Protein kinase C and cyclic AMP regulate reversible exposure of binding sites for fibrinogen on the glycoprotein IIB–IIIA complex of human platelets

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Platelet aggregation is mediated via binding of fibrinogen to sites on the membrane glycoprotein IIB–IIIA complex which become exposed when the cells are stimulated. We report here evidence of a dynamic and reversible exposure of binding sites for fibrinogen. In the absence of fibrinogen, exposed sites (B^*) gradually lose their capacity to bind fibrinogen and close (B^o). On stimulation with platelet-activating factor (PAF, 500 nM) at 22 °C, closing of B^* is enhanced by agents that raise cyclic AMP levels (10 ng of prostaglandin I_2 /ml; 5 mM-theophylline), inhibit protein kinase C (PKC; 25 μ M-sphingosine; 1 μ M-staurosporine), or disrupt the energy supply (30 mM-2-deoxy-D-glucose + 1 mM- CN^-), or by raising the temperature to 37 °C. Conversely, activation of PKC (1 μ M-1,2-dioctanoyl-*sn*-glycerol; 55 nM-phorbol 12-myristate 13-acetate) and an increase in intracellular $[Ca^{2+}]$ (100 nM-ionomycin + extracellular Ca^{2+}) oppose the disappearance of B^* . Phosphorylation of the 47 kDa protein illustrates the tight coupling between PKC and B^* under all conditions tested, except when the cyclic AMP level is raised, and B^* is converted to B^o without affecting PKC activity. Although the increase in PKC activity is much smaller with ADP or even absent upon stimulation with adrenaline, the control of B^* is equally sensitive to modulation of cyclic AMP and PKC activity. We conclude that PAF, ADP and adrenaline regulate exposure of fibrinogen binding sites through a common mechanism consisting of two independent pathways, one dominated by PKC and the other by an as yet unidentified cyclic AMP-sensitive step.

INTRODUCTION

The platelet membrane glycoprotein IIB–IIIA complex (GPIIB–IIIA) is a member of a family of Arg-Gly-Asp-specific receptors, the so-called cytoadhesins or integrins [1–3]. Similar, but not identical, glycoproteins have been identified on a variety of cell types [3–6], suggesting a major role for this glycoprotein complex in cell attachment phenomena.

Although GPIIB–IIIA is present at the outer leaflet of the plasma membrane [7–9], it is unable to bind fibrinogen unless the cells are stimulated with specific agonists such as ADP, thrombin or platelet-activating factor (PAF), which induce conformational changes in the glycoprotein complex [10,11] or alterations in its microenvironment [12,13], thereby making it accessible to fibrinogen [14–17], fibronectin [18–20], von Willebrand Factor [18,21], vitronectin [22] and thrombospondin [23]. Little is known about the mechanisms that control the exposure of these binding sites.

In platelets there are at least three signal-transducing pathways [24], the phospholipase A_2 (PLA_2) and phospholipase C (PLC) pathways, which both initiate platelet function, and the adenylate cyclase (AC) pathway, which opposes the activating pathways.

Indomethacin, an inhibitor of cyclo-oxygenase, and UK 38,385, an inhibitor of thromboxane synthetase, partly inhibit fibrinogen binding after stimulation with PAF [25], indicating that thromboxane A_2 (TxA_2), a product of the PLA_2 -pathway, plays a role in the exposure of fibrinogen binding sites. However, when platelets are made partly refractory to ADP, the remaining fibrinogen binding closely follows Ca^{2+} mobilization, indicating that the PLC pathway is also involved [26]. In saponin-treated platelets both TxA_2 and the PLC pathway are essential

for inducing fibrinogen binding, since complete inhibition can be obtained using either indomethacin or PKC inhibitors [27]. Activation of the AC pathway, which raises cyclic AMP levels, prevents the exposure of binding sites [28]. On the other hand, a decrease in the cyclic AMP level fails to affect fibrinogen binding, indicating that alterations in the cyclic AMP concentration as such fail to induce platelet function. The role of cyclic AMP has been sought in modulation of signals generated by platelet activators [29].

