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 γ(9) fibrinogen and γ(9) 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 GP Ib/IIa. Furthermore, as long as α-thrombin remains bound to the platelets, agonists that activate the PGI₂-receptor are unable to close GP Ib/IIa.

The present data show that although α-thrombin-induced GP Ib/IIa exposure shares the PKC- and cyclic AMP-sensitivity seen with weak agonists, its primary control occurs via different mechanisms that appear to be unique for this agonist.

MATERIALS AND METHODS

Materials. Sepharose 2B and CNBr-activated Sepharose 4B were from Pharmacia (Uppsala, Sweden). PAF, Fura 2-acetoxymethylester, 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 α-chymotrypsin were obtained from Sigma (St Louis, MO) and bovine serum albumin (BSA, demineralized) from Organon Teknika (Turnhout, Belgium). [3H]PIP₂ (specific radioactivity 314 TBq/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 (Bebringer, La Jolla, CA). The synthetic GRGDS-peptide was a kind gift of August Bekkers (Department of Biochemistry, University of Utrecht, The Netherlands). All other chemicals were of analytical grade.

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).

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GPIIB/IIA EXPOSURE BY \( \alpha \)-THROMBIN

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 Ca\(^{2+}\)-free Tyrode's solution (137 mmol/L NaCl, 2.68 mmol/L KCl, 0.42 mmol/L Na\(_2\)HPO\(_4\), 1.7 mmol/L MgCl\(_2\), 11.9 mmol/L NaHCO\(_3\), pH 7.25) containing 0.2% BSA and 0.1% glucose.

Preparation of \([^{32}P]\)-labeled fibrinogen. Fibrinogen 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 6.3 cm, equilibrated with 50 mmol/L Tris/HC1 pH 7.4) and washed with 1 mol/L NaCl in 50 mmol/L Tris/HC1 pH 7.4, followed by 1 mol/L urea. Fibrinogen was eluted with 6 mol/L urea in 0.1 mol/L citric acid, pH 4.2. All buffers contained 0.1 mol/L PMSF, 1 mmol/L benzamidine, 5 mmol/L \(\epsilon\)-aminocaproic acid, 10 mmol/L EDTA, and 0.02% (wt/vol) sodium azide. The fractions containing fibrinogen were pooled and dialyzed at 4°C against 50 mmol/L Tris/HC1 pH 7.4, for at least 24 hours. The fibrinogen was concentrated by dialysis at 4°C against 30% saturated ammonium sulfate for 16 hours. The precipitate was centrifuged (10 minutes, 3,000 g), dissolved in a small volume of Ca\(^{2+}\)-free Tyrode's solution, and dialyzed for 16 hours against the same buffer. Fibrinogen was stored at -20°C until use.

Electrophoresis on a 5% polyacrylamide gel, according to Laemmli,\(^{28}\) after reduction with \(\beta\)-mercaptoethanol, showed over 99% homogeneity. This fibrinogen preparation contained less than 10 ng von Willebrand factor per milligram of fibrinogen as determined with an enzyme-linked immunosorbent assay (ELISA).

The purified fibrinogen was radiolabeled with \(^{32}P\) by a modified iodogen method. Details of this procedure have been described elsewhere for fibrinogen.\(^{29}\)

Preparation of \([^{32}P]\)-labeled fibrinogen. \([^{32}P]\)-labeled fibrinogen was prepared as described elsewhere.

Platelet stimulation and fibrinogen/fibrinogen binding assay. Gel-filtered platelets ([200 to 300] x 10\(^{10}\) platelets/\(\mu\)L) were stimulated with 0.1 U/mL \(\alpha\)-thrombin or 500 mmol/L PAF in the absence of fibrinogen/fibrinogen and without stirring. After stimulation, samples were withdrawn at the times indicated in the Results section and incubated with 1 \(\mu\)mol/L \([^{32}P]\)-labeled fibrinogen/fibrinogen for 10 minutes at 22°C (without stirring). Under these conditions the concentrations of fibrinogen and fibrinogen 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). Fibrinogen/fibrinogen binding was measured by placing 200 \(\mu\)L of cell suspension (in triplicate) on top of 100 \(\mu\)L 25% (wt/vol) sucrose in Ca\(^{2+}\)-free Tyrode's solution in micro sedimentation tubes (Starledt, Vienna, Austria) and separating the cells from the medium by centrifugation (12,000g, 2 minutes, 22°C) in a Beckman Microfuge E (Beckman Instruments, Mydrecht, The Netherlands). The tip of the tube (pellet fraction) was cut off and the pellet and supernatant were counted in a \(\gamma\)-radiation counter. The number of molecules bound per platelet was calculated from the radioactivity in the pellet fraction, compared with the total activity in the pellet plus supernatant.

