimulation of the G_i-coupled P2Y₁₂ receptor or G_z-coupled α_{2A}-adrenergic receptor by 2-MeSADP and epinephrine, respectively, were able to cause Akt phosphorylation (Figure 2B). However, YFLLRNP-mediated G_{12/13} signaling in combination

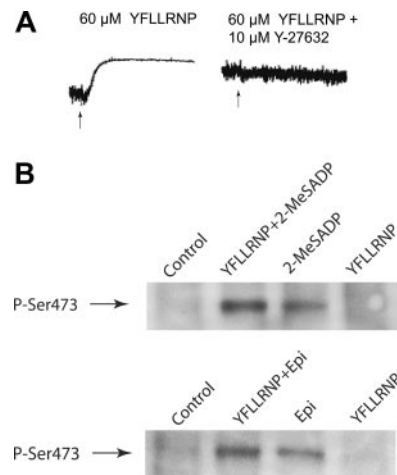


Figure 2. G_{12/13} signaling potentiates Akt phosphorylation mediated by G_i or G_z signaling in human platelets. (A) Aspirin-treated, washed human platelets preincubated with 10 μM Y-27632 were stimulated with 60 μM YFLLRNP, and platelet aggregation was measured as described in "Materials and methods." Arrows indicate the addition of agonist. (B) Washed platelets were stimulated at 37°C for 5 minutes with YFLLRNP (60 μM), 2-MeSADP (1 μM), or epinephrine (Epi; 10 μM). Platelets stimulated with 2-MeSADP were preincubated with 10 μM MRS-2179. The addition of YFLLRNP (60 μM) was made as indicated. The reaction was stopped by the addition of 3 × SDS sample buffer. Samples were separated by SDS-PAGE, Western blotted, and probed with anti-phospho-Akt (Ser⁴⁷³) antibody. The data shown are representative of 3 experiments.

with G_i/G_z signaling caused a 1.92- to 2.37-fold increase in Akt phosphorylation compared with those obtained with epinephrine or 2-MeSADP alone (Figure 2B). These results suggest that G_{12/13} signaling has a potentiating effect on Akt phosphorylation when combined with selective G_i or G_z signaling in human platelets.

Effect of Rho kinase in G_{12/13}- and G_i/G_z-mediated Akt phosphorylation

It has been shown that G_{12/13}-mediated platelet shape change is inhibited by the p160^{ROCK} inhibitor Y-27632 and is independent of PLC/Ca²⁺/calmodulin myosin light-chain kinase signaling.^{18,20} Thus, it is suggested that Rho kinase p160^{ROCK} is a key signaling molecule downstream of G_{12/13} pathways, and we investigated its role in the potentiation of Akt phosphorylation. Since G_{12/13}-induced platelet shape change was blocked by the Rho kinase inhibitor Y-27632, we tested the effect of Y-27632 on Akt phosphorylation induced by G_{12/13} and G_i/G_z pathways. Figure 3 shows that Y-27632 has no effect on Akt phosphorylation induced by costimulation of G_{12/13} and G_i/G_z signaling, indicating that Rho/Rho kinase does not have any role in potentiation of Akt

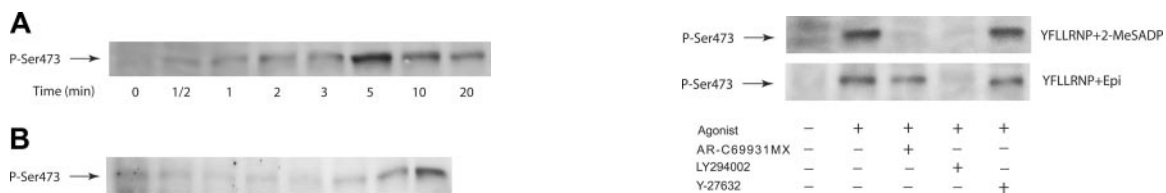


Figure 1. YFLLRNP-induced Akt phosphorylation in human platelets. (A) Time-dependent phosphorylation (P) of Akt at Ser⁴⁷³ with YFLLRNP. Washed platelets were stimulated at 37°C for the time points indicated with YFLLRNP (500 μM) without stirring. (B) Washed platelets were incubated with different concentrations of YFLLRNP for 5 minutes at 37°C without stirring. The reaction was stopped by the addition of 3 × SDS sample buffer. Samples were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and probed with anti-phospho-Akt (Ser⁴⁷³) antibody. Results are representative of 3 experiments.

