Platelet-activating factor (PAF-acether) induces high- and low-affinity binding of fibrinogen to human platelets via independent mechanisms

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When human platelets are incubated with 500 nM-PAF-acether (platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) under equilibrium conditions (60 min, 22 °C, non-stirred suspensions), two classes of fibrinogen binding sites are exposed: one class with a high affinity [K_d (7.2 ± 2.1) $\times 10^{-8}$ M, 2367 ± 485 sites/platelet, $n = 9$] and one class with a low affinity [K_d (5.9 ± 2.4) $\times 10^{-7}$ M, 26972 ± 8267 sites/platelet]. Preincubation with inhibitors of cyclo-oxygenase (acetylsalicylic acid, indomethacin) or thromboxane synthetase (UK 38,485) completely abolishes high-affinity binding, leaving low-affinity binding unchanged. In contrast, ADP scavengers (phosphocreatine/creatine kinase or phosphoenol pyruvate/pyruvate kinase) completely prevent low-affinity binding, leaving high-affinity binding unaltered. Initial binding studies (2–10 min incubation) confirm these findings with a major part of the binding being sensitive to ADP scavengers, a minor part sensitive to indomethacin and complete blockade with both inhibitors. Increasing the temperature to 37 °C decreases the number of low affinity-binding sites 6-fold without changing high-affinity binding. Aggregation, measured as the rate of single platelet disappearance, then depends on high-affinity binding at 10 nM-fibrinogen or less, whereas at 100 nM-fibrinogen or more low-affinity binding becomes predominant. These findings point at considerable platelet activation during binding experiments. However, arachidonate metabolism ($[^3\text{H}]$ arachidonate mobilization and thromboxane synthesis) and secretion ($[^{14}\text{C}]$ serotonin and β -thromboglobulin) are about 10% or less of the amounts found under optimal conditions (5 units of thrombin/ml 37 °C, stirring). We conclude that PAF-acether induces little platelet activation under binding conditions. The amounts of thromboxane A_2 and secreted ADP, however, are sufficient for initiating high- and low-affinity fibrinogen binding via mutually independent mechanisms.

INTRODUCTION

PAF-acether is a potent lipid mediator strongly implicated in the pathogenesis of acute allergic and inflammatory reactions (Pinckard *et al.*, 1982). It is produced by a variety of cell types such as blood cells (Vargaftig *et al.*, 1981), endothelial cells (Prescott *et al.*, 1984) and salivary glands (Cox *et al.*, 1981). *In vivo*, PAF-acether induces vasodilatation and hypotension (Goldstein *et al.*, 1984) with plasma exudation and it is thought to play an important role in asthma (Page *et al.*, 1984). Apart from pulmonary hypertension, airway constriction and oedema, intravenous infusion of PAF-acether induces thrombocytopenia and clumping of platelets in the lung capillaries leading to sudden death (Pinckard *et al.*, 1980; Handley *et al.*, 1984).

In vitro, PAF-acether is a potent activator of platelets and triggers shape change, aggregation and secretion of dense, α - and acid hydrolase granule contents (Chesney *et al.*, 1982; Klopogge *et al.*, 1983). Platelet aggregation is known to be mediated via exposure of the glycoprotein IIb–IIIa complex (Gogstad *et al.*, 1982; DiMinno *et al.*, 1983; Bennet & Vilaire, 1979), which binds fibrinogen in a Ca^{2+} - or Mg^{2+} -dependent reaction (Marguerie *et al.*, 1980). Exposure of fibrinogen binding sites occurs with different platelet agonists such as ADP, collagen,

thrombin, arachidonate, prostaglandin endoperoxides, adrenaline (DiMinno *et al.*, 1983; Bennet & Vilaire, 1979; Marguerie *et al.*, 1980; Kornecki *et al.*, 1981; Marguerie & Plow 1983) and also with PAF-acether (Marguerie & Plow, 1983; Klopogge & Akkerman, 1984), but the mechanism by which each agonist exposes the sites is subject to controversy. Marguerie *et al.* (1980) attribute a central role to ADP, and other agonists would only act via ADP, secreted from the dense granules. Indeed, prostaglandin endoperoxide-induced fibrinogen binding can be completely abolished by ADP scavengers (Morinelli *et al.*, 1983). On the other hand, Harfenist *et al.* (1982) demonstrated binding of fibrinogen to degranulated platelets treated with arachidonate, suggesting that other mechanisms play a role. The observation by Peerschke (1982) that adrenaline-induced fibrinogen binding is insensitive to ADP-removing enzymes is in concert with this and also the fact that adrenaline in combination with other agonists produces more binding than adrenaline alone (Peerschke, 1984) indicates that more than one mechanism must be involved.

Recently we have described that PAF-acether activates platelets via binding to a specific receptor which is saturated after about 20 min incubation with 0.5 nM-PAF-acether (Klopogge & Akkerman, 1984). At concentrations above 1.0 nM, PAF-acether is taken up by

the platelets and metabolized, but these processes are probably irrelevant for platelet activation (Pieroni & Hanahan, 1983). The present report describes the characteristics of PAF-acether-induced fibrinogen binding and will demonstrate that PAF-acether triggers exposure of the fibrinogen receptor via two mutually independent mechanisms.