To investigate GPIIB/IIA complexes that are present in the open capillary system (OCS) and the \(\alpha\)-granules, platelets were stimulated with 500 mmol/L PAF in the presence of the synthetic peptide GRGDS (5 \(\mu\)mol/L) for 45 minutes to occupy the binding sites exposed on the platelet membrane. After washing, the platelets were stimulated with 0.1 U/mL \(\alpha\)-thrombin in the absence of fibrinogen/fibrinogen and subsequently treated for analysis of exposed binding sites as outlined previously.

The data were corrected for nonspecific binding, defined as the binding of \([^{32}P]\)-fibrinogen/fibrinogen to unstimulated platelets. Data are expressed as means ± standard deviation (SD).

Secretion of \(\alpha\)-granule contents after a 30-minute stimulation, measured as the release of \(\beta\)-thromboglobulin, was maximally 60% (0.1 U/mL \(\alpha\)-thrombin) of the maximal secretable amount of \(\beta\)-thromboglobulin (5 U/mL \(\alpha\)-thrombin, 5 minutes, 37°C, with stirring). In PAF-treated platelets secretion was always less than 10% of the maximal secretable amount of \(\beta\)-thromboglobulin. Thus, the amount of fibrinogen secreted from the \(\alpha\)-granules was maximally 1.8 mg/10\(^{10}\) platelets.\(^{30}\) Theoretically, this amount can occupy 3% of the binding sites exposed by \(\alpha\)-thrombin, which is within the standard error of the binding studies.

Chymotrypsin treatment of platelets. The method used has been described by Peerschke and Coller.\(^{31}\) In short, acidified PRP was centrifuged (700g, 20 minutes) and the platelets were washed twice in HEPES-Tyrode buffer (129 mmol/L NaCl, 8.9 mmol/L NaHCO\(_3\), 0.8 mmol/L KH\(_2\)PO\(_4\), 0.8 mmol/L MgCl\(_2\), 5.6 mmol/L glucose, 10 mmol/L HEPES, pH 6.5) and resuspended in HEPES-Tyrode solution (pH 7.5; final platelet concentration 350 x 10\(^{10}\)/L). After incubation with \(\alpha\)-chymotrypsin (300 \(\mu\)g/mL, 5 minutes, 22°C) 0.8 mmol/L PMSF and 30 mmol/L PGI\(_2\) were added. The platelets were centrifuged (800g, 10 minutes) and resuspended in Ca\(^{2+}\)-free Tyrode's solution and used in binding experiments, as described previously.

Measurement of PKC activity. Platelets were labeled with 3.7 MBq carrier free \([^{32}P]\)PAF/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 Ca\(^{2+}\)-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. Ca\(^{2+}\)-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 \(3^{2}P\)-radioactivity incorporated in the 47-Kd protein, the major substrate for PKC in platelets, was determined as described elsewhere.\(^{32}\) The \(3^{2}P\)-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 phosphoinositide 4,5-bisphosphate (PIP\(_2\)). Changes in PIP\(_2\) were measured according to a modified procedure described by Jolles et al.\(^{30}\) for rat brain cells. In short, platelets were labeled with \(3^{2}P\)-orthophosphate and isolated by centrifugation as described previously. The platelets were stimulated with 500 mmol/L PAF or 0.1 U/mL \(\alpha\)-thrombin (22°C). The reaction was terminated at different timepoints by addition of 2 mL chloroform/methanol/13 mol/L HCl (100:50:1, by volume; 0°C). The lipids were extracted as described by Bligh and Dyer.\(^{33}\) Phospholipids were separated by high-performance thin-layer chromatography and the radioactivity was determined by autoradiography of the plate on Kodak X-Omat film. The spots were scraped off and counted for \(3^{2}P\) by liquid scintillation counting. The \(3^{2}P\)-content of PIP\(_2\) is expressed as a percentage of that of unstimulated platelets.

Measurement of intracellular \([^{32}P]\). Acidified PRP (pH 6.5) was incubated with 3 \(\mu\)mol/L Fura 2-acetoxy methyl ester for 45 minutes at 37°C. Platelets were centrifuged and resuspended in 1 mL HEPES-Tyrode (pH 6.5) containing 0.2% gelatin and 0.1% D-glucose and gel-filtered. Fluorescence was measured on a Hitachi F3000 fluorescence spectrophotometer (Hitachi, Ltd, Tokyo, Japan) after stimulation with 500 mmol/L PAF or 0.1 U/mL \(\alpha\)-thrombin (22°C) using an excitation wavelength of 345 nm and an emission wavelength of 495 nm. The suspension was mildly stirred (±50 rpm).
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,18,19 we have shown that the exposure of binding sites on GPIIb/IIIa can be deduced from the binding of [125I]-fibrinogen provided that the number of exposed binding sites is made rate limiting. As illustrated in Fig 1 and specified earlier,18,19 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.20 When [125I]-fibrinogen was replaced by [3H]-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 [125I]-fibrinogen (coagulation prevented by 30 nmol/L PPACK, added 3 minutes after α-thrombin stimulation) and to [125I]-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

