Akt Activation in Platelets Depends on $G_i$ Signaling Pathways*

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The serine-threonine kinase Akt has been established as an important signaling intermediate in regulating cell survival, cell cycle progression, as well as agonist-induced platelet activation. Stimulation of platelets with various agonists including thrombin results in Akt activation. As thrombin can stimulate multiple G protein signaling pathways, we investigated the mechanism of thrombin-induced activation of Akt. Stimulation of platelets with a PAR1-activating peptide (SFLRN), PAR4-activating peptide (AYPGKF), and thrombin resulted in Thr$^{308}$ and Ser$^{473}$ phosphorylation of Akt, which results in its activation. This phosphorylation and activation of Akt were dramatically inhibited in the presence of AR-C69931MX, a P2Y$_{12}$ receptor-selective antagonist, or GF 109203X, a protein kinase C inhibitor, but Akt phosphorylation was restored by supplemental G$_i$ or G$_z$ signaling. Unlike wild-type mouse platelets, platelets from G$_{o-}$-deficient mice failed to trigger Akt phosphorylation by thrombin and AYPGKF, whereas Akt phosphorylation was not affected by these agonists in platelets from mice that lack P2Y$_{1}$ receptor. However, ADP caused Akt phosphorylation in G$_{o-}$ and P2Y$_{1-}$deficient platelets, which was completely blocked by AR-C69931MX. In contrast, ADP failed to cause Akt phosphorylation in platelets from mice treated with clopidogrel, and thrombin and AYPGKF induced minimal phosphorylation of Akt, which was not affected by AR-C69931MX in these platelets. These data demonstrate that G$_i$ but not G$_o$ or G$_{2/12}$ signaling pathways are required for activation of Akt in platelets, and G$_i$ signaling pathways, stimulated by secreted ADP, play an essential role in the activation of Akt in platelets.

Akt (also known as protein kinase B (PKB)) is a 57-kDa serine/threonine kinase, which is the cellular homologue of the viral oncogene v-akt of the acutely transforming retrovirus Akt8 (1). Akt plays an important role in mediating the anti-apoptotic effect of many growth factors, and it is also overexpressed in several cancer forms (2, 3). Akt contains a pleckstrin homology domain adjacent to a centrally located catalytic domain that is connected to a short C-terminal tail (4). The catalytic domain of Akt contains a Thr$^{308}$ phosphorylation site and displays high homology to the catalytic domains of cAMP-dependent protein kinase A and protein kinase C. A second phosphorylation site, Ser$^{473}$, is located in the C-terminal tail (4). There are at least three different Akt isoforms identified in humans, which display more than 80% sequence homology and are named Akt1 (PKB$\alpha$), Akt2 (PKB$\beta$), and Akt3 (PKB$\gamma$) (5). It has been shown that Akt1 (PKB$\alpha$) and Akt2 (PKB$\beta$) are expressed in human platelets, whereas Akt3 (PKB$\gamma$) is not present in human platelets (6). Both translocation of Akt to cell membranes and phosphorylation of both Thr$^{308}$ and Ser$^{473}$ are required for full enzyme activity. Akt is activated by various agonists including platelet-derived growth factor, epidermal growth factor, insulin, nerve growth factor, U46619 (a thromboxane A$_2$ analogue), convulxin (a glycoprotein VI agonist), and thrombin (6–8). Phosphatidylinositol (PI) 3-kinase inhibitors prevent activation of Akt (9), indicating that PI 3-kinase is an upstream regulator of Akt. PI 3-kinase products PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ trigger the simultaneous phosphorylation of Akt by phosphatidylinositol-dependent kinases (PDK) 1 and 2. Membrane attachment of Akt is a prerequisite for phosphorylation of PDK1 and 2. PDK1 has been shown to phosphorylate Thr$^{308}$ of Akt1, and the phosphorylation of Ser$^{473}$ is independent of Thr$^{308}$ (10). Ser$^{473}$ is phosphorylated by a kinase whose identity is still obscure but has been proposed as PKD2. Some researchers propose that autophosphorylation at the Ser$^{473}$ site is the mechanism by which this serine residue is phosphorylated (11). Recently, the integrin-linked kinase has been shown to be an important regulator of Ser$^{473}$ phosphorylation (12). In addition to the PI 3-kinase-mediated activation of Akt, a phospholipase C$_{\beta}$-dependent pathway via a calcium-dependent PKC subtype has been known to increase activity of Akt1 by thrombin (6). Activation of PKC results in the PI 3-kinase-independent increase in Akt1 activity by selective phosphorylation of Ser$^{473}$, but selective phosphorylation of Ser$^{473}$ is insufficient for full PKB activation. Yan et al. (13) have also identified Ca$^{2+}$-dependent regulation of Akt in which Ca$^{2+}$/calmodulin-dependent protein kinase kinase can activate Akt in vitro through phosphorylation of Thr$^{308}$.

It is well established that ADP-induced platelet aggregation requires concomitant signaling from both the P2Y$_{12}$ and P2Y$_{12}$ receptors that couple to G$_i$ and G$_z$, respectively (14). Thrombin mediates its cellular effects primarily through a family of G$_i$-coupled protease-activated receptors (PARs), and we have

bovine serum albumin; PMA, phorbol 12-myristate 13-acetate; PtdIns, phosphatidylinositol; 2-MeSADP, 2-methylthio-ADP.