Figure 3. Role of Rho kinase in Akt phosphorylation induced by combined G_{12/13} and G_i/G_z signaling in platelets. Washed platelets preincubated with 1 μM AR-C69931MX, 25 μM LY294002, or 10 μM Y-27632 were stimulated at 37°C for 5 minutes with either YFLLRNP (60 μM) + 2-MeSADP (1 μM) or YFLLRNP (60 μM) + epinephrine (10 μM). Platelets stimulated with 2-MeSADP were preincubated with 10 μM MRS-2179. The reaction was stopped by the addition of 3 × SDS sample buffer. Equal amounts of proteins were analyzed by Western blot analysis with anti-Ser(P)⁴⁷³ antibody. The Western blot analysis shown is a representative of 3 independent experiments.

phosphorylation. However, AR-C69931MX, an antagonist at the G_i -coupled P2Y₁₂ receptor, inhibited concomitant $G_{12/13}$ - and G_i -mediated Akt phosphorylation. This result was substantiated in experiments where epinephrine reversed the inhibitory effect of AR-C69931MX on Akt phosphorylation. PI 3-kinase has been shown to be a key signaling molecule for Akt phosphorylation.⁹ LY294002, a PI 3-kinase inhibitor,²⁷ blocked Akt phosphorylation caused by costimulation of $G_{12/13}$ and G_i/G_z signaling, suggesting the important role of PI 3-kinase in this Akt phosphorylation. It appears that PI 3-kinase mediates its effect through the P2Y₁₂- or α_{2A} -adrenergic-stimulated G_i/G_z signaling pathways.

Contribution of $G_{12/13}$ signaling to Akt phosphorylation in mouse platelets

We compared Akt phosphorylation in response to AYPGKF, a PAR4-activating peptide, and thrombin in platelets from wild-type mice as well as from mice lacking G_{α_q} . Immunoblot analysis revealed that AYPGKF and thrombin failed to phosphorylate Akt at Ser⁴⁷³ and Thr³⁰⁸ in platelets from G_{α_q} knockout mice (Figure 4A). However, AYPGKF and thrombin caused phosphorylation of Ser⁴⁷³ and Thr³⁰⁸ in wild-type littermates, which was partially inhibited in the presence of AR-C69931MX. We have shown that thrombin

receptors fail to directly couple to G_i in platelets and depend on secreted ADP for G_i stimulation² and that thrombin-induced Akt phosphorylation depends on G_i pathways.³ Therefore, our data suggest the possible contribution of other G-protein-coupled signaling to Akt phosphorylation mediated by thrombin in platelets.

It has been shown that thrombin receptors couple to G_q and $G_{12/13}$ pathways.¹ To determine the contribution of $G_{12/13}$ pathways to Akt phosphorylation in mouse platelets, we selectively supplemented G_i or G_z stimulation to AYPGKF- and thrombin-induced Akt phosphorylation in G_{α_q} -deficient mouse platelets. Figure 4B illustrates that the activation of PAR receptors with thrombin and AYPGKF in G_{α_q} -deficient platelets, which couple to $G_{12/13}$ pathways, did not induce Akt phosphorylation. Simultaneous addition of either 2-MeSADP or epinephrine to thrombin- or AYPGKF-stimulated G_{α_q} -deficient platelets resulted in a significant increase (3.2- to 4.5-fold) in Akt phosphorylation compared with that induced by 2-MeSADP or epinephrine alone. These results suggest that $G_{12/13}$ signaling from thrombin receptors is capable of potentiating Akt phosphorylation mediated by G_i or G_z pathways in mouse platelets.

