Regulation of Glycoprotein IIB/IIIA Exposure on Platelets Stimulated With α-Thrombin

By Gijsbert van Willigen and Jan-Willem N. Akkerman

Previous studies have shown that binding sites for fibrinogen on platelets stimulated with platelet-activating factor (PAF), adenosine diphosphate or epinephrine rapidly close in the absence of fibrinogen. In the present study we investigated whether α-thrombin induced similar changes in the glycoprotein (GP) IIB/IIIA-complex. Whereas 80% of binding sites exposed by PAF closed within 30 minutes (22°C), α-thrombin (0.1 U/mL) triggered long-lasting exposure of binding sites for [125I]-fibrinogen and [125I]-fibronectin. Even removal of α-thrombin with an excess of hirudin failed to close the binding sites. Similar to PAF, α-thrombin–exposed sites rapidly closed after addition of the protein kinase C inhibitor staurosporine (1 μmol/L) or dibutyryl cyclic adenosine monophosphate (250 μmol/L). In contrast, prostacyclin (PGI₂, 10 ng/mL), which induced rapid closure of binding sites in platelets stimulated with PAF, failed to close the sites in α-thrombin–treated platelets. Removal of α-thrombin from the platelets restored the PGI₂-sensitivity. These data indicate that a short interaction between α-thrombin and platelets triggers long-lasting exposure of GPIIB/IIIA. Furthermore, as long as α-thrombin remains bound to the platelets, agonists that activate the PGI₂-receptor are unable to close GPIIB/IIIA.

THE PLATELET membrane glycoprotein IIB/IIIA-complex (GPIIB/IIIA) is a member of a family of Arg-Gly-Asp–specific receptors. Although this complex is present on the outer layer of the platelet plasma membrane, it is unable to bind adhesive proteins unless the cells make contact with platelet-stimulating agents such as platelet-activating factor (PAF), adenosine diphosphate (ADP), and epinephrine. On stimulation the complex becomes accessible for fibrinogen, fibronectin, von Willebrand factor, vitronectin, and thrombospondin.

When platelets are stimulated in the absence of fibrinogen, the cells gradually lose their capacity to bind fibrinogen. Evidence that this process is reversible came from a study by Peerschke in which platelets made refractory to ADP could be restimulated by thrombin. Also, when arachidonic acid-induced aggregation was interrupted by addition of prostacyclin (PGI₂), a mixture of epinephrine and arachidonic acid restored fibrinogen binding.

In previous publications we showed that binding sites exposed by weak agonists such as PAF, ADP, and epinephrine disappear in the absence of fibrinogen. A second stimulation with a different agonist restored the binding capacity, and exposure and disappearance of binding sites appeared reversible processes that were tightly controlled by protein kinase C (PKC) and a cyclic adenosine monophosphate (AMP)-sensitive step.

In the present study we investigated the mechanisms by which α-thrombin–exposed binding sites for adhesive proteins on GPIIB/IIIA. α-Thrombin is known to stimulate platelets via binding to at least two types of binding sites. One of these is susceptible to chymotrypsin.

Platelet isolation. Venous blood was drawn from healthy volunteers (with informed consent) and collected into tri-sodium citrate (0.1 vol of 130 mmol/L tri-sodium citrate). The donors claimed not to have taken any medication during the previous 10 days. Platelets were isolated by either gel-filtration or centrifugation (as described in the Materials and Methods section) and were washed twice in Tyrode’s buffer (NaCl, 129.9 mmol/L; KCl, 5.0 mmol/L; CaCl₂, 1.8 mmol/L; MgCl₂, 1.0 mmol/L; glucose, 11.1 mmol/L; HEPES, 20 mmol/L; pH 7.4).

Materials. Sepharose 2B and CNBr-activated Sepharose 4B were from Pharmacia (Uppsala, Sweden). PAF, Fura 2-acetoxyethyl ester, and staurosporine were purchased from Boehringer (Mannheim, Germany) and PGI₂ from Cayman Chemicals (Ann Arbor, MI). Human α-thrombin, benzamidine, phenylmethylsulfonyl fluoride (PMSF), dibutyryl cyclic AMP (dbcAMP), and chymotrypsin were obtained from Sigma (St Louis, MO) and bovine serum albumin (BSA, demineralized) from Organon Teknika (Turnhout, Belgium). Na[3H] (specific radioactivity 629 GBq/mmol) was purchased from New England Nuclear (Boston, MA). Fibrinogen (Grade L) was obtained from KABI (Stockholm, Sweden), hirudin from Pentapharm (Basel, Switzerland), and D-phenylalanyl-L-prolyl-L-arginine Chloromethyl ketone (PPACK) was purchased from Calbiochem (Bebrin, La Jolla, CA). The synthetic GRGD-peptide was a kind gift of August Bekkers (Department of Biochemistry, University of Utrecht, The Netherlands). All other chemicals were of analytical grade.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.
below). For the preparation of gel-filtered platelets citrated blood was centrifuged (200g, 10 minutes 22°C) and the platelet-rich plasma (PRP) was placed on a Sepharose 2B column, equilibrated in Ca²⁺-free Tyrode’s solution (137 mmol/L NaCl, 2.68 mmol/L KCl, 0.42 mmol/L NaH₂PO₄, 1.7 mmol/L MgCl₂, 11.9 mmol/L NaHCO₃, pH 7.25) containing 0.2% BSA and 0.1% glucose.

