Regulation of platelet glycoprotein IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) function via the thrombin receptor

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Binding sites on glycoprotein (GP) IIb/IIIa exposed by 0.5 unit/ml α -thrombin are insensitive to prostaglandin I_2 (PGI_2), in contrast with sites exposed by ADP or platelet-activating factor. Here we show that the thrombin receptor agonist peptide (TRAP) (SFLLRN; 15 μ M) opens almost the same number of GPIIb/IIIa molecules as 0.5 unit/ml α -thrombin (64840 ± 8920 compared with 81050 ± 6030 molecules of fibronectin bound/platelet), but these sites rapidly close on addition of PGI_2 . To investigate whether α -thrombin and TRAP initiate different signalling pathways, we measured phospholipase C (PLC)-mediated control of GPIIb/IIIa and its sensitivity to cyclic AMP. Optimal concentrations of α -thrombin and TRAP activated PLC maximally, but TRAP induced only about 50% protein kinase C (PKC) activation after 10 min stimulation compared with α -thrombin. These concentrations also suppressed PGI_2 -induced cyclic AMP accumulation, with α -thrombin inducing complete inhibition and TRAP about 10% less. Direct activation of PKC

by phorbol 12-myristate 13-acetate confirmed earlier observations that PGI_2 -induced cyclic AMP accumulation is partly inhibited via PKC. Applying different concentrations of α -thrombin, TRAP or a combination of α -thrombin and the thrombin receptor inhibitory peptide (TRIP) (Mpr-F-Cha-Cha-RKPNDK-NH₂; 800 μ M) (Mpr, 3-mercaptopropionic acid; Cha, cyclohexylalanine), we show that the different means of stimulating the thrombin receptor all suppressed PGI_2 -induced cyclic AMP accumulation via (i) activation of PKC and (ii) activation of the heterotrimeric G-protein, G_i . We conclude that complete inhibition of cyclic AMP accumulation requires activation of both PKC and G_i , as observed with 0.5 unit/ml α -thrombin. Although TRAP almost fully exposes GPIIb/IIIa, its activation of PKC is incomplete, enabling PGI_2 to raise cyclic AMP concentration from 1.4 ± 0.7 to 4.1 ± 1.3 nmol/ 10^{11} platelets ($P < 0.005$) which is sufficient to close exposed GPIIb/IIIa molecules.

INTRODUCTION

α -Thrombin is a potent inducer of platelet aggregation and secretion and stimulates platelets via the phospholipase A_2 (PLA_2) and phospholipase C (PLC) pathways, tyrosine kinases of the Src family [1], GDP–GTP exchange of Rap1B [2] and other poorly understood signal processing. At the same time it inhibits adenylate cyclase via the inhibitory heterotrimeric G-protein $G_{i2\alpha}$ [3,4], thereby preventing the accumulation of cyclic AMP induced by prostaglandin I_2 (PGI_2). Cyclic AMP is a potent platelet inhibitor and decreases the affinity of thrombin for its receptor [5], prevents the exposure of glycoprotein (GP) IIb/IIIa [6] and rapidly shifts exposed GPIIb/IIIa to the conformation that is inaccessible to fibrinogen or fibronectin [7].

A cDNA encoding a functional thrombin receptor has been isolated from the human megakaryocytic cell line DAMI [8]. mRNA coding for this receptor was detected in human platelets [8]. From the amino acid composition, seven transmembrane domains could be deduced, a structure that is characteristic of receptors coupled to heterotrimeric G-proteins. The large N-terminal extracellular extension of the receptor bears a thrombin-cleavage site carboxyl to R⁴¹ (LDPR/SFLLRN...) [8,9]. Cleavage by α -thrombin exposes a new N-terminus (SFLLRN...), which functions as a tethered peptide ligand that activates the receptor [8]. Synthetic peptides corresponding to the tethered ligand evoke many of the cellular responses induced by α -thrombin, such as activation of PLC, inhibition of adenylate

cyclase, a rise in cytosolic free Ca^{2+} concentration, an increase in tyrosine kinase and phosphatidylinositol 3-kinase activity as well as aggregation and secretion [4,8–13]. Peptides corresponding to the tethered peptide ligand but lacking the serine residue inhibit thrombin-induced aggregation [12,14], although some residual stimulatory activity remains [14]. Thus many of the responses induced by α -thrombin can be explained by the properties of the cloned thrombin receptor.

Platelet aggregation is mediated via binding of fibrinogen to sites on the GPIIb/IIIa complex that become exposed when the cells are activated [15]. Exposure of binding sites is the result of signal generation through the PLC pathway with little involvement of PLA_2 -catalysed reactions [7]. Exposure by ADP, platelet-activating factor and adrenaline is transient and sensitive to PGI_2 , which closes exposed binding sites on GPIIb/IIIa within seconds [7]. Surprisingly, when platelets are stimulated with a high concentration of α -thrombin (> 0.1 unit/ml), binding sites remained exposed even in the presence of PGI_2 [16].

In the present study we investigated the regulation of GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) via the thrombin receptor in more detail, making use of peptides that mimic the tethered peptide ligand of the seven-transmembrane-domain thrombin receptor [thrombin receptor agonist peptide (TRAP); amino acid sequence SFLLRN] or prevent activation of this receptor [thrombin receptor inhibitory peptide (TRIP); amino acid sequence Mpr-F-Cha-Cha-RKPNDK-NH₂] (Mpr, 3-mercaptopropionic acid; Cha, cyclohexylalanine). We found that TRAP was almost as

Abbreviations used: GPIIb/IIIa, glycoprotein IIb/IIIa; TRAP, thrombin receptor agonist peptide; PLC, phospholipase C; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TRIP, thrombin receptor inhibitory peptide; PGI_2 , prostaglandin I_2 ; PLA_2 , phospholipase A_2 ; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PRP, platelet-rich plasma; Boc, *t*-butoxycarbonyl; ACD, 2.5% trisodium citrate, 1.5% citric acid and 2.0% α -glucose.