When platelets are stimulated, fibrinogen binds to GPIIB–IIIA in a specific and saturable manner [14–17]. However, in the absence of fibrinogen, the cells gradually lose their capacity to bind fibrinogen [26,30]. In the course of our studies on the regulation of fibrinogen binding, we found that the disappearance of exposed sites provides an extremely sensitive means with which to investigate the factors that control the conversion between closed and exposed binding sites for fibrinogen. We also addressed the question of whether these changes are part of a reversible transition between closed and opened binding sites, or merely reflect a conversion to permanently inaccessible sites which serve no further role in platelet function.

EXPERIMENTAL

Materials

1,2-Dioctanoyl-*sn*-glycerol (diC_8), phorbol 12-myristate 13-acetate (PMA) and adrenaline were purchased from Sigma (St. Louis, MO, USA), and ionomycin and PAF were from Calbiochem (Behring, La Jolla, CA, U.S.A.). Sphingosine was obtained from Serva (Heidelberg, Germany), and staurosporine

Abbreviations used: GPIIIA, glycoprotein IIIA; GPIIB, glycoprotein IIB; GPIIB–IIIA, glycoprotein IIB–IIIA complex; PAF, platelet-activating factor; PMA, phorbol 12-myristate 13-acetate; diC_8 , 1,2-dioctanoyl-*sn*-glycerol; PGI_2 , prostaglandin I_2 ; PLA_2 , phospholipase A_2 ; PLC, phospholipase C; AC, adenylate cyclase; TxA_2 , thromboxane A_2 ; PRP, platelet-rich plasma; PKC, protein kinase C; B^* and B^o , exposed and closed fibrinogen-binding sites respectively.

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and ADP were from Boehringer (Mannheim, Germany). Prostaglandin I_2 (PGI_2) was obtained from Cayman Chemicals (Ann Arbor, MI, U.S.A.) and indomethacin was from Merck, Sharp and Dohme (Philadelphia, PA, U.S.A.). Sepharose 2B and gelatin Sepharose 4B were from Pharmacia (Uppsala, Sweden), and BSA (demineralized) was from Organon Teknika (Turnhout, Belgium). $Na^{125}I$ (specific radioactivity 629 GBq/mg) was obtained from Amersham International (Amersham, Bucks., U.K.), and [^{32}P]P (specific radioactivity 314 TBq/mmol) was from New England Nuclear (Boston, MA, U.S.A.). Fibrinogen (grade L) was purchased from KABI (Stockholm, Sweden). 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 previous 10 days. Platelets were isolated by either gel filtration or centrifugation (as described below). For the preparation of gel-filtered platelets, citrated blood was centrifuged (200 g, 10 min, 22 °C) and the platelet-rich plasma (PRP) was placed on a Sepharose 2B column equilibrated in Ca^{2+} -free Tyrode's solution (137 mM-NaCl, 2.68 mM-KCl, 0.42 mM- NaH_2PO_4 , 1.7 mM- $MgCl_2$, 11.9 mM- $NaHCO_3$, pH 7.25) containing 0.2% BSA and 0.1% glucose.

Preparation of ^{125}I -labelled fibrinogen

Fibrinogen was made fibrin- and fibronectin-free by passing it through a gelatin Sepharose 4B column. The purified fibrinogen was radiolabelled with $Na^{125}I$ by a modified Iodogen method as described elsewhere [25]. The final preparation contained, under reducing conditions, a pure mixture of $A\alpha$ -chains (68 kDa), $B\beta$ -chains (57 kDa) and γ -chains (M_r 49 kDa). Contamination by Factor VIII and von Willebrand Factor was less than 0.0015% on a molar basis, as measured with an e.l.i.s.a.