Preparation of [¹²⁵I]-labeled fibronectin. Fibronectin was isolated from fresh-frozen plasma (FFP) by affinity chromatography on gelatin-Sepharose 4B that was prepared according to the manufacturer’s description. FFP (300 mL) was thawed and passed through gelatin-Sepharose 4B (5 x 3.6 cm, equilibrated with 50 mmol/L Tris/HCl pH 7.4) and washed with 1 mol/L NaCl in 50 mmol/L Tris/HCl pH 7.4, followed by 1 mol/L urea. Fibronectin was eluted with 6 mol/L urea in 0.1 mol/L citric acid, pH 4.2. All buffers contained 0.1 mmol/L PMSF, 1 mmol/L benzamidine, 5 mmol/L e-aminocaproic acid, 10 mmol/L EDTA, and 0.02% (wt/vol) sodium azide. The fractions containing fibronectin were pooled and dialyzed at 4°C against 50 mmol/L Tris/HCl pH 7.4, for at least 24 hours. The fibronectin was concentrated by dialysis at 4°C against 30% saturated ammonium sulfate for 16 hours. The precipitate was collected by centrifugation (10 minutes, 3,000g, 22°C), dissolved in a small volume of Ca²⁺-free Tyrode’s solution, and dialyzed for 16 hours against the same buffer. Fibronectin was stored at −20°C until use.

Electrophoresis on a 5% polyacrylamide gel, according to Laemmli, after reduction with β-mercaptoethanol, showed over 99% homogeneity. This fibronectin preparation contained less than 10 ng von Willebrand factor per milligram of fibronectin as determined with an enzyme-linked immunosorbent assay (ELISA).

The purified fibronectin was radiolabeled with Na²¹³¹ by a modified iodogen method. Details of this procedure have been described elsewhere for fibrinogen.

Preparation of [¹²⁵I]-labeled fibronectin. [¹²⁵I]-labeled fibronectin was prepared as described elsewhere.

Platelet stimulation and fibronectin/fibrinogen binding assay. Gel-filtered platelets ([200 to 300] x 10⁹ platelets/µL) were stimulated with 0.1 U/mL α-thrombin or 500 mmol/L PAF in the absence of fibronectin/fibrinogen and without stirring. After stimulation, samples were withdrawn at the times indicated in the Result section and incubated with 1 µmol/L [¹²⁵I]-labeled fibronectin/fibrinogen for 10 minutes at 22°C (without stirring). Under these conditions the concentrations of fibronectin and fibronectin are in excess and a 10-minute incubation reflects the number of exposed binding sites with minimal influence of irreversible binding (for details see refs 18, 19, and 25). Fibronectin/fibrinogen binding was measured by placing 200 µL of cell suspension (in triplicate) on top of 100 µL 25% (wt/vol) sucrose in Ca²⁺-free Tyrode’s solution in micro sedimentation tubes (Starstedt, Vienna, Austria) and separating the cells from the medium by centrifugation (12,000g, 2 minutes, 22°C) in a Beckman Microfuge E (Beckman Instruments, Miami, The Netherlands). The tip of the tube (pellet fraction) was cut off and the pellet and supernatant were counted in a γ-radiation counter. The number of molecules bound per platelet was determined by calculating the ratio of radioactivity in the supernatant over the radioactivity in the pellet. The pellets were resuspended in Ca²⁺-free Tyrode’s solution (pH 7.25) and resuspended in Ca²⁺-free Tyrode’s solution (pH 7.25) and resuspended in Ca²⁺-free Tyrode’s solution (pH 7.25).

Labelled platelets were stimulated and after 5 minutes a modulator of the PLC or AC pathway was added at the concentrations given in the Results section. Ca²⁺-free Tyrode’s solution was added as a control. Samples were collected after 0-, 4-, 10-, and 20-minute incubations at 22°C and the radioactivity was determined by autoradiography of the platelets.

