The role of PAR4 in thrombin-induced thromboxane production in human platelets

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Summary
There are two protease-activated receptors (PARs), PAR1 and PAR4, in human platelets. It has been suggested that PAR1 mediates platelet responses to low concentrations of thrombin, whereas PAR4 mediates signaling only at high concentrations. In the present study, we used a selective PAR4 blocker, YD-3, to investigate the role of PAR4 in thrombin-induced thromboxane formation in human platelets. YD-3 completely prevented thromboxane production by either a low concentration of thrombin (0.1 U/ml) or the PAR4 agonist peptide GYPGKF. In contrast, YD-3 did not affect thromboxane production caused by the PAR1 agonist peptide SFLLRN, collagen or arachidonic acid. YD-3 also decreased [3H]arachidonic acid release from thrombin-stimulated platelets. Moreover, desensitization of platelets with GYPGKF prevented low thrombin-induced thromboxane formation. The decreased thromboxane production by YD-3 is linked to inhibition of calcium influx in thrombin-stimulated platelets. These results suggest that PAR4 plays an important role in the regulation of thromboxane formation in platelets responding to thrombin through prolonged elevation of [Ca2+]i and activation of phospholipase A2. These data also indicate that PAR4 can be activated by relatively low concentrations of thrombin in human platelets. The selective inhibition of thrombin-induced thromboxane production by YD-3 may be of therapeutic benefit for thrombotic diseases.

Keywords
Protease-activated receptor, thrombin, thromboxane, platelets, YD-3

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Introduction
Thrombin is a trypsin-like serine protease playing a central role in both hemostasis and thrombosis (1). In the blood coagulation cascade, thrombin is the final key enzyme, cleaving fibrinogen to form fibrin. Moreover, thrombin is the most potent agent which induces platelet activation and aggregation. These effects of thrombin are thought to be critical for platelet-dependent arterial thrombosis in unstable angina and myocardial infarction.

The ability of thrombin to activate platelets depends upon its proteolytic activity and appears to be largely mediated by a family of G protein-coupled protease-activated receptors (PARs). PAR1 and PAR4 direct the responses of human platelets to thrombin whereas mouse platelets utilize PAR3 and PAR4 to direct their responses to thrombin. PAR1 is the prototype of the thrombin receptor; thrombin binds to and cleaves the exodomain of PAR1 to create a new amino terminus, which can then act as a tethered ligand to activate the receptor (2-4). The synthetic peptide SFLLRN corresponding to the first six residues of this
new amino terminus, functions as a PAR1 agonist and mimics most stimulatory effects of thrombin on human platelets, including aggregation and secretion (5, 6). Furthermore, through inhibition of PAR1’s function by an antagonist, blocking antibodies or desensitization prevented platelet activation by low concentrations of thrombin, and modestly attenuated that by high thrombin (7-9). In contrast to PAR1, PAR4 lacks the hirudin-like thrombin-binding domain and is less sensitive to thrombin. Moreover, blockade of PAR4 alone with an antibody had no effect on platelet aggregation caused by low concentrations of thrombin. The inhibition of high thrombin-induced platelet activation by PAR4-blocking antibody becomes significant only when PAR1 has been simultaneously blocked (10-12). Therefore, PAR1 is considered as a “high-affinity” thrombin receptor which is necessary for responses to low thrombin, while PAR4 is a “low-affinity” receptor mediating responses only at high thrombin (12).

Although the functional role of PAR4 on human platelet aggregation has been well established, the involvement of PAR4 in other processes of platelet activation, such as arachidonic release and thromboxane production, is less clear. Activation of platelets by thrombin or other inducers increases intracellular calcium levels and then activates phospholipase A2, with the subsequent release of arachidonic acid and its metabolism to thromboxane A2. Released thromboxane A2 from the activated platelets is an important factor in amplification of the original stimulus due to recruitment of additional platelets from the circulation to the site of aggregation (13, 14). It has been reported that PAR1 alone cannot totally account for thrombin-induced thromboxane generation, since the PAR1 agonist peptide SFLLRN does not match the substantial release of arachidonic acid and thromboxane formation induced by thrombin and activates phospholipase A2 only partially compared with thrombin (15, 16). Recently, Henriksen and Hanks reported that the PAR4 agonist peptide AYPGKF can stimulate thromboxane production by human platelets and suggested that PAR4 accounts for the second pathway of thrombin-induced thromboxane production in human platelets (17).

