Platelet Activation by von Willebrand Factor Requires Coordinated Signaling through Thromboxane A$_2$ and Fc$_\gamma$RIIA Receptor*

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Interaction of von Willebrand Factor with glycoprotein Ib-IX-V induces platelet activation through a still poorly defined mechanism. Previous studies have suggested a possible role for the low affinity receptor for immunoglobulin, Fc$_\gamma$RIIA, in GPIb-IX-V signaling. Here we show that binding of vWF to platelets induces the tyrosine phosphorylation of Fc$_\gamma$RIIA by a Src kinase. Treatment of platelets with the anti-Fc$_\gamma$RIIA monoclonal antibody IV.3 specifically inhibits vWF-induced but not thrombin-induced pleckstrin phosphorylation and serotonin secretion. Moreover, vWF fails to induce pleckstrin phosphorylation in mouse platelets, lacking Fc$_\gamma$RIIA, and serotonin secretion is impaired. Pleckstrin phosphorylation and serotonin secretion in human platelets stimulated with vWF are blocked by the cyclooxygenase inhibitor acetylsalicylic acid. However, release of arachidonic acid and synthesis of TXA$_2$ induced by vWF are not affected by the anti-Fc$_\gamma$RIIA monoclonal antibody IV.3. Similarly, vWF-induced tyrosine phosphorylation of Fc$_\gamma$RIIA, as well as of Syk and PLC$_\gamma$2, occurs normally in aspirinized platelets. Inhibition of the tyrosine kinase Syk by piceatannol does not affect vWF-induced tyrosine phosphorylation of Fc$_\gamma$RIIA but prevents phosphorylation of PLC$_\gamma$2. Pleckstrin phosphorylation and platelet secretion induced by vWF, but not by thrombin, are also inhibited by piceatannol. Pleckstrin phosphorylation is also sensitive to the phosphatidylinositol 3-kinase inhibitor wortmannin. These results indicate that PLC$_\gamma$2 plays a central role in platelet activation by vWF and that the stimulation of this enzyme requires coordinated signals through endogenous TXA$_2$ and Fc$_\gamma$RIIA.

von Willebrand Factor (vWF) is a large glycoprotein synthesized by endothelial cells and megakaryocytes and plays an important role in platelet adhesion and thrombus formation (1). Under conditions of high shear stress or in the presence of modulators like ristocetin or botrocetin, vWF binds to the platelet membrane GPIb-IX-V and initiates signals leading to platelet activation (1). This process involves the activation of PLC and PLAr, the cytoskeleton reorganization and the interaction of several signaling molecules with the actin filaments, the tyrosine phosphorylation of several proteins, the activation of G protein, and the stimulation of ITAM-bearing receptors (2). For this reason, endogenous TXA$_2$ is believed to play a central role in platelet activation by vWF. However, other events, such as the activation of some tyrosine kinases, are independent of endogenous TXA$_2$ and are mediated by the binding of vWF to GP Ib-IX-V (3). Some of these events, such as the activation of PLC, have been demonstrated to be promoted by the secondary action of TXA$_2$ produced from arachidonic acid through the cyclooxygenase pathway (2). For this reason, endogenous TXA$_2$ is believed to play a central role in platelet activation by vWF. However, other events, such as the activation of some tyrosine kinases, are independent of endogenous TXA$_2$ and are mediated by the binding of vWF to GP Ib-IX-V. Pleckstrin phosphorylation is also sensitive to the phosphatidylinositol 3-kinase inhibitor wortmannin. These results indicate that PLC$_\gamma$2 plays a central role in platelet activation by vWF and that the stimulation of this enzyme requires coordinated signals through endogenous TXA$_2$ and Fc$_\gamma$RIIA.
In this work we further investigate the functional interplay between FcγRIIA and GPIb-IX-V in vWF-stimulated platelets. We show that FcγRIIA is actually tyrosine phosphorylated upon vWF binding to platelets by a mechanism independent of TxA2 production. Moreover, blockade of FcγRIIA by a specific mAb prevents PLC activation and platelet secretion induced by vWF under conditions in which the production of TxA2 occurs normally. These results indicate that, in addition to endogenous TxA2, FcγRIIA plays an essential role in platelet activation by vWF.