MATERIALS

Chemicals

PAF-acether, 1-*O*-hexadecyl/octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, purchased from Calbiochem-Behring, was dissolved in a Ca^{2+} -free Tyrode's solution (Walsh, 1972), containing bovine serum albumin (2.5 mg/ml) (Organon Teknika, Oss, The Netherlands) and stored at -70°C . Forskolin was also obtained from Calbiochem-Behring. The cyclo-oxygenase inhibitors indomethacin and acetylsalicylic acid were from Merck, Sharp & Dohm and Sigma respectively. The thromboxane synthetase inhibitor dazmegrel (UK 38,485) was from Pfizer and kindly provided by Dr. H. M. Typer, Department of Clinical Research, Pfizer, Sandwich, Kent, U.K. ADP scavengers used were phosphoenolpyruvate/pyruvate kinase (PEP/PK) and phosphocreatine/creatine kinase (CP/CPK), both purchased from Boehringer Mannheim. Before use, PK and CPK were dialysed for 14 h (0°C) against 500 vol. of Ca^{2+} -free Tyrode's solution to remove the contaminating $(\text{NH}_4)_2\text{SO}_4$.

Preparation of ^{125}I -labelled fibrinogen

Commercially available human fibrinogen (grade L, KABI, Stockholm, Sweden) was dissolved in water (30 mg/ml) in the presence of 10 mM-6-aminohexanoic acid (J. T. Baker Chemical Co.) and 1 mM benzamidine hydrochloride (Sigma). Contaminating fibrin and fibronectin were removed by chromatography on a gelatin-Sepharose 4B column (Pharmacia) (Engvall *et al.*, 1978). The column was equilibrated and eluted with Ca^{2+} -free Tyrode's solution (pH 7.25) which contained 10 mM-6-aminohexanoic acid and 1 mM-benzamidine hydrochloride. After dialysis four times against 40 vol. of Ca^{2+} -free Tyrode's buffer (8 h, 4°C), the fibrinogen solution was concentrated by dehydration against Sephadex G-200 (Pharmacia) and stored at -70°C . The protein concentration of the final preparation was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. Contamination by Factor VIII-von Willebrand factor was less than 0.015% on a molar basis, as measured by an enzyme immunoassay (Cejka, 1982). Analysis of fibrinogen under reducing conditions according to Laemmli (1970) revealed a pure preparation consisting of $\text{A}\alpha$ -chains (M_r 68000), $\text{B}\beta$ -chains (M_r 57000) and γ -chains (M_r 49000).

Fibrinogen was radiolabelled with Na^{125}I (Amersham International) by a modified iodogen procedure (Nieuwenhuizen *et al.*, 1980). Conical 3 ml glass minivials (Chrompack, Middelburg, The Netherlands) were coated with iodogen by transferring 200 μl of an iodogen solution (2 mg/100 ml of 1,2-dichloroethane; Merck) and evaporating the solvent under a stream of dry N_2 . Quantities of 7.9–9.6 nmol of fibrinogen were iodinated with 0.93–1.86 nmol of Na^{125}I (1 or 2 mCi) in borate buffer [0.125 M-disodium tetraborate (J. T. Baker

Chemical Co.)/0.075 M-NaCl (pH 8.2)] containing 3.3 nmol of KI in three subsequent incubations (20 min each, 0°C). The mixture was first dialysed against 500 vol. of non-radioactive KI (10 g/l in 0.15 M-NaCl) at 4°C for 14 h followed by three times against 500 vol. of Tyrode's solution (8 h, 4°C). Incorporation of ^{125}I into fibrinogen was $78 \pm 15\%$ ($n = 11$). The amount of free ^{125}I , measured after precipitating bound ^{125}I with 20% (w/v) trichloroacetic acid, was $3.4 \pm 2.1\%$ of total radioactivity, which remained unchanged for 3 weeks ($n = 33$). The clottability, measured with 5 units of thrombin/ml (La Roche, Basel, Switzerland), after 10 min at 37°C was $99.2 \pm 0.8\%$. The specific activity of the ^{125}I -labelled fibrinogen was 36–222 Ci/mmol. The ^{125}I -labelled fibrinogen solutions were stored at -80°C and used within 3 weeks of preparation.

Platelet isolation

Venous blood from healthy volunteers was collected (with informed consent) into 0.1 vol. of 130 mM-trisodium citrate. The donors claimed not to have taken any medicine during the 10 days prior to blood collection. Gel-filtered platelets were prepared, as described elsewhere (Klopogge *et al.*, 1983) and suspended in Ca^{2+} -free Tyrode's solution containing 0.2% bovine serum albumin and 5 mM-glucose (pH 7.25). The final platelet concentration was $(3\text{--}4) \times 10^8$ cells/ml. In all experiments cell lysis was minimal ($< 0.2\%$, based on the liberation of lactate dehydrogenase).