In the present work, a selective PAR4 antagonist YD-3 (18) was used to further investigate PAR4’s role in thromboxane generation by thrombin. We showed here that PAR4 is important for thromboxane formation induced by either low concentrations or high concentrations of thrombin. The biological significance of PAR4-mediated thromboxane formation was also assessed.

**Materials and methods**

**Materials**

YD-3 [1-benzyl-3-(ethoxycarbonylphenyl)-indazole] was synthesized based on the methods described previously (19). Human α-thrombin, arachidonic acid, collagen (type I, bovine Achilles tendon), and fluo-3/AM were obtained from Sigma Chem. Co. USA. The synthetic peptide SFLLRN-NH2, GYPGKF-NH2 and Phe-Pro-Arg-chloromethylketone (PPACK) were purchased from Bachem Co. USA. Thromboxane B2 ELISA kits and [3H]arachidonic acid were obtained from Amersham Co. USA. RWJ-56110 was kindly provided by Dr. Claudia K. Derian (Johnson & Johnson Pharmaceutical Research & Development, Spring House, Pennsylvania). All other chemicals were purchased from Sigma Chem. Co. USA.

**Preparation of washed platelets**

Human blood anticoagulated with acid citrate dextrose (ACD) was obtained from healthy human volunteers who had not taken any drugs within the last two weeks. The platelet suspension was then prepared according to the washing procedure described previously (20). Platelets were finally suspended in Tyrode’s solution containing Ca2+ (2 mmol/L), glucose (11.1 mmol/L) and bovine serum albumin (3.5 mg/ml) at a concentration of 3×10^8 platelets/ml.

**Measurement of platelet aggregation**

Platelet aggregation was measured turbidimetrically with a light-transmission aggregometer (Chrono-Log Co., U.S.A.) (21). The platelet suspension was incubated with dimethyl sulfoxide (DMSO, vehicle) or YD-3 at 37°C for 3 min under a stirring condition (1200 rpm) prior to the addition of the platelet stimulators. The extent of platelet aggregation was measured as the maximal increase of light transmission within 5 min after the addition of stimulators.

For PAR4 desensitization studies, platelets resuspended from the first platelet pellet were incubated with GYPGKF (1 mmol/L) in the presence of prostaglandin E1 (PGE1, 1 µmol/L) at room temperature for 30 minutes without stirring; the desensitized platelets were then washed by centrifugation and resuspended in PGE1-free Tyrode’s solution (12).

In the experiments of platelet recruitment, platelet aggregation was induced by thrombin for 5 min, PPACK was then added to neutralize the activity of thrombin, an equal portion of untreated platelets was added subsequently to the cuvette, which increased the turbidity of the solution and hence led to a reduction of light transmission. Aggregation of recruited platelets was then measured for 5 min (23).

**Measurement of thromboxane B2 formation**

Because thromboxane A2 is very unstable and rapidly converted to stable metabolite thromboxane B2, we thus measured the latter instead of thromboxane A2. After the challenge of platelets with stimulators for 5 min, EDTA (2 mmol/L) and indomethacin (50 µmol/L) were added to stop reactions. The platelet suspensions were centrifuged for 3 min at 13,000 rpm, the thromboxane B2 in the supernatants were assayed using ELISA kits according to the procedure described by the manufacturer.
Measurement of $[^3H]$arachidonic acid release

The method modified from that of Kramer et al. (15) was used. Platelet-rich plasma was labeled with 0.5 µCi/ml of $[^3H]$arachidonic acid for 2h at 30°C in the presence of aspirin (100 µmol/L). Platelets were then washed and resuspended in Tyrode’s solution. After stimulation, platelets were fixed at the indicated time with an equal volume of 6% glutaraldehyde solution. Samples were centrifuged at 13,000 g for 10 min, and the supernatant was counted for radioactivity by scintillation spectrometry.