Incorporation of ^{125}I into fibrinogen was $95 \pm 4\%$. The amount of ^{125}I that was not precipitated with 20% (w/v) trichloroacetic acid was $5 \pm 3\%$. Clotting ability (with 5 units of α -thrombin/ml, 10 min, 37 °C) was $99.2 \pm 0.8\%$.

Platelet stimulation and fibrinogen binding assay

Gel-filtered platelets [$(200-300) \times 10^3$ platelets/ μ l] were stimulated with 500 nM-PAF, 10 μ M-ADP or 10 μ M-adrenaline in the absence of fibrinogen and without stirring. After stimulation, samples were withdrawn at the times indicated in the Results section and incubated with 1 μ M ^{125}I -labelled fibrinogen for 10 or 60 min without stirring. All incubations were carried out at 22 °C unless indicated otherwise. Fibrinogen binding was measured by placing 200 μ l of cell suspension (in triplicate) on top of 100 μ l of 25% (w/v) sucrose in Ca^{2+} -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 Beckmann Microfuge B or E. The tip of the tube (pellet fraction) was cut off and the pellet and supernatant were counted for radioactivity 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 radioactivity in the pellet plus supernatant. The data were corrected for non-specific binding, defined as the binding of ^{125}I -fibrinogen to unstimulated platelets. Data are expressed as means $\pm$ s.d.

Interaction between signal-transducing pathways and regulation of fibrinogen binding sites

Gel-filtered platelets were stimulated, and after 5 min modulators of the PLA_2 , AC or PLC pathways were added at the concentrations given in the Results section. The control was

incubated with Ca^{2+} -free Tyrode's solution. Samples were collected at 0, 10 and 20 min after stimulation and fibrinogen binding was measured.

Effect of temperature on the disappearance of fibrinogen binding sites

For measuring reversible exposure at different temperatures, gel-filtered platelets were stimulated with 500 nM-PAF for 5 min at 22 °C. The samples containing stimulated platelets were then transferred to a water bath kept at either 4 °C or 37 °C. Samples were drawn at the times indicated in the results section and a fibrinogen binding assay was performed at these temperatures. Alternatively, the platelets were stimulated at 4 °C, 22 °C or 37 °C and the disappearance of exposed binding sites was investigated at the same temperature.

Measurement of PKC activity

Platelets were labelled with 3.7 MBq of carrier-free [^{32}P]P/ μ l of acidified PRP (pH 6.5) for 1 h at 37 °C. Platelets were isolated by centrifugation (700 g, 20 min, 22 °C) and resuspended in Ca^{2+} -free Tyrode's solution (pH 7.25).

Labelled platelets were stimulated and after 5 min a modulator of the PLC or the AC pathway was added at the concentrations given in the Results section. Ca^{2+} -free Tyrode's solution was added to controls. Samples were collected after 0, 4, 10 and 20 min, and the ^{32}P radioactivity in the 47 kDa protein (the major substrate for PKC in platelets) was determined as described elsewhere [30]. The ^{32}P content in the 47 kDa protein in stimulated platelets is expressed as a percentage of that in unstimulated platelets.

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

RESULTS

Induction of fibrinogen binding and disappearance of fibrinogen binding sites in PAF-stimulated platelets

When gel-filtered platelets were stimulated with 500 nM-PAF in the presence of 1 μ M- ^{125}I -fibrinogen 29400 ± 8700 ($n = 9$, different donors) molecules of ^{125}I -fibrinogen bound per platelet after 60 min of incubation. The binding was saturable, and reached a maximum at 15 min after stimulation (Fig. 1a). When platelets were stimulated with PAF in the absence of ^{125}I -fibrinogen and then ^{125}I -fibrinogen was added at different times after stimulation, a sharp decrease in binding was found (Fig. 1b). This property was not restricted to stimulation with PAF, but was also found after stimulation with adrenaline and ADP, although these agonists exposed more binding sites than PAF (Fig. 1b). The exposed sites disappeared until a small proportion [$29 \pm 8\%$ ($n = 10$)] of the initial binding remained after 30 min.