To confirm the contribution of $G_{12/13}$ signaling to potentiation of Akt phosphorylation, we exposed G_{α_q} -deficient platelets to different concentrations of AYPGKF in the presence of 2-MeSADP. Figure 4C shows that selective activation of $G_{12/13}$ signaling by AYPGKF induced a concentration-dependent increase in Akt phosphorylation mediated by 2-MeSADP-induced G_i stimulation, confirming the role of $G_{12/13}$ signaling in potentiation of Akt phosphorylation when combined with G_i signaling. Taken together with our data showing that potentiation of Akt phosphorylation induced by AYPGKF and thrombin in G_{α_q} -deficient platelets was much stronger than that induced by a low dose of YFLLRNP in human platelets, these results suggest that AYPGKF and thrombin in combination with G_i/G_z in G_{α_q} -deficient platelets induced more robust Akt phosphorylation through strong stimulation of $G_{12/13}$ pathways.

Contribution of G_q versus $G_{12/13}$ pathways to Akt phosphorylation

YM-254890, which is isolated from culture broth of *Chromobacterium* species, has been shown to inhibit ADP-induced platelet aggregation by selectively inhibiting G_q activation.²⁸ In order to compare the relative contribution of G_q and $G_{12/13}$ pathways, we compared Akt phosphorylation in the presence and absence of the G_q -selective inhibitor YM-254890.

To ensure that YM-254890 inhibits the G_q pathway specifically, we measured agonist-induced platelet aggregation in the presence and absence of 50 nM YM-254890 or MRS-2179, a G_q -coupled P2Y₁ receptor antagonist,²⁹ blocked 2-MeSADP-induced platelet shape change and aggregation (Figure 5A). However, YM-254890 did not block the 2-MeSADP-induced inhibition of cAMP levels in platelets (data not shown). AYPGKF, which couples to G_q and $G_{12/13}$ pathways, caused platelet shape change in the presence of YM-254890. This shape change was abolished by the addition of Rho kinase inhibitor Y-27632, which has been shown to block $G_{12/13}$ -induced shape change (Figure 5A). These data indicate that YM-254890 selectively inhibits G_q signaling in platelets.

It has been shown that ADP depends on stimulation of G_i , but not G_q , to activate Akt.^{3,22} Immunoblot analysis revealed that 2-MeSADP caused Akt phosphorylation in the presence and absence of MRS-2179 or YM-254890, whereas 2-MeSADP-induced Akt phosphorylation was abolished by AR-C69931MX (Figure 5B), confirming that YM-254890 is a G_q -selective inhibitor and does not have a nonselective effect on Akt phosphorylation.

