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Tyrosine Phosphorylation and Syk Activation Are Involved in Thrombin-Induced Aggregation of Epinephrine-Potentiated Platelets[1]

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Thrombin and epinephrine in combination exert synergistic effects on platelet activation. On the other hand, tyrosine phosphorylation and activation of tyrosine kinases including Syk have been shown to play a critical role in the induction of platelet responses to thrombin stimulation. This study investigated the role of tyrosine phosphorylation and Syk activation in the synergistic mechanisms between thrombin and epinephrine. Although epinephrine alone (4 μM) slightly induced protein-tyrosine phosphorylation and Syk activation, the presence of epinephrine caused a shift to the left in the dose-dependence of thrombin (0.01-0.5 U/ml)-induced tyrosine phosphorylation and Syk activation, as well as platelet aggregation. Phenoxybenzamine, an α-adrenoceptor antagonist, canceled this potentiation by epinephrine. Since platelets dominantly express α_2-adrenoceptor, this result indicates that epinephrine acts through the occupancy of α_2-adrenoceptor. Furthermore, pretreatment with a tyrosine kinase inhibitor, genistein, or a cAMP-elevating agent, prostacyclin (PGI_2), significantly reduced these synergistic effects of epinephrine. Taken together, our results suggested that the potentiation by epinephrine may be mediated *via* enhancement of tyrosine phosphorylation and Syk activation, in part through a decrease of intracellular cAMP levels.

Key words: epinephrine, potentiation, Syk, thrombin, tyrosine phosphorylation.

That one platelet agonist added to platelets at a concentration too low to cause aggregation enhances the response and leads to induction of aggregation upon addition of another platelet agonist at a similar subthreshold concentration has been demonstrated for several pairs of agonists (*1-5*). This phenomenon is of great interest, since experiments using low concentrations of several agonists may mimic the conditions under which thrombosis occurs *in vivo* (*6*). Epinephrine is a unique platelet agonist in that it potentiates platelet aggregation induced by other agonists such as thrombin, collagen, ADP, vasopressin, and thromboxane A_2 (*7*). Previous studies suggested that the potentiation of agonist-induced platelet activation by epinephrine is mediated *via* enhancement of inositol phospholipid hydrolysis and elevation of intracellular calcium concentration (*8-10*), but the mechanisms of this potentiation of agonist-induced platelet activation by epinephrine are largely unknown.

Tyrosine phosphorylation has been shown to play a critical role in the induction of cellular responses to extracellular stimuli in a variety of cell types (*11*). Upon platelet activation induced by various agonists, a set of proteins undergoes tyrosine phosphorylation (*12-14*). Furthermore, tyrosine kinase inhibitors suppress platelet activation, suggesting a critical role for these protein modifications throughout the platelet activation process (*15-17*). All platelet protein-tyrosine kinases (PTKs) reported to date are non-receptor types, Src and Syk being the most abundant (*13, 18*). In addition to Src and Syk, several PTKs including Fyn, Yes, Hck, Lyn, and Fak have been identified (*19, 20*). Although it is not clear which PTK is responsible for a particular tyrosine-phosphorylated protein, several kinases change their activities upon platelet activation. In particular, the activity of Syk is rapidly increased by 10-fold upon thrombin stimulation, reaching a maximum at 10 s (*21*). Platelet activation also elevates the Src activity to a lesser degree with a slower time course (*22*). The activity of Fak appears to be modified even later by fibrinogen binding to glycoprotein IIb/IIIa on the platelet membrane (*20*). These lines of evidence suggest that Syk is actively engaged in the regulation of platelet functions at the initial phase of platelet activation.

The aims of the present study were to investigate the effects of epinephrine on the transduction processes activated by thrombin and to examine the changes in protein-tyrosine phosphorylation and Syk activity in thrombin-induced aggregation of epinephrine-potentiated platelets. In the present study, we found that in human platelets, epinephrine potentiated thrombin-induced tyrosine phosphorylation and Syk activation correlated with platelet aggregation. Our data provide evidence for an important

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Abbreviations: PTK, protein-tyrosine kinase; PGI_2, prostacyclin; PLC, phospholipase C; DG, diacylglycerol; IP_3, inositol trisphosphate.

role of tyrosine phosphorylation and Syk activation in the potentiation by epinephrine.