Binding studies with ^{125}I -fibrinogen

Duplicate samples of gel-filtered platelets were incubated at 22°C (without stirring), with different amounts of ^{125}I -fibrinogen. Exactly 5 s later the platelets were stimulated with different concentrations of PAF-acether as indicated in the Results section. At different incubation times, triplicate samples (unless stated otherwise) of cell suspension (200 μl each) were collected and placed on 100 μl of 20% (w/v) sucrose in Tyrode's solution, in polypropylene tubes (0.4 ml tubes for microsedimentation; Sarstedt, Wien, Austria). Immediately thereafter, the samples were centrifuged in a Beckman Microfuge (12000 g , 22°C) for 2 min and the tips of the tubes were cut off just above the pellet. The number of molecules of fibrinogen bound per platelet was calculated from the specific radioactivity of the ^{125}I -labelled fibrinogen in the pellet fraction compared with the total radioactivity in pellet plus supernatant. The amount of non-specific binding was measured by parallel incubation with ^{125}I -fibrinogen in the absence of PAF-acether. Control studies between 3 nM- and 67 nM- ^{125}I -fibrinogen have shown that those cells bound as much ^{125}I -fibrinogen as did PAF-acether-treated platelets incubated with ^{125}I -fibrinogen in the presence of 100-fold molar excess of non-radiolabelled fibrinogen (at higher concentrations of ^{125}I -fibrinogen, a 100-fold molar excess of non-radiolabelled fibrinogen did not easily dissolve).

Patients

A few experiments were carried out with platelets from a patient with Glanzmann thrombasthenia, known to be severely deficient in the glycoprotein IIb-IIIa complex, the putative binding site for fibrinogen. In another series of experiments platelets were used from a patient with storage pool disease, which are severely deficient in granule-bound ATP and ADP. These

platelets contained < 0.02 and $0.18 \mu\text{mol}$ of granule-bound ATP and ADP/ 10^{11} platelets, respectively (patient no. 5 in Akkerman *et al.*, 1983).

Analysis of platelet responses

Platelets were incubated at 22°C with PAF-acether (0.5 nM or 500 nM), with fibrinogen (40 nM or 500 nM) or with combinations of both. Functional parameters were measured after 30 s, when the cell suspension was stirred (900 rev./min) or after 60 min (non-stirring conditions).

Aggregation of gel-filtered platelets was measured by counting the disappearance of single platelets (Frojmovic *et al.*, 1983; Klopogge & Akkerman, 1984), after fixing samples of platelet suspension in 9 vol. of a mixture of cold (0°C) 0.5% glutaraldehyde (Fluka) in 0.15 M-NaCl . The platelets were counted in a Platelet Analyzer 810 (Baker Instruments, Allentown, PA, U.S.A.) with apertures set at 3.2 and $16 \mu\text{m}^3$. In unstimulated platelet suspensions $94 \pm 2\%$ (means $\pm$ S.D., $n = 36$) of the total platelets were counted between these settings.

Secretion was measured by collecting samples of 0.15 vol. of $1.025 \text{ M-formaldehyde}$ (BDH) in 0.15 M-NaCl at 0°C (freshly prepared). For analysis of serotonin secretion, platelets were preincubated with $1 \mu\text{M-5-hydroxy[side chain-2-}^{14}\text{C]tryptamine}$ ($[^{14}\text{C}]$ serotonin; sp. radioactivity 58 Ci/mol ; The Radiochemical Centre, Amersham) for 30 min at 37°C . Imipramine ($2.5 \mu\text{M}$; Geigy) was added prior to the incubation to prevent re-uptake of $[^{14}\text{C}]$ serotonin. The collected samples were centrifuged (10000 g , 1 min, 0°C). The supernatants were used for analysis of $[^{14}\text{C}]$ serotonin, a marker for the dense granules (measured according to standard procedures) and β -thromboglobulin, a marker for the α -granules (measured with a radioimmunoassay kit from The Radiochemical Centre, Amersham).

Thromboxane synthesis was measured with a radioimmunoassay kit for thromboxane B_2 (New England Nuclear) after collecting samples of platelet suspensions in 10 vol. of assay buffer (Calbiochem-Behring) (Halushka *et al.*, 1981).

Effect of cyclo-oxygenase inhibitors, thromboxane-synthetase inhibitor and ADP scavengers

Gel-filtered platelets were preincubated with the cyclo-oxygenase inhibitors aspirin ($1 \text{ mM-acetylsalicylic acid}$) or indomethacin ($30 \mu\text{M}$) for 30 min at 37°C prior to the binding experiments or platelet function studies. For inhibition of thromboxane synthetase, platelets were preincubated (30 min, 37°C) with $2.5 \mu\text{M-dazmegrel}$ (UK 38,485), a specific thromboxane synthetase inhibitor (Randall *et al.*, 1981). The influence of ADP scavengers was measured by using 0.28 mM-PEP with 3 units of PK/ml or a combination of 5 mM-CP with 40 units of CPK/ml, added 1 min before the binding studies or the aggregation and secretion experiments.