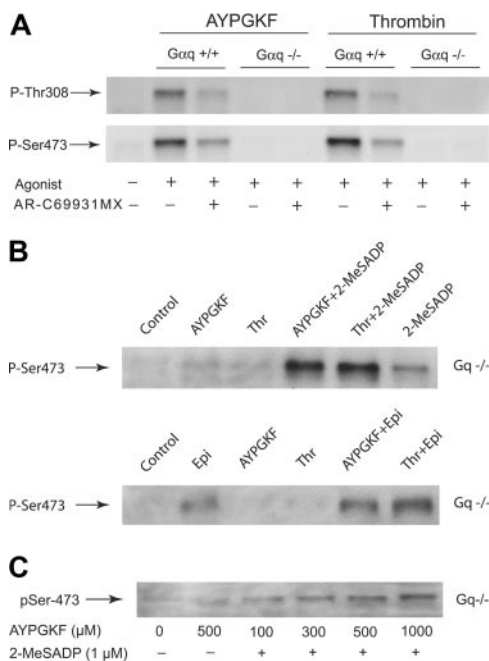


Figure 4. Role of $G_{12/13}$ signaling in potentiation of Akt phosphorylation in G_{α_q} -deficient mice. (A) Akt phosphorylation in response to AYPGKF and thrombin in G_{α_q} -deficient mice. Wild-type platelets and G_{α_q} -deficient platelets preincubated in the absence and presence of 1 μ M AR-C69931MX were stimulated at 37°C for 5 minutes with either AYPGKF (500 μ M) or thrombin (0.5 unit/mL). The reaction was stopped by the addition of 3 \times SDS sample buffer. Equal amounts of proteins were analyzed by Western blot analysis with anti-Thr(P)³⁰⁸ or anti-Ser(P)⁴⁷³. The data shown are representative of 3 experiments. (B) $G_{12/13}$ signaling potentiates Akt phosphorylation in G_{α_q} -deficient platelets. G_{α_q} -deficient platelets were stimulated at 37°C for 5 minutes with either AYPGKF (500 μ M) or thrombin (Thr; 0.5 unit/mL) without stirring. The addition of 2-MeSADP (1 μ M) or epinephrine (10 μ M) was made as indicated. The reaction was stopped by the addition of 3 \times SDS sample buffer. Samples were separated by SDS-PAGE, Western blotted, and probed with anti-phospho-Akt (Ser⁴⁷³) antibody. The data shown are representative of 3 experiments. (C) Concentration-dependent Akt phosphorylation induced by combined $G_{12/13}$ and G_i signaling in G_{α_q} -deficient platelets. G_{α_q} -deficient platelets were stimulated at 37°C for 5 minutes with different concentrations of AYPGKF in the presence of 2-MeSADP (1 μ M). The reaction was stopped by the addition of 3 \times SDS sample buffer. Platelet proteins were separated by SDS-PAGE, Western blotted, and probed with anti-phospho-Akt (Ser⁴⁷³) antibody.

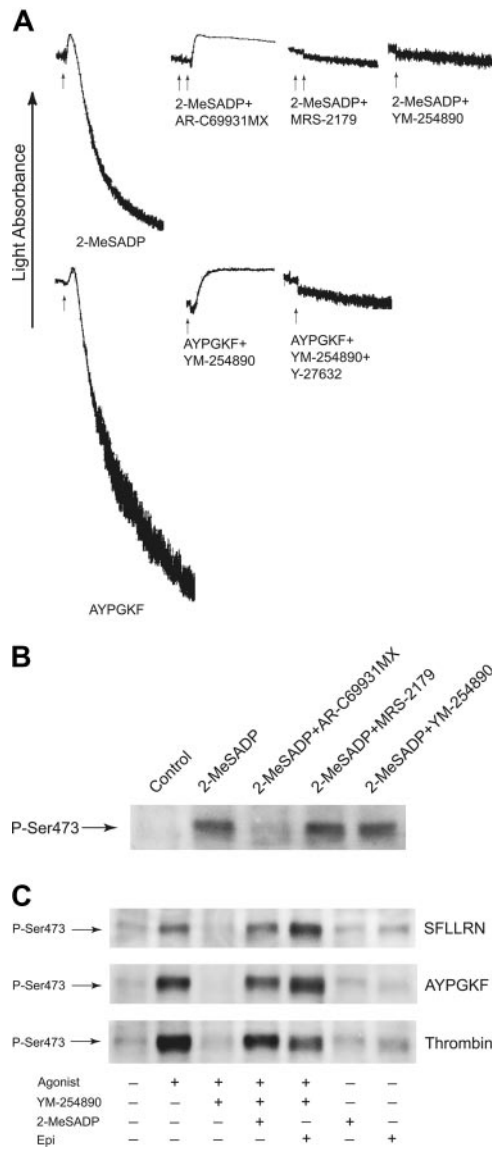


Figure 5. G_{12/13} signaling is required for thrombin- and thrombin receptor-activating peptide-induced Akt phosphorylation. (A) YM-254890 does not inhibit G_{12/13}-induced platelet shape change. Aspirin-treated, washed human platelets preincubated with 10 μM Y-27632 or 50 nM YM-254890 were stimulated with agonists as noted, and platelet aggregation was measured as described in "Materials and methods." The arrows indicate the addition of agonists and antagonists. Ten micromolar MRS-2179 and 1 μM AR-C69931MX was added to samples prior to stimulation with agonists. Tracings are representative of 3 experiments from 3 different donors. (B) Effect of YM-254890 on 2-MeSADP-induced Akt phosphorylation. Washed human platelets were stimulated with 1 μM 2-MeSADP in the presence or absence of reagents as indicated. Platelet proteins were separated by SDS-PAGE, Western blotted, and probed with anti-phospho-Akt (Ser⁴⁷³) antibody. (C) Selective G_{12/13} signaling restores PAR agonist-induced Akt phosphorylation in the presence of G_i/G_z signaling. Washed human platelets preincubated in the absence and presence of 50 nM YM-254890 were stimulated at 37°C for 5 minutes with either SFLLRN (10 μM), AYPGKF (500 μM), or thrombin (0.5 unit/mL). The addition of 1 μM 2-MeSADP or 10 μM epinephrine was made as indicated. Samples were separated by SDS-PAGE, Western blotted, and probed with anti-phospho-Akt (Ser⁴⁷³) antibody. Results are representative of 3 experiments.