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effective at exposing binding sites on GPIIb/IIIa as α -thrombin, but could not prevent their closure by PGI₂, suggesting that TRAP and α -thrombin induce different activation mechanisms.

MATERIALS AND METHODS

Materials

The protein kinase C (PKC) inhibitor Ro-31-8220 was kindly given by Dr. G. Lawton, Roche Research Centre, Welwyn Garden City, Herts., U.K. Sepharose 2B and CNBr-activated Sepharose 4B were from Pharmacia Biotech (Brussels, Belgium). PGI₂ and the thromboxane analogue U46619 were purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Human α -thrombin, benzamidine, phorbol 12-myristate 13-acetate (PMA), pertussis toxin, imipramine, PMSF and dibutyl cyclic AMP were obtained from Sigma (St. Louis, MO, U.S.A.), and BSA (demineralized) from Organon Teknika (Turnhout, Belgium). Na¹²⁵I (specific radioactivity 629 GBq/mg), 5-hydroxy[*side chain*-2-¹⁴C]tryptamine creatinine sulphate ([¹⁴C]serotonin; 1.85–2.29 GBq/mmol) and the dual-range cyclic AMP radioimmunoassay kit were from Amersham International (Amersham, Bucks., U.K.). [³²P]P_i (specific radioactivity 314 TBq/mmol) and [³²P]NAD⁺ (specific radioactivity 29.6 TBq/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Phosphoenolpyruvate (PEP), pyruvate kinase (PK), ATP, GTP and NAD⁺ were from Boehringer-Mannheim (Mannheim, Germany). *N*-Boc-L-asparagine resin (where Boc is *t*-butoxycarbonyl) was obtained from Saxon Biochemicals (Hannover, Germany), Boc-L-phenylalanine from VEGA Biotechnology (Tucson, AZ, U.S.A.), Boc-L-leucine hydrate from Du Pont and *N*-Boc-*N*^ε-tosyl-L-arginine and *N*-Boc-benzyl-L-serine from Bachem (Bubendorf, Switzerland). Saponin was from BDH (Poole, Dorset, U.K.). All other chemicals were of analytical grade.

Platelet isolation

Freshly drawn venous blood from healthy volunteers (with informed consent) was collected into 0.1 vol. of 130 mM trisodium citrate. The donors claimed not to have taken any medication during the preceding 10 days. Citrated blood was centrifuged (200 *g*; 10 min; 22 °C), and platelets were isolated from the platelet-rich plasma (PRP) by gel filtration or centrifugation. For gel filtration, PRP was placed on a Sepharose 2B column, equilibrated in Ca²⁺-free Tyrode's solution (137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH₂PO₄, 1.7 mM MgCl₂, 11.9 mM NaHCO₃, pH 7.25) containing 0.2% BSA and 0.1% D-glucose. Washed platelets were obtained by centrifugation (700 *g*; 15 min; 22 °C) of PRP acidified to pH 6.5 with ACD (2.5% trisodium citrate, 1.5% citric acid and 2.0% D-glucose). The platelet pellet was resuspended in HEPES/Tyrode's buffer (145 mM NaCl, 5 mM KCl, 0.5 mM NaH₂PO₄, 1 mM MgSO₄, 10 mM HEPES, pH 7.2) containing 0.1% D-glucose and 0.2% BSA. The platelet number was 2×10^8 – 3×10^8 cells/ml, unless stated otherwise.

Peptides

TRAP (SFLLRN) was synthesized with a semiautomatic peptide synthesizer (Labotec AG SP650) by the solid-phase method as described by van Scharrenburg et al. [17] using chloromethylpolystyrene/1% divinylbenzene resin and *N*-Boc-protected amino acids. TRAP showed an EC₅₀ for platelet aggregation of 1.4 ± 0.2 μ M in PRP (results not shown). TRIP [17a] was obtained from Neosysteme Laboratoire (Strasbourg, France). This peptide

mimics FLLRNPNDK representing the tethered peptide ligand but lacking the S⁴² residue which is important for activation of the receptor. It showed an IC₅₀ of 5 μ M in inhibiting the ATP secretion induced by 1 unit/ml α -thrombin. The purity of the peptides was confirmed by reversed-phase HPLC and amino acid analysis.

Preparation of ¹²⁵I-labelled fibronectin

Fibronectin was isolated from fresh frozen plasma by affinity chromatography on gelatin-Sepharose 4B as described elsewhere [16]. Electrophoresis on a 5% polyacrylamide gel, as described by Laemmli [18], after reduction with 2-mercaptoethanol, showed more than 99% homogeneity. The fibronectin preparation contained less than 10 ng of von Willebrand factor/mg of fibronectin as determined with a von Willebrand factor ELISA. The purified fibronectin was radiolabelled with Na¹²⁵I by a modified Iodogen method, as described in detail elsewhere for fibrinogen [19].