Measurement of PKC activity. Platelets were labelled with 3.7 MBq carrier free [³²P]P₂O₄/mL of acidified PRP (pH 6.5) for 1 hour at 37°C. Platelets were isolated by centrifugation (700g, 20 minutes, 22°C) and resuspended in Ca²⁺-free Tyrode’s solution (pH 7.25) and resuspended in Ca²⁺-free Tyrode’s solution (pH 7.25).

Labeled platelets were stimulated and after 5 minutes a modulator of the PLC or AC pathway was added at the concentrations given in the Results section. Ca²⁺-free Tyrode’s solution was added as a control. Samples were collected after 0-, 4-, 10-, and 20-minute incubations at 22°C and the radioactivity was determined by autoradiography of the platelets.

Measurement of phosphoinositol-4,5-bisphosphate (PIP₂). Changes in PIP₂ were measured according to a modified procedure described by Jolles et al for rat brain cells. In short, platelets were labeled with [³²P]orthophosphate and isolated by centrifugation as described previously. The [³²P]-content of the 47-Kd protein, the major substrate for PKC in platelets, was determined as described elsewhere. The [³²P]-content of the 47-Kd protein is expressed as a percentage of that of unstimulated platelets.

In control experiments no differences in PKC activity could be detected between centrifuged platelets and gel-filtered platelets (results not shown).

Measurement of phosphoinositol-4,5-bisphosphate (PIP₂). Changes in PIP₂ were measured according to a modified procedure described by Jolles et al for rat brain cells. In short, platelets were labeled with [³²P]orthophosphate and isolated by centrifugation as described previously. The [³²P]-content of the 47-Kd protein, the major substrate for PKC in platelets, was determined as described elsewhere. The [³²P]-content of the 47-Kd protein is expressed as a percentage of that of unstimulated platelets.

Measurement of inositol trisphosphate ([³⁸I]IP₃). Acidified PRP (pH 6.5) was incubated with 3 µmol/L Fura 2-acetoxymethylester for 45 minutes at 37°C. Platelets were centrifuged and resuspended in 1 mL HEPES-Tyrode buffer (129 mmol/L NaCl, 8.9 mmol/L NaHCO₃, 0.8 mmol/L KH₂PO₄, 0.8 mmol/L MgCl₂, 5.6 mmol/L glucose, 10 mmol/L HEPES, pH 6.5) and resuspended in Ca²⁺-free Tyrode’s solution (pH 7.5; final platelet concentration 350 x 10⁶/µL). After incubation with α-thrombin (300 µg/mL, 5 minutes) 0.8 mmol/L PMSF and 30 mmol/L PGI₂ were added. The platelets were centrifuged (800g, 10 minutes) and resuspended in Ca²⁺-free Tyrode’s solution and used in binding experiments, as described previously.

Measurement of PKC activity. Platelets were labelled with 3.7 MBq carrier free [³²P]P₂O₄/mL of acidified PRP (pH 6.5) for 1 hour at 37°C. Platelets were isolated by centrifugation (700g, 20 minutes, 22°C) and resuspended in Ca²⁺-free Tyrode’s solution (pH 7.25). The [³²P]-content of the 47-Kd protein is expressed as a percentage of that of unstimulated platelets.

In control experiments no differences in PKC activity could be detected between centrifuged platelets and gel-filtered platelets (results not shown).

Measurement of phosphoinositol-4,5-bisphosphate (PIP₂). Changes in PIP₂ were measured according to a modified procedure described by Jolles et al for rat brain cells. In short, platelets were labeled with [³²P]orthophosphate and isolated by centrifugation as described previously. The [³²P]-content of the 47-Kd protein, the major substrate for PKC in platelets, was determined as described elsewhere. The [³²P]-content of the 47-Kd protein is expressed as a percentage of that of unstimulated platelets.

Measurement of inositol trisphosphate ([³⁸I]IP₃). Acidified PRP (pH 6.5) was incubated with 3 µmol/L Fura 2-acetoxymethylester for 45 minutes at 37°C. Platelets were centrifuged and resuspended in 1 mL HEPES-Tyrode buffer (pH 6.5) for 1 hour at 37°C. Platelets were isolated by centrifugation (700g, 20 minutes, 22°C) and resuspended in Ca²⁺-free Tyrode’s solution (pH 7.25). After incubation with α-thrombin (300 µg/mL, 5 minutes) 0.8 mmol/L PMSF and 30 mmol/L PGI₂ were added. The platelets were centrifuged (800g, 10 minutes) and resuspended in Ca²⁺-free Tyrode’s solution and used in binding experiments, as described previously.

