Synergistic Effect of Thrombin on Collagen-Induced Platelet Procoagulant Activity Is Mediated Through Protease-Activated Receptor-1

Jeffrey F.W. Keuren, Simone J.H. Wielders, Hans Ulrichts, Tilman Hackeng, Johan W.M. Heemskerk, Hans Deckmyn, Edouard M. Bevers, Theo Lindhout

Objective—In the blood coagulation process, the rate of thrombin formation is critically dependent on phosphatidylserine (PtdSer) at the surface of activated platelets. Thrombin synergistically enhances the collagen-induced platelet procoagulant response. The objective of this study is to elucidate the mechanism of this synergistic action with a focus on the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]), and the various platelet receptors for thrombin.

Methods and Results—We demonstrate that procoagulant activity is related to a sustained increased [Ca$^{2+}$], which in turn depends on extracellular Ca$^{2+}$ influx. Increased PtdSer exposure coincides with increased [Ca$^{2+}$], and was observed in a subpopulation (~14%) of the platelets after stimulation with thrombin plus collagen. 2D2-Fab fragments against the thrombin binding site on GPIbα made clear that this receptor did not signal for platelet procoagulant activity. Inhibition of protease-activated receptor 1 (PAR-1) and PAR-4 by selective intracellular inhibitors and selective desensitization of these receptors revealed that PAR-1, but not PAR-4, activation is a prerequisite for both sustained elevations in [Ca$^{2+}$], and procoagulant activity induced by collagen plus thrombin.

Conclusions—The interaction of thrombin with PAR-1 mediates a synergistic effect on collagen-induced procoagulant activity by inducing a sustained elevation in [Ca$^{2+}$], in a subpopulation of platelets. (Arterioscler Thromb Vasc Biol. 2005;25:1499-1505.)

Key Words: thrombin ■ collagen ■ platelets ■ procoagulant activity ■ protease-activated receptors

Platelet membranes have an asymmetrical distribution of phospholipids over the two membrane leaflets with aminophospholipids almost exclusively present in the cytoplasmic leaflet. This asymmetrical distribution can be dissipated by the action of a phospholipid scramblase, resulting in surface exposure of phosphatidylserine (PtdSer). Presence of PtdSer in the exoleaflet of the platelet plasma membrane is of physiological importance because it enhances thrombin formation, which is essential to the formation of a stable hemostatic plug or which contributes to the formation of stable thrombi that may occlude blood vessels.1

For years it has been shown that a combination of collagen and thrombin is a far more potent agonist in generating procoagulant platelet surfaces than each of the individual agonists alone.2 The mechanism behind this synergistic action of thrombin and collagen, however, remains to be elucidated. A key element in the process is the level of the cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]).3 It has been suggested that the synergistic effect of thrombin and collagen in exposing PtdSer is based on the ability of this combination of agonists to induce a high calcium influx that persists over a sufficiently long period to stimulate scramblase that transfers PtdSer to the outer-leaflet and to inhibit aminophospholipid translocase to prevent PtdSer from being pumped back to the inner leaflet.4,5 We hypothesize that thrombin and collagen must act together through distinct platelet receptors and signaling pathways that reinforce their individual capacities in elevating the platelet intracellular calcium level.

Glycoprotein (GP)VI is the major collagen-signaling receptor that induces platelet procoagulant activity (reviewed by Nieswandt and Watson6). However, in spite of the increasing knowledge of receptor-related platelet activation, the contribution of the different thrombin-dependent signal pathways leading to exposure of PtdSer is still a matter of debate. The platelet thrombin receptors, PAR-1,7,8 PAR-4,7 and GPIb-IX-V9–11 are all identified as potential contributors to the thrombin-induced procoagulant response. A possible explanation for these different findings could be the neglected role of GPIbα as a cofactor in the thrombin-catalyzed activation of PAR-1.12 On the other hand it was reported that...
PAR-4 activation peptide acts synergistically with PAR-1 activation peptide in the generation of collagen-mediated procoagulant activity. The objective of this study is to delineate the relative contributions of PAR-1, PAR-4, and GPIb-IX-V in thrombin and thrombin plus collagen–induced platelet procoagulant activity using specific intracellular PAR-1,4 antagonists and a monoclonal antibody that blocks thrombin–GPIbα interaction. In addition, we explored the relationship between increases in intracellular calcium and the generation of a procoagulant surface induced by thrombin receptor agonists.