Platelet stimulation and fibronectin-binding assay

The exposure of binding sites on GPIIb/IIIa was measured by determining the specific binding of ¹²⁵I-fibronectin. Gel-filtered platelets were stimulated at 22 °C in the absence of fibronectin with α -thrombin or TRAP at concentrations indicated in the Results section with or without the inhibitory peptide TRIP (800 μ M). To some samples PGI₂ (10 ng/ml final concentration) was added 5 min after stimulation, thereby leaving the initial activation of the thrombin receptor undisturbed. At different times after stimulation, samples were withdrawn and incubated with 1 μ M ¹²⁵I-labelled fibronectin for 10 min at 22 °C (without stirring). Under these conditions fibronectin is in excess and a 10 min incubation reveals the number of exposed binding sites with minimal interference by irreversible binding [7,19,20]. Fibronectin binding was measured by placing a 100 μ l platelet suspension (in triplicate) on top of 100 μ l of 25% (w/v) sucrose in Ca²⁺-free Tyrode's solution in micro-sedimentation tubes (Sarstedt, Vienna, Austria) and separating the cells from the medium by centrifugation (12000 *g*; 2 min; 22 °C) in a Beckman Microfuge E (Beckman, Palo Alto, CA, U.S.A.). 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 calculated from the radioactivity in the pellet fraction, compared with the total in the pellet plus supernatant. The data were corrected for non-specific binding, defined as the binding of ¹²⁵I-fibronectin to unstimulated platelets. The same non-specific binding was found with stimulated platelets in the presence of a 10-fold excess of unlabelled fibronectin (results not shown).

Measurement of phosphatidic acid formation

The formation of phosphatidic acid was measured by a modification of the procedure described by Jolles et al. [21] for rat brain cells. In short, PRP was acidified with ACD and labelled with 3.7 MBq of carrier-free [³²P]P_i/ml for 1 h at 37 °C. Platelets were isolated by centrifugation (700 *g*; 20 min; 22 °C) and resuspended in HEPES/Tyrode's buffer containing 0.1% D-glucose and 0.2% BSA. The platelets were stimulated with 0.05 or 0.5 unit/ml α -thrombin [in some experiments in combination with TRIP (800 μ M)] or 15 μ M TRAP (22 °C). In some experiments PGI₂ (10 ng/ml final concentration) was added 5 min after stimulation. The reaction was terminated after 10 min by the addition of 2 ml of chloroform/methanol/13 M HCl (100:50:1, by vol.; 4 °C). The lipids were extracted as described by Bligh

and Dyer [22]. Phospholipids were separated by high-performance TLC and the radioactivity was determined by autoradiography on Kodak Royal X-Omat film. The spots were scraped off and counted for ^{32}P by liquid-scintillation counting.

Measurement of PKC activity

The activity of PKC was deduced from the phosphorylation of the 47 kDa protein pleckstrin, a major substrate of PKC [23]. Platelets were labelled with ^{32}P as described above. The cells were stimulated at 22 °C with 0.005–0.5 unit/ml α -thrombin in the absence or presence of TRIP (800 μM), with 0.15–100 μM TRAP or 0.25–25 nM PMA. In some experiments platelets were pretreated for 1 min with 10 μM Ro-31-8220 [24]. PGI_2 (10 ng/ml final concentration) was added 5 min after stimulation. Samples were taken 10 min after stimulation and the ^{32}P radioactivity incorporated into pleckstrin was determined as described elsewhere [25].

Measurement of cyclic AMP concentration

Washed platelets were stimulated at 22 °C with 0.005–0.5 unit/ml α -thrombin in the absence or presence of TRIP (800 μM), stimulated with 0.15–100 μM TRAP or 0.25–25 nM PMA. In some experiments platelets were pretreated with 10 μM Ro-31-8220 for 1 min. PGI_2 was added 5 min after stimulation, and samples were taken 10 min after stimulation. Cyclic AMP was measured by radioimmunoassay performed according to the manufacturer's instructions. To exclude an effect of secreted ADP on the inhibition of cyclic AMP accumulation, in some experiments platelets were treated with 0.02 vol. of PEP/PK (14 mM PEP, 150 units/ml PK), added 1 min before stimulation.

[^{32}P]ADP-ribosylation of pertussis-toxin-sensitive G-proteins

ADP-ribosylation was performed in saponin-treated platelets as described by Wilhelm and Siess [26], with minor modifications. In short, washed platelets in Hepes/Tyrod's buffer, pH 7.1 (7.5×10^8 cells/ml), were stimulated with 0.005–0.5 unit/ml α -thrombin or 0.5–15 μM TRAP at 22 °C, without stirring. PGI_2 was added after 5 min and 5 min later 80 μl of platelet suspension was added to 20 μl of ADP-ribosylation cocktail {0.25 mg/ml saponin, 50 mM thymidine, 5 mM ATP, 5 mM EDTA, 0.5 mM GTP, 5 mM NAD^+ , 5 mM leupeptin, 100 $\mu\text{g/ml}$ aprotinin, 40 $\mu\text{g/ml}$ pertussis toxin (activated with 1 mM dithiothreitol at 37 °C) and 100 μM [^{32}P]NAD $^+$ }. Incubation was carried out for 1 h at 37 °C with gentle shaking. Then platelets were centrifuged for 5 min (22 °C; 15000 g) and the pellet was resuspended in 90 μl of Laemmli sample buffer and boiled for 3 min. Proteins were separated by electrophoresis through an 11% polyacrylamide gel and the amount of radioactivity in the 41–42 kDa protein was detected on an autoradiogram.

Dense-granule secretion and granule-deficient platelets

Secretion of dense granules was measured by analysing the liberation of [^{14}C]serotonin as described elsewhere [27]. PRP acidified to pH 6.5 was incubated with 1 μM [^{14}C]serotonin (30 min; 37 °C) and gel-filtered; 2 μM imipramine was then added to prevent re-uptake of secreted serotonin. The cells were stimulated and, after 5 min, PGI_2 (10 ng/ml) was added; 5 min later samples were collected into 0.15 vol. of 1.035 M formaldehyde (0 °C). After centrifugation (10000 g ; 4 °C), the supernatants were counted for radioactivity. Secreted [^{14}C]serotonin was expressed as a percentage of total label in the platelets.