Measurement of PKC activity. Platelets were labelled with 3.7 MBq carrier free [³²P]P₂O₄/mL of acidified PRP (pH 6.5) for 1 hour at 37°C. Platelets were isolated by centrifugation (700g, 20 minutes, 22°C) and resuspended in Ca²⁺-free Tyrode’s solution (pH 7.25). After incubation with α-thrombin (300 µg/mL, 5 minutes) 0.8 mmol/L PMSF and 30 mmol/L PGI₂ were added. The platelets were centrifuged (800g, 10 minutes) and resuspended in Ca²⁺-free Tyrode’s solution and used in binding experiments, as described previously.
*Measurements of cyclic AMP-level.* Cyclic AMP-levels in the platelets were measured using a radioimmune assay, performed according to the manufacturer's instructions.

**RESULTS**

**GPIIb/IIIa exposure by α-thrombin.** In previous studies, we have shown that the exposure of binding sites on GPIIb/IIIa can be deduced from the binding of $[^{125}I]$-fibrinogen provided that the number of exposed binding sites is made rate limiting. As illustrated in Fig 1 and specified earlier, a relatively high concentration of PAF exposed a maximal number of binding sites within the first few minutes, which was followed by a gradual decrease in available binding sites. Similar findings were obtained after stimulation with ADP and epinephrine. When $[^{125}I]$-fibrinogen was replaced by $[^{125}I]$-fibronectin a similar pattern was seen, indicating that the disappearance of binding sites was independent of the nature of the adhesive protein and reflected a property of GPIIb/IIIa.

Surprisingly, when platelets were stimulated with 0.1 U/mL α-thrombin, exposed binding sites remained accessible to $[^{125}I]$-fibrinogen (coagulation prevented by 30 nmol/L PPACK, added 3 minutes after α-thrombin stimulation) and to $[^{125}I]$-fibronectin for as long as 30 minutes (Fig 1) or longer (results not shown).

It is well known that in contrast to PAF, α-thrombin exposes not only GPIIb/IIIa complexes present on the outer-leaflet of the membrane, but also complexes that are present in the OCS and the α-granules. To investigate whether these two pools of GPIIb/IIIa show a similar behavior, platelets were stimulated with 500 nmol/L PAF in the presence of the synthetic peptide GRGDS (5 μmol/L, 45 minutes) that saturated exposed surface located GPIIb/IIIa. Subsequently, platelets were stimulated with 0.1 U/mL α-thrombin in the absence of radiolabeled ligand, to expose the second pool originating from the α-granules and the OCS (Fig 2). This treatment exposed an almost equal number of binding sites as seen after the first treatment with PAF (in the absence of GRGDS), which remained accessible over a long period of time. These data indicate that the internal pool of GPIIb/IIIa and the complexes on the plasma membrane show similar properties after exposure by α-thrombin.

**Control of binding site exposure on GPIIb/IIIa.** In platelets stimulated by PAF, ADP, or epinephrine the PLC pathway is crucial in exposing binding sites on GPIIb/IIIa because it initiates the activation of PKC, which correlates quantitatively with the number of exposed binding sites. On the other hand, increasing cyclic AMP rapidly makes the binding sites inaccessible. As illustrated in Table 1, binding sites exposed by α-thrombin were equally susceptible to the inhibitor of PKC, staurosporine (1 μmol/L) and to dbcAMP (250 μmol/L), suggesting that α-thrombin controls GPIIb/IIIa exposure via similar mechanisms as PAF.
ADP, and epinephrine. In contrast, PGI2 (10 ng/mL), which very effectively closed PAF-exposed binding sites, failed to induce alterations in GPIIB/IIA when α-thrombin was the agonist. To investigate whether the maintenance of exposed GPIIB/IIA was due to persistent signal generation by α-thrombin receptors, platelets were first stimulated with 0.1 U/mL α-thrombin and 60 seconds later a 30-fold molar excess of hirudin was added. As also shown in Table 1, this treatment failed to affect the number of exposed binding sites for [125I]-fibronectin, which still remained accessible up to 30 minutes after activation. Despite the abolishment of signal generation the exposed binding sites remained sensitive to the PKC inhibitor staurosporine (1 pmol/L) and an increase in cyclic AMP content (dbcAMP, 250 μmol/L). However, the removal of α-thrombin by hirudin restored the sensitivity to PGI2 (10 ng/mL), which now rapidly induced closure of virtually all binding sites.