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§ The abbreviations used are: PKB, protein kinase B; G$_i$, heterotrimeric GTP-binding protein which stimulates phospholipase C; G$_o$, heterotrimeric GTP-binding protein which inhibits adenyl cyclase; G$_{12/13}$, heterotrimeric GTP-binding proteins 12 and 13; P2Y$_{12}$, platelet ADP receptor coupled to stimulation of phospholipase C; PAR, protease-activated receptor; PKC, protein kinase C; PI 3-kinase, phosphatidylinositol 3-kinase; GSK-3, glycogen synthase kinase-3; PDK, phosphatidylinositol-dependent kinase; PIP$_2$, platelet-rich plasma; dimethyl-BAPTA, 5,5′-dimethyl-bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; RT, room temperature; BSA, bovine serum albumin; PMA, phorbol 12-myristate 13-acetate; PtdIns, phosphatidylinositol; 2-MeSADP, 2-methylthio-ADP.
Akt activation is largely dependent on signaling through the thrombin- and thrombin receptor-activating peptide-mediated activation of Akt. In human and mouse platelets, we have found that platelets is stimulated by Gq-o rG i-coupled pathways. signal transduction mechanisms of Akt activation are less actions as a downstream effector of PI 3-kinase. However, the other reagents were reagent-grade, and deionized water was used throughout.

Aggregometry—Aggregation of 0.5 ml washed platelets was analyzed using a P.I.C.A. lumiggregometer (Chrono-log Corp., Havertown, PA). Aggregation was measured using light transmission under stirring conditions (900 rpm) at 37 °C. Each sample was allowed to aggregate for at least 3 min. The chart recorder (Kipp and Zonen, Bohemia, NY) was set for 0.2 mm/s.

Measurement of Akt Phosphorylation—Platelets were stimulated with agonists under non-stirring conditions for the appropriate time, and the reaction was stopped by the addition of 3× SDS sample buffer. In some experiments, GF 109203X (10 μM), a PKC inhibitor, and di-methyl-BAPTA (10 μM), a calcium chelator, were added and incubated for 5 min at 37 °C without stirring before agonist stimulation. Platelet samples were boiled for 5 min, and proteins were separated on 10% SDS-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 60 min at RT, and membranes were incubated overnight at 4 °C with primary antibody (1:1000 in TBST, 2% BSA) with gentle agitation. After three washes for 5 min each with TBST, the membranes were probed with the alkaline phosphatase-labeled goat anti-rabbit IgG (1:5000 in TBST, 2% BSA) for 1 h at RT. After additional washing steps, membranes were then incubated with a CDP-Star® chemiluminescent substrates for 10 min at RT, and immunoreactivity was detected using Fujifilm Luminescent Image Analyzer (model LAS-1000 CH, Japan).

In Vitro Kinase Assay for Akt—Akt kinase activity was measured using a modification of the Akt kinase assay kit from Cell Signaling Technology (Beverly, MA). Briefly, platelets were stimulated with agonists under non-stirring conditions for 3 min, and the reaction was stopped by the addition of 50% ice-cold cell lysis buffer (100 mM Tris (pH 7.5), 750 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5% Triton X-100, 12.5 mM sodium pyrophosphate, 5 mM β-glycerol phosphate, 5 mM Na3VO4, 5 μg/ml leupeptin, and 5 mM phenylmethylsulfonyl fluoride). Resuspended immobilized Akt antibody slurry (20 μl) was added to 200 μl of platelet lysates to selectively immunoprecipitate Akt from the lysate with gentle rocking 2 h at 4 °C. The pellet was washed twice with 500 μl of 1× lysis buffer and twice with 500 μl of 1× kinase buffer (25 mM Tris (pH 7.5), 5 mM β-glycerol phosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, and 10 mM MgCl2). The immunoprecipitated pellet was then incubated with 40 μl of 1× kinase buffer supplemented with 200 μM ATP and 1 μM of GSK-3 fusion protein for 30 min at 30 °C allowing immunoprecipitated Akt to phosphorylate GSK-3. The reaction was terminated with 20 μl of 3× SDS sample buffer. Samples were boiled for 5 min and loaded on 14% SDS-PAGE gel. Akt-induced phosphorylation of GSK-3 was detected by Western blotting using phospho-GSK-3a/b (Ser21/9) antibody.

RESULTS

SFLLRN, AYPGKF, and Thrombin Induce Akt Phosphorylation in a Dose- and Time-dependent Manner—It has been shown that thrombin induces phosphorylation of Akt in platelets (6, 19). To determine the kinetics of Akt phosphorylation, Ser21/9 phosphorylation was monitored over a time range of 0.5–20 min. Fig. 1A illustrates that a rapid increase in Akt phosphorylation in response to thrombin was detectable as early as 30 s after stimulation. The level of phosphorylation peaked at around 3 min and remained stable after 20 min of stimulation.

Because thrombin signals via PAR1 and PAR4 receptors in human platelets, we examined Akt phosphorylation in response to SFLLRN, a PAR1-activating peptide, AYPGKF, a PAR4-activating peptide, and thrombin. We exposed platelets to different concentrations of the agonists, and Ser21/9 phosphorylation was measured at 3 min after the addition of agonist. Fig. 1B shows that all three agonists induced a concentration-dependent increase in Akt phosphorylation. An increase in Ser21/9 phosphorylation was detectable at concentrations above 3 μM SFLLRN, 200 μM AYPGKF, or 0.1 unit/ml thrombin; furthermore, higher concentrations revealed further phosphorylation that peaked at concentrations above 5 μM SFLLRN, 500 μM AYPGKF, or 0.5 unit/ml thrombin. A similar pattern of concentration-dependent phosphorylation of Thr308 in response to SFLLRN, AYPGKF, and thrombin was also detected (data not shown).