Granule-depleted platelets were prepared by incubating washed platelets (10^9 cells/ml) with 5 mM EDTA (15 min; 37 °C)

followed by stimulation with 10 μM U46619 with stirring in a glass cuvette at 37 °C for 3 min. After gel filtration through Sepharose 2B in Tyrod's solution, the suspension was standardized at 2×10^8 cells/ml. Total ATP and ADP concentrations, measured by the firefly technique [27], were 4.4 and 1.5 $\mu\text{mol}/10^{11}$ platelets respectively, reflecting a dense-granule depletion of about 50%.

Presentation of data

Data are expressed as means $\pm$ S.D. Statistical significance was determined by Student's t test for paired data where appropriate or for unpaired data and considered significant at $P < 0.05$.

RESULTS

Exposure of binding sites on GPIIb/IIIa via the thrombin receptor

A high concentration of α -thrombin (0.5 unit/ml) induced maximal exposure of GPIIb/IIIa and binding of 81050 ± 6030 molecules of fibronectin/platelet ($n = 4$). As reported previously [16], these sites remained accessible for as long as 20 min and were insensitive to PGI_2 (Figure 1a). A 10-fold lower con-

Figure 1 Exposure of binding sites on GPIIb/IIIa by α -thrombin: effect of PGI_2

(a) Time course. Platelets were stimulated with 0.5 ($\circ$) or 0.05 ($\triangle$) unit/ml α -thrombin (22 °C) and the number of fibronectin-binding sites on GPIIb/IIIa was determined at different times after stimulation. Concurrently, samples were stimulated with α -thrombin followed by addition of PGI_2 (10 ng/ml final concentration) at 5 min after stimulation ($\bullet$, $\blacktriangle$ respectively). Alternatively, platelets were treated with 0.5 unit/ml α -thrombin followed by addition of 10 mM dibutyl cyclic AMP after 5 min ($\blacksquare$). (b) Dose-dependence. Platelets were stimulated with different concentrations of α -thrombin and the number of fibronectin-binding sites was determined 20 min after stimulation ($\circ$). Concurrently, samples were stimulated with α -thrombin followed by addition of PGI_2 (10 ng/ml) 5 min after stimulation ($\bullet$). Data (means $\pm$ S.D.) are expressed as percentage of maximal exposure of fibronectin-binding sites with 0.5 unit/ml α -thrombin (100% = 81050 ± 6030 molecules of fibronectin/platelet; $n = 4$).

Table 1 Exposure of binding sites on GPIIb/IIIa by α -thrombin and TRAP

Platelets were stimulated with 0.5 or 0.05 unit/ml α -thrombin, with or without the inhibitory peptide TRIP (800 μ M) and with the activating peptide TRAP (15 μ M and 150 μ M). Concurrently, samples were treated with PGI₂ (10 ng/ml) added at 5 min after stimulation. The number of exposed GPIIb/IIIa sites was determined after 20 min stimulation. Data (means $\pm$ S.D., $n = 4$) are expressed as percentage of maximal exposure with 0.5 unit/ml α -thrombin (81 050 $\pm$ 6030).

Additions	Presence of TRIP	Exposed sites on GPIIb/IIIa (%)	
		– PGI ₂	+ PGI ₂
α -Thrombin			
0.5 unit/ml	–	100	87 $\pm$ 12
	+	21 $\pm$ 10	16 $\pm$ 13
0.05 unit/ml	–	101 $\pm$ 12	34 $\pm$ 9
	+	21 $\pm$ 11	13 $\pm$ 12
TRAP			
15 μ M	–	80 $\pm$ 11	15 $\pm$ 2
150 μ M	–	81 $\pm$ 4	16 $\pm$ 8

centration of α -thrombin exposed a similar number of binding sites (81 860 $\pm$ 9730), but these sites gradually became inaccessible on addition of PGI₂. As expected this was due to accumulation of cyclic AMP, as confirmed by closure of GPIIb/IIIa on addition of dibutyl cyclic AMP (Figure 1a). Figure 1(b) shows that between 0.023 and 2.3 unit/ml α -thrombin exposed the same number of binding sites, suggesting that the signalling pathways leading to GPIIb/IIIa exposure were already maximally stimulated at low α -thrombin concentrations. In contrast, the insensitivity to PGI₂ seen at 0.5 unit/ml α -thrombin or more gradually disappeared at lower α -thrombin concentrations, and at 0.023 unit/ml α -thrombin about 80 % of the exposed sites had disappeared after 15 min incubation with PGI₂. As these dose-response relationships suggested that α -thrombin exposed binding sites on GPIIb/IIIa and inhibited PGI₂-induced cyclic AMP accumulation via different mechanisms, we searched for means of studying these effects separately, making use of the activating peptide TRAP, which mimics the tethered ligand of the cloned thrombin receptor, and the inhibitory peptide TRIP, which interferes with receptor activation by this ligand.

Table 1 further illustrates the maximal exposure of binding sites on GPIIb/IIIa by 0.5 and 0.05 unit/ml α -thrombin and the different sensitivity to PGI₂. Activation by 15 μ M TRAP, which is 10-fold the EC₅₀ value for optimal aggregation (not shown), exposed slightly fewer sites (80 $\pm$ 11 %; $n = 4$; $P = 0.02$), and raising the concentration 10-fold did not expose any more. Sites exposed by 15 μ M TRAP rapidly became inaccessible on addition of PGI₂, leaving only 15 $\pm$ 2 % accessible after 15 min; a 10-fold higher concentration also failed to change the sensitivity to PGI₂. The inhibitory peptide TRIP (800 μ M) reduced GPIIb/IIIa exposure by both α -thrombin concentrations to 21 $\pm$ 10 % ($n = 3$).