An alternative explanation for the persistent exposure of binding sites is that α-thrombin proteolytically cleaved GPIIB/IIA, thereby making binding sites accessible to fibrinogen or fibronectin. When α-thrombin was first incubated with PPACK (5 minutes, 4°C) to block its proteolytic activity and thereafter added to platelets, no binding sites were exposed, confirming that proteolysis is vital for α-thrombin-induced exposure of GPIIB/IIA (results not shown). This process was mimicked by treating platelets with α-chymotrypsin (300 μg/mL, 5 minutes, 22°C) which induced a similar long-lasting exposure of fibrinogen binding sites. However, in contrast to α-thrombin-exposed sites, binding sites made accessible by α-chymotrypsin were insensitive to inhibition of PKC (staurosporine, 1 μmol/L) or treatment with dbcAMP (250 μmol/L), indicating that they were uncoupled from intracellular control mechanisms. Also, PGI2 (10 ng/mL) failed to affect the α-chymotrypsin-exposed binding sites.

The observation that binding sites exposed by α-thrombin and PAF both required an active PKC and a low level of cyclic AMP raised the question of whether the differences in binding site exposure seen between both agonists were due to a different modulation of the PLC and AC pathway. As shown in Fig 3A, exposure and closure of binding sites in platelets stimulated by PAF (500 nmol/L) closely paralleled the phosphorylation of the 47-Kd protein, which is a major substrate of PKC. The mobilization of intracellular Ca2+ was transient and did not match these changes in GPIIB/IIA, and the kinetics of 32P-PIP2 accumulation (Fig

---

**Table 1. Modulation of Signal Transduction**

<table>
<thead>
<tr>
<th>Effector</th>
<th>α-Thrombin</th>
<th>α-Thrombin + Hirudin</th>
<th>α-Chymotrypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 6.4</td>
<td>98.2 ± 7.8</td>
<td>96.2 ± 6.1</td>
</tr>
<tr>
<td>Stauroporine</td>
<td>7.1 ± 6.8</td>
<td>4.9 ± 1.3*</td>
<td>95.7 ± 8.5</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>9.6 ± 7.2</td>
<td>7.3 ± 3.9*</td>
<td>101.3 ± 4.6</td>
</tr>
<tr>
<td>PGI2</td>
<td>95.9 ± 3.8</td>
<td>7.9 ± 7.8*</td>
<td>98.4 ± 3.9</td>
</tr>
</tbody>
</table>

*Significantly different from control (P < .01, Student's t-test).

---

**Fig 3.** GPIIB/IIA-exposure versus signal generation. Gel-filtered platelets were stimulated with 500 nmol/L PAF (A) or 0.1 U/mL α-thrombin (B) and the disappearance of exposed binding sites on GPIIB/IIA (method see Fig 1) and the radioactivity in the 47-Kd protein of [32P]-labeled platelets was measured. The phosphorylation data are expressed as percentage of 32P in the 47-Kd protein of unstimulated platelets (mean ± SD, n = 3). In the inserts, the changes in Fura-2 fluorescence and in 32P-PIP2 (B) after stimulation are shown (measured as described in Materials and Methods). The Fura-2 fluorescence is expressed in relative fluorescence units (rfu). The changes in 32P-PIP2 are expressed as percentage of 32P in PIP2 of unstimulated platelets. Shown are experiments representative for at least four other experiments.
3A) and other phosphatidylinositol metabolites (result not shown) failed to correlate with the changes in binding properties. Similar measurements in platelets stimulated with α-thrombin (0.1 U/mL) also failed to show a correlation between binding site exposure and changes in cytosolic Ca²⁺ or [³²P]-PIP₂ (Fig 3B). In contrast, 47-Kd phosphorylation again paralleled the more permanent exposure of binding sites on stimulation with α-thrombin. Thus, the differences in the control of GPIIb/IIIa by PAF and α-thrombin is due to differences in the regulation of PKC.

Figure 4 illustrates a comparison between binding sites exposed by α-thrombin and the concentration of cyclic AMP. The persistent exposure of GPIIb/IIIa was paralleled by low and stable levels of cyclic AMP. When PGF₂α (10 ng/mL) was added 5 minutes after activation by α-thrombin (0.1 U/mL) neither the number of exposed binding sites nor the concentration of cyclic AMP changed. However, removal of α-thrombin by an excess of hirudin (3 U/mL) before PGF₂α treatment restored the prostacyclin sensitivity and induced both a rapid closure of exposed binding sites and a concurrent increase in cyclic AMP concentration (results not shown). Also, when platelets were first treated with PGF₂α and hirudin was added at various intervals thereafter, PGF₂α sensitivity was restored (Fig 4). As more time elapsed between the two additions, both the accumulation of cyclic AMP and the decrease in the number of exposed binding sites became slower, probably because of the poor stability of PGF₂α at neutral pH.