Re-exposure of fibrinogen binding sites

Fig. 2 illustrates that after a first exposure of binding sites induced by PAF followed by the disappearance of exposed sites, a second and even a third exposure could be induced by successive additions of adrenaline and ADP. Thus the major part of the fibrinogen binding could be restored a second and even a third time, using different agonists and a 10 min incubation time with ^{125}I -fibrinogen, without exhausting the binding sites. Similar patterns were seen after a 60 min incubation with labelled fibrinogen; in each case re-stimulation triggered binding of about 30000–50000 molecules of ^{125}I -fibrinogen per platelet, depending on the type of agonist (results not shown). A particular agonist always induced more fibrinogen binding when given initially than when used as a second or third stimulator. Although the

Fig. 1. Exposure and disappearance of fibrinogen binding sites
(a) Platelets were stimulated with 500 nM-PAF in the presence of 1 μ M-¹²⁵I-fibrinogen at 22 °C. After different incubation times samples were analysed for specific fibrinogen binding. Data are means $\pm$ S.D., $n = 3$. (b) Platelets were stimulated with 500 nM-PAF (●), 10 μ M-adrenaline (▼) or 10 μ M-ADP (△) with simultaneous addition of 1 μ M-¹²⁵I-fibrinogen or with different intervals (5–30 min) between the stimulator (first addition) and ¹²⁵I-fibrinogen (second addition). Specific binding was measured after 60 min incubations at 22 °C. Data are means $\pm$ S.D., $n = 6$.

Fig. 2. Re-exposure of fibrinogen binding sites
Platelets suspended in fibrinogen-free medium were stimulated at $t = 0$ with 500 nM-PAF, 20 min later with 10 μ M-adrenaline and 40 min later with 10 μ M-ADP. At the times indicated in the Figure, samples were withdrawn and incubated for 10 min with 1 μ M-¹²⁵I-fibrinogen. The data are expressed as percentages of specific binding induced by PAF at $t = 0$ (100% = 22361 binding sites per platelet). Data are means $\pm$ S.D., $n = 4$.

secretion of α -granule contents was always below 5 % [as assessed by the release of β -thromboglobulin (results not shown)], it cannot be excluded that endogenous released fibrinogen interfered with the binding assays, leading to lower binding after repeated exposure and disappearance.

Interaction between signal-transducing pathways and regulation of fibrinogen binding sites

The disappearance of exposed binding sites was extremely sensitive to compounds that interfered with signal transduction through the AC and PLC pathways. After stimulation of gel-filtered platelets with 500 nM-PAF in the absence of ¹²⁵I-fibrinogen, addition of PGI₂ (10 ng/ml) or theophylline (5 mM) 5 min later triggered the rapid disappearance of exposed binding sites within the next 5 min, whereas untreated platelets still possessed about 60 % of the initial number of binding sites (Table 1). Addition of the PKC inhibitors sphingosine (25 μ M) or staurosporine (1 μ M) greatly enhanced the disappearance of exposed binding sites also required metabolic energy. A mixture of 30 mM-2-deoxy-D-glucose and 1 mM-CN⁻, which under these conditions lowered the metabolic ATP/ADP content to about 25 % of resting values within 10 min [31], decreased the number of exposed sites to 3–4 % in the same period.

Table 1 also illustrates that the disappearance of exposed binding sites could be delayed by adding activators of PKC, e.g. diC₈ (1 μ M) or PMA (55 nM) after a 5 min stimulation with PAF. This effect was only transient, and 10 min later some of the re-exposed sites had again disappeared. Similar data were obtained by addition of ionomycin (100 nM) in the presence of extracellular Ca²⁺ (50–100 μ M), with more re-exposure as the extracellular Ca²⁺ concentration was increased. Ionomycin had no effect in the absence of extracellular Ca²⁺.