Measurements of $[^3\text{H}]$ arachidonate metabolism

Arachidonic acid liberation in platelets was measured by incubating platelet-rich plasma at 37°C for 60 min with $0.01 \mu\text{M-}[^3\text{H}]$ arachidonic acid (90 Ci/mmol ; Amersham International) followed by gel filtration. After incubation of the cell suspension (60 min, 22°C) with PAF-acether (0.5 or 500 nM) and fibrinogen ($1 \mu\text{M}$), lipids were extracted according to Bligh & Dyer (1959). A 1.0 ml sample was mixed with 3.6 ml of chloroform/methanol ($1:2$, v/v). Then, water (1.2 ml) and chloro-

Fig. 1. Comparison of binding between ^{125}I -fibrinogen and non-radiolabelled fibrinogen

Relative binding of different ratios of ^{125}I -labelled fibrinogen and unlabelled fibrinogen to gel-filtered platelets, stimulated with $500 \text{ nM-PAF-acether}$ for 60 min, is shown. Total fibrinogen concentration was 106 nM . Data are means of duplicate measurements (correlation coefficient $r = 0.984$). Similar data were obtained at shorter incubation times.

form (1.2 ml) were added under vigorous shaking and the mixture was centrifuged at 2000 g (22°C) for 1 min to enhance phase separation. The lower phase was collected, concentrated to dryness under N_2 flow, and dissolved in $25 \mu\text{l}$ of chloroform/ 10^8 platelets. The mixture was chromatographed at room temperature on silica gel 60 (Merck) with the upper phase of ethyl acetate/acetic acid/ $2,2,4$ -trimethylpentane (iso-octane)/water ($9:2:5:10$, by vol.) according to Hong & Levine (1976). The lipids were located with I_2 vapour. Spots containing free arachidonic acid and its metabolites were scratched off and the radioactivity was counted directly in toluene-based scintillation fluid.

RESULTS

Comparison between binding affinity of native and ^{125}I -labelled fibrinogen

Platelets were incubated with $500 \text{ nM-PAF-acether}$ for 1 h, in the presence of fibrinogen. The ratio of ^{125}I -labelled and non-labelled fibrinogen was varied, keeping the total concentration constant (106 nM). Fig. 1 shows that there is no difference in binding of labelled and unlabelled fibrinogen to the platelets. At an equivalent molar ratio of labelled and unlabelled fibrinogen, 50% of the radioactivity of the ^{125}I was associated with the platelets.

Equilibrium kinetics of PAF-acether-induced fibrinogen binding

The specific binding of fibrinogen to platelets stimulated with PAF-acether increased with time and reached equilibrium after 30 (at $\geq 1 \mu\text{M}$ fibrinogen, Fig. 2) to 60 min (at $< 1 \mu\text{M}$ fibrinogen, results not shown). A 60 min incubation period was therefore chosen to analyse the kinetics of equilibrium binding. Fibrinogen binding at equilibrium depended on the concentration of PAF-acether. In the presence of a high concentration of ^{125}I -fibrinogen ($1 \mu\text{M}$) 60% of maximal binding was

Fig. 2. Time course of specific ^{125}I -fibrinogen binding to platelets induced by PAF-acether

The ^{125}I -fibrinogen concentration was $.1.1 \mu\text{M}$, PAF-acether concentrations were 0.49 nM (●), 0.97 nM (△) and 500 nM (○) respectively. Data are means $\pm$ S.D. ($n = 6$) from one cell suspension, representative of four similar experiments.

Fig. 3. Binding of ^{125}I -fibrinogen to platelets induced by 500 nM -PAF-acether

Data are means $\pm$ S.D. of triplicate samples and show total binding (○), non-specific binding (■) and specific binding (●) following 60 min incubation.

found with 0.5 nM -PAF-acether, 75% with 1 nM -PAF-acether whereas maximal binding was found with 100 nM -PAF-acether or more (Fig. 2). The kinetics of fibrinogen binding to platelets stimulated with 500 nM -PAF-acether are illustrated in Fig. 3. The specific binding increased in the range between 7.1 nM - and $1 \mu\text{M}$ -fibrinogen. Above $1 \mu\text{M}$ -fibrinogen, saturation of a finite number of binding sites was reached and no further increase in specific binding could be measured. Scatchard analysis of these data (Fig. 4) revealed two types of fibrinogen binding: a high-affinity binding with a K_d of about 70 nM and 2400 sites/platelet and a low-affinity binding with a K_d of about 590 nM and 27000 sites/platelet (Table 1).

Experiments with platelets from a patient with Glanzmann disease failed to show specific fibrinogen binding, both in the high- and low-affinity binding range. Those platelets showed only non-specific binding similar to the non-specific binding of normal platelets (results not shown).

The role of endoperoxide/thromboxane formation and ADP secretion in PAF-acether-induced fibrinogen binding

In order to evaluate the role of endoperoxide/thromboxane formation in PAF-acether-induced fibrinogen binding, platelets were pretreated with aspirin or indomethacin and subsequently stimulated with 500 nM -PAF-acether. This treatment led to a slight but significant decrease in specific fibrinogen binding. In contrast, when the role of secreted ADP was investigated by treating platelets with ADP scavengers a considerable decrease in fibrinogen binding was found (Fig. 5). However, a small but significant amount of fibrinogen remained bound to the platelets. This part could only be abolished by treating the platelets with indomethacin or aspirin. Under those conditions only aspecific binding remained. These findings indicated that the major part of PAF-acether-induced fibrinogen binding was mediated via ADP while the remainder was the result of endoperoxide/thromboxane formation.