Effect of Secretion and Gi Signaling Pathways in Akt Phosphorylation and Activation by SFLLRN, AYPGKF, and Thrombin in Human Platelets—Immunoblot analysis revealed that
Akt is phosphorylated on Ser\(^{473}\) and Thr\(^{308}\) in response to SFLLRN, AYPGKF, and thrombin in human platelets (Fig. 2). SFLLRN induced a lower extent of Ser\(^{473}\) and Thr\(^{308}\) phosphorylation, whereas AYPGKF induced a similar extent of Akt phosphorylation as thrombin. These results are consistent with a previous study in which phosphorylation of Thr\(^{308}\) and Ser\(^{473}\) induced by SFLLRN in human platelets is 50% of the response induced by thrombin (6). However, they failed to activate Akt completely in response to the PAR4-activating peptide GYPGKF, which is in contrast with our data, wherein dual phosphorylation and activation of Akt occur in response to AYPGKF. These results indicate that both PAR1 and PAR4 receptors can induce concurrent phosphorylation of Ser\(^{473}\) and Thr\(^{308}\), and AYPGKF and thrombin are more potent activators of Akt phosphorylation than SFLLRN.

Activation of PAR1 or PAR4 causes release of dense granule contents, and the released ADP activates the G\(_i\) pathways through stimulation of the P2Y\(_{12}\) receptor (15). To determine whether PAR1 and PAR4 receptors can activate Akt independently of secreted ADP and G\(_i\) signaling pathways, we utilized several complementary approaches to evaluate the role of G\(_i\) signaling in Akt phosphorylation. 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 (20, 21). To assess the role of secreted ADP to the Akt activation upon stimulation of platelets with SFLLRN, AYPGKF, and thrombin, we used GF 109203X (22), a selective inhibitor of PKC isoforms, and AR-C69931MX, an antagonist at the G\(_i\)-coupled P2Y\(_{12}\) receptor (23). In the presence of AR-C69931MX or GF 109203X, we found that both Ser\(^{473}\) and Thr\(^{308}\) phosphorylations were dramatically inhibited suggesting the important role of secretion and G\(_i\)-coupled P2Y\(_{12}\) receptor in Akt phosphorylation. It has been shown that intracellular calcium increases also have a role in platelet secretion (24). To investigate the role of calcium in Akt phosphorylation, the high affinity calcium chelator dimethyl-BAPTA was used to block intracellular calcium increases. The presence of dimethyl-BAPTA significantly, but not completely, inhibited both Ser\(^{473}\) and Thr\(^{308}\) phosphorylation in response to all agonists. As dimethyl-BAPTA interferes with platelet secretion, inhibition of Akt phosphorylation in dimethyl-BAPTA-treated platelets might be due to the loss of G\(_i\) stimulation by secreted ADP. It is known that PI 3-kinase is the main upstream regulator of Akt, and PI 3-kinase plays an important role in the G\(_i\)-dependent potentiation of platelet aggregation. SFLLRN-, AYPGKF-, and thrombin-induced Akt phosphorylation were inhibited in the presence of the PI 3-kinase inhibitor, LY294002, suggesting that phosphorylation of Akt is PI 3-kinase dependent. These results indicate that secreted ADP and G\(_i\)-coupled P2Y\(_{12}\) receptors are important in SFLLRN-, AYPGKF-, and thrombin-induced phosphorylation of Akt, and PI 3-kinase is a key element in the G\(_i\)-coupled pathway for Akt phosphorylation.

It has been known that phosphorylation of both Thr\(^{308}\) and Ser\(^{473}\) is required for full activation of Akt (4). Akt activity was
measured by in vitro kinase assay that detects phosphorylation of GSK-3 induced by immunoprecipitated Akt. Phosphorylation of GSK-3 was measured by Western blotting using a phospho-GSK-3α/β (Ser21/9) antibody, and GSK-3α/β kinase activity has been shown to be negatively regulated by Akt via phosphorylation at Ser21/9. Our results of Akt kinase activity in human platelets (Fig. 3) correspond to Akt phosphorylation shown in Fig. 2. In the presence of dimethyl-BAPTA, activation of Akt in response to SFLLRN, AYPGKF, and thrombin was diminished but not completely abolished. Furthermore, Akt activation was nearly abolished in the presence of AR-C69931MX and GF 109203X confirming that Gi stimulation by secretion is essential for Akt phosphorylation in response to ADP, AYPGKF, and thrombin. However, as in wild-type mouse platelets, ADP caused Akt phosphorylation in the presence or absence of MRS-2179, a P2Y1 receptor antagonist (26), whereas ADP-activated Akt phosphorylation was abolished by AR-C69931MX suggesting the essential role of Gq-coupled P2Y12 receptor in Akt activation in platelets. This provides further evidence that the absence of Akt phosphorylation in Gq-, lacking P2Y1, or wild-type mice. In wild-type littermates, AYPGKF and thrombin caused phosphorylation of Ser473, which was significantly inhibited in the presence of AR-C69931MX. However, AYPGKF- and thrombin failed to phosphorylate Akt at Ser473 in platelets from Gαq knockout mice (Fig. 5A). It has been shown that platelets from Gαq knockout mice fail to release their granule contents in response to thrombin (25), and we have shown that PAR1 and PAR4 fail to directly couple to Gq in platelets and depend on secreted ADP for Gq stimulation (15). To determine whether absence of Akt phosphorylation in Gαq-, deficient platelets is due to direct effect of Gq on Akt or indirectly through the absence of Gq stimulation by secretion, Akt phosphorylation in response to ADP was compared in Gαq-, deficient mice and their wild-type littermates (Fig. 5B). In both wild-type and Gαq-, deficient mouse platelets, ADP caused Akt phosphorylation in the presence or absence of MRS-2179, a P2Y1 receptor antagonist (26), whereas ADP-activated Akt phosphorylation was abolished by AR-C69931MX suggesting the essential role of Gq-coupled P2Y12 receptor in Akt activation in platelets. This provides further evidence that the absence of Akt phosphorylation in Gαq-, deficient platelets in response to AYPGKF and thrombin is due to the absence of Gq stimulation by secretion. A similar approach was used with P2Y12-, deficient mouse platelets, and Akt phosphorylation was measured in response to ADP, AYPGKF, and thrombin. P2Y12- deficient mouse platelets do not aggregate in response to ADP with abolished calcium response, whereas P2Y12 receptor coupled to the inhibition of adenyl cyclase was unaffected (17, 18). Consistent with the published results (17, 18), platelets from the P2Y1 receptor-deficient mice, developed in our laboratory, failed to aggregate in response to ADP (Fig. 6A). However, AYPGKF-induced aggregation was unaffected in platelets from these mice (not shown). The presence or absence of P2Y1 receptor did not affect the activity of Akt in response to ADP, AYPGKF, and thrombin. However, as in wild-type mouse platelets, AR-C69931MX also abolished ADP-induced Akt phosphorylation in the P2Y1,-deficient mouse platelets (Fig. 6B). The absence of P2Y1 did not affect the phosphorylation of Akt in response to ADP, providing further evidence that Gαq is not required for
Akt activation in platelets whereas G\textsubscript{i}-coupled P2Y\textsubscript{12} receptor is necessary and sufficient for the stimulation of Akt.