Measurement of intracellular Ca$^{2+}$ mobilization

Platelets pelleted from platelet-rich plasma were resuspended in Ca$^{2+}$-free Tyrode’s solution, then incubated with fluo-3/AM (2 µmol/L) at 37°C for 30 min. In order to prevent leakage of dye, probenecid (2.5 mmol/L) was added to the buffers throughout the experiments. After washing twice, the fluo-3-loaded platelets were finally suspended in Ca$^{2+}$-free Tyrode’s solution at a concentration of 5 x 10$^7$ platelets/ml. Fluorescence (Ex 505 nm, Em 530 nm) was measured with a fluorescence spectrophotometer (Model F2500; Hitachi, Tokyo, Japan) at 37°C.

Statistics

Results are expressed as the mean ± standard error of the mean (S.E.M.) and comparisons were made using Student’s t test. A probability of 0.05 or less was considered significant.

Results

Effect of YD-3 on thromboxane production in platelets by thrombin and PAR4 agonist peptide

According to our previous works (18), YD-3 completely inhibited PAR4 agonist peptide GYPGKF-induced aggregation of human platelets with an IC$_{50}$ value of about 0.2 µmol/L. However, even up to 100 µmol/L, YD-3 had no effect on thrombin (0.1 U/ml)-induced platelet aggregation. This result is consistent with the observation that PAR4 is not necessary for platelet aggregation by thrombin. We therefore sought to investigate whether other processes of platelet activation, such as thromboxane formation, were affected by PAR4 blockade. To our surprise, YD-3 (0.1-10 µmol/L) concentration-dependently inhibited thrombin (0.1 U/ml)-induced thromboxane formation to the basal levels with an IC$_{50}$ value of 0.6 ± 0.2 µmol/L (Fig. 1). When the concentration of thrombin was raised to 0.5 U/ml, YD-3 (10 µmol/L) still partly decreased the thromboxane formation (ng/ml: 72.5 ± 18.9 v.s. control 278.2 ± 29.4, n = 3, p < 0.001). This effect is specific for thrombin and GYPGKF, because thromboxane formation induced by PAR1 agonist peptide SFLLRN and collagen was not significantly affected by YD-3 (Fig. 2). Furthermore, the metabolism of exogenous arachidonic acid (200 µmol/L) to thromboxane in platelets was also not influenced by 10 µmol/L of YD-3 (ng/ml: 447.3 ± 82.5 v.s. control 487.2 ± 48.3, n=3). YD-3 also had no effect on platelet aggregation by SFLLRN, collagen and arachidonic acid (data not shown).

Figure 1: Inhibitory effects of YD-3 on thrombin-induced platelet thromboxane B$_2$ formation. Washed human platelets were stirred with DMSO (0.5 %, control) or various concentrations of YD-3 at 37°C for 3 min, then thrombin (0.1 U/ml) was added to stimulate platelets for 5 min. The reaction was stopped, platelets were then pelleted and supernatants were assayed for TxB$_2$ by ELISA. The amount of thrombin-induced thromboxane B$_2$ produced in platelets treated with DMSO (control) was 22.4 ± 4.2 ng/ml platelets. Percent inhibitions of control are presented as mean ± SEM (n = 3).
Effect of YD-3 on the release of arachidonic acid from thrombin-stimulated platelets

To examine whether the inhibition of YD-3 on thromboxane formation results from a reduction in the release of arachidonic acid, we prelabeled aspirinized platelets with [3H]arachidonic acid and assessed the effect of YD-3 on the release of radio-labeled arachidonic acid (including 12-lipoxygenase metabolites) from thrombin-stimulated platelets. As shown in Figure 3, YD-3 (10 µmol/L) significantly inhibited the mobilization of arachidonic acid by 0.5 U/ml of thrombin. These results are parallel to the inhibition of thromboxane production indicating that YD-3’s action is due to prevention of the activation of cytosolic phospholipase A2 (cPLA2), an enzyme responsible for thrombin-induced arachidonic acid release.