These findings were further substantiated in binding studies designed to analyse the two types of fibrinogen binding in more detail. Scatchard plots revealed that aspirin or indomethacin specifically abolished high-affinity fibrinogen binding, leaving the kinetics of low-affinity binding unaltered (Fig. 6a, Table 1). A similar effect was found with the thromboxane synthetase inhibitor UK 38.485 (Table 1). In contrast, PEP/PK (Fig. 6b, Table 1) or CP/CPK (results not shown) specifically abolished the low-affinity binding characteristics but left the high-affinity binding basically unchanged. This indicated that low-affinity fibrinogen binding was mediated via ADP. Since the most likely sources for ADP was secretion from the dense granules,

Fig. 4. Scatchard plot of specific binding of ^{125}I -fibrinogen to platelets induced by 500 nM -PAF-acether

Low affinity: $K_d = 6.74 \times 10^{-7} \text{ M}$ and 24600 binding sites/platelet; high affinity: $K_d = 6.29 \times 10^{-8} \text{ M}$ and 2700 binding sites/platelet.

Table 1. Kinetics of PAF-acether-induced fibrinogen binding

Platelets were stimulated for 60 min with 0.5 or 500 nM-PAF-acether in the absence of inhibitors and with 500 nM-PAF-acether in the presence of different concentrations of 125 I-fibrinogen with or without the cyclo-oxygenase inhibitor indomethacin (30 μ M), the thromboxane synthetase inhibitor UK 38,485 (5 μ M) or the ADP scavenger PEP/PK (0.28 mM + 3 units/ml). Data are means $\pm$ S.D. with number of experiments (*n*) and are significantly different between high- and low-affinity binding (*t*-test, *P* < 0.05). In contrast, neither the high-affinity constants nor the low-affinity constants differed significantly between untreated platelets (0.5 and 500 nM-PAF-acether) and suspensions treated with inhibitors (500 nM-PAF-acether; *P* > 0.05).

Conditions	High-affinity binding		Low-affinity binding		<i>n</i>
	K_d (M)	Binding sites/ platelet	K_d (M)	Binding sites/ platelet	
500 nM-PAF-acether	$7.17 (\pm 2.11) \times 10^{-8}$	2367 ± 485	$5.86 (\pm 2.40) \times 10^{-7}$	26972 ± 8267	9
Indomethacin	—	No binding	$5.31 (\pm 2.04) \times 10^{-7}$	19482 ± 4743	4
UK 38,485	—	No binding	2.51×10^{-7}	23123	2
PEP/PK	$5.20 (\pm 3.24) \times 10^{-8}$	1572 ± 464	—	No binding	4
Indomethacin + PEP/PK	—	No binding	—	No binding	4
0.5 nM-PAF-acether	$5.91 (\pm 1.20) \times 10^{-8}$	1733 ± 346	$5.11 (\pm 2.30) \times 10^{-7}$	19501 ± 8509	3

Fig. 5. Binding kinetics of 125 I-fibrinogen in the presence of ADP scavengers

Platelets were preincubated with PEP/PK (0.28 mM + 3 units/ml), and stimulated with 500 nM-PAF-acether. Data are means $\pm$ S.D. (*n* = 6) and show total binding (○), non-specific binding (■) and specific binding (●).

the binding studies were repeated with storage pool deficient platelets, which contained minimal amounts of granule-bound ADP. As expected, these platelets bound much less fibrinogen than did normal cells and Scatchard analysis revealed that binding occurred only in the range of the high-affinity binding sites (Fig. 6c).

When these experiments were repeated using 0.5 nM-PAF-acether instead of the higher dose, similar results were obtained. Although the amount of specific binding was lower (Fig. 2), this could not be related to a specific decrease in a single binding type. Instead, both high- and low-affinity binding remained present (Table 1), each susceptible to inhibitors according to the characteristics found at 500 nM-PAF-acether (results not shown). Hence, these results indicate that under equilibrium conditions PAF-acether triggers the exposure of the fibrinogen

binding sites via two mutually independent mechanisms, each leading to different binding characteristics towards fibrinogen. One mechanism is mediated via endoperoxide/thromboxane formation and is responsible for high-affinity binding, while the other is mediated via ADP secreted from the dense granules and results in low-affinity binding of fibrinogen.

Non-equilibrium kinetics

Displacement studies with excess of unlabelled fibrinogen have revealed that bound 125 I-fibrinogen gradually becomes irreversibly attached to the platelets. Fig. 7 illustrates that initially binding was completely reversible, as measured following forskolin addition (10 μ M final concentration), which enabled displacement studies in a range where excess of unlabelled fibrinogen proved unsuitable due to the poor solubility of fibrinogen. At longer incubation times, however, a gradual decrease in reversible binding became apparent leading to $65 \pm 3\%$ (*n* = 6) dissociable binding after 60 min, the time used for equilibrium measurements. In order to evaluate whether this property interfered with the characteristics of fibrinogen binding found at equilibrium, binding studies were repeated in the range where complete reversibility was observed. Fig. 8 illustrates the initial binding of 125 I-fibrinogen to platelets stimulated with 500 nM-PAF-acether. Again fibrinogen binding was sensitive to indomethacin and ADP scavengers. Pretreatment with indomethacin reduced the binding by about 20% whereas PEP/PK led to an inhibition of about 80%. The combination of both inhibitors abolished all specific fibrinogen binding. Although under non-equilibrium conditions the remaining indomethacin-sensitive binding was too small to measure accurately, its contribution could be estimated from the difference between total and PEP/PK-sensitive binding (Fig. 8, broken line).