Control of α-thrombin-induced GPIIb/IIIa exposure by PKC and cyclic AMP. In an earlier publication a strong, approximately linear, correlation was described between the number of exposed binding sites for fibrinogen and PKC activity, in platelets stimulated with PAF and ADP. The only deviation from this correlation was seen on increasing the cyclic AMP level, which closed the binding sites without affecting PKC activity. Table 2 illustrates that exposure of binding sites by α-thrombin was accompanied by a fourfold activation of PKC. Addition of staurosporine (1 µmol/L) induced an equally rapid closure of exposed binding sites and dephosphorylation of the 47-Kd protein to resting levels. PGF₂α alone, which failed to close binding sites, was also unable to change the phosphorylation of the 47-Kd protein. However, addition of PGF₂α after removing α-thrombin with hirudin triggered rapid closure of binding sites. Interestingly, addition of hirudin to α-thrombin-treated platelets neither affected the exposed binding sites nor PKC activity, indicating that both properties were independent of concurrent signal generation. Thus, under all conditions tested the continuous exposure of GPIIb/IIIa corresponded with the persistent phosphorylation of the 47-Kd protein, except when the cyclic AMP level was.

### Table 2. Exposed Binding Sites and Protein Kinase-C Activity in α-Thrombin-Stimulated Platelets

<table>
<thead>
<tr>
<th>Additions</th>
<th>Exposed Sites t = 10 min</th>
<th>% Increase in PKC Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrode</td>
<td>100.0 ± 5.4</td>
<td>325</td>
</tr>
<tr>
<td>Stauroporine</td>
<td>7.1 ± 5.6</td>
<td>0</td>
</tr>
<tr>
<td>PGF₂α</td>
<td>95.9 ± 3.8</td>
<td>300</td>
</tr>
<tr>
<td>Hirudin + PGF₂α</td>
<td>7.9 ± 7.8</td>
<td>280</td>
</tr>
<tr>
<td>Hirudin</td>
<td>98.2 ± 7.3</td>
<td>310</td>
</tr>
</tbody>
</table>

[³²P]-labeled platelets were stimulated with 0.1 U/mL α-thrombin. At t = 1 min 3 U/mL hirudin or an equal volume Tyrode’s solution was added. At t = 5 min PGF₂α (10 ng/mL) or staurosporine (1 µmol/L) was added. The radioactivity in the 47-Kd protein was measured 10 min after stimulation, as described in Materials and Methods. The fibrinectin binding was detected as described in Fig 1. The phosphorylation data are expressed as the percent increase in [³²P] in the 47-Kd protein as compared with unstimulated platelets. Fibrinectin binding data are expressed as the percent specific binding induced at t = 0 (data are means ± SD, n = 3, 100% = 81,908 exposed binding sites). The data are representative for three similar experiments.
virtually identical binding properties that are seen for
without affecting the phosphorylation patterns. The latter
raised. High cyclic AMP-levels closed all binding sites
for a low level of cyclic AMP; and (3) the insensitivity to
modulation of the phospholipase A_2 pathway (ref 19, results
not shown). This indicates that the pathways via PLC and
AC appear predominant in the control of GPIIB/IIIA. The
virtually identical binding properties that are seen for
for ['^32P]-fibrinogen, although the absolute number of ex-
posed sites differed. A maximal stimulation at 22°C trig-
ger rapid exposure of GPIIB/IIIA within less than 5
minutes and was followed by an exponential disappearance
of binding sites, provided that fibrinogen was absent and
could not form a stable [fibrinogen]-[GPIIB/IIIA]-com-
plex. Subsequent restimulation with a different agonist led
to re-exposure, and exposed and closed binding sites
appeared to be two different states of a single population of
binding sites initially pointed to a role for proteolytic
processes similar to exposure of binding sites by α-chymo-
trypsin and pronase. Indeed, blockade of the active site
by TLCK or PPACK (results not shown) without inhibiting
binding to the platelets, confirmed that proteolysis was
an essential step in the exposure of GPIIB/IIIA. However,
addition of hirudin after α-thrombin had completed bind-
ing site exposure had no effect. Also, α-thrombin–exposed
binding sites closed rapidly when PKC was inhibited (sta-
urosporine) or the cyclic AMP level was increased (dbc-
AMP), whereas α-chymotrypsin exposed sites were insen-
sitive to those treatments. These observations indicate that
other processes mediate the α-thrombin–controlled binding
site exposure. The persistent exposure of binding sites seen
after stimulation with α-thrombin could also be due to
binding of fibrinogen to secreted thrombospondin. This
would also explain why removal of α-thrombin from the
platelet by hirudin had no effect. However, virtually all
binding sites for ['^32P]-fibrinogen/fibronectin closed after
addition of dbcAMP and staurosporine, making this expla-
nation unlikely.