In order to verify the relative contribution of G_q and G_{12/13} pathways to PAR-induced Akt phosphorylation, we investigated the effect of combined activation of G_{12/13} and G_i/G_z signaling in the presence of YM-254890. As shown in Figure 5C, SFLLRN-, AYPGKF-, and thrombin-induced Akt phosphorylation was abolished in the presence of YM-254890, confirming our previous study that PAR receptors cannot directly couple to G_i signaling.² However, supplemental G_i or G_z

signaling by 2-MeSADP or epinephrine in the presence of YM-254890 restored Akt phosphorylation to the extent achieved by these agonists. These results provide further evidence that G_q signaling does not have any direct effect on Akt phosphorylation and that G_{12/13} signaling downstream of PARs is essential to achieve normal extent of Akt phosphorylation by thrombin and thrombin receptor-activating peptides in platelets.

Role of Src family kinase in G_{12/13}- and G_i/G_z-mediated Akt phosphorylation

As the Rho kinase inhibitor Y-27632 did not block Akt phosphorylation, we suggest that there is a separate pathway downstream of G_{12/13} stimulation that contributes to potentiation of Akt phosphorylation. Immunoblot analysis of the phosphorylation of Src tyrosine 416, which correlates with Src activity, revealed that AYPGKF and thrombin were able to induce an increase in Src phosphorylation in Gα_q-deficient platelets (Figure 6A), suggesting that selective G_{12/13} activation results in activation of Src family kinase. SFLLRN, AYPGKF, and thrombin were also able to induce an increase in Src phosphorylation in the presence of 50 nM YM-254890 in human platelets (data not shown). Y-27632 had no effect on G_{12/13}-induced Src phosphorylation, which was blocked by a selective inhibitor of Src family tyrosine kinase PP2,³⁰ indicating that Rho kinase does not have any role in G_{12/13}-induced Src phosphorylation. To determine the role of Src kinase in Akt phosphorylation, we measured the effects of PP2 on Akt phosphorylation induced by combined G_{12/13} and G_i/G_z stimulation. As