**EXPERIMENTAL PROCEDURES**

**Materials—**Sepharose CL-2B was from Amersham Pharmacia Biotech. Thrombin, ristocetin, botrocetin, acetylsalicylic acid, indomethacin, piceatannol, wortmannin, protein A-Sepharose, and mouse IgG were from Sigma. PP1 was purchased from Alexis. von Willebrand Factor (Hemate P) was obtained from Behringwerke (Marburg, Germany). [32P]orthophosphate, [14C]serotonin (57 mCi/mmol), and the endogenous TxA2, FcγRIIA was achieved by incubation of platelets with the anti-FcγRIIA mAb IV.3 or with 2 μg/ml unrelated mAb IV.3 and 30 μg/ml unrelated serum from vWF-stimulated platelets. Moreover, a tyrosine-phosphorylated protein with a similar electrophoretic mobility and reactivity to anti-phosphotyrosine was immunoprecipitated and identified.

**Measurement of FcγRIIA Phosphorylation—**Samples of resting and stimulated platelets (0.4 ml) were lysed by addition of an equal volume of immunoprecipitation buffer 2× (100 mM Tris/HCl pH 7.4, 200 mM NaCl, 2 mM EGTA, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 2 μM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, 2 mM NaF, 2% Nonidet P40, 0.5% sodium deoxycholate). The cleared lysates were immunoprecipitated with 2 μg of anti-FcγRIIA mAb IV.3 or with 2 μg of the anti-Syk and anti-PLCγ2 antisera, as previously described (7). Upon electrophoresis on 10 or 7.5% acrylamide gels, immunoprecipitated proteins were transferred to nitrocellulose and probed with the anti-phosphotyrosine antibody 4G10 as previously described (7). All the shown results are representative of at least three different experiments.

**Results—**Tyrosine Phosphorylation of FcγRIIA in vWF-stimulated Platelets—We have previously shown that FcγRIIA mediates the translocation of the small G-proteins rap1b and rap2b to the cytoskeleton and the tyrosine phosphorylation of selected substrates including Syk and PLCγ2 in vWF-stimulated platelets (7). To clarify the role of FcγRIIA in platelet stimulation by vWF, we examined whether FcγRIIA itself was actually activation on stimulation of human platelets with vWF. Activation of FcγRIIA involves the phosphorylation of tyrosine residues in its cytoplasmatic ITAM that enables the receptor to bind and activate Syk leading to the tyrosine phosphorylation of downstream substrates, such as PLCγ2 (12, 16, 17). Gel-filtered platelets were treated with buffer or stimulated with 10 μg/ml vWF in the presence of 0.5 mg/ml ristocetin or with 10 μg/ml vWF and 10 nM ristocetin for 3 min under constant stirring. The reaction was stopped by brief centrifugation, and the [14C]serotonin released in the supernatant was determined by liquid scintillation counting. Stimulation of 32P-labeled platelets (0.05-ml samples) was performed for increasing times and was stopped by addition of an equal volume of SDS-sample buffer. Phosphorylation of pleckstrin was analyzed by SDS-PAGE on 10–20% acrylamide gradient gels followed by autoradiography.

**Phosphorylation of Syk—**We show that FcγRIIA is actually tyrosine phosphorylated upon vWF binding to platelets by a mechanism independent of TxA2 production. Moreover, blockade of FcγRIIA by a specific mAb prevents PLC activation and platelet secretion induced by vWF under conditions in which the production of TxA2 occurs normally. These results indicate that, in addition to endogenous TxA2, FcγRIIA plays an essential role in platelet activation by vWF.

**Measurement of [32P]Serotonin Secretion—**[32P]Serotonin-labeled platelets (0.1-ml samples) were stimulated with the appropriated agonist in the presence of 5 μM imipramine. A 10-μl aliquot was withdrawn before addition of the agonist to evaluate the total incorporated radioactivity. Stimulation was stopped by addition of 0.9 ml of 1.2% paraformaldehyde and 100 mM EDTA and cooling on ice. Platelets were recovered by centrifugation at 10,000 × g for 3 min, and the radioactivity of [32P]serotonin released in the supernatant was determined by liquid scintillation counting.
clearly weaker than that produced by direct activation of the receptor, which most likely represents the maximal response.