MATERIALS AND METHODS

Materials and Chemicals—Human blood was obtained from healthy donors. Epinephrine, human thrombin, and phenoxybenzamine were purchased from Sigma. Genistein and herbimycin A were from Extrasynthese and Funakoshi Pharmaceutical, respectively. Prostacyclin (PGI_2) and anti-Syk monoclonal antibody (101) were from Wako Chemicals. The anti-phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology.

Platelet Isolation—After informed consent had been obtained, human blood was drawn from adult volunteers, who claimed to have taken no aspirin or other drugs for two weeks prior to the donation. Blood was collected from a forearm vein, ten volumes of blood into 1 volume of acid-citrate-dextrose anticoagulant. Platelet-rich plasma was obtained by centrifugation of the blood at $200\times g$ for 10 min at room temperature and then supplemented with prostaglandin E_1 to a final 280 nM, 1 mM aspirin, and 1 U/ml apyrase. The washed platelet pellets were suspended in modified Tyrode-Hepes buffer containing 1 mM Ca^{2+}.

Measurement of Platelet Aggregation—Isolated platelets (2×10^8 cells/ml) were incubated at 37°C with stirring before and after the addition of agonists. The degree of platelet aggregation was examined using a Lumi-Aggregometer model 600 (Chrono-Log) as described previously (*15*).

Immunoblot Analysis—Samples from total cell lysates were separated using 12.5% SDS-PAGE, blotted onto Immobilon P (Millipore) and analyzed with anti-phosphotyrosine antibody (4G10) as described previously (*14*).

Stimulation and Immunoprecipitation Kinase Assay—After stimulation of platelets (2×10^8 cells/ml) with agonists for 30 s, cells were pelleted, lysed with 500 μl of lysis buffer (2% Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 100 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin) and then sonicated for 5 s. The lysates were clarified by centrifugation at $100{,}000\times g$ for 10 min and immunoprecipitated with anti-Syk antibody. Immunoprecipitates were washed three times with lysis buffer, once with 10 mM Hepes/NaOH pH 8.0 containing 0.5 M NaCl and finally with 10 mM Hepes/NaOH pH 8.0. The immunoprecipitates were incubated in a reaction mixture containing 100 mM Hepes/NaOH pH 8.0, 10 μM Na_3VO_4, 50 mM $MgCl_2$, 5 mM $MnCl_2$, and 1 μM [γ-^{32}P]ATP (200 cpm/fmol) for 10 min at 30°C. The reactions were terminated by boiling for 3 min with SDS-sample buffer and subjected to 12.5% SDS-PAGE followed by autoradiography. Phosphoimage values of Syk activities were quantitated by digital optical scanning on a BAS-2000 (Fujix, Tokyo).

RESULTS

Synergistic Effect of Epinephrine on Thrombin-Induced Aggregation and Inhibition by α-Adrenoceptor Antagonist—Since it is well known that subthreshold concentrations of epinephrine can potentiate the stimulatory effects of other agonists such as thrombin, we confirmed the synergistic effect of epinephrine on thrombin-induced aggregation of human platelets and tested whether epinephrine acts through α-adrenergic receptor activation. Figure 1A shows the low level (0.05 unit/ml) of thrombin-induced platelet aggregation in the absence or presence of epinephrine in human platelets. A low concentration of thrombin alone induced only a slow aggregation response, while epinephrine alone at the concentration of 4 μM did not cause any platelet aggregation. However, thrombin-induced aggregation was markedly enhanced by 4 μM epinephrine (Fig. 1A) and this enhancement was reduced in a dose-dependent fashion by phenoxybenzamine, an α-adrenoceptor inhibitor (Fig. 1B). Since platelets dominantly express α_2-adrenergic receptor, these results indicated that epinephrine can potentiate platelet aggregation in response to thrombin through interaction with the α_2-adrenergic receptor.