A further comparison between fully and partially dissociable fibrinogen binding could be made by calculating the number of binding sites on the basis of binding studies at 5, 10 and 60 min incubation. As demonstrated elsewhere (Klopprogge *et al.*, 1986), at 500 nM-PAF-acether, exposure of binding sites is complete within 2–5 min and thereafter the sites behave as

Fig. 6. Scatchard analysis of PAF-acether (500 nM)-induced fibrinogen binding in the presence of indomethacin or PEP/PK

(a) shows the effect of 30 μM -indomethacin (closed symbols). Data are from a single platelet suspension ($K_d = 5.44 \times 10^{-7}$ M, 19961 sites/platelet) and are representative of four similar experiments. The open symbols show the binding in the absence of indomethacin both in the high- and low-affinity binding range (means $\pm$ S.D., $n = 3$). (b) shows the effect of PEP/PK (0.28 mM + 3 units/ml). From the data (means $\pm$ S.D., $n = 6$), $K_d = 12.03 \times 10^{-8}$ M and 765 binding sites/platelet can be calculated (representative of four similar experiments). (c) shows the binding to storage pool deficient platelets (granule-bound ADP < 0.2 $\mu\text{mol}/10^{11}$ cells). (Representative of two similar experiments.)

Fig. 7. Dissociation of bound fibrinogen by forskolin

Platelets were incubated with 500 nM-PAF-acether in the presence of 1 μM - ^{125}I -fibrinogen. At the times indicated by the arrows 10 μM -forskolin was added. The symbols refer to forskolin addition after 0.5 (∇), 5 ($\blacksquare$), 15 ($\bullet$), 25 ($\blacktriangle$), 35 ($\square$), 45 ($\circ$) and 55 ($\triangle$) min and are means $\pm$ S.D. from six determinations.

normal acceptors. The binding of fibrinogen can then be analysed by using the equation:

$$\frac{d[\text{FB}]}{dt} = k_{+1}[\text{F}][\text{B}] - k_{-1}[\text{FB}] \quad (\text{I})$$

where at a given time, [F], [B] and [FB] stand for the respective concentrations of free ligand, free binding sites and complex; k_{+1} and k_{-1} are the rate constants for association and dissociation, respectively.

The three variables in eqn. (I) are mutually dependent with the total number of binding sites, $[\text{B}]_{\text{total}}$, representing a constant. Thus $[\text{B}] = [\text{B}]_{\text{total}} - [\text{FB}]$ and

can be substituted in eqn. (I). Integration of eqn. (I) results in:

$$[\text{FB}]_t = \frac{[\text{F}][\text{B}]_{\text{total}}}{[\text{F}] + K_d} (1 - e^{-(k_{+1}[\text{F}] + k_{-1})t}) \quad (\text{II})$$

where $[\text{B}]_{\text{total}}$ stands for the concentration of binding sites at 5 min following PAF-acether addition and the term:

$$1 - e^{-(k_{+1}[\text{F}] + k_{-1})t}$$

describes the time course with which equilibrium is achieved. The equation:

$$[\text{FB}]_{\text{eq.}} = \frac{[\text{F}][\text{B}]_{\text{total}}}{[\text{F}] + K_d} \quad (\text{III})$$

describes the complex concentration at equilibrium and is related to the total number of binding sites $[\text{B}]_{\text{total}}$.

Applying eqn. (II) to the non-equilibrium measurements (5 and 10 min) leads to a ratio, which is independent of the total number of binding sites $[\text{B}]_{\text{total}}$:

$$\frac{[\text{FB}]_{t=10 \text{ min}}}{[\text{FB}]_{t=5 \text{ min}}} = (1 + e^{-(k_{+1}[\text{F}] + k_{-1})t}) \times 300$$

Together with the known value for K_d ($K_d = k_{-1}/k_{+1}$; Table 1) these two equations can be solved for k_{+1} and k_{-1} . When these values (Table 2) are used for calculating the amount of fibrinogen bound to indomethacin-treated platelets at 5, 10 and 60 min of incubation with the help of eqn. (II), the calculated values fall within the range of experimentally obtained data (Table 3). Hence, the number of bound fibrinogen molecules remains unchanged when a 5–10 min incubation period, with almost 100% dissociable fibrinogen, is extended to 60 min incubation with about 60% dissociable fibrinogen. Assuming that the difference between untreated platelets and indomethacin-treated platelets reflects the PEP/PK-sensitive part, a similar comparison between calculated and measured binding data can be made (Table 3). Again no differences are found, suggesting that both types of

Fig. 8. Initial binding of fibrinogen to PAF-acether-stimulated platelets

Platelets were stimulated with 500 nM-PAF-acether in the presence of $1 \mu\text{M}$ - ^{125}I -fibrinogen without (○) and with (●) pretreatment with $30 \mu\text{M}$ -indomethacin. The broken line represents the difference between both types of binding and is an estimation of indomethacin-sensitive binding.

fibrinogen binding are unaffected by the gradual decrease in reversible binding.