Indomethacin (30 μ M) failed to affect the disappearance of exposed binding sites (results not shown), indicating that the TxA₂ does not play a major role in the disappearance of exposed binding sites.

Table 1. Effectors that modulate the disappearance of exposed binding sites

Gel-filtered platelets were stimulated with 500 nM-PAF in the absence of ¹²⁵I-fibrinogen. After 5 min, PGI₂ (10 ng/ml), theophylline (5 mM), sphingosine (25 μ M), staurosporine (1 μ M), 2-deoxyglucose (DG) + CN⁻ (30 μ M + 1 mM respectively) diC₈ (1 μ M), PMA (55 nM) or ionomycin (100 nM; in the presence or the absence of extracellular Ca²⁺) was added. ¹²⁵I-Fibrinogen was added at 10 min ($\Delta t = 10$) or 20 min ($\Delta t = 20$) later and the incubation was continued for 10 min. The data are expressed as percentages of specific binding induced by PAF at $t = 0$ (100% = 35379 binding sites per platelet). Data are means $\pm$ S.D., $n = 3$ (except control, $n = 15$). All results are significantly different from the control ($P < 0.01$, Student's t test).

Effector	Exposed sites after stimulation with PAF (% of control)	
	$\Delta t = 10$ min	$\Delta t = 20$ min
Control	63.3 $\pm$ 3.8	47.9 $\pm$ 3.1
PGI ₂	1.2 $\pm$ 2.0	0.8 $\pm$ 1.1
Theophylline	20.0 $\pm$ 1.9	19.2 $\pm$ 1.4
Sphingosine	0.2 $\pm$ 0.9	0.2 $\pm$ 1.7
Staurosporine	9.2 $\pm$ 0.6	0.2 $\pm$ 1.3
DG + CN ⁻	4.4 $\pm$ 0.6	3.0 $\pm$ 1.0
diC ₈	81.0 $\pm$ 1.3	72.0 $\pm$ 1.3
PMA	82.7 $\pm$ 2.7	69.6 $\pm$ 0.6
Ionomycin	66.2 $\pm$ 2.9	45.6 $\pm$ 1.2
Ionomycin + 50 μ M-Ca ²⁺	81.0 $\pm$ 0.8	68.2 $\pm$ 1.4
Ionomycin + 100 μ M-Ca ²⁺	87.0 $\pm$ 6.7	75.9 $\pm$ 4.1

Results similar to those seen in PAF-stimulated platelets were obtained after stimulation with $10\ \mu\text{M}$ -ADP (results not shown). On stimulation with $10\ \mu\text{M}$ -adrenaline the exposed binding sites showed a similar sensitivity to modulation of the cyclic AMP level. Surprisingly, sphingosine and staurosporine also enhanced the disappearance of adrenaline-induced binding sites (results not shown), although this agonist is known to induce little [32] or no [33] activation of PKC.

Temperature-dependence of the exposure and disappearance of fibrinogen binding sites

When after a 5 min stimulation at $22\ ^\circ\text{C}$ the platelet suspension was rapidly cooled to $4\ ^\circ\text{C}$ or warmed to $37\ ^\circ\text{C}$, the decrease in exposed binding sites was much steeper at these temperatures than at $22\ ^\circ\text{C}$ (Fig. 3). At $4\ ^\circ\text{C}$ the disappearance stopped at $20 \pm 4\%$ of the initial binding, whereas at $37\ ^\circ\text{C}$ the decrease continued until all exposed sites had disappeared. Similar disappearance characteristics were obtained when both exposure and disappearance were measured at $4\ ^\circ\text{C}$, $22\ ^\circ\text{C}$ and $37\ ^\circ\text{C}$, despite the fact that the maximal number of exposed binding sites differed greatly between these temperatures. At $4\ ^\circ\text{C}$, 9731 ± 1895 binding sites were exposed ($n = 3$), compared with 35100 ± 3217 ($n = 3$) at $22\ ^\circ\text{C}$ and 36790 ± 2918 ($n = 3$) at $37\ ^\circ\text{C}$.