**Experimental Procedures**

For a detailed Methods section, please see http://atvb.ahajournals.org.

**Results**

**Thrombin-Induced Intracellular Calcium Response and Platelet Prothrombinase Activity**

Thrombin is considered as the most potent physiological platelet agonist. Although this is true for elevating the platelet intracellular calcium concentration, its effectiveness to induce a procoagulant response is a matter of debate. Therefore, both responses were measured in a buffer system as a function of the thrombin concentration. Low amounts of thrombin (0.2 to 2 nmol/L) cause a rapid and transient rise in platelet [Ca$^{2+}$], whereas higher thrombin concentrations (>2 nmol/L) induce a rapid but more sustained rise in [Ca$^{2+}$], (Figure IA and Table I, available online at http://atvb.ahajournals.org). Δ[Ca$^{2+}$]$_{peak}$ values of ≥1 μmol/L were obtained with thrombin concentrations ≥5 nmol/L. The Δ[Ca$^{2+}$]$_{25%}$ value increased proportionally with the thrombin concentration, reflecting the role of PAR-4 in sustaining Δ[Ca$^{2+}$] levels. Our data also reveal that at saturating concentrations, thrombin induces a higher Δ[Ca$^{2+}$]$_{25%}$ than each of the specific PAR-1 and PAR-4 activation peptides. This difference might be attributed to the presence of a third thrombin receptor, namely GPIb-IX-V.

To study the participation of GPIbα, we made use of a monoclonal antibody against the thrombin-binding site on GPIbα (MoAb 2D2). Both MoAb 2D2 and 2D2-Fab completely inhibited low dose (0.2 to 0.6 nmol/L) α-thrombin–induced platelet aggregation and had no inhibitory effect on platelet aggregation induced by higher α-thrombin concentrations (>1 nmol/L). Aggregation by ADP, collagen, ristocetin, botrocetin, or the PAR-1 agonist SFLLRN was unaffected by MoAb 2D2. 2D2-Fab (10 μg/mL) inhibited the thrombin (2 nmol/L)-induced Δ[Ca$^{2+}$]$_{peak}$ by 40% but had no effect on Δ[Ca$^{2+}$]$_{25%}$. Because higher concentrations of 2D2-Fab did not result in a more reduced calcium flux, it is unlikely that the affinity of 2D2-Fab for GPIbα is too low to compete with thrombin (2 nmol/L) for the binding site on GPIbα. When platelets were stimulated with 0.5 nmol/L thrombin in the presence of 2D2-Fab (10 μg/mL), we found a 70% reduced Δ[Ca$^{2+}$]$_{peak}$ and a 40% reduced Δ[Ca$^{2+}$]$_{25%}$ value. It is apparent that the relative contribution of GPIbα in the calcium response increases with decreasing thrombin concentrations.

The capacity of thrombin-treated platelet suspensions to support prothrombin activation by the factor Xa-factor Va complex is plotted in Figure IB and C as a function of Δ[Ca$^{2+}$]$_{peak}$ and Δ[Ca$^{2+}$]$_{25%}$, respectively. It is clearly shown that while Δ[Ca$^{2+}$]$_{peak}$ and Δ[Ca$^{2+}$]$_{25%}$ values increase with thrombin concentrations above 0.5 nmol/L, prothrombinase activity (closed circles) does not increase more than 2-fold (P<0.05) compared with resting platelets (closed square). PAR-1 and PAR-4 activation peptides SFLLRN (open square) and AYPGKF (open triangle), used at concentrations that elicit a maximal calcium response, were unable to generate procoagulant platelet surfaces. Both SFLLRN and AYPGKF generated Δ[Ca$^{2+}$]$_{peak}$ values that are higher than the threshold thrombin concentration of 0.5 nmol/L. AYPGKF, but not SFLLRN, elicited an increase in Δ[Ca$^{2+}$]$_{25%}$ comparable with that induced by thrombin (0.5 nmol/L).