Tam et al^23 reported that an excess of hirudin rapidly
removed α-thrombin from its receptors on platelets. This
treatment interrupted phosphorylation of the 47-Kd and
20-Kd proteins,^24 phosphatidic acid formation,^19,25 arach-
donic acid liberation,^26 and Ca^{2+}-mobilization. In contrast,
shape change^27,28 and the formation of phosphatidylinosi-
tol^29 required a short period of stimulation and continued
unchanged when receptor occupancy was terminated. Inter-
ference with a-thrombin receptor interaction also inter-
rupted optical aggregation and secretion of lysosomal
granules,^30,31 indicating that these functions depend on
concurrent signal generation to be completed. The same is
valid for α-thrombin–induced GPIIB/IIIA exposure be-
cause addition of hirudin at intervals of less than 15 seconds
reduced the number of exposed binding sites (results not
shown). However, when exposure of binding sites was
completed, an intact [α-thrombin]-[receptor]-complex was
no longer required to maintain GPIIB/IIIA in the exposed
configuration. The observation that 15-second stimulation
suffices for maximal binding site exposure accords with a
similar requirement for optimal secretion of α- and dense-
granule contents and optical aggregation. In contrast to
α-thrombin, removal of PAF and ADP from their receptor
led to a diminished exposure of GPIIB/IIIA. Addition of
BN52021, a PAF antagonist, to PAF-stimulated platelets
interrupted fibrinogen binding. Addition of ADP scav-
engers during ADP stimulation resulted in reversible aggrega-
tion and inhibition of fibrinogen binding. Recently, Vu et
al^32 described that the activation of a thrombin receptor on
Xenopus oocytes occurs via a novel mechanism in which
α-thrombin cleaves part of the N-terminal region of this
receptor liberating a new N-terminus that activates its own
receptor even in the absence of α-thrombin. A similar
mechanism would explain why binding sites exposed by
α-thrombin on platelets are insensitive to hirudin treat-
ment. A comparison between changes in cytosolic Ca^{2+},
3′-P-PIP_2 and its metabolites, and PKC in platelets stimu-
lated with PAF and α-thrombin showed that the exposure
of GPIIB/IIIA correlated with the activity of PKC, mea-
sured as the phosphorylation of the 47-Kd protein. In
contrast, the other second messengers had either returned
to resting levels or differed profoundly in activation pat-
terns compared with the exposure of GPIIB/IIIA. These
observations indicate that exposure of binding sites on
GPIIB/IIIA is tightly controlled by PKC. In line with these
findings is the persistent phosphorylation of the 47-Kd
protein after dissociation of α-thrombin from its receptors.
However, when cyclic AMP was increased, exposed binding
sites closed without a concurrent change in PKC activity.
Thus, an increase in cyclic AMP uncouples the interaction
between PKC and exposed binding sites, indicating that

DISCUSSION
The present data show a few similarities and some
notable differences in the control of GPIIB/IIIA exposure
initiated by α-thrombin and weak activators such as PAF,
ADP, and epinephrine. Common features are: (1) the
strong dependence on an active PKC; (2) the requirement
for a low level of cyclic AMP; and (3) the insensitivity to
interference by PAF, ADP, and epinephrine. Common features are: (1) the
strong dependence on an active PKC; (2) the requirement
for a low level of cyclic AMP; and (3) the insensitivity to
interference by PAF, ADP, and epinephrine. Common features are: (1) the
strong dependence on an active PKC; (2) the requirement
for a low level of cyclic AMP; and (3) the insensitivity to
interference by PAF, ADP, and epinephrine.
cyclic AMP is dominant over PKC activity in the regulation of exposure of binding sites.

Recently, Parise et al. reported that stimulation of platelets with α-thrombin triggers the phosphorylation of GPIIIA. Thrombin caused a 4.1-fold increase in GPIIIA phosphorylation within 3 minutes. The time course of this phosphorylation was similar to other events, e.g., the exposure of fibrinogen binding sites.3 The mechanism by which GPIIIA was phosphorylated was staurosporine-sensitive. The amino acid residue that was phosphorylated was a threonine, possibly threonine 758 or 762 of the cytoplasmic domain.4 Therefore, the phosphorylation of GPIIIA is probably mediated via PKC, a serine/threonine-dependent protein kinase. Because GPIIIA consists of only one polypeptide chain, phosphorylation may induce a conformational change, leading to receptor activation.6 Thus, an explanation for the inducible binding properties of GPIIB/IIIA must be sought in part in the control of PKC. Apparently, control of PKC differs markedly between PAF- and α-thrombin–treated platelets. Possibly, PAF and α-thrombin activate different subtypes of PKC or other threonine/serine kinases that are able to phosphorylate the 47-Kd protein and GPIIIA. Another possibility is that α-thrombin inhibits protein phosphatases, thereby inhibiting dephosphorylation. Indeed, inhibition of phosphatases by cooling platelets to 4°C resulted in a continuous binding site exposure and stable levels of phosphorylated 47-Kd protein.7 Thus, it is likely that the phosphorylation of GPIIIA is the trigger for receptor exposure.