These data indicated that exposure of binding sites on GPIIb/IIIa by α -thrombin could also be induced by TRAP and was inhibited by TRIP. This agrees with the concept that activation of the seven-transmembrane-domain receptor generated the signals that caused the exposure. In contrast, the resistance to PGI₂ observed at high α -thrombin concentration could not be induced with TRAP, suggesting an alternative mechanism for signal processing.

Table 2 PKC activation by α -thrombin and TRAP

Platelets were stimulated with α -thrombin or TRAP and 10 min later [³²P]pleckstrin was determined. Concurrently, samples were treated with PGI₂ (10 ng/ml) added 5 min after stimulation. Data (means $\pm$ S.D.; $n = 3$) are expressed as percentage of maximal pleckstrin phosphorylation with 0.5 unit/ml α -thrombin.

Additions	Presence of TRIP	Activation of PKC (%)	
		– PGI ₂	+ PGI ₂
None	–	22 $\pm$ 2	–
α -Thrombin			
0.5 unit/ml	–	100	88 $\pm$ 6
	+	45 $\pm$ 9	–
0.05 unit/ml	–	75 $\pm$ 13	62 $\pm$ 11
	+	38 $\pm$ 6	–
TRAP (15 μ M)	–	57 $\pm$ 7	46 $\pm$ 13

Activation of PLC and PKC

Although the intracellular mechanisms that regulate the accessibility of GPIIb/IIIa are largely obscure, signalling elements downstream of PLC might be implicated. Therefore we searched for differences in the control of this pathway after activation by α -thrombin, TRAP or a combination of α -thrombin and TRIP. Stimulation with α -thrombin for 10 min induced an almost 3-fold increase in [³²P]phosphatidic acid at 0.5 unit/ml and a 2-fold increase at 0.05 unit/ml. TRAP was equally as effective as 0.5 unit/ml α -thrombin ($P = 0.2$). The combination of α -thrombin and TRIP lowered the accumulation of [³²P]phosphatidic acid to about 40 %. These patterns were not significantly altered in the presence of PGI₂, suggesting that the different sensitivities to PGI₂ of GPIIb/IIIa exposure by α -thrombin and by TRAP were not due to different methods of modulation of PLC-mediated reactions (results not shown).

Measurement of [³²P]pleckstrin as an indication of PKC activity showed a 5-fold increase at 0.5 unit/ml and a 3.5-fold increase at 0.05 unit/ml α -thrombin (Table 2). The phosphorylation by TRAP was 2.7-fold higher than in resting platelets, illustrating a much lower PKC activation at 10 min after stimulation than at the high α -thrombin concentration. TRIP inhibited PKC activation by α -thrombin at both concentrations (up to about 55 %). Table 2 also shows that, in the presence of PGI₂, pleckstrin phosphorylation was slightly lower. Thus α -thrombin and TRAP activated the same steps in the PLC pathway, but activation of PKC was much weaker after stimulation with TRAP than with α -thrombin. Furthermore, PGI₂ inhibited pleckstrin phosphorylation only to a minor extent, suggesting that a rise in cyclic AMP concentration affected GPIIb/IIIa primarily at steps downstream of PKC.

Modulation of cyclic AMP accumulation

In order to clarify why PGI₂ closed binding sites on GPIIb/IIIa after stimulation with a low α -thrombin concentration or TRAP but not with a high α -thrombin concentration, we measured the PGI₂-induced increase in cyclic AMP under the different binding conditions. Resting platelets contained 1.4 $\pm$ 0.7 nmol of cyclic AMP/10¹¹ platelets ($n = 9$) and addition of 10 ng/ml PGI₂ (final concn.) induced a 17-fold increase to 23.4 $\pm$ 6.2 nmol/10¹¹ platelets after 5 min (Figure 2). Stimulation with 0.5 unit/ml

Figure 2 Inhibition of PGI₂-induced cyclic AMP accumulation by α -thrombin and TRAP

Platelets were stimulated with α -thrombin with or without TRIP (800 μ M) and with TRAP. PGI₂ (10 ng/ml) was added after 5 min and cyclic AMP was measured 10 min after stimulation. Data (means $\pm$ S.D.; $n = 6$) are expressed as nmol of cyclic AMP/ 10^{11} platelets. $\blacksquare$, Cyclic AMP concentration in the absence of PGI₂; $\square$, cyclic AMP concentration after the addition of PGI₂; ----, cyclic AMP concentration of unstimulated platelets.