These data indicate that neither SFLLRN nor AYPGKF mimics thrombin with respect to generation of procoagulant platelet membranes, in spite of inducing a marked increase in Δ[Ca$^{2+}$]$_{peak}$. Because of the demonstrated participation of GPIbα in the thrombin (≥0.5 nmol/L)-induced calcium response, platelet procoagulant activity was also measured under the same conditions but in the presence of 2D2-Fab (10 μg/mL). It is apparent that GPIbα is not involved in platelet procoagulant activity induced by low thrombin concentrations because 2D2-Fab (10 μg/mL) does not inhibit the thrombin-induced generation of platelet procoagulant activity (data not shown).

**Thrombin Receptor–Mediated Calcium Flux Synergistically Enhances Collagen Receptor–Mediated Generation of Platelet Procoagulant Activity**

Collagen induces a rise in [Ca$^{2+}$], through a GPVI-mediated pathway, which appears to be associated with an increase in platelet procoagulant activity. Whereas thrombin alone induces a rather low procoagulant activity, it acts synergistically in combination with collagen, but the precise mechanism of this synergism remains to be elucidated. We therefore investigated the effect of increasing thrombin concentrations on collagen-mediated [Ca$^{2+}$], changes and generation of platelet procoagulant activity. In the absence of thrombin, collagen (10 μg/mL) causes a modest but rather sustained [Ca$^{2+}$], level (Figure 1A, bottom trace). Low concentrations of thrombin greatly increase the collagen-mediated initial rate of the calcium flux. In addition, thrombin dose dependently increases the sustained [Ca$^{2+}$], levels (Table II, available online at http://atvb.ahajournals.org). It is remarkable that thrombin induces sustained calcium levels in view of the relative short half-lives of the activated thrombin receptors PAR-1 and PAR-4. In addition, neither SFLLRN nor AYPGKF could elicit a similar sustained calcium influx (Figure 1A).

Figure 1B and 1C depict the capacity of thrombin plus collagen–activated platelets to support prothrombin activation as a function of Δ[Ca$^{2+}$]$_{peak}$ and Δ[Ca$^{2+}$]$_{25%}$, respectively. In the absence of thrombin, collagen generates a 5× higher prothrombinase activity when compared with nonstimulated platelets, while in the presence of thrombin concentrations ≥0.5 nmol/L a 15-fold increase was found (Figure 1B). Although this increase is associated with a significant increase in Δ[Ca$^{2+}$]$_{25%}$, the Δ[Ca$^{2+}$]$_{peak}$ is not significantly affected when the thrombin concentration is increased from 0
PAR-1 and PAR-4 agonists did not significantly enhance the platelet procoagulant response when compared with that of collagen alone (data not shown).

**Calcium Influx and Generation of Procoagulant Platelet Surfaces in Individual Cells**

Our interpretations, thus far, did not take into account the possibility that treatment of platelets in suspension with collagen plus thrombin might result in the appearance of platelet subpopulations with different calcium fluxes and variable extents of PtdSer exposure.\(^5,16,17\) To examine the relationship between changes in intracellular calcium and PtdSer exposure for single platelets activated with thrombin, collagen, or thrombin plus collagen, binding of Alexa Fluor 647-annexin A5 was compared with changes in fluorescence intensity of the calcium probe Fura-Red using a flow cytometer. Flow cytometry analysis (Figure 2) showed that treatment of platelets with the calcium ionophore, ionomycin, made 90% of the platelets annexin A5 positive with an increased \([\text{Ca}^{2+}]_i\). Stimulation of platelets with collagen (10 \(\mu\text{g/mL}\)) plus thrombin (0.5 \(\text{nmol/L}\)) resulted in a minor fraction of platelets (14%) that bound annexin A5 and had elevated \([\text{Ca}^{2+}]_i\). The majority of platelets (74%) remained low in \([\text{Ca}^{2+}]_i\), and did not bind annexin A5. In addition, Figure 2 also shows that when platelets were activated with either thrombin or collagen alone, the subpopulation of platelets that bound annexin A5 and showed increased \([\text{Ca}^{2+}]_i\), was \(\approx\)2- to 3-fold smaller than in a platelet suspension that is stimulated with collagen plus thrombin.