**Akt Phosphorylation in Clopidogrel-dosed Mouse Platelets** — Clopidogrel is a thienopyridine derivative, which blocks activation of platelets by selectively targeting P2Y\textsubscript{12} receptor (27). Clopidogrel has been shown to selectively inhibit ADP-induced platelet aggregation and inhibition of adenylyl cyclase in rat platelets 2 h after oral administration at doses ranging from 1 to 25 mg/kg (28). In order to confirm the role of secreted ADP and the P2Y\textsubscript{12} receptor in Akt activation, we used platelets from mice dosed with clopidogrel and compared phosphorylation of Ser\textsuperscript{473} in response to ADP, AYPGKF, and thrombin. The inhibitory effects of ADP on PGE\textsubscript{1}-stimulated cAMP levels were abolished in clopidogrel-dosed (30 mg/kg/day for 2 days) mouse platelets, whereas platelet calcium response by ADP was not affected by clopidogrel treatment (data not shown). In platelets from mice dosed with clopidogrel, ADP-induced plate-
let aggregation was completely inhibited without an effect on shape change (Fig. 7A). As shown in Fig. 7B, ADP caused an increase in Ser\(^{473}\) phosphorylation in the presence or absence of MRS-2179 in control mouse platelets, and the presence of AR-C69931MX significantly inhibited phosphorylation at this site. However, ADP failed to induce Akt phosphorylation in platelets from mice dosed with clopidogrel confirming that G\(_i\)-coupled P2Y\(_{12}\) receptor is essential for Akt activation. The level of Ser\(^{473}\) phosphorylation was nearly abolished in response to AYPGKF and thrombin in clopidogrel-dosed mouse platelets, which was not affected in the presence of AR-C69931MX. The residual phosphorylation of Akt could be due to G\(_i\) stimulation by secreted chemokines because \(\alpha\) granules can release small quantities of chemokines that can activate G\(_i\) pathways (29). Taken together, these results confirm that G\(_i\) stimulation is necessary for Akt activation, and the G\(_i\)-coupled P2Y\(_{12}\) receptor, which is stimulated by secreted ADP, has a significant role in Akt activation by thrombin and thrombin receptor-activating peptides.

**PKC-dependent Akt Phosphorylation in Platelets Depends on Secretion**—In order to clarify the role of secreted ADP, we evaluated the effect of P2Y\(_{12}\) receptor antagonist on Akt phosphorylation in response to the PKC activator PMA. Immunoblot analysis revealed that Akt is phosphorylated on both Ser\(^{473}\) and Thr\(^{308}\) in response to PMA in human platelets (Fig. 8). AR-C69931MX inhibited the PMA-induced Akt phosphorylation suggesting the important role of the G\(_i\)-coupled P2Y\(_{12}\) receptor activation in Akt phosphorylation. This conclusion was substantiated in experiments with epinephrine, where activation of separate G\(_i\)-coupled receptor by epinephrine restored Akt phosphorylation when platelets are stimulated in the presence of AR-C69931MX. As shown in Fig. 8, phosphorylation of Thr\(^{308}\) was more dramatically inhibited by the AR-C69931MX than Ser\(^{473}\) phosphorylation, suggesting that G\(_i\) signaling might play a differential role in the regulation of these two phosphorylation sites. These results indicate that secreted ADP and G\(_i\)-coupled P2Y\(_{12}\) receptors are important in activation of Akt induced by direct activation of PKC with PMA.