α -thrombin completely abolished the accumulation of cyclic AMP and concentrations typical of resting platelets were found (1.8 ± 0.8 nmol/ 10^{11} platelets; $n = 7$; $P = 0.2$). Also after stimulation with 0.05 unit/ml α -thrombin most of the cyclic AMP accumulation was prevented but the reduction was only about 90% and cyclic AMP concentrations about twice the control values were found (3.3 ± 1.2 nmol/ 10^{11} cells; $n = 7$; $P = 0.0013$). After stimulation with 15 μ M TRAP or a 10-fold excess, inhibition was incomplete and respective cyclic AMP concentrations of 4.1 ± 1.3 and 5.1 ± 0.4 nmol/ 10^{11} cells were measured, which were significantly higher than in resting ($P < 0.005$) and thrombin (0.5 unit/ml)-stimulated ($P = 0.002$) platelets. A further increase in TRAP concentration to 1000 μ M did not result in further suppression of cyclic AMP accumulation (4.0 ± 0.7 nmol/ 10^{11} cells; $P = 0.01$). The inhibitory peptide TRIP partially abolished the effect of α -thrombin, resulting in cyclic AMP concentrations of 13.3 ± 0.7 and 14.0 ± 2.9 nmol/ 10^{11} platelets with 0.5 and 0.05 unit/ml α -thrombin respectively. The strong inhibition of cyclic AMP accumulation by α -thrombin, the interference by TRIP and the fact that TRAP could replace α -thrombin indicated that activation of the seven-transmembrane-domain receptor interfered with adenylate cyclase regulation. However, there was an important difference between the complete suppression by 0.5 unit/ml α -thrombin and the 90% suppression by 0.05 unit/ml α -thrombin and 15–150 μ M TRAP. This is illustrated in Figure 3, which shows the decrease in exposed GPIIb/IIIa when the cyclic AMP concentration rose above the level in resting platelets.

Interaction between PKC activation and the adenylate cyclase system

Earlier studies have indicated that α -thrombin interferes with PGI₂-induced cyclic AMP accumulation via three mechanisms: (i) inhibition of adenylate cyclase by PKC [28,29], possibly via phosphorylation of the enzyme [30] or one of its trimeric G-proteins [31,32]; (ii) secretion of ADP followed by receptor binding and inhibition of adenylate cyclase [33]; (iii) direct

Figure 3 Correlation between cyclic AMP concentration and the exposure of binding sites on GPIIb/IIIa

Platelets were stimulated with 0.005–2.3 units/ml α -thrombin, and then PGI₂ was added after 5 min. After 20 min stimulation, the number of exposed GPIIb/IIIa sites was determined. Under the same conditions, samples were taken to determine the cyclic AMP concentration. ¹²⁵I-fibronectin-binding values are expressed as percentage of the maximal exposure with 0.5 unit/ml α -thrombin without PGI₂. Data are means of three separate experiments. ----, Cyclic AMP concentration in resting platelets.

activation of the inhibitory G-protein G_{i2} by the thrombin receptor [3,4]. The PKC-mediated control of adenylate cyclase was investigated by comparing the inhibition of cyclic AMP accumulation by α -thrombin and TRAP with direct activation by PMA. As illustrated in Figure 4, increasing concentrations of PMA induced a dose-dependent rise in [³²P]pleckstrin concentration and reduction in cyclic AMP accumulation. Maximal pleckstrin phosphorylation was obtained at 5 nM PMA or more, which inhibited the rise in cyclic AMP accumulation by about 70%. The remaining cyclic AMP concentration (8.0 ± 1.3 nmol/ 10^{11} platelets) was significantly higher than in resting platelets ($P = 0.0003$), illustrating that maximal PKC activation alone was insufficient for complete inhibition of cyclic AMP accumulation. Stimulation with α -thrombin gave a much stronger inhibition at

Figure 4 Comparison between pleckstrin phosphorylation and PGI₂-induced cyclic AMP accumulation

Platelets were stimulated with different concentrations of PMA (0.25–25 nM; $\bullet$), α -thrombin (0.005–0.5 unit/ml; $\circ$) and TRAP (0.15–100 μ M; $\blacktriangledown$). At 5 min after stimulation, 10 ng/ml PGI₂ (final concentration) was added and 5 min later samples were collected for determination of [³²P]pleckstrin (expressed as a percentage of that in untreated platelets) and cyclic AMP (expressed as percentage inhibition of PGI₂-induced cyclic AMP accumulation). Data are means of duplicate measurement in three separate experiments. For reasons of clarity S.D. values were omitted; they were between 5% (for cyclic AMP) and 15% (for pleckstrin phosphorylation).

Table 3 Role of secreted ADP and PKC in the inhibition of PGI₂-induced cyclic AMP accumulation

Platelets were stimulated with α -thrombin or TRAP and after 5 min treated with PGI₂. After 10 min of stimulation, samples were taken to determine the cyclic AMP concentration. PEP/PK, Platelets treated with PEP/PK added 1 min before stimulation; DGP, dense-granule-deficient platelets; Ro, platelets treated with the PKC inhibitor Ro-31-8220 (10 μ M) 1 min before stimulation. Data are expressed as percentage of PGI₂-induced cyclic AMP accumulation and are means $\pm$ S.D. of three separate experiments. N.D., Not determined. * $P < 0.05$, compared with control.

Additions	Inhibition (%)			
	Control	PEP/PK	DGP	Ro
α -Thrombin				
0.5 unit/ml	98 $\pm$ 3	96 $\pm$ 2	96 $\pm$ 1	90 $\pm$ 3*
0.05 unit/ml	91 $\pm$ 5	92 $\pm$ 5	N.D.	67 $\pm$ 13*
TRAP				
15 μ M	87 $\pm$ 6	85 $\pm$ 7	84 $\pm$ 14	84 $\pm$ 6
1.5 μ M	77 $\pm$ 4	80 $\pm$ 3	N.D.	61 $\pm$ 9*

Figure 5 ADP-ribosylation of pertussis-toxin-sensitive G-proteins in platelets stimulated with α -thrombin and TRAP