Enhancement of Thrombin-Induced Protein-Tyrosine Phosphorylation by Epinephrine and Inhibition by α-Adrenoceptor Antagonist—Next, we studied whether epinephrine can potentiate thrombin-induced tyrosine phosphorylation. In the studies shown in Fig. 2, platelets were stimulated by 4 μM epinephrine plus various concentrations of thrombin for 30 s and then whole tyrosine phosphorylation was examined. Epinephrine (4 μM) itself did not cause detectable tyrosine phosphorylation of platelets as judged by immunoblotting probed with anti-phosphotyrosine Ab (4G10). However, the presence of epinephrine caused a shift to the left in the dose-dependence of thrombin (0.01-0.5 U/ml)-induced tyrosine phosphorylation. For instance, the combination of 4 μM epinephrine and 0.05 unit/ml thrombin induced tyrosine phosphorylation to the same extent as the stimulation with 0.5 unit/ml thrombin alone. Next, we examined the effect of phenoxybenzamine on the potentiation of thrombin-induced tyrosine phosphorylation by epinephrine (Fig. 3). When platelets were pre-incubated with phenoxybenzamine at various concentrations (10-40 μM) for 2 min and then stimulated

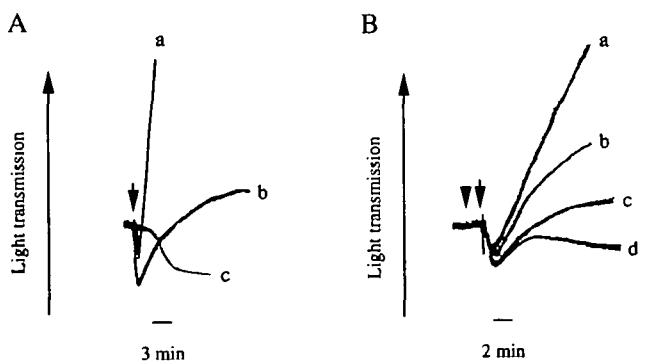

Fig. 1. **Synergistic effect of epinephrine on thrombin-induced platelets aggregation and inhibition by phenoxybenzamine.** Human platelets (2×10^8/ml) were stimulated with thrombin or epinephrine alone, or the combination of thrombin and epinephrine in the absence or presence of phenoxybenzamine, and then platelet aggregation was monitored as described under "MATERIALS AND METHODS." (A) a, 4 μM epinephrine plus 0.05 U/ml thrombin; b, 0.05 U/ml thrombin alone; c, 4 μM epinephrine alone. (B) a, 4 μM epinephrine plus 0.05 U/ml thrombin; b, c, and d, pretreatment with 10, 20, and 40 μM phenoxybenzamine for 2 min, respectively. The arrows and arrowhead indicate the addition of agonists and antagonist, respectively.

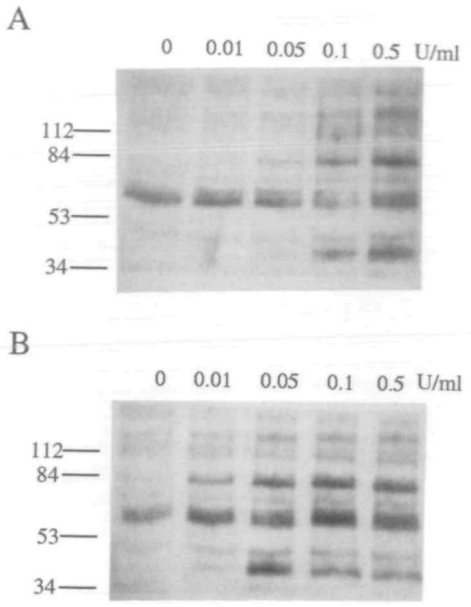

Fig. 2. **Enhancement of thrombin-induced tyrosine phosphorylation by epinephrine.** Human platelets (2×10^8/ml) were stimulated with the indicated concentrations of thrombin in the absence (A) or presence (B) of 4 μM epinephrine for 30 s at 37°C. The samples were analyzed by anti-phosphotyrosine antibody (4G10) immunoblotting as described under "MATERIALS AND METHODS." Positions of molecular markers are shown to the left in kDa.