**Restoration of SFLLRN-, AYPGKF-, or Thrombin-induced Akt Phosphorylation Blocked by GF 109203X or AR-C69931MX**—In order to verify the important role of secreted ADP in SFLLRN-, AYPGKF-, or thrombin-induced Akt phosphorylation, we investigated the effect of selective activation of G\(_i\)- or G\(_z\)-coupled receptor stimulation in the presence of GF 109203X. As shown in Fig. 9A, selective activation of G\(_i\)- or G\(_z\)-coupled receptor by ADP or epinephrine reversed the effects of secretion blockade on SFLLRN-, AYPGKF-, or thrombin-induced Akt phosphorylation. Epinephrine also reversed the inhibitory effects of AR-C69931MX on the Akt phosphorylation induced by these agonists (Fig. 9B). These results provide further evidence that P2Y\(_{12}\)-dependent signaling by secreted ADP is largely responsible for Akt activation by SFLLRN-, AYPGKF-, or thrombin.

**DISCUSSION**

It has been shown that stimulation of platelets with various agonists including thrombin results in Akt activation. Although it is known that Akt functions as one of several downstream effectors of PI 3-kinase (9, 30), the molecular mechanism involved in Akt activation in platelets is not well established. Thus we have investigated the signaling events involved in Akt activation mediated by thrombin and thrombin receptor-activating peptides in platelets. In particular, we focused on the role of secretion and G\(_i\) pathways in Akt activation utilizing several complementary approaches including utilizing selective P2 receptor antagonists and PI 3-kinase inhibitors, blocking granule secretion, and the use of platelets from mice lacking Gr\(_\alpha\)q, lacking P2Y\(_1\), or treated with clopidogrel. Our study has led to the conclusion that G\(_i\) signaling is required for activation of Akt in platelets and that G\(_i\) stimulation by secreted ADP is essential for Akt activation in response to throm-
bin and thrombin receptor-activating peptides, which activate G<sub>q</sub> and G<sub>i</sub>/G<sub>i2</sub>/G<sub>i12</sub> pathways.

Our experiments reveal that Akt was phosphorylated on Ser<sup>473</sup> and Thr<sup>308</sup> in response to SFLLRN, AYPGKF, and thrombin, and this response was found to be time-dependent (Fig. 1) confirming dual phosphorylation of Akt in platelets by these agonists. Kroner et al. (6) reported that phosphorylation of Thr<sup>308</sup> and Ser<sup>473</sup> induced by SFLLRN is 50% of the response induced by thrombin, whereas GYPGKF fails to activate Akt completely. This observation with GYPGKF is in contrast with our data, which shows dual phosphorylation of Akt in response to AYPGKF. This seems due to the difference in PAR4-activating peptides wherein AYPGKF has shown to be a selective and more potent PAR4-activating peptide (31). Akt activity in stirred platelets stimulated with thrombin has been shown to be biphasic, which mirrors the biphasic activation of PI 3-kinase, where PtdIns(3,4,5)P<sub>3</sub> is generated upon initial stimulation and PtdIns(3,4)P<sub>2</sub> is generated later in platelet aggregation (19). Because platelets were prevented from aggregating during kinetic studies of Akt phosphorylation, a biphasic activation pattern of Akt was not observed in our study.

Thrombin mediates its cellular effects primarily through a family of G<sub>q</sub>-coupled PARs, whereas the G<sub>i</sub> pathway is activated by the released ADP through activation of the P2Y<sub>12</sub> receptor (15). However, it has not been clear whether activation of Akt by thrombin is through G<sub>q</sub> or G<sub>i</sub> pathways. Akt has been shown to become activated in response to mutationally activated G<sub>q</sub>/H<sub>H9251</sub> and G<sub>i</sub>/H<sub>H9251</sub> in COS cells (32). Recently, G<sub>q</sub>-coupled receptors have been shown to activate Akt in astrocytoma cells, which involves phospholipase C- and Ca<sup>2+</sup>/H<sub>11001</sub>-mediated PI 3-kinase activation (33). Others reported that Gi-coupled receptors can activate Akt by G protein heterodimers in a PI 3-kinase-dependent manner, whereas activated G<sub>q</sub> has a prominent inhibitory effect on Akt activation in COS and HEK-293 cells (34). A recent report (35) indicates that activated G<sub>q</sub> inhibits p110<sub>α</sub> PI 3-kinase activity and blocks Akt activation in HEK-293 cells. To determine whether thrombin and thrombin receptor-activating peptides can activate Akt independently of se-

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**Fig. 8.** Phosphorylation of Akt induced by direct activation of PKC with PMA. Platelets preincubated with 1 μM AR-C69931MX, 10 μM GF 109203X, or 25 μM LY294002 were stimulated at 37 °C for 3 min with PMA (0.5 μM). The addition of epinephrine (20 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 (Ser<sup>473</sup> and Thr<sup>308</sup>) antibody. The data shown are representative of three independent experiments.