![Figure 1](https://www.bloodjournal.org)  
**Fig 1.** Disappearance of binding sites for fibrinogen and fibronectin on GPIIb/IIIa. Gel-filtered platelets were stimulated with 500 nmol/L PAF (○, ○) or 0.1 U/mL α-thrombin (Δ, △) with simultaneous addition (t = 0) of [125I]-fibrinogen (1 μmol/L, closed symbols) or [125I]-fibronectin (1 μmol/L, open symbols) or different intervals (t = 5 to 30 minutes) between stimulator (first addition) and [125I]-ligand (second addition). The specific binding was measured after a 10-minute incubation at 22°C (data are means ± SD, n = 3). Data are expressed as the percentage of the maximal number of α-thrombin exposed binding sites (100% = 87,292 binding sites per platelet).

![Figure 2](https://www.bloodjournal.org)  
**Fig 2.** Disappearance of binding sites for fibronectin on GPIIb/IIIa present in the OCS. To investigate GPIIb/IIIa present in the OCS, GPIIb/IIIa-complexes present on the platelet surface were first saturated with GRGDS (see Materials and Methods). These platelets were subsequently stimulated with 0.1 U/mL α-thrombin (○, ○) in the absence of [125I]-fibrinogen. Control platelets were stimulated with 500 nmol/L PAF (○, ○) or 0.1 U/mL α-thrombin (△, △) in the absence of radiolabeled fibrinogen. [125I]-Fibronectin (1 μmol/L) was added directly after stimulation (t = 0) or different intervals (t = 5 to 30 minutes) between stimulator (first addition) and the ligand (second addition). The specific binding was measured after a 10-minute incubation at 22°C (data are means ± SD, n = 3). Data are expressed as the percentage of the maximal number of α-thrombin exposed binding sites (100% = 87,292 binding sites per platelet).

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.20 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,
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Complexes of a-thrombin with 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 incubated with 0.1 U/mL a-thrombin or 0.1 U/mL a-thrombin plus 3 U/mL hirudin at t = 1 min. The GPIIB/IIA complex was also exposed with α-chymotrypsin (see Materials and Methods). After a 5-min incubation, staurosporine (1 pmol/L), dbcAMP (250 μmol/L), or PGI2 (10 ng/mL) was added. Samples were drawn at t = 10 min and incubated for 10 min with 1 μmol/L [32P]Pi-brain. The data are expressed as percent of specific binding measured at t = 0 (means ± SD, n = 3, 100% = 81,908 and 21,687 exposed binding sites per platelet for a-thrombin and α-chymotrypsin, respectively).

Table 1. Modulation of Signal Transduction

<table>
<thead>
<tr>
<th>Effector</th>
<th>a-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>Staurosporine</td>
<td>7.1 ± 5.6*</td>
<td>4.9 ± 1.3*</td>
<td>95.7 ± 8.5</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>9.8 ± 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).

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 [32P]-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 (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

![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 (C) of 32P-labeled platelets were 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.](http://www.bloodjournal.org)
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 PGI₂ (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 PGI₂ 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 PGI₂ and hirudin was added at various intervals thereafter, PGI₂ sensitivity was restored (Fig 4). As more time elapse 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 PGI₂ 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. PGI₂ alone, which failed to close binding sites, was also unable to change the phosphorylation of the 47-Kd protein. However, addition of PGI₂ 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

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

<table>
<thead>
<tr>
<th>Additions</th>
<th>Exposed Sites</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>PGI₂</td>
<td>95.9 ± 3.8</td>
<td>300</td>
</tr>
<tr>
<td>Hirudin + PGI₂</td>
<td>7.9 ± 7.8</td>
<td>280</td>
</tr>
<tr>
<td>Hirudin</td>
<td>98.2 ± 7.8</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 PGI₂ (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 fibronectin 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. Fibronectin 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.
raised. High cyclic AMP-levels closed all binding sites without affecting the phosphorylation patterns. The latter observation indicates that an increase in cyclic AMP is always dominant over PKC activity.

**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 modulation of the phospholipase A₂ 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 [³⁵S]-fibrinogen and [¹²⁵I]-fibronectin illustrate that under these conditions the nature of the adhesive proteins is of minor importance. Major differences between the effect of α-thrombin on GPIIB/IIIA and previously reported exposure by PAF, ADP, and epinephrine include: (1) a long-lasting exposure in contrast to the transient patterns seen with weak stimuli; (2) the observation that exposed binding sites do not require continuous receptor occupancy and signal processing; and (3) the blockade of receptor-mediated activation of AC as long as the [α-thrombin]-[receptor]-complexes are left intact.