Binding of α-thrombin to platelets not only exposes GPIIB/IIIA, but also inhibits AC by activation of an inhibitory GTP-binding protein (G_i) coupled to AC.21,40 The PAF receptor is not coupled to this G_i.46 This explains the differences in sensitivity to PGI_1 between α-thrombin– and PAF-exposed binding sites. In our study, the inhibition of AC could be abolished by removing α-thrombin from the platelets with hirudin. This observation is in contrast to the irreversible inhibition of G_i reported by Aktories and Jakobs.47

The present study is based on modulations of GPIIB/IIIA at 22°C under virtually fibrinogen-free conditions, although a fibrinogen binding study was used to evaluate the state of the binding sites. Obviously, this condition differs greatly from the in vivo situation in which fibrinogen is present at a 10-fold higher concentration and the temperature is higher. However, binding studies under more physiologic conditions show that it takes at least a few seconds before all exposed GPIIB/IIIA complexes are occupied, leaving a short period of time in which GPIIB/IIIA may be susceptible to the regulatory mechanisms described previously. The implications of our data for the in vivo situation would be threefold: first, even a short contact between α-thrombin and platelets would lead to a long-lasting exposure of GPIIB/IIIA; second, interference with signal generation is bound to have little effect once GPIIB/IIIA has been exposed; and third, as long as α-thrombin is bound to the platelets, agents that interfere with platelet aggregation via activation of the PG12-receptor have no effect. The generation of thrombin is generally considered one of the earliest events in the generation of thrombosis, and is the basis of antithrombotic therapy with heparin in situations of deep venous thrombosis and pulmonary embolism. It follows from the present data that once α-thrombin has exposed GPIIB/IIIA, subsequent maintenance of exposed binding sites and occupation by fibrinogen, fibronectin, and probably other cytoadhesions is beyond control by α-thrombin and its possible neutralization by [heparin]-[antithrombin III]-complexes. Also, attempts to impair platelet activation in vivo by inhibiting cyclooxygenase (aspirin, sulphinpyrazone) or blocking the thromboxane A_2-receptor (AH 23848, GR 32191, BM 13177) are bound to have little effect once platelets have been activated by α-thrombin. Finally, attempts to impair platelet aggregation in vivo with stable analogues of PG12 (iloprost) or agents that depend on enhanced cyclic AMP production (phosphodiesterase inhibitors such as dipyridamole) will not be successful if [α-thrombin]-[receptor]-complexes are preserved in vivo. Obviously, each of these treatments may be effective if applied before platelet activation in what has been called the prethrombotic state. However, at later stage treatment with PG12-analogues might successfully impair platelet aggregation when applied in combination with hirudin thereby abolishing the G_i-mediated blockade of AC. This would provide a means to modulate activated platelets in vivo. Alternatively, approaches based on exposed GPIIB/IIIA and the Arg-Gly-Asp-containing peptides. It follows from the present data that specific inhibition of platelet PKC might also be a basis for future antithrombotic therapy.

ACKNOWLEDGMENT

The authors thank Gertie Gorter for her valuable technical assistance.

REFERENCES
17. Peerschke EIB: Ca2+ mobilization and fibrinogen binding of platelets refractory to adenosine diphosphate stimulation. J Lab Clin Med 106:1111, 1985
27. Peerschke EIB, Coller BS: A murine monoclonal antibody that blocks fibrinogen binding to normal platelets also inhibits fibrinogen interactions with chymotrypsin-treated platelets. Blood 64:59, 1984
46. Brass LF, Woolkalis MJ, Manning DR: Interactions in platelets between G proteins and the agonists that stimulate


49. Weiss HJ, Lages B: Evidence for tissue factor dependent activation of the classic extrinsic coagulation mechanism in blood obtained from bleeding time wounds. Blood 71:629, 1988

Regulation of glycoprotein IIB/IIIA exposure on platelets stimulated with alpha-thrombin

G van Willigen and JW Akkerman