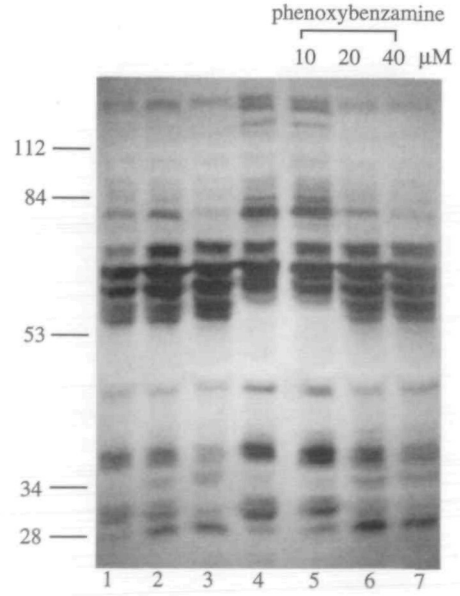

Fig. 3. **Inhibition of thrombin and epinephrine-induced tyrosine phosphorylation by phenoxybenzamine.** Human platelets (2×10^8/ml) were stimulated for 30 s with vehicle (lane 1), 0.05 U/ml thrombin (lane 2), 4 μM epinephrine (lane 3), or the combination of thrombin and epinephrine in the absence (lane 4) or presence of indicated concentrations of phenoxybenzamine (lanes 5–7). The samples were separated by a longer gel size using 10% SDS-PAGE and analyzed by anti-phosphotyrosine antibody (4G10) immunoblotting as described under "MATERIALS AND METHODS." Positions of molecular markers are shown to the left in kDa.

with 0.05 U/ml thrombin plus 4 μM epinephrine, the enhanced tyrosine phosphorylations of several proteins were suppressed in a dose-dependent fashion. These results indicated that epinephrine can potentiate tyrosine phosphorylation as well as platelet aggregation in response to thrombin through interaction with the α_2-adrenergic receptor.

Enhancement of Thrombin-Induced Syk Activation by Epinephrine and Inhibition by α-Adrenoceptor Antagonist—We previously demonstrated that the activity of Syk is rapidly increased by 10-fold upon thrombin stimulation, reaching a maximum at 10 s (*21*). In contrast, the activation of other PTKs including Src and Fak occurs to a lesser degree and with a slower time course (*20, 22*). Therefore, we focused on the Syk activation and monitored the changes in Syk activity during potentiation of thrombin-induced platelet aggregation by epinephrine. Platelets were stimulated by 0–0.5 U/ml thrombin alone or with 4 μM epinephrine for 30 s following immunoprecipitation kinase assay with anti-Syk monoclonal antibody (Fig. 4). Epinephrine (4 μM) alone caused little Syk activation, but in the presence of 4 μM epinephrine, the Syk activation induced by thrombin was clearly enhanced in a narrow range of thrombin concentrations (0.01–0.1 U/ml) (Fig. 4). To understand the