Thrombin-induced thromboxane formation is decreased in PAR4-desensitized platelets

To further identify the role of PAR4 in thrombin-induced thromboxane formation, washed platelets were pretreated with a high concentration of GYPGKF (1 mmol/L) to desensitize PAR4. As shown in Figure 4, the second addition of GYPGKF did not elicit any thromboxane formation in GYPGKF-pretreated platelets indicating PAR4 had been desensitized. Furthermore, these platelets could still respond to SFLLRN suggesting this desensitization was specific to PAR4. Desensitization of platelets with GYPGKF totally prevented low thrombin (0.1 U/ml)-induced thromboxane production, as did blockade of PAR4 by YD-3.

Figure 2: Effect of YD-3 on platelet thromboxane B2 formation caused by various inducers. Washed human platelets were stirred with DMSO (0.5 %, control, solid bars) or YD-3 (10 µmol/L, open bars) at 37 °C for 3 min, then thrombin (0.1 U/ml), GYPGKF (500 µmol/L), SFLLRN (5 µmol/L) or collagen (10 µg/ml) was added to stimulate platelets for 5 min. Thromboxane B2 formation in these platelets was measured as described in Figure 1. Results are presented as mean ± SEM (n = 3-4). ***P < 0.001 as compared with the respective control.

Figure 3: Effect of YD-3 on thrombin-induced release of [3H]arachidonic acid ([3H]AA) in platelets. Aspirinized platelets prelabeled with [3H]AA were incubated with thrombin (0.5 U/ml) in the presence of DMSO (0.5 %, control, ○) or YD-3 (10 µmol/L, □) at 37 °C. The reaction was stopped at indicated time, and released [3H]AA (including 12-lipoxygenase products) was measured by scintillation spectrometry. Results are presented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01 as compared with control.

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Additive inhibition of thromboxane production by PAR1 antagonist and YD-3

Because it has been demonstrated that PAR1 is the major receptor responsible for thrombin’s actions in human platelets and since YD-3 only partially inhibited high thrombin (0.5 U/ml)-induced thromboxane formation, we used a selective PAR1 antagonist RWJ-56110 (25, 26) to examine the interaction between PAR1 and PAR4 on thromboxane production. At 10 µmol/L, which is about 50-fold its IC₅₀ value for SFLLRN-induced platelet aggregation (25, 26), RWJ-56110 markedly prevented 0.1 U/ml of thrombin-induced thromboxane formation, but had much less inhibitory effect on 0.5 U/ml of thrombin (Fig. 5).

**Figure 4:** Effect of PAR4-desensitization on thrombin-induced thromboxane B₂ formation in platelets. Platelets were pretreated with GYPGKF (1 mmol/L) in the presence of PGE₁ (1 µmol/L) at room temperature for 30 minutes without stirring, then washed by centrifugation. Native platelets (solid bars) and PAR4-desensitized platelets (open bars) were stimulated with GYPGKF (1 mmol/L), SFLLRN (10 µmol/L) or thrombin (0.1 U/ml) for 5 min at 37 °C. Thromboxane B₂ formation in these platelets was measured as described in Figure 1. Results are presented as mean ± SEM (n = 3). **P < 0.01 as compared with the respective control.

**Figure 5:** Synergistic effects of YD-3 and RWJ-56110 on thrombin-induced thromboxane formation. Washed human platelets were preincubated with DMSO (0.5 %, control), YD-3 (10 µmol/L) or/and RWJ-56110 (RWJ), 10 µmol/L, thrombin (0.5 U/ml) was then added to induce thromboxane formation. Results are presented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 as compared with the respective controls.
When platelets were pretreated simultaneously with YD-3 and RWJ-56110, thromboxane formation in response to high thrombin was abolished (Fig. 5). In contrast, although the rate of platelet aggregation was delayed, the maximal extent of platelet aggregation in response to high thrombin was not significantly affected by the combination of these two compounds (data not shown).