**Fig. 9.** Restoration of Akt phosphorylation by selective stimulation of either G<sub>q</sub>- or G<sub>i</sub>-coupled receptor. A, platelets preincubated in the absence and presence of 10 μM GF 109203X were stimulated at 37 °C for 3 min with either SFLLRN (10 μM), AYPGKF (500 μM), or thrombin (0.5 unit/ml). The addition of 2-MeSADP (1 μM) or epinephrine (20 μM) was made as indicated. B, platelets preincubated with 1 μM AR-C69931MX were stimulated at 37 °C for 3 min with either SFLLRN (10 μM), AYPGKF (500 μM), or thrombin (0.5 unit/ml) in the absence and presence of 20 μM epinephrine. 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 (Ser<sup>473</sup>) antibody. Results are representative of three experiments.
creted ADP and G\textsubscript{i} signaling pathways, we have used GF 109203X, a selective inhibitor of PKC isofoms, and AR-C69931MX, an antagonist at the G\textsubscript{i}-coupled P2Y\textsubscript{12} receptor. PKC has been shown to play a major role in the induction of platelet secretion. In the presence of GF 109203X, Akt activation in response to SFLLRN, AYPGKF, and thrombin was dramatically blocked, suggesting that these agonists depend on secretion to activate Akt. Inhibition of Akt activation by AR-C69931MX also indicates an essential role of G\textsubscript{i}-coupled P2Y\textsubscript{12} receptor by secreted ADP in PAR1- and PAR4-induced Akt activation. Consistent with our observations, Tilton et al. (36) demonstrated that G\textsubscript{i}-coupled receptors can activate Akt by PI 3-kinase in human phagocytes. The observation that activation of Akt was inhibited in the presence of dimethyl-BAPTA suggests the involvement of the calcium-dependent PKC subtypes.

Previous reports show calcium-dependent regulation of Akt by Ca\textsuperscript{2+}/calmodulin-dependent protein kinase kinase on the Thr\textsuperscript{308} phosphorylation (13) or by calcium-dependent PKC on the level of Ser\textsuperscript{473} phosphorylation (6). The latter observation is in contrast with our data, which show the inhibition of both Thr\textsuperscript{308} and Ser\textsuperscript{473} phosphorylation by dimethyl-BAPTA. Because it is known that intracellular calcium increase has a role in platelet secretion (37), it is conceivable that dimethyl-BAPTA interferes with platelet secretion resulting in the inhibition of G\textsubscript{i} stimulation by secreted ADP and inhibits both Thr\textsuperscript{308} and Ser\textsuperscript{473} phosphorylation. The role of calcium on Akt activation is further defined in the experiment by using platelets from G\textsubscript{\alpha}\textsubscript{q}- and P2Y\textsubscript{12}-deficient mice.

Some degree of thrombin-induced Akt phosphorylation occurs even in the absence of P2Y\textsubscript{12} signaling. There are two possibilities. First, platelet granules contain several other agonists, such as chemokines, that could activate G\textsubscript{i} pathways (29). Thus, thrombin could activate G\textsubscript{i} pathways even in the absence of P2Y\textsubscript{12} receptor by these other G\textsubscript{i}-activating agonists. This could result in a small Akt phosphorylation by thrombin even in the absence of the P2Y\textsubscript{12} receptor. The second possibility is that thrombin could phosphorylate Akt to a very small extent independently of G\textsubscript{i} pathways. Our experiments do not distinguish these two scenarios.

Even though the role of PI 3-kinase in Akt activation is well known (9, 30), several reports have suggested a PI 3-kinase-independent activation of Akt. Stimulation of \beta\textsubscript{\alpha}-adrenergic receptor, which couples to both G\textsubscript{i} and G\textsubscript{\alpha} proteins (38), has been shown to cause the stimulation of Akt which is not dependent on PI 3-kinase in rat adipose cells (39). It is also shown that cAMP-elevating agents or cellular stress such as heat shock and hyperosmolarity can increase the activity of Akt through a pathway that is independent of PI 3-kinase (40, 41). Our study reveals that PI 3-kinase activation is necessary for thrombin- and thrombin receptor-activating peptide-mediated activation of Akt in platelets. Our data are also consistent with other studies, where thrombin-induced Akt activation is dependent on PI 3-kinase activity in platelets (6), airway smooth muscle cells (42), and IIC9 cells (43). It is conceivable that the alternative pathways that stimulate PI 3-kinase-independent activation of Akt are present in some other cell preparations, which are activated by agonists linked to G\textsubscript{i}-coupled receptors.

Our data using knockout mouse platelets demonstrate that ADP induces Akt phosphorylation in P2Y\textsubscript{12}- or G\textsubscript{\alpha}\textsubscript{q}-deficient platelets. However, ADP-induced Akt phosphorylation was completely abolished in the presence of AR-C69931MX in these platelets further indicating that G\textsubscript{\alpha}\textsubscript{q} is not required for Akt activation in platelets, whereas G\textsubscript{\alpha}-coupled P2Y\textsubscript{12} receptor is necessary and sufficient for activation of Akt (Figs. 5B and 6B). It is known that thrombin receptors couple to G\textsubscript{\alpha} and G\textsubscript{12/13} pathways (44). However, AYPGKF and thrombin fail to induce Akt phosphorylation in G\textsubscript{\alpha}\textsubscript{q}-deficient platelets (Fig. 5A), suggesting that G\textsubscript{12/13} pathways do not have any role in Akt activation in platelets. It also has been shown that platelets from G\textsubscript{\alpha}\textsubscript{q}-deficient mice fail to release their granule contents in response to thrombin (25), and we have shown that thrombin fails to directly couple to G\textsubscript{\alpha} in the absence of secretion (15). Thus, the complete inhibition of Akt phosphorylation by AYPGKF and thrombin in G\textsubscript{\alpha}-deficient platelets may not be due to the direct inhibitory effect of G\textsubscript{\alpha} but due to the absence of secretion to stimulate G\textsubscript{\alpha}, supporting the idea that thrombin-induced Akt activation depends on secretion/G\textsubscript{\alpha} pathways.