Role of PKC in the regulation of fibrinogen binding sites

The observations depicted in Table 1 indicated that PKC may play a crucial role in the regulation of the exposure and disappearance of fibrinogen binding sites on GPIIb-IIIa. In order to investigate a possible coupling between PKC and GPIIb-IIIa, the phosphorylation of the 47 kDa protein, a major substrate for PKC in platelets, was compared with the disappearance of exposed binding sites. Fig. 4 illustrates that the addition of the PKC inhibitors sphingosine and staurosporine not only induced the closure of the exposed binding sites, but also lowered the phosphorylation of the 47 kDa protein to the levels found in unstimulated platelets. On the other hand, an increase in PKC activity was observed when the disappearance of binding sites was delayed by addition of diC_8 or PMA, as well as by increasing the intracellular Ca^{2+} concentration (ionomycin + extracellular Ca^{2+}). Thus, under these conditions, alterations in the number of exposed binding sites closely paralleled changes in PKC activity. However, when PGI_2 was added, exposed

Fig. 3. Effect of temperature on the disappearance of fibrinogen binding sites

Gel-filtered platelets were stimulated with $500\ \text{nM}$ -PAF for 5 min at $22\ ^\circ\text{C}$. Samples containing stimulated platelets were transferred to an incubation temperature of $4\ ^\circ\text{C}$ (Δ), $22\ ^\circ\text{C}$ ($\bullet$) or $37\ ^\circ\text{C}$ ($\square$). ^{125}I -Fibrinogen was added at different time intervals (0–30 min) after stimulation. Data are expressed as percentages of the specific binding induced by PAF at $t = 0$ and $22\ ^\circ\text{C}$ ($100\% = 35405$ binding sites per platelet). Data are means $\pm$ S.D., $n = 3$.

Fig. 4. Correlation between PKC activity and exposure of fibrinogen binding sites

^{32}P -labelled platelets were stimulated with $500\ \text{nM}$ -PAF, and 5 min thereafter an effector was added. The radioactivity in the 47 kDa protein was measured 10 min after stimulation, as described in the Experimental section. The data are plotted against the fibrinogen binding of experiments under identical conditions (see Table 1). The effectors used were control ($\bullet$), $25\ \mu\text{M}$ -sphingosine ($\circ$), $1\ \mu\text{M}$ -staurosporine ($\blacksquare$), $1\ \mu\text{M}$ - diC_8 ($\blacktriangle$), $55\ \text{nM}$ -PMA ($\square$), $100\ \text{nM}$ -ionomycin + $100\ \mu\text{M}$ - Ca^{2+} (Δ) and PGI_2 (∇). The phosphorylation data are expressed as percentages of the ^{32}P present in the 47 kDa protein of unstimulated platelets [data are means $\pm$ S.D., $n = 3$ except control ($n = 6$)]. Fibrinogen data are expressed as percentages of specific binding induced by PAF at $t = 0$ [data are means $\pm$ S.D., $n = 3$ except control ($n = 15$)].

binding sites closed without a concurrent change in phosphorylation of the 47 kDa protein, indicating that a rise in the cyclic AMP concentration uncoupled the interaction between PKC and exposed binding sites.

Fig. 5 shows the amount of phosphorylation of the 47 kDa protein at various stages of repeated exposure and disappearance of binding sites induced by PAF, adrenaline and ADP. Although the number of exposed sites was approximately the same (Fig. 2) the extent of 47 kDa protein phosphorylation by these agonists varied widely from none to a 4-fold increase after stimulation with adrenaline or PAF respectively. The tight coupling between PKC activity and the number of exposed binding sites also held when exposure and disappearance were studied at different temperatures. After stimulation with PAF at $22\ ^\circ\text{C}$, a rise in