**Effect of YD-3 on thrombin-elicited intracellular Ca\(^{2+}\) mobilization**

Previous studies have demonstrated that Ca\(^{2+}\) plays a critical role in the activation of cPLA\(_2\) as it promotes association of cPLA\(_2\) with its membrane phospholipid substrate (27, 28). We therefore wanted to examine if the decreased TxA\(_2\) formation and AA release by YD-3 result from inhibition of calcium mobilization in platelets. As shown in Figure 6A and 6B, both 0.1 and 0.5 U/ml thrombin elicited a calcium spike followed by a prolonged phase. When platelets were treated with YD-3, the thrombin calcium signal still had a spike-type profile but largely lost the prolonged phase, thus the elevated calcium signal was rapidly decaying towards the baseline. In contrast, the PAR1 antagonist RWJ-56110 eliminated the calcium spike with less effect on the subsequent prolonged response to thrombin. Addition of RWJ-56110 in combination with YD-3 led to a decrease in both the spike calcium response and the prolonged response elicited by thrombin.

In the absence of extracellular calcium, thrombin induced only a transient calcium spike which was little affected by YD-3 (Fig. 6C, D).

**Effect of YD-3 on thromboxane-dependent platelet recruitment by thrombin**

To assess whether PAR4-mediated thromboxane-release plays a role in platelet recruitment, a method described by Freeman and colleagues was used (23). Washed platelets were challenged with thrombin to induce irreversible aggregation, following addition of PPACK and an equal portion of untreated platelets, a secondary aggregation (recruitment) was observed. As shown in Figure 7, this recruitment largely depended on thrombin-induced thromboxane formation, since pretreatment of indomethacin significantly inhibited it. Similar to indomethacin, YD-3 and RWJ-56110 also markedly prevented platelet recruitment in these conditions.

**Discussion**

In previous studies (10, 12), thrombin activated PAR4 with an EC\(_{50}\) about 50-fold higher than the corresponding figure for PAR1 in a Xenopus oocytes system. Moreover, the anti-PAR4 antibody does not affect platelet aggregation even with low concentrations (1 mmol/ml \(\cong\) 0.1 U/ml) of thrombin and has only a small effect on ATP secretion from platelets. The inhibition of PAR4-blocking antibody becomes significant only when PAR1 has been simultaneously blocked. It was thus suggested that platelet activation and aggregation induced by low concentrations of thrombin is mediated by PAR1, whereas PAR4 is significantly activated only in response to high concentrations of thrombin. Surprisingly, we observed in the present study that the PAR4 antagonist YD-3 completely prevented thromboxane formation.
formation induced by the low concentration of thrombin without affecting platelet aggregation. These data imply that PAR4 may be involved in some processes of platelet activation by low thrombin.

To further explore the mechanism involved in YD-3’s action on thrombin-induced thromboxane formation, we first identified whether the inhibition of YD-3 is specific to thrombin and PAR4 agonist peptide. YD-3 selectively and completely prevented either 0.1 U/ml of thrombin or GYPGKF-induced thromboxane production without affecting that by SFLLRN or collagen. Moreover, the conversion of exogenous arachidonic acid to thromboxane A₂ in platelets was not inhibited by YD-3. These data indicate that YD-3’s action results from specific blockade of PAR4 but not from inhibition of thromboxane synthesis. This suggestion is further supported by the observation that YD-3 markedly decreases the release of arachidonic acid from thrombin-stimulated platelets and this effect is also parallel to inhibition of thromboxane production.

By using PAR4-desensitized platelets, we could further identify the role of PAR4 in mediating thrombin-induced thromboxane production. Pretreatment of human platelets with GYPGKF led to selective desensitization of PAR4. Under this condition, 0.1 U/ml of thrombin failed to elicit any thromboxane production, as it did in YD-3-treated platelets. These data strongly indicate that PAR4 could be activated even with low thrombin and also involved in thrombin-induced thromboxane production in human platelets. Our results also correlate with those of Henriksen and Hanks (17) in that pretreatment of platelets with AYPGKF partially desensitizes thromboxane production in response to high (100 nmol/L ≅ 10 U/ml) thrombin.