We next tried to identify the kinase responsible for tyrosine phosphorylation of FcRIIA induced by vWF. Gel-filtered platelets were preincubated with 20 μg/ml of the Syk-selective inhibitor piceatannol or with 10 μM of the Src kinases inhibitor PP1 and then stimulated with vWF and ristocetin. The tyrosine phosphorylation of immunoprecipitated FcRIIA was evaluated by immunoblotting with an anti-phosphotyrosine antibody. Fig. 2 shows that vWF-induced tyrosine phosphorylation of FcRIIA was not affected by piceatannol, but was completely inhibited by the preincubation of platelets with PP1. These results indicate that a Src kinase is responsible for the tyrosine phosphorylation of FcRIIA in vWF-stimulated platelets. Moreover, the lack of effects of piceatannol is in agreement with the evidence that Syk lies downstream of the activated FcRIIA (12, 16).

It is known that many events related to platelet activation by vWF are actually mediated by TxA2 produced from arachidonic acid through the cyclooxygenase pathway (2). To investigate the possible role of TxA2 on vWF-induced tyrosine phosphorylation of FcRIIA, we preincubated platelets with two different cyclooxygenase inhibitors, acetylsalicylic acid and indomethacin. Fig. 3 shows that neither of the inhibitors affected vWF-induced tyrosine phosphorylation of FcRIIA. Moreover, tyrosine phosphorylation of FcRIIA induced by vWF occurred normally in platelets preincubated with the TxA2 receptor antagonist SQ29,548 (data not shown). Therefore, activation of FcRIIA is directly promoted by vWF and does not require the action of produced TxA2.

We have previously shown that tyrosine phosphorylation of 32P-labeled platelets preincubated with the Syk-selective inhibitor piceatannol. Fig. 5 shows that tyrosine phosphorylation of PLCγ2 induced by vWF was totally prevented when Syk was inhibited by piceatannol. These results identify a signaling pathway that is activated in vWF-stimulated platelets by a mechanism independent of TxA2 and which involves FcRIIA, the tyrosine kinase Syk, and PLCγ2.

Effect of the Blockage of FcRIIA and of the Inhibition of Syk on vWF-induced PLC Activation and Platelet Secretion—We next investigated the role of FcRIIA and Syk on two important events promoted by vWF binding to GPIb-IX-V: activation of PLC and serotonin secretion. Activation of PLC leads to the generation of diacylglycerol, which activates protein kinase C. The main platelet substrate for protein kinase C is the 47 kDa protein pleckstrin whose phosphorylation represents a reliable marker for PLC activation. Fig. 6 shows the phosphorylation of pleckstrin induced by vWF in 32P-labeled platelets. Such phosphorylation was not affected by preincubation of intact platelets with control IgG but was almost totally blocked by the anti-FcRIIA mAb IV.3. Fig. 6 also shows that pleckstrin phosphorylation induced by thrombin was not affected by either control IgG or the IV.3 mAb.
pleckstrin phosphorylation. It shows that inhibition of PI 3-kinase by wortmannin totally prevented vWF-induced PLC activation and the consequent serotonin secretion in mouse platelets that can bind to vWF, but do not express FcγRIIA. Because mouse platelets have been shown to interact with human vWF activated by botrocetin but not by ristocetin (21), stimulation was performed with 10 μg/ml vWF and 0.5 mg/ml ristocetin (vWF +), or with 1 unit/ml thrombin (THR). Total platelet proteins were separated on a 5–15% acrylamide gradient gel. The figure represents an autoradiography of the dried gel. The positions of the molecular mass markers are indicated on the left. The arrow on the right shows the position of phosphorylated pleckstrin.

![Fig. 6. Inhibition of vWF-induced pleckstrin phosphorylation by the IV.3 mAb.](http://www.jbc.org/)

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We have shown that inhibition of the tyrosine kinase Syk by piceatannol prevented vWF-induced tyrosine phosphorylation of PLCγ2 (Fig. 5). As shown in Fig. 7, piceatannol also completely prevented vWF-induced activation of PLC, as revealed by the inability of the agonist to promote phosphorylation of pleckstrin. By contrast, piceatannol had no effects on pleckstrin phosphorylation induced by thrombin (data not shown). These results suggest that PLCγ2 represents the main PLC isoenzyme activated by vWF. It has been reported that tyrosine phosphorylation of PLCγ2 is not sufficient to support its activation, and that additional factors are required. Among these factors are the lipid products of PI 3-kinase (19, 20). Fig. 8 shows that inhibition of PI 3-kinase by wortmannin totally prevented vWF-induced PLC activation and the consequent pleckstrin phosphorylation.