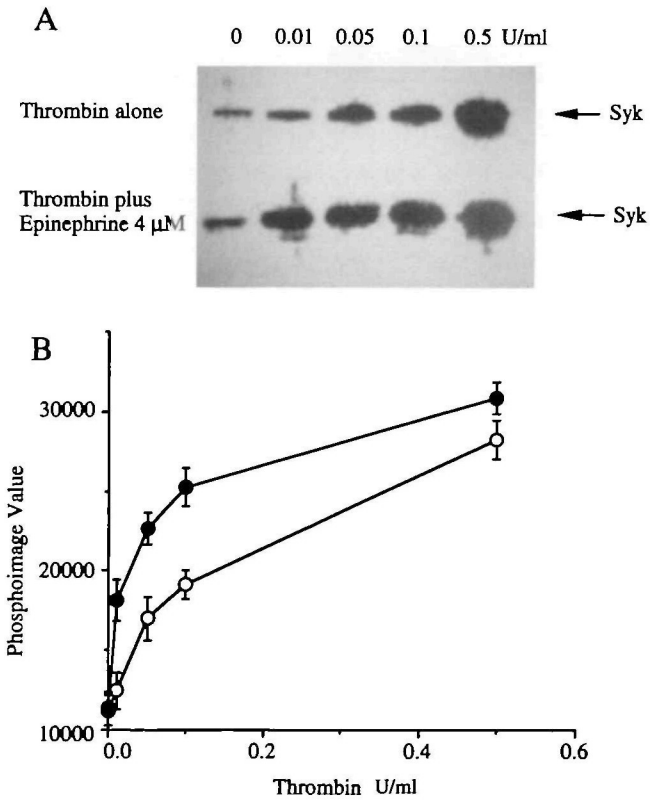

Fig. 4. **Enhancement of thrombin-induced Syk activation by epinephrine.** Human platelets (2×10^8/ml) were stimulated for 30 s with the indicated concentrations of thrombin in the absence or presence of 4 μM epinephrine, and then the activities of Syk were assessed in an immunoprecipitation kinase assay as described under "MATERIALS AND METHODS." (A) An autoradiogram of representative results of three independent experiments. (B) The graph shows the changes of phosphoimage values of Syk activities. Results are the mean ± SD of three independent experiments. ○, thrombin; ●, epinephrine plus thrombin.

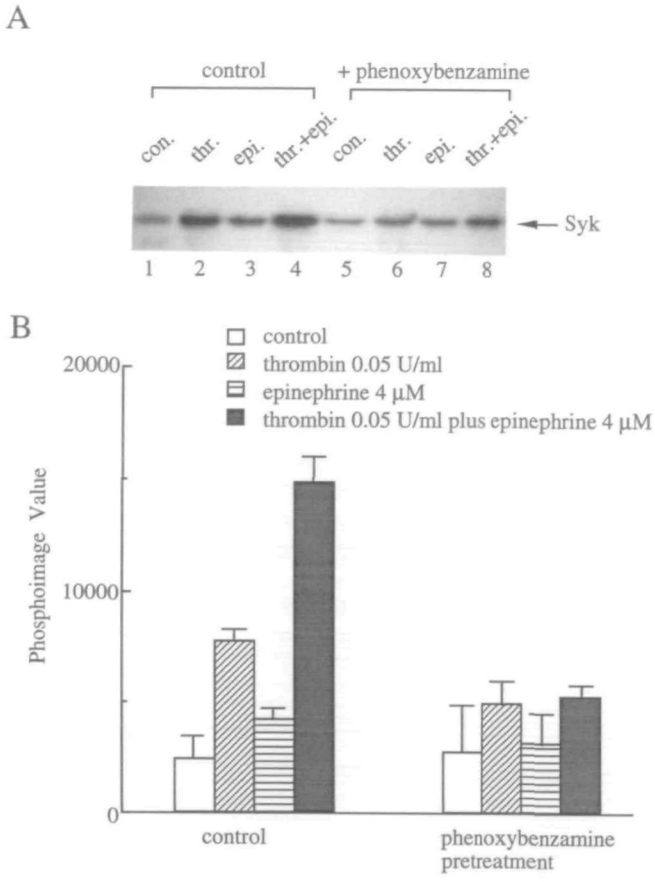

Fig. 5. **Inhibition of thrombin and epinephrine-induced Syk activation by phenoxybenzamine.** (A) Human platelets (2×10^8/ml) were stimulated for 30 s with vehicle (lanes 1 and 5), 0.05 U/ml thrombin (lanes 2 and 6), 4 μM epinephrine (lanes 3 and 7), or the combination of thrombin and epinephrine (lanes 4 and 8) in the absence (lanes 1-4) or presence of 20 μM phenoxybenzamine (lanes 5-8). The activities of Syk were assessed in an immunoprecipitation kinase assay as described under "MATERIALS AND METHODS." (A) An autoradiogram of representative results of three independent experiments. (B) The graph shows the changes of phosphoimage values of Syk activities. Results are the mean ± SD of three independent experiments.