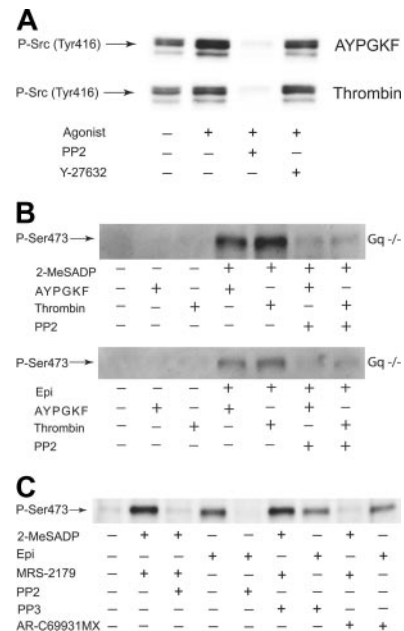


Figure 6. Src kinase-dependent Akt phosphorylation induced by combined G_{12/13} and G_i/G_z signaling in platelets. (A) Gα_q-deficient platelets preincubated with either 10 μM Y-27632 or 10 μM PP2 were stimulated at 37°C for 2 minutes with either AYPGKF (500 μM) or thrombin (0.5 unit/mL). Platelet proteins were separated by SDS-PAGE, Western blotted, and probed for phospho-Tyr⁴¹⁶ Src. (B) Gα_q-deficient platelets preincubated in the absence and presence of 10 μM PP2 were stimulated at 37°C for 5 minutes with agonists as noted. The reaction was stopped by the addition of 3 × SDS sample buffer. Platelet proteins were analyzed by Western blot analysis with anti-Ser(P)⁴⁷³ antibody. (C) Washed human platelets preincubated with 1 μM AR-C69931MX, 10 μM PP2, or 10 μM PP3 were stimulated at 37°C for 5 minutes with either 2-MeSADP (1 μM) or epinephrine (10 μM). Platelets stimulated with 2-MeSADP were preincubated with 10 μM MRS-2179. Platelet proteins were separated by SDS-PAGE, Western blotted, and probed with anti-phospho-Akt (Ser⁴⁷³) antibody. The Western blot analysis shown is a representative of 3 independent experiments.

shown in Figure 6B, Akt phosphorylation induced by simultaneous addition of either 2-MeSADP or epinephrine to thrombin or AYPGKF in G_{α_q} -deficient platelets was abolished in the presence of PP2. This suggests that a Src-dependent pathway in the $G_{12/13}$ signaling pathway plays an important role in this potentiation of Akt phosphorylation in the presence of G_i/G_z signaling in platelets.

Recently, we have suggested that both G_i and G_z signaling can cause Src activation in platelets.³¹ In order to verify the role of Src in Akt phosphorylation mediated by G_i/G_z stimulation, we tested the effect of PP2 and PP3, a control compound that does not inhibit tyrosine kinase activity, on Akt phosphorylation induced by G_i or G_z alone. Whereas PP2 abolished Akt phosphorylation induced by G_i and G_z signaling, the control compound PP3 had no effect (Figure 6C), indicating that Src activity plays a role in Akt phosphorylation mediated by selective G_i or G_z signaling.

Discussion

It has been shown that Akt is activated by various agonists including ADP, epinephrine, and thrombin in platelets.^{3,7,21,22} We have shown that ADP causes Akt phosphorylation independently of G_q stimulation, that thrombin- and thrombin receptor-activating peptide-induced Akt phosphorylation depends predominantly on G_i stimulation through P2Y₁₂ receptor activation by secreted ADP, and that epinephrine substitutes for the P2Y₁₂ receptor-mediated effects through G_z signaling cascade.³ However, the activation of P2Y₁₂ or α_{2A} -adrenergic receptors with 2-MeSADP and epinephrine, which couple to G_i and G_z , respectively, induces a much smaller extent of Akt phosphorylation than thrombin or thrombin receptor-activating peptides in platelets. Akt phosphorylation in stirred platelets has been shown to be biphasic, first through inside-out signaling and then through outside-in signaling.³² Thus, when aggregation is allowed to occur, Akt phosphorylation caused by thrombin could be stronger because thrombin can cause platelet aggregation to a greater extent than ADP and hence could cause stronger Akt phosphorylation. However, platelets were prevented from aggregating in our study to eliminate the contribution of outside-in signaling, and yet thrombin causes more Akt phosphorylation than ADP. Hence, it is conceivable that there might be some potentiating contribution of other G-protein signaling to Akt phosphorylation mediated by G_i or G_z pathways. In particular, we have focused on the potentiating effect of $G_{12/13}$ pathways on the Akt phosphorylation mediated by G_i/G_z pathways.

It has been shown that thrombin receptors couple to G_q and $G_{12/13}$ pathways.¹ YFLLRNP binds to PAR1 and causes shape change without activating the G_q pathways when used at low concentrations, and this shape change is mediated by the $G_{12/13}$ pathways. When human platelets are stimulated with a low concentration of YFLLRNP, YFLLRNP fails to cause Akt phosphorylation, suggesting that $G_{12/13}$ signaling is unable to induce Akt phosphorylation independently. However, YFLLRNP significantly increases the Akt phosphorylation in combination with selective activation of either G_i or G_z pathways, suggesting that $G_{12/13}$ pathways play an important role in potentiating Akt phosphorylation mediated by G_i/G_z pathways in human platelets. In addition, AYPGKF and thrombin fail to induce Akt phosphorylation in G_{α_q} -deficient platelets, confirming that $G_{12/13}$ signaling alone does not have any role in Akt phosphorylation in mouse platelets. When we selectively supplemented G_i or G_z signaling to AYPGKF- and thrombin-induced Akt phosphorylation in G_{α_q} -deficient platelets, Akt phosphorylation was dramatically potentiated. These results

further confirm that $G_{12/13}$ signaling has a potentiating contribution to G_i/G_z -induced Akt phosphorylation in both human and mouse platelets.