Fig. 5. PKC activity on re-exposure of fibrinogen binding sites

^{32}P -labelled platelets were repeatedly stimulated as described in the legend to Fig. 2. At the times indicated the radioactivity in the 47 kDa protein was measured as described in the Experimental section. The data are expressed as percentages of the ^{32}P present in the 47 kDa protein of unstimulated platelets. Shown is a representative experiment.

temperature to 37 °C led to rapid dephosphorylation of the 47 kDa protein, which paralleled the rapid disappearance of exposed binding sites (Fig. 3). On the other hand, cooling to 4 °C, known to stabilize a limited number of exposed sites (Fig. 3), led to almost stable levels of ³²P-labelled 47 kDa protein (results not shown). When both exposure and disappearance were studied at different temperatures, a similar correlation with exposed binding sites was observed: at 37 °C much faster 47 kDa protein phosphorylation was observed, which rapidly decreased after 4 min, a period during which all exposed sites were closed. On the other hand, stable levels of phosphorylated 47 kDa protein were seen at 4 °C in periods during which the number of exposed sites remained stable.

DISCUSSION

We [30] and others [26] have previously reported that exposed binding sites on platelets gradually become inaccessible for fibrinogen. Here we present data indicating that these closed sites can be made accessible again for fibrinogen a second and even a third time on stimulation with different agonists. The maximal number of binding sites found after repeated stimulation is of the same magnitude as the number of GPIIb-IIIa complexes on the platelet membrane, as assessed with monoclonal antibodies [34,35], which supports the concept of a single population of binding sites that can be repeatedly exposed. Other evidence for such a reversibility comes from a study by Peerschke, in which platelets made refractory for ADP could be restimulated by thrombin [26]. Also, when arachidonic acid-induced aggregation is interrupted by addition of prostacyclin, a mixture of adrenaline and arachidonic acid restores fibrinogen binding, a process that is accompanied by parallel changes in ultrastructural appearance, cytoskeletal assembly and aggregation [36].

The present data can be explained by assuming a model in which platelet stimuli convert a closed configuration of GPIIb-IIIa (B^o) to an exposed state (B^{*}), which rapidly returns to B^o in the absence of fibrinogen. The characteristics of the B^o↔B^{*} interconversion are similar in platelets stimulated with PAF, ADP and adrenaline, although the absolute numbers of B^{*} may differ. The conversion of B^{*} to B^o is enhanced by: (1) agents that raise the cyclic AMP concentration, (2) inhibitors of PKC, (3) inhibitors of ATP generation, and (4) raising the incubation temperature from 22 °C to 37 °C. These data are in agreement with earlier work which demonstrated that exposure can be abolished by a high cyclic AMP content [28], inhibitors of metabolic ATP generation [31,37] and inhibitors of PKC [38]. Thus the conversion of the closed to the exposed configuration (B^o→B^{*}) requires a low cyclic AMP content and an activated PKC. An increase in cytosolic Ca²⁺ content also favours the B^{*} state, possibly by facilitating PKC activity, as this is a Ca²⁺-dependent protein kinase [39]. Thus both states of GPIIb-IIIa can be induced via modulation of PKC activity. Although an increase in cyclic AMP favours B^o, a decreased cyclic AMP level induced by treatment with 2',5'-dideoxyadenosine does not expose binding sites (results not shown). Therefore cyclic AMP only acts as a modulator of activating mechanisms, which is in concert with the permissive role found in other models of stimulus-response coupling [29]. A likely explanation may be sought in the activation of protein kinase A, a cyclic AMP-dependent protein kinase, which occurs in parallel with the inhibition of platelet aggregation [40,41]. Lerea *et al.* [42] reported that an increase in cyclic AMP content reduced the affinity of the thrombin receptor, with possible attenuation of its signal-generating properties. The PAF receptor appears unresponsive to changes in cyclic AMP, since phosphorylation of the 47 kDa protein (Fig. 4) as well as the binding of ³H-labelled

PAF (results not shown) remain constant upon modulation of the cyclic AMP concentration.