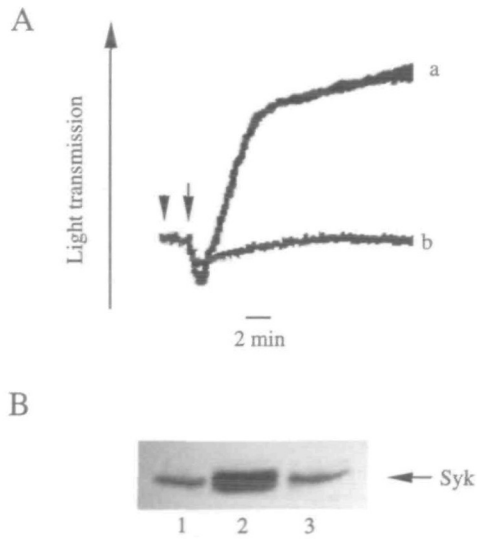

Fig. 6. **Effect of tyrosine kinase inhibitor on the potentiation by epinephrine.** (A) Human platelets (2×10^8/ml) were incubated for 2 min with vehicle (a) or 50 μg/ml genistein (b) and then stimulated with the combination of 0.05 U/ml thrombin and 4 μM epinephrine. Platelet aggregation was monitored as described under "MATERIALS AND METHODS." The arrow and arrowhead indicate the addition of agonists and inhibitor, respectively. (B) Human platelets (2×10^8/ml) were incubated for 2 min with vehicle (lanes 1 and 2) or 50 μg/ml genistein (lane 3) and then stimulated with vehicle (lane 1) or 0.05 U/ml thrombin plus 4 μM epinephrine (lanes 2 and 3). The activities of Syk were assessed in an immunoprecipitation kinase assay as described under "MATERIALS AND METHODS."

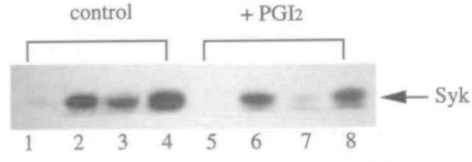

Fig. 7. **Effect of PGI_2 on the potentiation of Syk activation by epinephrine.** Human platelets (2×10^8/ml) were incubated with 1 μM PGI_2 or with an equal volume of dimethylsulfoxide as the control for 2 min, and then stimulated for 30 s with vehicle (lanes 1 and 5), 0.05 U/ml thrombin (lanes 2 and 6), 4 μM epinephrine (lanes 3 and 7), or the combination of thrombin and epinephrine (lanes 4 and 8). The activities of Syk were assessed in an immunoprecipitation kinase assay as described under "MATERIALS AND METHODS."

mechanism of potentiation of thrombin-induced Syk activation by epinephrine, we examined the effect of phenoxybenzamine on the potentiation of thrombin-induced Syk activation by epinephrine (Fig. 5). When platelets were preincubated with phenoxybenzamine (20 μM) for 2 min and then stimulated by 0.05 U/ml thrombin plus 4 μM epinephrine, the Syk activation was significantly suppressed. These results indicated that epinephrine can potentiate Syk activation in response to thrombin through interaction with the α_2-adrenergic receptor.

Effect of Tyrosine Kinase Inhibitor and PGI_2 on the Potentiation by Epinephrine—In order to understand the role of tyrosine phosphorylation and Syk activation in the potentiation by epinephrine, the effect of a PTK inhibitor, genistein, on the platelet aggregation was examined (Fig. 6). Preincubation with genistein completely canceled both thrombin-induced platelet aggregation and Syk activation potentiated by epinephrine. In parallel with these inhibitory effects, this inhibitor also significantly attenuated thrombin-induced tyrosine phosphorylation potentiated by epinephrine (data not shown). The same result was also obtained by preincubation with another PTK inhibitor, herbimycin A (data not shown). These results suggest that tyrosine phosphorylation induced by PTK(s) including Syk plays an important role in the platelet aggregation potentiated by epinephrine. To understand further the action of epinephrine, we used PGI_2, a cAMP-elevating agent, to investigate the mechanism of potentiation of epinephrine in thrombin-induced Syk activation. As shown in Fig. 7, pretreatment of platelets with 1 μM PGI_2 for 2 min partially inhibited Syk activation induced by thrombin and completely inhibited the potentiation by epinephrine. This result suggested that the elevation of intracellular cAMP may negatively regulate Syk activation.