Even though G_i signaling represents the main signaling pathway leading to Akt phosphorylation, some degree of thrombin-induced Akt phosphorylation has been shown in platelets even in the absence of P2Y₁₂ receptor activation. Since thrombin receptors directly couple to G_q and activate G_i by secreted ADP,² it has raised the speculation that thrombin can mediate Akt phosphorylation by the G_q -dependent pathways that do not require secreted ADP.²² This may be due to the fact that they did not consider the contribution of $G_{12/13}$ signaling to Akt phosphorylation. Platelet granules contain several other agonists, such as chemokines and epinephrine, that could activate G_i/G_z pathways. Hence, thrombin could activate G_i/G_z pathways even in the absence of P2Y₁₂ receptor by these other granule contents. Taken together with our data showing that Akt phosphorylation mediated by G_z signaling with epinephrine was potentiated by selective activation of $G_{12/13}$ signaling, our study strongly suggests that this partial Akt phosphorylation that is independent of ADP secretion may not be due to the direct effect of G_q but to potentiating contribution of $G_{12/13}$ signaling to Akt phosphorylation mediated by G_z signaling. Our study also reveals that the level of PAR-induced Akt phosphorylation is not affected by the G_q -selective inhibitor YM-254890 when $G_{12/13}$ is supplemented with G_i/G_z signaling. These results further confirm that $G_{12/13}$, but not G_q , signaling downstream of PARs is essential to achieve a normal extent of Akt phosphorylation. It also confirms that the absence of Akt phosphorylation in the absence of G_q signaling is due not to the direct effect of G_q on Akt phosphorylation, but to the absence of G_i/G_z stimulation by secretion.

It is controversial whether coupling of PAR1 to G_i is direct or indirect.²³ Several groups have shown that PAR1 activates both G_q and G_i pathways whereas PAR4 couples to only the G_q pathway.³³⁻³⁵ If PAR1 can directly couple to G_i pathways independently of secretion, PAR1 could activate both $G_{12/13}$ and G_i pathways in the absence of G_q pathways in human platelets. Thus, we expect the normal Akt phosphorylation in response to PAR1 agonist SFLLRN in the absence of G_q pathways because coactivation of both $G_{12/13}$ and G_i pathways leads to normal Akt phosphorylation. In our hands, SFLLRN failed to induce Akt phosphorylation in the presence of YM-254890 (Figure 5C), suggesting that PAR1 can

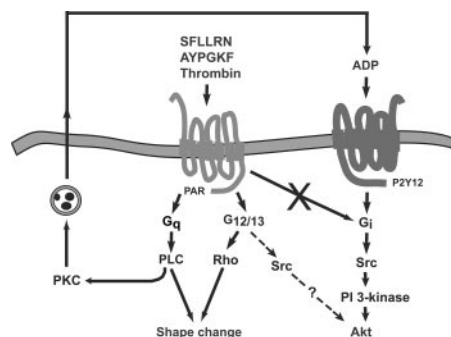


Figure 7. Outline of the signaling events leading to PAR-mediated protein kinase B/Akt activation in platelets. Thrombin and thrombin receptor-activating peptides induce Akt activation in platelets through secreted ADP that stimulates G_i signaling pathways in which Src kinase and PI 3-kinase play an important role. $G_{12/13}$ signaling potentiates Akt phosphorylation mediated by G_i pathways, and costimulation of $G_{12/13}$ and G_i signaling can induce the same extent of Akt phosphorylation achieved by PAR agonists in normal platelets. Activation of $G_{12/13}$ signaling might mediate its potentiating effects through Src kinase activation, and Src kinase plays an important role in ADP- and thrombin-mediated Akt phosphorylation. The mechanism and the specific member of Src family kinases contributing to Akt phosphorylation are not clear. Note that consistent with our previous observations, PAR stimulation in platelets does not lead to direct G_i activation.²

only couple to G_{12/13} and not G_i pathways in the absence of G_q in human platelets. Hence, this study confirms our previous findings² and, using a different downstream signaling molecule, clearly demonstrates that PAR1 does not directly stimulate G_i pathways in platelets.