Clopidogrel has been shown to selectively target the P2Y\textsubscript{12} receptor (27). To confirm the role of secreted ADP and G\textsubscript{i} stimulation in Akt activation, we have used clopidogrel-dosed mouse platelets. In these platelets, ADP fails to induce Akt phosphorylation confirming that G\textsubscript{i}-coupled P2Y\textsubscript{12} receptor is required for Akt activation in platelets. Furthermore, AYPGKF and thrombin fail to cause significant Akt phosphorylation. Thus, AYPGKF and thrombin cannot cause Akt stimulation independently of secreted ADP. These findings correlate with previous findings and provide further evidence that G\textsubscript{i} stimulation by secreted ADP is required for thrombin- and thrombin receptor-activating peptide-induced Akt activation in platelets.

The fact that ADP can cause Akt phosphorylation and, in the absence of secreted ADP, thrombin cannot cause (strong) Akt phosphorylation is surprising, as thrombin is a more potent agonist of platelets than ADP. A number of studies have shown that PI 3-kinases can be activated by thrombin downstream of PARs (6, 15, 45). As PI 3-kinases are known to be upstream of Akt, one would expect Akt phosphorylation to occur downstream of thrombin, even in the absence of ADP. But thrombin causes a very small Akt phosphorylation in the absence of the P2Y\textsubscript{12}-Thrombin might activate several PI 3-kinases (\alpha, \beta, and \gamma), whereas ADP might activate only PI 3-kinase \gamma. Activation of PI 3-kinase may not immediately result in Akt phosphorylation without some events regulated by G\textsubscript{i} stimulation. Thus G\textsubscript{i} pathways might be regulating some signaling events downstream of PI 3-kinases and upstream of Akt. Consistent with our conclusions, Hirsch et al. (46) showed that ADP fails to cause Akt phosphorylation in PI 3-kinase \gamma knockout mouse platelets, but thrombin causes Akt phosphorylation in these platelets. Thus, in PI 3-kinase \gamma knockout mouse platelets, phosphatidylinositol 1,4,5-trisphosphate accumulation downstream of thrombin can lead to Akt phosphorylation as G\textsubscript{i} signaling events regulating Akt activation are unaffected. However, ADP-induced phosphatidylinositol 1,4,5-trisphosphate formation is affected in these platelets as ADP causes phosphatidylinositol 1,4,5-trisphosphate formation through PI 3-kinase \gamma, which is missing in these platelets.

ADP causes much smaller extent of Akt phosphorylation than thrombin or TRAPs. This could be due to a potentiating effect of G\textsubscript{\alpha} pathways on G\textsubscript{i}-mediated Akt phosphorylation. PARs, through strong stimulation of G\textsubscript{\alpha} pathways, could potentiate Akt phosphorylation mediated by G\textsubscript{i} pathways and hence cause much stronger Akt phosphorylation than ADP. Akt phosphorylation occurs biphasically, first through inside-out signaling and then through outside-in signaling (19). In our conditions, we eliminated the contribution of outside-in signaling and its contribution to Akt phosphorylation. Thus, when aggregation is allowed to occur, thrombin-induced Akt phosphorylation could be stronger because thrombin can cause aggregation to a greater extent than ADP and hence could cause stronger Akt phosphorylation. However, under our conditions, the contribution of outside-in signaling is eliminated, and still thrombin causes more Akt phosphorylation than ADP. Hence, there might be some potentiating contribution of G\textsubscript{\alpha} to Akt phospho-
Gq Pathways Activate Akt in Platelets

The activation of PI3-kinase downstream of Gq pathways is implicated in irreversible platelet aggregation (15, 45). It is also suggested that PI3-kinase γ exerts a significant role in ADP-induced platelet aggregation (46). Even though the importance of PI3-kinase in platelet aggregation is well established. Because a key enzyme in Akt activation is PI3-kinase, we can speculate that Akt activation downstream of Gi/PI 3-kinase may play an important role in platelet activation. Furthermore, the absolute requirement of Gi pathways for Akt activation suggests that Akt may play a role in irreversible platelet aggregation, inactivation of Gq signaling by thrombin or PAR-activating peptide-induced Akt phosphorylation under the conditions of secretion blockade (Fig. 9A) or P2Y12 receptor blockade (Fig. 9B). Supplemental Gq/Gi signaling restored Akt phosphorylation to the levels achieved by thrombin stimulation. These results further confirm that Gq signaling regulates Akt phosphorylation, and epinephrine, through Gq signaling cascade, can substitute for the P2Y12 receptor-mediated effects.