Earlier studies on GPIIB/IIIA regulation in platelets activated by PAF, ADP, and epinephrine⁶⁶,⁶⁹ showed almost identical kinetics of exposure and closure of binding sites for [³⁵S]-fibrinogen, although the absolute number of exposed sites differed. A maximal stimulation at 22°C triggered 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]-complex. 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 GPIIB/IIIA complexes.⁷⁶ The equilibrium between both forms of the complex favored the closed configuration, unless activation of platelets with PAF, ADP, and epinephrine triggered a transient increase in PKC activity, as reflected by the phosphorylation of the 47-Kd protein. In contrast to the phosphorylation of the 47-Kd protein, leading to a transient exposure of binding sites. The present observation that α-thrombin triggers continuous exposure of binding sites initially pointed to a role for proteolytic processes similar to exposure of binding sites by α-chymotrypsin⁷⁷ and pronase.¹ Indeed, blockade of the active site by TLCK⁵² 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 binding site exposure had no effect. Also, α-thrombin–exposed binding sites closed rapidly when PKC was inhibited (stau-rosporine) or the cyclic AMP level was increased (dbcAMP), whereas α-chymotrypsin exposed sites were insensitive 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 [³⁵S]-fibrinogen/fibronectin closed after addition of dbcAMP and staurosporine, making this explanation unlikely.

Tam et al⁵³ 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,²⁶ phosphatidic acid formation,³⁵,³⁶ arachidonic acid liberation,⁴⁶ and Ca²⁺ mobilization. In contrast, shape change⁷⁵,⁷⁶ and the formation of phosphatidylinosi-to⁷⁷ required a short period of stimulation and continued unchanged when receptor occupancy was terminated. Interference with α-thrombin receptor interaction also interrupted optical aggregation and secretion of lysosomal granules,⁶⁶,⁷⁷ indicating that these functions depend on concurrent signal generation to be completed. The same is valid for α-thrombin–induced GPIIB/IIIA exposure because 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.³⁶,⁷⁷ 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.³⁸ Addition of ADP scavengers during ADP stimulation resulted in reversible aggregation and inhibition of fibrinogen binding.³⁸ Recently, Vu et al⁶³ 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 treatment. A comparison between changes in cytosolic Ca²⁺,³⁷-PIP₂ and its metabolites, and PKC in platelets stimulated with PAF and α-thrombin showed that the exposure of GPIIB/IIIA correlated with the activity of PKC, measured 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 patterns 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
cyclic AMP is dominant over PKC activity in the regulation of exposure of binding sites.

Recently, Parise et al\textsuperscript{44} 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, eg, the exposure of fibrinogen binding sites.\textsuperscript{42} 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.\textsuperscript{43} 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,\textsuperscript{44,45} leading to receptor activation.\textsuperscript{46} Thus, an explanation for the inducible binding properties of GPIIIB/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\textsuperscript{44} or other threonine/serine kinases\textsuperscript{46} 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.\textsuperscript{49} Thus, it is likely that the phosphorylation of GPIIIA is the trigger for receptor exposure.

Binding of α-thrombin to platelets not only exposes GPIIIB/IIIA, but also inhibits AC by activation of an inhibitory GTP-binding protein (G\textsubscript{i}) coupled to AC.\textsuperscript{21,46} The PAF receptor is not coupled to this G\textsubscript{i}.\textsuperscript{46} This explains the differences in sensitivity to PGI\textsubscript{2} 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\textsubscript{i} reported by Aktories and Jakobs.\textsuperscript{47}

The present study is based on modulations of GPIIIB/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 GPIIIB/IIIA complexes are occupied, leaving a short period of time in which GPIIIB/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 GPIIIB/IIIA; second, interference with signal generation is bound to have little effect once GPIIIB/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 PGI\textsubscript{2}-receptor have no effect. The generation of thrombin is generally considered one of the earliest events in the generation of thrombosis\textsuperscript{50,49} 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 GPIIIB/IIIA, subsequent maintenance of exposed binding sites and occupation by fibrinogen, fibronec, 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\textsubscript{2}-receptor (AH 23848, GR 32191, BM 13177)\textsuperscript{50} 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 PGI\textsubscript{2} (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 stages treatment with PGI\textsubscript{2}-analouges might successfully impair platelet aggregation when applied in combination with hirudin thereby abolishing the G\textsubscript{i}-mediated blockade of AC. This would provide a means to modulate activated platelets in vivo. Alternatively, approaches based on exposed GPIIIB/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.

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

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