Figure 7: Effect of YD-3 on platelet recruitment following thrombin stimulation. (A) Representative tracings of platelet recruitment experiments. Platelet aggregation was induced by thrombin (T, 0.5 U/mL) for 5 minutes in the presence of DMSO (control), YD-3 (10 µmol/L) or RWJ-56110 (RWJ), 10 µmol/L after which PPACK (P, 2 µmol/L) and an equal portion of untreated platelets (PS) was added. Aggregation of recruited platelets was then measured for another 5 min. (B) Quantitative data from the recruitment experiments as described in (A). Aggregation of recruited platelets is measured as the maximal increase of light transmission within 5 min after the addition of untreated platelets (PS). Percent inhibitions of control are presented as mean ± SEM (n = 3).
It has been suggested that PAR1 is the major thrombin receptor in human platelets which mediates almost all of the known processes in platelet activation by thrombin, including thromboxane production (29). We showed here that the selective PAR1 antagonist RWJ-56110 significantly inhibited thrombin-induced thromboxane formation indicating that PAR1 is also necessary for this process. On the other hand, however, neither PAR1 nor PAR4 blockade alone could completely eliminate thromboxane formation by high thrombin. This indicates that maximal thromboxane formation elicited by thrombin needs co-activation of both PAR1 and PAR4. In fact, the combination of YD-3 and RWJ-56110 totally prevented high thrombin-induced thromboxane production.

Intracellular calcium increase is the major regulatory mechanism for cPLA_{2} activation as it promotes association of cPLA_{2} with its membrane phospholipid substrate (27, 28). Therefore, we would like to examine if the decreased arachidonic acid release and thromboxane formation by the PAR4 antagonist YD-3 are due to inhibition of thrombin-induced calcium mobilization in platelets. It has been demonstrated that PAR1 and PAR4 elicited calcium signaling with distinct temps in human platelets (22, 30); PAR1 triggered a rapid and transient increase in intracellular calcium while PAR4 triggered a slower but more prolonged response. The differences in the kinetics of PAR1 and PAR4 signaling imply that the two PARs may play a distinct role in the early and late events of platelet activation (22, 30). In the present work, we showed that thrombin elicited a calcium spike followed by a plateau phase in the presence of 1 mM extracellular calcium and, pretreatment of platelets with RWJ-56110 and YD-3 inhibited mainly the spike and the plateau response, respectively. These data confirm that PAR1 mediates the rapid and transient phase while PAR4 mediates the sustained phase of thrombin-induced calcium signaling. Moreover, because the prolonged elevation of [Ca^{2+}]_{i} in platelets stimulated with thrombin is largely due to the continuing influx of external Ca^{2+} (31), and that blockade of PAR4 by YD-3 has only little effect on thrombin-induced Ca^{2+} spike in the absence of extracellular calcium, we suggest that PAR4 plays an important role in mediating thrombin-elicited calcium influx. It is well known that sustained increase of [Ca^{2+}]_{i} is crucial to cPLA_{2} activation, as mobilization of arachidonic acid in human platelets by thrombin is diminished in the absence of extracellular Ca^{2+} (32). Moreover, SFLLRN is unable to evoke a sustained increase in [Ca^{2+}]_{i} and only partially activates phospholipase A_{2} in platelets. On the other hand, co-addition of SFLLRN and GYPGKF at subthreshold concentrations can evoke a more pronounced and lasting Ca^{2+} signal than that induced by either peptide alone (18, 30). Therefore, it is likely that PAR4 serves as an important auxiliary factor for PAR1 to maintain the high [Ca^{2+}]_{i} levels that support the activation of cPLA_{2}. In low thrombin-stimulated platelets, the PAR1 Ca^{2+} signal is too small to meet the threshold of cPLA_{2} activation and requires action with the subsequent inputs from PAR4, since blockade of either receptor leads to the abolition of platelet thromboxane production. In contrast, in high thrombin-stimulated platelets, the Ca^{2+} signal from PAR1 is strong enough to partially activate cPLA_{2}, but only when it acts in concert with the PAR4-mediated Ca^{2+} signal resulting in optimal activation of cPLA_{2}. Thus, blockade of either receptor markedly attenuates thrombin-induced increase of [Ca^{2+}]_{i} and thromboxane production in platelets.