To see whether the intracellular calcium indicator Fura-Red affected annexin A5 binding, similar experiments were performed with platelets not loaded with Fura-Red. The respective percentages of annexin V binding were similar to that found for Fura-Red loaded platelets (data not shown), indicating that neither the affinity of Fura-Red for \(\text{Ca}^{2+}\) ions \((K_0=140 \text{nmol/mL})\) nor its intracellular concentration did decrease the free cytosolic \(\text{Ca}^{2+}\) concentration below the threshold value that is critical for PtdSer exposure at the platelet surface.

**The Relative Contributions of Thrombin Receptors to the Collagen Plus Thrombin–Stimulated Generation of Procoagulant Platelet Surfaces**

The relative contribution of thrombin-activated PAR-1 and PAR-4 to the thrombin plus collagen stimulation of platelet procoagulant activity was investigated by using the intracellular PAR-1 and PAR-4 antagonists, P1pal-12 and P4pal-10, at a concentration (5 \(\mu\text{mol/L}\)) that was sufficient to completely inhibit the calcium response induced with 0.5 \(\text{nmol/L}\) thrombin. P1pal-12 and P4pal-10 decreased significantly \((P<0.05)\) the thrombin plus collagen–induced \([\text{Ca}^{2+}]_i\) and \([\text{Ca}^{2+}]_{\text{peak}}\). Desensitization of PAR-1, but not of PAR-4, also decreased \([\text{Ca}^{2+}]_i\) and \([\text{Ca}^{2+}]_{\text{peak}}\) elicited by thrombin plus collagen (Figure 3A and 3B). The apparent contradictory results obtained with P4pal10 and desensitization of PAR-4 is readily explained by the property of P4pal10 to block also PAR-1 signaling.\(^{18}\) The collagen-induced calcium response was not inhibited by P1pal-12 and P4pal-10 (data not shown).
Figure 3 shows that reduction of the intracellular calcium mobilization is associated with a decreased ability of the stimulated platelets to support prothrombin activation. Both P1pal-12 and P4pal-10 reduced the prothrombinase activity to the level of that induced by collagen alone. Of note, P1pal-12 or P4pal-10 did not inhibit prothrombinase that is assembled on procoagulant vesicles composed of 20 mol% PtdSer and 80 mol% PtdCho (data not shown). As expected, desensitization of PAR-1, but not that of PAR-4, reduced prothrombinase activity.

As reported in a previous section, blocking the interaction between thrombin (0.5 nmol/L) and GPIbα with the 2D2-Fab significantly reduced the thrombin-induced calcium flux. The calcium response of platelets that were stimulated with collagen plus thrombin was not inhibited by 2D2-Fab (Figure 3A and 3B). Furthermore, 2D2-Fab did not inhibit the generation of platelet procoagulant surfaces (Figure 3C). These findings suggest that a thrombin–GPIbα interaction is not required for the synergistic effect of thrombin on the collagen-induced procoagulant response.
measurements require the presence of Ca\(^{2+}\) influx of extracellular calcium. Of note, prothrombinase synergistic effect of thrombin is critically dependent on the exposure does not require a high and sustained level of stimulation should be addressed whether the prothrombinase measurement should be addressed whether the prothrombinase measurements require the presence of Ca\(^{2+}\). Thus, the question should be addressed whether the prothrombinase measurements on platelets that are activated in the absence of extracellular calcium had a rapid but transient increase in [Ca\(^{2+}\)](i) peak, or its perseverance (Δ[Ca\(^{2+}\)](i)-5). Thrombin, SFLLRN, and AYPGKF had a strong additional effect on collagen-induced Δ[Ca\(^{2+}\)](i). However, only thrombin induced, dose dependently, a high sustained [Ca\(^{2+}\)]. While collagen alone increased prothrombinase activity 5-fold, saturating thrombin (0.5 nmol/L) caused an additional 3-fold increase. Again, compared with thrombin, SFLLRN and AYPGKF or a combination of SFLLRN and AYPGKF were unable to enhance the collagen-induced generation of platelet procoagulant activity. These findings confirm that the generation of platelet procoagulant activity requires sustained high [Ca\(^{2+}\)](i), during at least 5 minutes and that a rapid but transient increase of [Ca\(^{2+}\)], is not sufficient. However, our results do not support the suggestion that transient [Ca\(^{2+}\)] peak values of 0.8 μmol/L and higher, as induced by SFLLRN, are required to maximally generate platelet procoagulant activity. The synergistic effect of thrombin was already maximal at 0.5 nmol/L, giving rise to a Δ[Ca\(^{2+}\)](i)-5 between 0.3 and 0.4 μmol/L. Thus, in spite of increasing Δ[Ca\(^{2+}\)] peak and Δ[Ca\(^{2+}\)]-5 values with increasing thrombin concentrations above 0.5 nmol/L, the procoagulant activity did not further increase. These findings are compatible with the role of intracellular calcium as a second messenger in a nonlinear signaling cascade with the procoagulant response at a distal point. It is thus quite feasible that small concentrations of thrombin are saturating with respect to the procoagulant response but not for a maximal increase in Δ[Ca\(^{2+}\)] peak or Δ[Ca\(^{2+}\)]-5.