We next investigated the involvement of FcγRIIA and of the tyrosine kinase Syk on platelet secretion induced by vWF. In time course experiments, we found that release of [14C]serotonin induced by vWF was rapid and maximal after 3 min of stimulation (data not shown). Pretreatment of platelets with the anti-FcγRIIA antibody IV.3 but not with unrelated IgG, totally inhibited the ability of vWF to induce release of [14C]serotonin (Fig. 9). By contrast, the blocking of FcγRIIA had no effect on [14C]serotonin release induced by thrombin (Fig. 9). The release of [14C]serotonin induced by vWF, but not by thrombin was also inhibited in a dose-dependent manner by the Syk inhibitor piceatannol (Fig. 10). Therefore, both FcγRIIA and the tyrosine kinase Syk are involved in the signaling pathway leading to PLC activation and platelet secretion induced by vWF.

To clarify the physiological significance of FcγRIIA in platelet activation by vWF we analyzed pleckstrin phosphorylation and serotonin secretion in mouse platelets that can bind to vWF, but do not express FcγRIIA. Because mouse platelets have been shown to interact with human vWF activated by botrocetin but not by ristocetin (21), stimulation was performed with 10 μg/ml vWF and 6 μg/ml botrocetin. Fig. 11 shows that stimulation of mouse platelets with 1 unit/ml thrombin caused a rapid and strong phosphorylation of pleckstrin that was already maximal after 30 s. By contrast, treatment of mouse platelets with vWF for up to 10 min did not cause any significant phosphorylation of pleckstrin (Fig. 11). Even when 10-fold higher doses of vWF were used, no significant pleckstrin phos-
phorylation was observed (data not shown). We next investigated the release of \([14C]\) serotonin. Treatment of mouse platelets with vWF caused the release of about 50% of the incorporated serotonin (Fig. 12). This response was clearly impaired when compared with that elicited by thrombin, which was able to promote the release of more than 90% of the incorporated serotonin. Interestingly, secretion of \([14C]\) serotonin induced by thrombin and vWF in human platelets was comparable and almost maximal (Fig. 12). Even treatment of platelets with 10-fold higher concentrations of vWF and botrocepin did not elicit a response comparable with that observed upon stimulation with thrombin or that measured in human platelets. These results indicate that vWF-induced serotonin secretion and PLC activation in mouse platelets lacking FcyRIIA are impaired.

**Correlation among FcγRIIA, TxA2, PLC Activation, and Platelet Secretion**—It has been previously shown that pleckstrin phosphorylation induced by vWF is inhibited by indomethacin, demonstrating that PLC activation is totally dependent on arachidonic acid metabolism through the cyclooxygenase pathway (2). We confirmed these results, and we found that pretreatment of platelets with the cyclooxygenase inhibitor acetylsalicylic acid totally prevented pleckstrin phosphorylation induced by vWF (Fig. 13A). Moreover, as shown in Fig. 13B, the release of \([14C]\) serotonin induced by vWF was blocked in acetylsalicylic acid-treated platelets. Finally, vWF-induced serotonin secretion was also inhibited by the TxA2 receptor antagonist SQ29,548 (data not shown).

**FIG. 8.** Wortmannin inhibits vWF-induced pleckstrin phosphorylation. \(^{32}P\)-labeled platelets were incubated in the absence or in the presence of 10 nM wortmannin for 15 min and then stimulated with 10 \(\mu\)g/ml vWF and 0.5 mg/ml ristocetin for the indicated times. Phosphorylation of pleckstrin was evaluated by autoradiography of the total platelet proteins separated on 5–15% acrylamide gradient gels. The arrow on the right indicates the position of phosphorylated pleckstrin.