In addition to Ca^{2+}, cPLA_{2} is regulated by protein phosphorylation. It has been reported that incubation of platelets with thrombin induced the phosphorylation of cPLA_{2}, thereby increasing its intrinsic activity in a reconstitution assay (33). Subsequent studies indicated that p38, but not ERK, is responsible for thrombin-induced cPLA_{2} phosphorylation (34, 35). However, the importance of cPLA_{2} phosphorylation in the release of arachidonic acid from stimulated platelets remains controversial (33, 35, 36). Kramer and his colleagues (15, 35) indicated that SFLLRN is less effective than thrombin in inducing PLA_{2} phosphorylation and p38 activation. Although it is unclear if PAR4 also mediates PLA_{2} phosphorylation and p38 activation in human platelets, AYPGKF has been reported to induce the activation of Src and p38 in cardiomyocytes (37). Therefore, the possibility that the PAR4-mediated cPLA_{2} activation is through enhancing the phosphorylation of the enzyme cannot be ruled out. Further studies on this issue are needed.

In contrast to marked inhibition of thrombin-elicited thromboxane production by a PAR4 antagonist, other studies indicate that blockade of PAR4 does not lead to significant inhibition of thrombin-induced ATP release (12). The reason for this difference may be that platelet dense granule release is an early event of platelet activation and precedes both PAR4 activation and extracellular calcium influx (38-40), whereas thromboxane production, as mentioned above, requires the PAR4-mediated Ca^{2+} signal to reach the maximal levels. Therefore, it is apparent that a rapid and transient PAR1 signal sufficiently accounts for the early events of thrombin-induced platelet activation; however, a combination of sequential activation of PAR1 and PAR4 is necessary for the late events which require long-lasting signaling.

Besides thromboxane production, the slower but prolonged signaling mediated by PAR4 may also be important for other late events of platelet activation such as compaction of platelet aggregates (38) or platelet recruitment. It is well known that released thromboxane following platelet stimulation is critical for recruitment of nearby unactivated platelets; these recruited platelets in turn amplify thrombus growth in the injured vessel (13, 14). Therefore, we would like to assess if PAR4-mediated thromboxane formation is important for platelet recruitment. The data shown here indicated that YD-3 did not affect the original platelet aggregation caused by thrombin, but markedly decreased the following platelet recruitment. Because indomethacin exhibited a similar effect in recruitment experiments,
these results suggested PAR4-mediated thromboxane may enhance platelet recruitment and hence amplify thrombus formation.

In the present study, we used a selective PAR4 blocker, YD-3, to investigate the role of PAR4 in thromboxane formation by thrombin. Our data demonstrated that PAR4 serves as an important auxiliary factor for PAR1 to maintain the high [Ca^{2+}]i levels that support the activation of cPLA2. Blockade of PAR4 is as effective, at least, as blockade of PAR1 on inhibition of thrombin-induced thromboxane production and platelet recruitment. The fact that YD-3 inhibits low thrombin-induced Ca^{2+} signal and thromboxane production, also indicates that PAR4 can be activated not only by high but also by low concentrations of thrombin. Because both thrombin and thromboxane play important roles in pathological thrombosis, selective inhibition of thrombin-induced thromboxane production by YD-3 may be of therapeutic benefit for thrombotic diseases and may have less adverse effects with respect to vascular endothelium and the GI tract than aspirin, because the prostaglandins formed in these cells will be spared.

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References