DISCUSSION

Platelets respond to thrombin stimulation by a rapid turnover of the inositol phospholipids through phospholipase C (PLC) activation, with the concomitant formation of inositol trisphosphate (IP_3) and diacylglycerol (DG). Previous studies demonstrated that the potentiation of thrombin-induced platelet activation by epinephrine results from an increase in the concentration of cytoplasmic second messengers including DG and IP_3 working *via* activation of protein kinase C and mobilization of intracellular Ca^{2+} respectively (*8-10*). These lines of evidence suggested that the mechanisms of the potentiation by epinephrine may involve the regulation of upstream molecule(s) such as thrombin receptor, GTP-binding protein, and PLC, which control phosphatidylinositol turnover. On the other hand, thrombin treatment also causes a dramatic increase in the level of phosphotyrosine on multiple proteins (*12-14*). PTK inhibitors such as genistein and erbstatin have been shown to block several platelet events, including platelet aggregation, phosphatidylinositol turnover, and serotonin secretion, in addition to tyrosine phosphorylation (*15-17*). In addition, inhibition of protein tyrosine phosphatases with pervanadate causes a dramatic increase in tyrosine phosphorylation and a concurrent induction of phosphatidylinositol turnover and platelet aggregation, suggesting that tyrosine phosphorylation plays a critical role in platelet activation through the regulation of the hydrolysis of phosphatidylinositol (*23*). Indeed, it was reported that thrombin activation of human platelets causes tyrosine phosphorylation of PLC-γ2 (*24*). However, the platelet thrombin receptor is a seven-transmembrane-domain receptor similar in overall structure to a number of Gq-protein-coupled receptors which appear to regulate the activity of PLC-β (*25*). How tyrosine phosphorylation events induce hydrolysis of phosphatidylinositol in thrombin-activated platelets remains unresolved, though it is possible that the synergistic mechanism between thrombin and epinephrine is involved in the induction of tyrosine phosphorylation. This hypothesis is supported by our observations that epinephrine potentiated thrombin-induced tyrosine phosphorylation and a PTK inhibitor canceled this effect of epinephrine on thrombin-induced platelet aggregation.

The hypothesis that tyrosine phosphorylation plays a critical role in the potentiation by epinephrine has led to characterization of the candidate kinases that may be responsible for these events. We previously demonstrated that platelets express a large amount of Syk and the activity of Syk is rapidly increased by 10-fold upon thrombin stimulation (*21*). Subsequently, we reported that Syk-deficient chicken B cell shows complete abolition of both IP_3 generation and tyrosine phosphorylation of PLCγ2 upon B cell receptor activation, indicating that Syk regulates hydrolysis of phosphatidylinositol (*26*). In addition, in the present study, we observed the potentiation by epinephrine of thrombin-induced Syk activation. Taken together, these results suggested that Syk is one of the key candidate kinases that may be responsible for thrombin-induced tyrosine phosphorylation potentiated by epinephrine.

How does epinephrine potentiate thrombin-induced tyrosine phosphorylation and Syk activation? Human platelets possess both α2- and β2-adrenergic receptors (*7*). Since phenoxybenzamine, an α-adrenoceptor antagonist, canceled this potentiation by epinephrine, it is clear that the potentiating effect of epinephrine is dependent on the occupancy of the α2-adrenergic receptor. It is well established that epinephrine may decrease an already elevated level of intracellular cAMP by inhibition of adenylate cyclase through the interaction with Giα-2, a GTP-binding protein (*7*). On the other hand, it has been demonstrated that PGI_2, which increases cAMP levels in platelets, inhibits thrombin-induced tyrosine phosphorylation, as well as platelet aggregation and secretion (*27*). Quite recently, we also reported that cAMP-dependent protein kinase negatively regulated fMLP-stimulated Syk activation in neutrophils (*28*). Furthermore, in the present study, we observed that PGI_2 significantly reduced the synergistic effects of epinephrine on thrombin-induced Syk activation. Thus, it is likely that the potentiation of tyrosine phosphorylation and Syk activation by epinephrine may be mediated, in part, through a decrease of intracellular cAMP levels.

Taken together, our results suggested that tyrosine phosphorylation and Syk activation are involved in the potentiation of thrombin-induced platelet aggregation by epinephrine. Further investigations are currently under way to clarify the mechanisms of thrombin-induced tyrosine phosphorylation and Syk activation potentiated by epinephrine and also their role in the signal transduction and physiological function of platelets.

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