The marked difference in both calcium and prothrombinase responses induced by the different thrombin receptor agonists suggests a differential involvement of thrombin on the one hand and PAR activation peptides on the other hand. We clearly demonstrated that when PAR-1 signaling is inhibited,
collagen plus thrombin–induced calcium signal and generation of platelet procoagulant activity are reduced to the level of that obtained with collagen alone. The involvement of PAR-1, but not of PAR-4, is also indicated by the finding that a low thrombin concentration (0.5 nmol/L) is already sufficient to induce maximal platelet prothrombinase activity. We note that our observations are in contrast with those recently reported by Dorsam et al. These investigators showed that the combined action of a PAR-1 activation peptide and a PAR-4 activation peptide is as effective as thrombin to enhance collagen-induced generation of platelet procoagulant activity.

Unlike thrombin, SFLLRN and AYPGKF do not interact with the high affinity thrombin receptor GPIbα. Differences in the response of these agonists are therefore likely to reflect the interaction of thrombin with GPIbα. Using 2D2-Fab directed against the thrombin exosite II binding site on GPIbα, we confirmed that this receptor is involved in calcium signaling and platelet aggregation induced by low thrombin concentrations (≤0.5 nmol/L). However, saturating amounts of the 2D2-Fab did not affect thrombin- or thrombin plus collagen–induced generation of procoagulant activity, excluding a significant role of thrombin-GPIbα interaction in this particular platelet function. In view of a recent study demonstrating that the role of GPIbα in the activation of the GTPase Rap1B is masked by ADP receptor signaling, we examined whether this is also true for the function of GPIbα in generating platelet procoagulant activity. The finding that platelets in the presence of the ADP scavenger apyrase responded equally to the thrombin plus collagen–induced generation of platelet procoagulant activity, either in the presence or absence of 2D2-Fab, apparently excludes the possibility that under the conditions of our experiments signaling through GPIbα remains unnoticed because of ADP receptor signaling (data not shown).

Earlier published work demonstrated a heterogeneity among individual platelets in responding to agonist-induced increases in [Ca2+]i, and PtdSer exposure. We also investigated the heterogeneous response of platelets but then for a lower thrombin concentration (0.5 nmol/L) and under stirring conditions. The latter appears to be necessary for optimal collagen-induced calcium signaling and prothrombinase activity. Indeed, in platelet suspensions stimulated with either collagen (10 μg/mL) or thrombin (0.5 nmol/L), the percentage of platelets that have both exposed PtdSer, as probed by annexin A5-binding, and elevated calcium levels is 4% to 6%. When platelets are activated with collagen plus thrombin, the percentage of these platelets increased 3-fold. Because this increase is in agreement with the increase in procoagulant activity, we conclude that only a small fraction of thrombin plus collagen–treated platelets are procoagulant. This once more supports the notion that sustained elevations in [Ca2+]i are required for platelets to lose their phospholipid membrane asymmetry.