**FIG. 9.** Effect of the mAb IV.3 on vWF- and thrombin-induced serotonin secretion. Platelets were labeled with \([14C]\) serotonin and incubated with buffer (none), 20 \(\mu\)g/ml mAb IV.3 or 20 \(\mu\)g/ml unrelated IgG for 3 min, and then stimulated with either 10 \(\mu\)g/ml vWF and 0.5 mg/ml ristocetin (vWF) or 1 unit/ml thrombin (THR). The release of \([14C]\) serotonin in the supernatant is reported as percentage of the total incorporated radioactivity. Data represent the mean ± S.D. of three different experiments.

**FIG. 10.** Effect of piceatannol on vWF- and thrombin-induced serotonin secretion. \([14C]\) serotonin-labeled platelets were incubated with the indicated amounts of piceatannol for 15 min. Upon stimulation with 10 \(\mu\)g/ml vWF and 0.5 mg/ml ristocetin (vWF) or 1 unit/ml thrombin (THR), the \([14C]\) serotonin released was determined by liquid scintillation counting and reported as percentage of the total incorporated radioactivity. The results represent the mean ± S.D. of three different experiments.

**FIG. 11.** Analysis of pleckstrin phosphorylation in mouse platelets. \(^{32}P\)-labeled mouse platelets were stimulated with 10 \(\mu\)g/ml vWF and 6 \(\mu\)g/ml botrocepin (vWF), or with 1 unit/ml thrombin (THR) for the indicated times. Total platelet proteins were separated on 10–20% acrylamide gradient gels, and phosphorylation of pleckstrin (indicated by the arrow on the right) was visualized by autoradiography.

**FIG. 12.** Serotonin secretion in mouse and human platelets. \([14C]\) serotonin-labeled mouse and human platelets were stimulated with 1 unit/ml thrombin (THR), 10 \(\mu\)g/ml vWF and 6 \(\mu\)g/ml botrocepin (vWF; 10 \(\mu\)g/ml), or with 1 unit/ml thrombin (THR) for 3 min. The \([14C]\) serotonin released, determined by liquid scintillation counting, is reported as percentage of the total incorporated radioactivity. Data represent the mean ± S.D. of 3–4 different determinations.
Taken together, these results indicate that PLC activation and platelet secretion induced by vWF require not only the Fc\(\gamma\)RIIA, but also the action of TxA\(_2\). Therefore, we investigated the correlation between the Fc\(\gamma\)RIIA- and TxA2-dependent signaling pathways. We have shown in this study that activation of Fc\(\gamma\)RIIA by vWF is independent of the production of TxA\(_2\) (Fig. 3). We examined whether the signaling pathway initiated by activation of Fc\(\gamma\)RIIA could lead to the release of arachidonic acid by PLA\(_2\) and its conversion to TxA\(_2\). A significant accumulation of the stable metabolite TxB\(_2\) in vWF-stimulated platelets versus resting platelets was measured by an immunoassay method (Fig. 14A). However, the accumulation of TxB\(_2\) was not reduced when Fc\(\gamma\)RIIA was blocked by the IV.3 mAb. We also measured directly the release of arachidonic acid from \[^{3}H\]arachidonate-labeled platelets (Fig. 14B). Even in this case no significant differences in the release of \[^{3}H\]arachidonate were observed in platelets stimulated with vWF in the absence or in the presence of the anti-Fc\(\gamma\)RIIA mAb IV.3. Therefore, we conclude that the generation of TxA\(_2\) induced by vWF is independent of Fc\(\gamma\)RIIA activation.

**DISCUSSION**

Stimulation of PLC plays a central role in platelet activation by extracellular agonists and is essential to promote the release of internal granules. Platelet stimulation by vWF induces the activation of PLC by a mechanism that has been recognized to be totally dependent on the release of arachidonic acid and its conversion to TxA\(_2\) (2). In this work we have demonstrated that production of TxA\(_2\) is necessary, but not sufficient for vWF to induce PLC activation and granule secretion. We have described the activation of an additional signaling pathway in vWF-stimulated platelets, involving the membrane Fc\(\gamma\)RIIA, the tyrosine kinase Syk and PLC\(\gamma\)2. Such a pathway, in addition to the generation of TxA\(_2\), is absolutely required for the agonist-induced PLC activation and granule secretion. These data confirm the essential role of Fc\(\gamma\)RIIA in mediating platelet response to vWF. Such a role has been initially hypothesized based on the association between Fc\(\gamma\)RIIA and GPIb-IX-V, the main vWF receptor on the platelet membrane (13, 14). We have previously shown that vWF-induced translocation of the small GTP-binding proteins rap1b and rap2b to the cytoskeleton as well as tyrosine phosphorylation of selected substrates, including Syk and PLC\(\gamma\)2 are dependent on Fc\(\gamma\)RIIA (7). In the