An alternative explanation for the decrease in binding sites may be desensitization of the PAF receptor, as described by Crouch & Lapetina [43]. However, the faster time course of this process (2–5 min) argues against such a mechanism. In addition, α -thrombin-exposed binding sites remain open even after abolishment of signal generation by the α -thrombin receptor, indicating that control of binding site exposure occurs close to GPIIb-IIIa (G. van Willigen, unpublished work).

Thus the interference with signal processing between the receptors for PAF, ADP and adrenaline and the exposure of GPIIb-IIIa points to two independent control mechanisms: one in which PKC plays a central role and another, cyclic-AMP-sensitive, step. Direct measurement of the phosphorylation of the 47 kDa protein confirms the tight coupling between PKC and the B^{*} configuration. Although sphingosine and staurosporine have a number of side-effects [44–46], the phosphorylation studies point to a role for PKC. A more precise comparison between the phosphorylation of the 47 kDa protein and fibrinogen binding reveals an almost quantitative relationship between the activity of PKC and the B^{*} configuration when a single agonist is considered. This is especially evident in the temperature studies. Compared with the situation at 22 °C, stimulation at 37 °C triggers a faster increase in 47 kDa protein phosphorylation, followed by an almost equally fast decrease to resting values. Although exposure of binding sites is approximately the same at both temperatures, the conversion from B^{*} to B^o closely parallels the pattern of phosphorylation of the 47 kDa protein. Furthermore, when the temperature is kept constant during stimulation, a subsequent increase from 22 °C to 37 °C leads to the same increase in B^o formation and loss of 47 kDa protein radioactivity. Unexpectedly, we found considerable fibrinogen binding after stimulation of platelets at 4 °C. Again, the pattern of 47 kDa protein phosphorylation mirrored this observation. Purified protein kinases remain active at 4 °C, whereas protein phosphatases are inactive [47,48]. A previous finding that binding site exposure is accompanied by phosphorylation of GPIIIa on a threonine residue [49], one of the amino acids that can be phosphorylated by PKC [50], raises the possibility that PKC directly phosphorylates GPIIb-IIIa. The disappearance of exposed sites may therefore reflect the dephosphorylation of the complex, which would explain the stable binding sites at 4 °C when the 47 kDa protein phosphorylation remains constant, as well as the other dephosphorylation patterns which closely resemble the closure of binding sites.

The quantitative correlations between PKC activity and exposed GPIIb-IIIa, seen in experiments with a single agonist, disappear completely when patterns are compared between platelets stimulated with PAF, ADP and adrenaline. Although the amount of B^{*} is roughly similar, there is considerably less 47 kDa protein phosphorylation in ADP-treated platelets compared with those stimulated by PAF. Even more striking are adrenaline-stimulated platelets, in which phosphorylation of the 47 kDa protein is extremely low [32] or virtually absent ([33], Fig. 5), but the amount of B^{*} is even higher than that found after PAF addition. Still, the susceptibility to PKC inhibitors illustrates that in adrenaline-stimulated platelets also the B^{*} configuration depends on an active PKC. Possibly the low PKC activity seen in resting platelets is sufficient to mediate adrenaline-induced fibrinogen binding, but attempts to further lower this activity with different types of inhibitors were not successful. These results are in contrast with the findings of Banga *et al.* [51], who postulated that, in adrenaline-stimulated platelets, exposure of GPIIb-IIIa precedes signal processing via PLC and PKC.

In conclusion, in platelets stimulated with PAF, ADP or

adrenaline, binding sites for fibrinogen can undergo a reversible transition between closed and exposed states. This transition is controlled by two independent mechanisms: a PKC mediated step and a cyclic AMP-sensitive step. The participation of the PKC-mediated pathway differs greatly between PAF-, ADP- and adrenaline-induced fibrinogen binding. Whether this reflects different modulation by the second, cyclic AMP-sensitive, pathway or is the result of a third, unknown, mechanism remains a subject for further study.

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