It has been shown that the Rho kinase p160^{ROCK} is an important signaling molecule involved in G_{12/13}-induced shape change.¹⁸ Although the p160^{ROCK} inhibitor Y-27632 can block G_{12/13}-mediated platelet shape change, our study reveals that Y-27632 fails to inhibit Akt phosphorylation caused by concomitant G_{12/13} and G_i/G_z signaling, suggesting that the Rho A/Rho kinase (p160^{ROCK})-dependent signaling cascade does not play any significant role in potentiating Akt phosphorylation although most G_{12/13} to date has involved the Rho A/Rho kinase (p160^{ROCK})-dependent pathway. Furthermore, it suggests that there is a separate pathway downstream of G_{12/13} contributing to potentiation of Akt phosphorylation that is independent of the Rho A/Rho kinase pathway.

Src family tyrosine kinases participate in the signal transduction pathways of various hematopoietic cells.³⁶ Six of 8 members of Src family kinases are present in platelets.³⁷ It has been shown that the Src family kinases Lyn and Fyn play a role in glycoprotein VI-mediated platelet activation.³⁸ It has also been described that stimulation of G-protein-coupled receptors induces activation of Src kinases.³⁹ However, the functional roles of Src family kinases in platelet activation have not been well defined. We show here that thrombin receptor-mediated activation of G_{12/13} leads to tyrosine phosphorylation of Src family kinase in platelets, suggesting that Src kinase is an important downstream molecule of G_{12/13} signaling. Consistent with our observations, it has been shown that constitutively active G_{α12}-expressing MDCK (Madin-Darby canine kidney) cells have an increased Src tyrosine kinase activity.⁴⁰

It is also shown that G_{α13} stimulates Src kinase activity in Rat-1 fibroblasts, suggesting the involvement of a Src-dependent pathway in G_{α13}-mediated cell proliferation and transformation.⁴¹ We then measured the effect of the Src kinase inhibitor PP2 on concomitant G_{12/13}⁻ and G_i/G_z-mediated Akt phosphorylation in G_{αq}-deficient platelets and found that PP2 blocked Akt phosphorylation induced by selective activation of either G_i or G_z signaling in the presence of G_{12/13} pathways. It has recently been reported that Src kinase is activated primarily by the G_q-coupled P2Y₁ receptor in platelets,⁴² but G_q-mediated Src activation may not have any contribution to Akt phosphorylation since G_q signaling does not have any role in Akt phosphorylation in platelets. Our previous work has shown that both G_i and G_z signaling can cause Src activation,³¹ and we have found that the Src kinase inhibitor PP2 inhibits G_i/G_z-mediated Akt phosphorylation. Thus, our study demonstrates that Src kinase downstream of G_i and G_{12/13}, but not G_q, is a key signaling molecule in the thrombin-mediated Akt phosphorylation, and G_{12/13} signaling cascade might mediate its potentiating effect on Akt phosphorylation through Src-dependent pathways (Figure 7). Further studies are required to identify which member of Src family kinases plays a role in this Akt phosphorylation.

In conclusion, we demonstrate that G_{12/13}, but not G_q, signaling downstream of PARs is essential to achieve normal Akt phosphorylation by thrombin and thrombin receptor-activating peptides in platelets. While G_q signaling does not have a direct effect on Akt phosphorylation, it contributes to G_i stimulation by secreted ADP that is also required for thrombin-induced Akt phosphorylation. Furthermore, Src family kinases are essential for ADP- and thrombin-induced Akt phosphorylation in platelets.

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