Sustained elevations in [Ca2+]i, resulting in a procoagulant response require a net influx of extracellular calcium. The precise mechanism by which this influx is regulated remains to be elucidated. We demonstrated that for platelets challenged with collagen, thrombin, or a combination of both in the absence of extracellular calcium, [Ca2+]i, slightly increased and then rapidly returned to that of resting platelets (Figure II), confirming that independent of the agonist used, extracellular calcium influx is required to obtain sustained [Ca2+]i. If the procoagulant response depends on the sustained level of intracellular calcium, then one would expect a diminished ability to support prothrombin activation under these conditions. Indeed, in the absence of extracellular calcium, the collagen plus thrombin–induced response was significantly reduced (P < 0.01) but not completely absent. As a matter of fact, the residual procoagulant activity equals that of collagen-stimulated platelets. Our data further show that the absence of extracellular calcium does not reduce the collagen-induced procoagulant response (P = 0.25). The notion that collagen might stimulate platelet procoagulant activity in a calcium-independent manner is supported by a recent study demonstrating that the GPVI/FcRgamma/SLP-76 signal pathway, essential for calcium signaling, is not essential for collagen-induced platelet procoagulant activity. The synergistic effect of thrombin on collagen-induced procoagulant response can therefore be attributed to a thrombin-mediated influx of extracellular calcium that increases [Ca2+]i.

In conclusion, treatment of platelets with collagen plus thrombin causes a sustained increase in [Ca2+]i in 14% of the whole platelet population. Only these platelets had exposed PtdSer at their cell surfaces and supported prothrombin activation. The sustained elevation in [Ca2+]i, is the result of an increased influx of extracellular calcium, mediated by a thrombin-dependent signaling pathway through PAR-1 activation. We found no evidence for a role of PAR-4 and GPIbα. It remains, however, to be elucidated why thrombin and not the PAR-1 activation peptide SFLLRN exerts such a synergistic action. Besides cleaving the N-terminal region of PAR-1 resulting in the release of a 41-aa peptide, thrombin also cleaves GPV at the platelet surface. Although the exact roles of the 41-aa peptide and cleaved GPV in hemostasis are largely unknown, both cleavage products were shown to positively mediate platelet adhesion and activation reactions.

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References


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Material & Methods; Figures I, II, Tables I, II

Experimental Procedures

Materials
Bovine serum albumin (BSA) and apyrase were from Sigma. Chromogenic substrate for thrombin (S2238) was from Chromogenix. Fibrillar HORM-type collagen was from Nycomed. Alexa Fluor 647-conjugated Annexin A5, Fura Red-AM and Fura-2-AM were from Molecular Probes. Lactadherin was a kind gift of Prof. Jan Trige Rasmussen (University of Aarhus, Denmark) and was conjugated with FITC. Human factor Xa, human prothrombin, human thrombin and bovine factor Va, were purified as described before. MoAb 2D2 is directed against the N-terminal globular domain of GPIbα and completely blocks thrombin interaction with glycocalcin. The palmitoylated peptides pal-RCLSSSAVANRS (PAR-1 antagonist; P1pal-12³) and pal-SGRRYGHALR (PAR-4 antagonist; P4pal-10³) and the PAR-1 activating peptide SFLLRN-NH₂ and the PAR-4 activating peptide AYPGKF-NH₂ were prepared in our laboratory by solid-phase peptide synthesis using the in situ neutralization/HBTU activation procedure for tBoc chemistry as previously described.⁴

Platelet preparation
Blood (1 volume) was collected into 1/6 volume of 80 mmol/L trisodium citrate, 52 mmol/L citric acid and 180 mmol/L glucose. Platelet-rich plasma (PRP) was obtained by centrifugation at 190 x g for 15 minutes. For calcium measurements platelets in plasma were loaded with Fura-2 (3 µmol/L) or Fura-Red (20 µmol/L) during 45 minutes at 37 °C. Platelets were then spun down by centrifugation, washed with Hepes buffer pH 6.6 (136 mmol/L NaCl, 2.7 mmol/L KCl, 5 mmol/L Hepes, 2 mmol/L MgCl₂, 10 mmol/L glucose, 0.1% BSA) and finally resuspended in Hepes buffer pH 7.5, as described before. The percentage of platelets that bound Alexa Fluor 647-conjugated Annexin A5 varied around 2%.