Platelets (7.5×10^8 cells/ml) were stimulated with α -thrombin or TRAP (22 $^{\circ}$ C). PGI₂ was added 5 min later and 10 min after stimulation 80 μ l of platelets was added to 20 μ l of ADP-ribosylation buffer, containing 0.25 mg/ml saponin. After 1 h of incubation (37 $^{\circ}$ C) permeabilized platelets were collected by centrifugation (15000 g ; 5 min; 22 $^{\circ}$ C) and solubilized in 3 $\times$ concentrated Laemmli sample buffer. ADP-ribosylation was visualized by autoradiography after SDS/PAGE (11% acrylamide).

relatively low levels of [32 P]pleckstrin. Thus, besides inhibiting via PKC, α -thrombin activated a second inhibitory pathway, which became more important at increasing concentrations of α -thrombin. A similar pattern was seen with different concentrations of TRAP, suggesting that the two means of receptor activation affected adenylate cyclase via the same pathways. A quantitative difference remained, however, with a maximal concentration of TRAP inducing an increase of 420 ± 80 % [32 P]pleckstrin and 92 % inhibition and α -thrombin inducing an

increase of 600 ± 120 % ($n = 3$) [32 P]pleckstrin and complete inhibition of adenylate cyclase.

Concurrent measurement of [14 C]serotonin secretion showed 38 ± 2 % release at 25 nM PMA, 57 ± 17 % at 15 μ M TRAP and 80 ± 9 % at 0.5 unit/ml α -thrombin ($n = 4$). Thus one of the reasons for the stronger inhibition of adenylate cyclase by 0.5 unit/ml α -thrombin might be the higher secretion of ADP. However, three observations argued against a major role for secreted ADP: (i) the ADP scavenger PEP/PK did not alter the inhibition (Table 3); (ii) partially degranulated platelets showed the same inhibition as normal platelets; (iii) weak activation triggered little secretion but preserved the inhibition, such as with 2 nM PMA and 1.5 μ M TRAP where 2.5 ± 1.3 and 1.8 ± 1.1 % secretion were accompanied by 68.4 ± 8.1 and 87.7 ± 3.1 % inhibition of cyclic AMP accumulation respectively. Thus activation of PKC and G_i were the main mechanisms for adenylate cyclase inhibition by the thrombin receptor.

The relative contributions of the PKC- and G_i-mediated inhibition were investigated using the PKC inhibitor Ro-31-8220, which not only prevented pleckstrin phosphorylation (not shown) but also reduced dense-granule secretion to less than 3 %. As shown in Table 3, Ro-31-8220 reduced the inhibition of adenylate cyclase at 0.5 unit/ml α -thrombin by about 10 %. In contrast, at low α -thrombin concentrations there was 25 % less inhibition than in control platelets, illustrating that the involvement of PKC in the inhibition of cyclic AMP accumulation became greater at lower agonist concentrations. The same was observed with TRAP, which at low concentrations showed about 20 % involvement of PKC whereas at a high concentration the contribution was insignificant.

Effect of α -thrombin and TRAP on pertussis-toxin-induced ADP-ribosylation

The activation of pertussis-toxin-sensitive trimeric G-proteins by α -thrombin and TRAP was compared in ADP-ribosylation studies. Both TRAP and α -thrombin inhibited ADP-ribosylation dose-dependently (Figure 5), illustrating the direct activation of G_i-proteins by both of these compounds. With high α -thrombin concentration there was complete inhibition of the pertussis-toxin-induced ADP-ribosylation. Complete inhibition was also found with 15 μ M TRAP indicating that this compound also induced complete activation of G_i.

DISCUSSION

The major findings of this study are as follows. (i) A high concentration of α -thrombin (≥ 0.5 unit/ml) exposes binding sites on GPIIb/IIIa and suppresses their closure by PGI₂; at decreasing α -thrombin concentrations the sites remain exposed but their sensitivity to PGI₂ is gradually restored. (ii) TRAP is almost as potent as α -thrombin in exposing GPIIb/IIIa but cannot prevent their closure by PGI₂. (iii) Both α -thrombin and TRAP suppress PGI₂-induced cyclic AMP accumulation via activation of PKC and the inhibitory G-protein G_i but only a high concentration of α -thrombin triggers complete inhibition. (iv) A relatively small elevation of the cyclic AMP concentration leads to closure of GPIIb/IIIa.

Previous studies have shown that fibrinogen- and fibronectin-binding sites on GPIIb/IIIa exposed by ADP, adrenaline or platelet-activating factor rapidly closed on addition of PGI₂ [7]. In contrast, sites exposed by a maximal concentration of α -thrombin were resistant to PGI₂ treatment [16]. Here we demonstrate that lowering the α -thrombin concentration gradually

restores the sensitivity to PGI_2 without reducing the number of initially exposed GPIIb/IIIa molecules. These different dose-response relationships suggest the presence of separate properties of the thrombin receptor or, alternatively, two different receptors that independently control the exposure of GPIIb/IIIa and suppression of adenylate cyclase. The use of TRAP made it possible to activate the receptor while bypassing agonist binding to the hirudin-like domain and liberation of the tethered ligand [8]. TRAP exposed almost the same number of GPIIb/IIIa molecules as α -thrombin, but these sites were sensitive to PGI_2 , suggesting that GPIIb/IIIa exposure required receptor activation by the tethered ligand whereas suppression of adenylate cyclase depended on a second property for which maximal receptor occupancy by native α -thrombin was essential.