![Figure 13. Effect of acetylsalicylic acid on vWF-induced pleckstrin phosphorylation and serotonin secretion. A, \(^{32}\)P-labeled platelets were incubated with or without acetylsalicylic acid (ASA) for 30 min and then stimulated with 10 \(\mu\)g/ml vWF and 0.5 mg/ml ristocetin for the indicated times. Total platelet proteins were separated on 5–15% acrylamide gradient gel, and phosphorylation of pleckstrin (indicated by the arrow on the right) was evaluated upon autoradiography of the dried gel. B, \(^{14}\)C]serotonin-labeled platelets were treated without (–) with (+) acetylsalicylic acid (ASA) and then stimulated with 10 \(\mu\)g/ml vWF and 0.5 mg/ml ristocetin for 3 min. The release of \[^{14}\)C]serotonin in the supernatant is expressed as percentage of the total incorporated radioactivity. Results are the mean ± S.D. of three separate experiments.

![Figure 14. Effect of the mAb IV.3 on vWF-induced TxA\(_2\) production and arachidonic acid release. A, resting platelets and platelets stimulated with 10 \(\mu\)g/ml vWF and 0.5 mg/ml ristocetin for 3 min in the absence or in the presence of 20 \(\mu\)g/ml mAb IV.3 or unrelated IgG, were centrifuged, and the supernatants used for determination of TxB\(_2\), a stable metabolite of TxA\(_2\), by an enzyme immunoassay system. Results represent the mean ± S.D. of 3–5 different experiments. B, \[^{3}H\]arachidonic acid-labeled platelets were incubated with buffer, IV.3, or unrelated IgG as described above, and then stimulated with 10 \(\mu\)g/ml vWF and 0.5 mg/ml ristocetin for 3 min. Upon centrifugation of the cells, \[^{3}H\]arachidonic acid in the supernatant was measured by liquid scintillation counting. The radioactivity measured in the supernatants from resting platelets was subtracted to the values obtained from vWF-treated cells and reported in the figure. The results are the mean ± S.D. of 3–5 experiments.](http://www.jbc.org/Downloaded from http://www.jbc.org/)
Phosphorylation of FcγRIIA induced by vWF does not require the secondary action of synthesized TxA2 and is probably mediated by a tyrosine kinase belonging to the Src family. Although the identity of this kinase is not known, possible candidates include Fyn and Lyn, which have been proposed to be involved in Fc receptor γ-chain phosphorylation induced by collagen (22).

The molecular mechanism of FcγRIIA activation following vWF binding to platelets is unclear. Normally FcγRIIA is activated upon clustering induced by immunocomplexes through the phosphorylation of two tyrosine residues in its intracellular ITAM (12). vWF does not directly bind to FcγRIIA, and we have previously demonstrated that its ability to initiate platelet activation through FcγRIIA is not because of potential contaminating immunoglobulins (7). However, vWF is a multimeric adhesive protein and its ability to stimulate platelets is correlated to the size of the multimers. Because of its multimeric nature, the platelet-activating vWF is most likely able to promote the clustering of its receptor, GPIb-IX-V, on the platelet surface. Based on the reported physical proximity between GPIb-IX-V and FcγRIIA, it is possible for FcγRIIA itself to be incorporated into these clusters and, as a consequence of this, become activated. In this context, the inhibitory effect of the anti-FcγRIIA mAb IV.3 on platelet activation reported in the present work, may derive from an interference with the clustering process as a consequence of its activation induced by thrombin (20). This is consistent with our findings that vWF can actually induce some release of serotonin in mouse platelets. Despite this, the compared analysis of vWF- and thrombin-induced secretion in mouse and human platelets revealed that this response is clearly impaired in mouse platelets lacking FcγRIIA. Such defective activation of mouse platelets by vWF is even more evident from the analysis of pleckstrin phosphorylation, which is almost undetectable. These results strengthen the importance of FcγRIIA on the platelet surface for efficient activation by vWF. The signaling pathway responsible for serotonin secretion induced by vWF in mouse platelets could be related to the Fc receptor γ-chain that is expressed in these cells and has been proposed to play a role in vWF stimulation of human platelets (11). It is also possible that the contribution of the Fc receptor γ-chain in vWF-induced activation of mouse platelets may be more relevant than in human cells, thus partially compensating for the absence of FcγRIIA.