Analysis of secretion and arachidonate metabolism under binding conditions

The strong interference of cyclo-oxygenase/thromboxane synthetase inhibitors and ADP scavengers with the PAF-acether-induced binding of fibrinogen suggested that a considerable degree of secretion and arachidonate metabolism took place under the conditions of the binding experiments. Table 4 lists the secretion of dense and α -granule contents as well as the concurrent formation of thromboxane B_2 and free $[^3\text{H}]$ arachidonate (including its metabolites). In the presence of 500 nM-PAF-acether only minimal secretion occurred during a 60 min incubation period, leading to about 10% liberation of dense and α -granule contents compared with maximal stimulation with thrombin (5 units/ml, 37°C , stirred suspensions). Slightly less secretion took place in the presence of ADP scavengers, whereas the cyclo-oxygenase/thromboxane synthetase inhibitors had no effect on the secretion characteristics. In the absence of inhibitors only about 4 nmol of thromboxane $\text{B}_2/10^{11}$

Table 3. Comparison between calculated and measured number of bound fibrinogen molecules

The number of bound fibrinogen molecules was measured at 5, 10 and 60 min following stimulation with 500 nM-PAF-acether and $1 \mu\text{M}$ - ^{125}I -fibrinogen and were compared with the calculated data using eqn. (II) (see the text) and the rate constants listed in Table 2. Data are from platelets from a single donor (representative for three similar experiments) and are expressed as number of fibrinogen molecules bound/platelet (means $\pm$ s.d., $n = 24$) for indomethacin-treated platelets and for the differences between these platelets and untreated cells. Differences between calculated and measured data were not significant ($P > 0.05$).

Time (min)	Fibrinogen bound (molecules/platelet)			
	Indomethacin-treated platelets (ADP-mediated binding)		Difference between untreated and indomethacin-treated platelets (thromboxane A_2 -mediated binding)	
	Calculated	Measured	Calculated	Measured
5	5653 ± 1300	7823 ± 1818	2368 ± 129	1999 ± 410
10	6462 ± 1173	9263 ± 1688	2576 ± 163	2188 ± 449
60	9599 ± 483	9588 ± 482	2558 ± 464	2208 ± 452

cells was formed, which is about 2% of the amounts formed after maximal stimulation with thrombin (5 units/ml, 37°C , stirred suspensions). The mobilization of $[^3\text{H}]$ arachidonate was equally small and less than 6% of the amounts formed with thrombin. ADP scavengers did not change these data, but inhibition of cyclo-oxygenase enhanced the accumulation of free $[^3\text{H}]$ arachidonate and almost completely abolished thromboxane B_2 synthesis. A similar blockade of thromboxane B_2 formation was found with UK 38,485. When the PAF-acether concentration was lowered to 0.5 nM, secretion and arachidonate metabolism became almost negligible in the presence and absence of ADP scavengers and inhibitors of thromboxane formation.

Comparison between fibrinogen binding and aggregation

The finding that one type of fibrinogen binding could be abolished while leaving the other type intact opened the possibility to evaluate the role of each type of

Table 2. Non-equilibrium kinetics of PAF-acether-induced fibrinogen binding

Gel-filtered platelets were incubated with $1 \mu\text{M}$ - ^{125}I -fibrinogen, without and with pretreatment with indomethacin. Fibrinogen binding was analysed at 5, 10 and 60 min following stimulation with 500 nM-PAF-acether. From these data the rate constants for association (k_{+1}) and dissociation (k_{-1}) were calculated as described in the text (means $\pm$ s.d., $n = 24$). The rate constants for the difference between untreated and indomethacin-treated platelets were thought to reflect thromboxane A_2 -mediated binding.

	Indomethacin-treated platelets (ADP-mediated binding)	Difference between untreated and indomethacin-treated platelets (thromboxane A_2 -mediated binding)
k_{+1}	$3.46 (\pm 1.70) \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$	$7.79 (\pm 2.40) \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$
k_{-1}	$2.04 (\pm 0.45) \times 10^{-3} \text{ s}^{-1}$	$5.61 (\pm 2.60) \times 10^{-4} \text{ s}^{-1}$

Table 4. Secretion and arachidonate metabolism during PAF-acether-induced fibrinogen binding

Platelets were stimulated with 0.5 or 500 nM-PAF-acether in the presence of 1 μ M-fibrinogen with and without the inhibitors defined in the legend of Table 1 under the conditions of the fibrinogen binding studies (22 °C, non-stirred). Secretion of dense and α -granule contents was analysed after 60 min by measuring [14 C]serotonin and β -thromboglobulin, respectively. These data (mean $\pm$ S.D., $n = 6$) were expressed as percentages of maximal secretable amounts found extracellularly following 5 min stimulation with 5 units of thrombin/ml at 37 °C in stirred suspensions (900 rev./min). Thromboxane B₂ formation after 60 min was expressed as nmol/10¹¹ cells ($n = 4$) and the formation of free [3 H]arachidonate (including its metabolites, $n = 4$) after 60 min was expressed as a percentage of the amounts found with 5 units of thrombin/ml (5 min, 37 °C).