Measurement of [Ca²⁺], in platelet suspension
Changes in [Ca$^{2+}$]$_i$ of Fura-2 loaded platelets were measured at 37 °C under continuous stirring by dual excitation fluorometry in an SLM-Aminco 8100 spectrofluorometer (SLM Instruments). Ratio values of fluorescence at 340 and 380 nm were converted to levels of [Ca$^{2+}$], as described. Experiments were performed in triplicate. Peak amplitudes of the calcium signal were defined as $\Delta$[Ca$^{2+}$]$_{\text{peak}}$, representing the maximal increase in [Ca$^{2+}$]$_i$ with respect to [Ca$^{2+}$]$_i$ of non-stimulated cells. The increase in [Ca$^{2+}$]$_i$ with respect to [Ca$^{2+}$]$_i$ of non-stimulated cells after 5 minutes of activation with agonist is defined as $\Delta$[Ca$^{2+}$]$_{t=5}$.

**Combined assay of [Ca$^{2+}$]$_i$ and PtdSer exposure in single platelets**

A suspension (0.5 mL) of Fura-Red loaded platelets (1.3x10$^7$/mL) was stimulated with indicated agonists at 37 °C under continuous stirring with a small stirring bar (200 rpm). After exactly 5 minutes a sample (50 µL) was taken and diluted 10-fold in Hepes-buffer pH 7.5 containing 3 mmol/L CaCl$_2$ and a 1:250 dilution of Alexa Fluor 647-conjugated Annexin A5. After 2 minutes incubation at room temperature (sufficient to obtain maximal binding of annexin A5) samples were analyzed by flow cytometry (Beckton Dickinson, FACScan flow cytometer). Platelets were identified by using analytical gates based on FS and SS log signals. Fura-Red was used to determine changes in [Ca$^{2+}$]$_i$. Alexa Fluor 647-annexin A5 was excited with the HeNe laser at 633 nm. The instrument was set to measure forward angle scattered light (FS), side angle scattered light (SS), Fura-Red (FL3, 670 nm long pass filter) and Alexa Fluor 647-annexin A5 (FL4, 661±16 nm bandpass filter). Data of 10,000 individual platelets in FL1, FL2 and FL3 were collected and analysed using WinMDI software (http://facs.scripps.edu/software.html). To detect PtdSer exposure of platelets in the absence of extracellular calcium, FITC-lactadherin (50 nmol/L) was used under the same conditions as described for Alexa Fluor 647-annexin A5. Fluorescence was collected in FL1 through 530±30 nm bandpass filter.

**Prothrombinase assay**

Platelets (1.3x10$^7$/mL) in a volume of 50 µL were stimulated with agonists for 5 minutes at 37 °C under continuous stirring with a small stirring bar (200 rpm). A solution (45 µL) of factor Xa and factor Va in Heps buffer containing 3 mmol/L Ca$^{2+}$ was added and after 30 seconds 5 µL prothrombin was added. The final concentrations were: 40 pmol/L factor Xa, 100 pmol/L factor Va, 3 mmol/L CaCl$_2$, 100 nmol/L prothrombin and 6.5x10$^6$ platelets/mL. Thrombin generation...
was stopped after 1 minute by addition of Hepes buffer pH 7.5 containing 20 mmol/L EDTA. Thrombin was measured with the chromogenic substrate S2238 as described.\(^7\) When thrombin was used as an agonist, changes in OD at 405 nm were corrected for substrate conversion by exogenous thrombin. Factor Va was added to make the rate of thrombin formation independent on release and activation of platelet factor V. The assay conditions were chosen such that the rate of thrombin formation was linear in time (up to 5 minutes), linear with the factor Xa concentration and platelet count (up to 7x10\(^6\) sonicated platelets/mL).

**Desensitization of PAR-1 and PAR-4**
Platelets (1.3x10\(^7\)/mL) suspended in Hepes buffer pH 7.5 containing 3 mmol/L CaCl\(_2\) were incubated with 10 \(\mu\)mol/L SFLLRN or 0.5 mmol/L AYPGKF for 30 minutes at 37 °C under non-stirring conditions. Desensitization of PAR-1 and PAR-4 was confirmed by the inability of these platelets to increase \([\text{Ca}^{2+}]_i\), when challenged again with SFLLRN and AYPGKF, respectively.