The complete inhibition of pleckstrin phosphorylation and granule secretion by the mAb IV.3 or by piceatannol in human platelets suggests that PLCγ2 may be the main PLC isoenzyme activated by vWF. It is known that activation of PLCγ2 requires, in addition to its tyrosine phosphorylation, a number of factors, including the lipid products of PI 3-kinase (19, 20). In this context, it is interesting to note that inhibition of PI 3-kinase by wortmannin completely blocks vWF-mediated pleckstrin phosphorylation.

Previous studies have demonstrated that PLC activation induced by vWF is totally dependent on the secondary action of endogenous TxA2 (2). We also have confirmed the essential role of TxA2 in platelet response to vWF, because we have shown in this work that inhibition of cyclooxygenase totally prevents pleckstrin phosphorylation as well as granule secretion. However, our results stimulate a revaluation of the role of TxA2 in platelet activation by vWF. First of all, we have shown that tyrosine phosphorylation of FcγRIIA, Syk, and PLCγ2 induced by vWF occurs independently of TxA2 synthesis. Moreover, the total inhibition of vWF-induced PLC activation and granule secretion in platelets pretreated with the mAb IV.3, under conditions in which production of TxA2 is not affected, indicates that the endogenous TxA2 is not per se sufficient to support platelet activation when the FcγRIIA-mediated signaling pathway here described is blocked. Such inability of endogenous TxA2 to fully support platelet activation may be related to its low concentration. Based on the measurement of the amount of accumulated TxB2 we calculated that endogenous TxA2 reached the concentration of about 50 nM. It is possible that such concentrations allow TxA2 to behave like a weak agonist that needs costimulatory factors to elicit its effects. The lack of effects of the mAb IV.3 on arachidonic acid release and TxB2 accumulation induced by vWF, together with the evidence that tyrosine phosphorylation of FcγRIIA (as well as of Syk and PLCγ2) is not affected by inhibition of cyclooxygenase, indicate that TxA2 and FcγRIIA-mediated signaling pathways are independent events, both of which are directly promoted by bind-
ing of vWF to its platelet receptor. We have shown here that a Src kinase is responsible for FcγRIIA phosphorylation, and we have obtained evidence that the Src kinase inhibitor PP1 is also able to prevent vWF-induced release of arachidonic acid from labeled platelets (data not shown). A central role for the Src tyrosine kinases in platelet activation promoted by recruitment of GPIb-IX-V has also been suggested by the evidence that PP1 inhibits alloaggregrin-induced platelet secretion and aggregation as well as phosphorylation of PLCγ2 (11). Therefore, it seems most likely that binding of vWF to GPIb-IX-V stimulates by a still unknown mechanism a Src kinase, which simultaneously promotes the activation of FcγRIIA, leading to PLCγ2 tyrosine phosphorylation and the activation of PLA2, leading to the release of arachidonic acid and to its conversion to TxA2. Integration of signals generated by the activated FcγRIIA and the synthesized TxA2 is required to elicit full platelet activation. The molecular mechanism of this integration is not known. One possibility arises from the consideration that activation of PLCγ2 requires the meeting of several events and factors, including the lipid products of PI 3-kinase and the proteins LAT and SLP-76 (19, 20, 27, 28). Although we have demonstrated that phosphorylation of PLCγ2 induced by vWF is dependent on FcγRIIA-mediated signaling, other factors necessary for the full activation of the enzyme could be recruited specifically through the action of endogenous TxA2. This possibility, however, deserves further investigation.

In conclusion, our results demonstrate for the first time that the release of endogenous TxA2 is required but not sufficient to support full platelet activation induced by vWF and recognize a novel essential role for FcγRIIA in this process.

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