The thrombin receptor on platelets is known to activate PLC leading to Ca^{2+} mobilization and activation of PKC which either directly or indirectly contributes to exposure of GPIIb/IIIa molecules [7]. Taking the accumulation of [^{32}P]phosphatidic acid as a reflection of PLC activity, we found that both α -thrombin and TRAP activated PLC. The inhibitory peptide TRIP reduced the α -thrombin-induced [^{32}P]phosphatidic acid formation. This is in accord with earlier findings in which omission of the serine residue in TRAP or replacement by a tyrosine residue abolished most of the activity and inhibited α -thrombin-induced platelet aggregation [12,14]. The inhibition was not complete, in agreement with some residual activation by the inhibitory peptide [14]. Similarly, α -thrombin-induced pleckstrin phosphorylation was inhibited by TRIP. TRAP also activated PKC but, in contrast with the activation by α -thrombin, [^{32}P]pleckstrin accumulation was transient and after 10 min stimulation was about 50 % lower than with 0.5 unit/ml α -thrombin (results not shown) in accord with a previous report [34]. Hence, apart from these differences, TRAP had the same effect on these steps in the PLC pathway as α -thrombin.

Activation of the thrombin receptor was also compared with the control of adenylate cyclase by measuring PGI_2 -induced cyclic AMP accumulation. Ligand binding to the PGI_2 receptor activates the stimulatory G-protein G_s and adenylate cyclase, whereas G_i opposes the activation [4]. In intact platelets, G_i is activated by the thrombin and α_{2A} -adrenergic receptor [35]. In addition to regulation by G-proteins, adenylate cyclase is known to be regulated by PKC, and both stimulation and inhibition has been described for different cell types (for a review see [36]). In intact human platelets the effect of PKC is predominantly inhibitory [28]. To discriminate between the roles of PKC and G_i , we compared the inhibition of adenylate cyclase by α -thrombin and TRAP with the effect of the phorbol ester PMA which directly activates PKC. The results confirmed that PKC activation alone inhibited cyclic AMP accumulation [28]. Both α -thrombin and TRAP induced greater inhibition than expected on the basis of PKC activity, probably reflecting the role of $\text{G}_{i2\alpha}$. These experiments together with determinations of cyclic AMP in the presence of Ro-31-8220 led to the following conclusions about the roles of PKC and G_i in the suppression of PGI_2 -induced cyclic AMP accumulation: (i) complete suppression requires the involvement of both PKC and G_i as activation of PKC alone (studies with PMA) or G_i alone (0.5 unit/ml α -thrombin in the presence of Ro-31-8220) induced a maximal inhibition of 70 and 90 % respectively; (ii) the complete suppression induced by 0.5 unit/ml α -thrombin is primarily due to G_i activation; (iii) at lower agonist concentrations the contribution of PKC increases, but remains below 20 %; (iv) the fact that TRAP cannot prevent PGI_2 -induced closure of GPIIb/IIIa binding sites is due to incomplete activation of PKC, as a combination of TRAP (15 μM) and PMA (25 nM) makes GPIIb/IIIa insensitive to

PGI_2 (results not shown). The incomplete suppression of cyclic AMP accumulation seen with TRAP might also be found with other agonists that are weak PKC activators. Indeed, the PGI_2 -sensitive GPIIb/IIIa exposed by 10 μM adrenaline becomes PGI_2 -resistant when adrenaline is added in combination with 5 nM PMA (results not shown).

Previous publications have reported that cyclic AMP reduced the affinity of the thrombin receptor [5], the hydrolysis of phosphatidylinositol 4,5-bisphosphate and activation of PKC [37,38]. As these are steps in the control of GPIIb/IIIa , this would explain the closure of fibronectin-binding sites under conditions of incomplete inhibition of adenylate cyclase. Our present set-up left the initial 5 min stimulation undisturbed, and the subsequent rise in cyclic AMP concentration induced by PGI_2 had little effect on [^{32}P]phosphatidic acid formation and [^{32}P]pleckstrin accumulation. Siess and Lapetina [39] demonstrated that PGI_2 inhibits platelet aggregation at steps distal to activation of PKC and Ca^{2+} -dependent protein kinase. The extreme sensitivity of exposed GPIIb/IIIa to minor increases in cyclic AMP is probably the result of a cyclic AMP-sensitive step close to this integrin. Above a threshold of 4–5 nmol/ 10^{11} platelets, all exposed GPIIb/IIIa complexes close. This is about 10 % of the increase in cyclic AMP induced by 10 ng/ml PGI_2 . Gray and Heptinstall [40] and Zahavi et al. [41] postulated the presence of two pools of cyclic AMP [41–43], a small pool that is important for inhibition of platelet aggregation and takes part in cyclic AMP-dependent phosphorylation processes in the membranes [44,45] and a second cytoplasmic pool that is unimportant for aggregation. The observation that GPIIb/IIIa exposure has a threshold of about 2 nmol of cyclic AMP/ 10^{11} platelets might indicate that membrane-bound cyclic AMP must be exhausted before binding sites on GPIIb/IIIa can be exposed.

Vassallo et al. [12] showed that in human platelets the seven-transmembrane-domain receptor is coupled to both phosphoinositide hydrolysis and inhibition of adenylate cyclase. Our present results are in line with this finding and do not support the concept of multiple types of thrombin receptors on human platelets with different signal-generating properties [46,47]. However, a difference between receptor activation by the native ligand and the activating peptide is the weaker signal generation by TRAP. Apparently, better targeting of the ligand to the receptor and/or the proteolytic cleavage generates additional signals that help to activate the PKC-mediated pathway.

This study was supported in part by the Netherlands Organization for Scientific Research/Netherlands Heart Foundation (grant 900-526-094/940-50-102), and by the NATO Collaborative Research Grants Programme (CRG 900964). We thank Gertie Gorter and Glenda Heynen for valuable technical assistance, and August Bekkers of the Center for Biochemistry and Lipid Enzymology, University of Utrecht, for help with synthesizing TRAP.

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