**Statistics**
To determine the statistical significance of differences, P-values were obtained with a non-parametric test for two independent variables (Mann-Whitney Test). Differences were defined significant when P<0.05. Data are expressed as mean values ± SD of at least 3 independent experiments.

**References**


Figure I: Effect of thrombin, SFLLRN and AYPGKF on platelet [Ca^{2+}]_i responses and platelet supported prothrombinase activity. A) Fura-2 loaded washed platelets in Hepes buffer containing 3 mmol/L Ca^{2+} were incubated in the absence of agonist (■) and in the presence of thrombin (0.2-10 nmol/L; ●), SFLLRN (10 µmol/L; □) or AYPGKF (0.5 mmol/L; ◄). Changes in [Ca^{2+}]_i were recorded as described. The calcium responses $\Delta$[Ca^{2+}]_peak (B) and $\Delta$[Ca^{2+}]_t=5 (C) were plotted versus corresponding prothrombinase activities. For details, see Experimental Procedures. Values are means and SD of three independent experiments.
**Figure II:** Changes in platelet $[\text{Ca}^{2+}]_i$ after stimulation with collagen, thrombin and collagen plus thrombin in the absence of extracellular calcium and after recalcification. Fura-2 loaded platelets were activated in the presence of 1 mmol/L EGTA with 0.5 nmol/L thrombin (T), 10 µg/ml collagen (C) or a combination of both agonists (C+T). CaCl$_2$ (4 mmol/L) was added after a 5-min activation time. Shown is one representative experiment out of three performed.
Table I. Changes in intracellular calcium peak values $[\text{Ca}^{2+}]_{\text{peak}}$ and intracellular calcium concentration after 5 min incubation $[\text{Ca}^{2+}]_{t=5}$ with thrombin, SFLLRN and AYPGKF.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Concentration</th>
<th>$\Delta[\text{Ca}^{2+}]_{\text{peak}}$ (nmol/L)</th>
<th>$\Delta[\text{Ca}^{2+}]_{t=5}$ (nmol/L)</th>
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<td>Thrombin</td>
<td>0.2 nmol/L</td>
<td>159±22</td>
<td>34±4</td>
</tr>
<tr>
<td></td>
<td>0.5 nmol/L</td>
<td>286±121</td>
<td>65±18</td>
</tr>
<tr>
<td></td>
<td>2 nmol/L</td>
<td>544±30</td>
<td>229±70</td>
</tr>
<tr>
<td></td>
<td>5 nmol/L</td>
<td>917±111</td>
<td>290±34</td>
</tr>
<tr>
<td></td>
<td>10 nmol/L</td>
<td>988±26</td>
<td>360±21</td>
</tr>
<tr>
<td>SFLLRN</td>
<td>10 µmol/L</td>
<td>1002±36</td>
<td>36±1</td>
</tr>
<tr>
<td>AYPGKF</td>
<td>500 µmol/L</td>
<td>450±75</td>
<td>71±11</td>
</tr>
</tbody>
</table>

Table II. Changes in intracellular calcium peak values $[\text{Ca}^{2+}]_{\text{peak}}$ and intracellular calcium concentration after 5 min incubation $[\text{Ca}^{2+}]_{t=5}$ with collagen plus either thrombin, SFLLRN or AYPGKF.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Concentration</th>
<th>$\Delta[\text{Ca}^{2+}]_{\text{peak}}$ (nmol/L)</th>
<th>$\Delta[\text{Ca}^{2+}]_{t=5}$ (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>10 µg/ml</td>
<td>441±4</td>
<td>198±33</td>
</tr>
<tr>
<td></td>
<td>plus thrombin</td>
<td>0.2 nM</td>
<td>441±123</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 nM</td>
<td>484±51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 nM</td>
<td>589±94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 nM</td>
<td>718±20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 nM</td>
<td>748±6</td>
</tr>
<tr>
<td></td>
<td>plus SFLLRN</td>
<td>10 µM</td>
<td>829±136</td>
</tr>
<tr>
<td></td>
<td></td>
<td>plus AYPGKF</td>
<td>598±142</td>
</tr>
</tbody>
</table>