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REFERENCES
1. Bellacosa, A., Testa, J. R., Staal, S. P., and Tsichlis, P. N. (1991) Science 254, 274–277.
2. Cheng, J. Q., Godwin, A. K., Bellacosa, A., Taguchi, T., Franke, T. F., Hamilton, T. C., Tischler, P. N., and Testa, J. R. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9267–9271.
3. Nakatani, K., Thompson, D. A., Barthel, A., Sakane, H., Liu, W., Weigel, R. J., and Roth, R. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 97, 2618–2622.
4. Alesi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) EMBO J. 15, 6541–6551.
5. Jin, J., and Kunapuli, S. P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4171–4175.
6. Kroner, C., Ebyrechts, K., and Akkerman, J. W. (2000) J. Biol. Chem. 275, 27790–27796.
7. Barry, F. A., and Gibbins, J. M. (2002) J. Biol. Chem. 277, 12847–12878.
8. Cho, M. J., Pestina, T. I., Stewart, S. A., Lowell, C. A., Jackson, C. W., and Gartner, T. K. (2002) Blood 99, 2442–2447.
9. Burgering, B. M., and Coffer, P. J. (1995) Nature 376, 599–602.
10. Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F., and Hawkins, P. T. (1997) Science 276, 567–570.
11. Toker, A., and Newton, A. C. (2000) J. Biol. Chem. 275, 8271–8274.
12. Troussard, A. A., Mawji, N. N., Ong, C., Mui, A., St. Arnaud, R., and Dedhar, S. (2003) J. Biol. Chem. 278, 23747–23755.
13. Yano, S., Tokumitsu, H., and Soderling, T. R. (1998) Nature 396, 584–597.
14. Jin, J., and Kunapuli, S. P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8070–8074.
15. Kim, S., Foster, C., Leechi, A., Quinton, T. M., Prosser, D. M., Jin, J., Cattaneo, M., and Kunapuli, S. P. (2002) Blood 99, 3629–3636.
16. Cho, M. J., Liu, J., Pestina, T. I., Stewart, S. A., Thomas, D. W., Coffman, T. M., Wang, D., Jackson, C. W., and Gartner, T. K. (2003) Blood 101, 2646–2651.
17. Fabre, J. E., Nguyen, M., Latour, A., Keifer, J. A., Audoly, L. P., Coffman, T. M., and Roller, B. H. (1999) Nat. Med. 5, 1189–1202.
18. Leon, C., Hechler, B., Freund, M., Eckly, A., Vial, C., Ohlmann, P., Dierich, A., LeMeur, M., Cazenave, J. P., and Gachet, C. (1999) J. Clin. Investig. 104, 1731–1737.
19. Banfic, H., Downes, C. P., and Rittenhouse, S. E. (1998) J. Biol. Chem. 273, 11630–11637.
20. Sheng, T., Takai, Y., Yamanishi, J., and Nishizuka, Y. (1983) J. Biol. Chem. 258, 2010–2013.
21. Geiger, B., Brich, J., Henig-Liedl, P., Eigenhauer, M., Schimanscher, P., Herbert, J. M., and Walter, U. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 2067–2071.
22. Savio, P., Laplace, M. C., Mauffrand, J. P., and Herbert, J. M. (1994) J. Pharmacol. Exp. Ther. 269, 772–777.
23. Kowalska, M., Ratajczak, M. Z., Majka, M., Jin, J., Kunapuli, S., Brass, L., and Ponzeh, M. (2000) Blood 96, 50–57.
24. Franke, T. F., Yang, S. I., Cho, M. J., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) Cell 81, 727–736.
25. Faraqui, T. R., Weiss, E. J., Shapiro, M. J., Huang, W., and Coughlin, S. R. (1996) J. Biol. Chem. 271, 10798–10807.
26. Marugo, C., Laguinge, L., Wetker, R., Cuadrado, A., and Guitkun, J. S. (1998) J. Biol. Chem. 273, 19080–19085.
27. Tang, X., Batty, I. H., and Downes, C. P. (2002) J. Biol. Chem. 277, 338–344.
28. Eppenhuizen, R. K., Visvakar, S., and Simonds, W. F. (2000) J. Biol. Chem. 275, 3870–3876.
29. Ballo, M., Lin, H. Y., Fan, G., Jiang, Y., and Lin, R. Z. (2000) J. Biol. Chem. 275, 24572–24574.
30. Tilton, B., Andjelkovic, M., Didichenko, S. A., Hemmings, B. A., and Thelen, M. (1997) J. Biol. Chem. 272, 28096–28101.
31. Quinton, T. M., Kim, S., Dangelmaier, C., Dorsam, R. T., Jin, J., and Kunapuli, S. P. (2002) Biochem. J. 368, 535–543.
32. Chaudhry, A., MacKenzie, R. G., Georgic, L. M., and Granneman, J. G. (1994) Cell. Signal. 6, 457–465.
33. Moule, S. K., Welsh, G. L., Edgell, N. J., Foulstone, E. J., Proud, C. G., and Denton, R. M. (1997) J. Biol. Chem. 272, 7175–7179.
34. Sahle, C. L., Filippa, N., Hemmings, B., and Van Obberghen, E. (1997) FEBS Lett. 409, 253–257.
35. Konishi, H., Matsuzaki, H., Tanaka, M., Ohe, Y., Tukumana, C., Kuroda, S., and Kikkawa, U. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7639–7643.
42. Walker, T. R., Moore, S. M., Lawson, M. F., Panetieri, R. A., Jr., and Chilvers, E. R. (1998) *Mol. Pharmacol.* **54**, 1007–1015
43. Phillips-Mason, P. J., Raben, D. M., and Baldassare, J. J. (2000) *J. Biol. Chem.* **275**, 18046–18053
44. Offermanns, S., Laugwitz, K. L., Spicher, K., and Schultz, G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 504–508
45. Trumel, C., Payrastre, B., Plantavid, M., Hechler, B., Viala, C., Presek, P., Martinson, E. A., Cazenave, J. P., Chap, H., and Gachet, C. (1999) *Blood* **94**, 4156–4165
46. Hirsch, E., Bosco, O., Tropel, P., Laffargue, M., Calvez, R., Altruda, F., Wymann, M., and Montrucchio, G. (2001) *FASEB J.* **15**, 2019–2021
47. Hu, Y., Qiao, L., Wang, S., Rong, S. B., Meuillet, E. J., Berggren, M., Gallegos, A., Powis, G., and Rozikowski, A. P. (2000) *J. Med. Chem.* **43**, 3045–3051
48. Smith, U., Carvalho, E., Mosialou, E., Beguinot, F., Formisano, P., and Ron- dimone, C. (2000) *Biochem. Biophys. Res. Commun.* **268**, 315–320
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