Conditions	Secretion (%)		Arachidonate metabolism (%)	
	[14 C]Serotonin	β -Thromboglobulin	Thromboxane B ₂	Free [3 H]arachidonate + metabolites
500 nM-PAF-acether	9 $\pm$ 6	13 $\pm$ 7	4.1 $\pm$ 0.1	3.4 $\pm$ 1.6
Indomethacin	12 $\pm$ 7	10 $\pm$ 8	0.2 $\pm$ 0.2	5.4 $\pm$ 1.0
UK 38,485	10 $\pm$ 5	14 $\pm$ 9	< 0.2	2.9 $\pm$ 0.9
PEP/PK	7 $\pm$ 4	12 $\pm$ 7	3.9 $\pm$ 1.0	4.9 $\pm$ 1.7
Indomethacin + PEP/PK	7 $\pm$ 2	7 $\pm$ 3	< 0.2	5.9 $\pm$ 0.6
0.5 nM-PAF-acether	2 $\pm$ 1	4 $\pm$ 2	2.8 $\pm$ 2.1	0.9 $\pm$ 0.1
Thrombin (5 units/ml)	(100)	(100)	295 $\pm$ 27	(100)

Fig. 9. Role of high- and low-affinity fibrinogen binding in platelet aggregation

Platelets were stimulated with 500 nM-PAF-acether in the presence of different concentrations of fibrinogen without (○) or with pretreatment with indomethacin, revealing low-affinity binding (●) or PEP/PK, revealing high-affinity binding (■).

binding in platelet aggregation. Under the assumption that stirring did not change the binding characteristics, aggregation (measured as single platelet disappearance) was measured with and without inhibitors, both at 22 °C and at 37 °C. Studies at 22 °C showed that aggregation following stimulation with 0.5 and 500 nM-PAF-acether completely depended on low-affinity binding both at high (500 nM) and low (10 nM) concentrations of fibrinogen. Similar findings were obtained at 37 °C when aggregation was measured in the presence of high concentrations of fibrinogen. In contrast, at a fibrinogen concentration of about 100 nM aggregation was slightly slower when high-affinity binding was abolished, suggesting that in this range both types of binding were involved. At lower fibrinogen concentrations, high-affinity binding became increasingly important and between 2 and 10 nM aggregation completely depended on high-affinity fibrinogen binding (Fig. 9). The different involvement of the

two types of binding seen at 22 °C and 37 °C suggested that the binding characteristics differed between both temperatures. Scatchard analysis revealed a significant decrease in low-affinity binding at 37 °C, whereas high-affinity binding remained unchanged. Specifically, the number of low-affinity binding sites decreased 6-fold and became close to the number of high-affinity binding sites (Table 5).

DISCUSSION

Earlier reports have demonstrated that platelets possess a specific receptor for PAF-acether (Klopogge & Akkerman, 1984; Valone *et al.*, 1982). The receptor is saturated within 20 min incubation with 0.5 nM-PAF-acether (22 °C), which leads to a variety of cellular responses including exposure of fibrinogen binding sites (Klopogge & Akkerman, 1984). The present study evaluates the characteristics of PAF-acether-induced fibrinogen binding. Such a study is complicated by the dynamic nature of fibrinogen binding sites. During platelet stimulation these sites shift from an inaccessible state to an exposed state with which fibrinogen can interact. In the absence of fibrinogen, however, the sites undergo a second alteration which makes them again inaccessible to fibrinogen (Klopogge *et al.*, 1986). Thus, characterization of fibrinogen binding requires fibrinogen to be present at the time of PAF-acether addition as well as optimal exposure of binding sites during the binding studies. As reported elsewhere (Klopogge *et al.*, 1986), binding site exposure is complete within 5 min following stimulation with 500 nM-PAF-acether, but takes about 30 min at 0.5 nM or less. The optimal conditions (incubation times of 5–60 min with 500 nM-PAF-acether and 60 min with 0.5 nM-PAF-acether) were therefore chosen for characterization of fibrinogen binding.

A comparison of fibrinogen binding at different concentrations of PAF-acether showed that 30 min incubation with 0.5 nM-PAF-acether induced only two-thirds of maximal binding, despite the fact that these conditions are sufficient for complete occupancy of PAF-acether receptors (Klopogge & Akkerman, 1984). This effect is only observed within platelets from a single

Table 5. Kinetics of PAF-acether induced fibrinogen binding to platelets

Platelets were incubated with 500 nM-PAF-acether in the presence of different concentrations of ^{125}I -fibrinogen (5–500 nM) at 37 °C for 60 min without stirring. Via Scatchard analysis the dissociation constants and the number of binding sites were obtained and compared with the values of 22 °C (Table 1). Data are means $\pm$ S.D. from four (37 °C) and nine (22 °C) experiments; differences were evaluated by Student's *t*-test.

Parameter	22 °C	37 °C	<i>P</i> value
Thromboxane A_2 -mediated binding			
K_d (M)	$7.2 (\pm 2.1) \times 10^{-8}$	$6.1 (\pm 1.0) \times 10^{-8}$	0.420
Binding sites	2367 ± 485	1632 ± 1123	0.285
ADP-mediated binding			
K_d (M)	$5.9 (\pm 2.4) \times 10^{-7}$	$2.1 (\pm 0.5) \times 10^{-7}$	0.025
Binding sites	26972 ± 8267	4499 ± 2348	0.001

donor, since different donors show great variations in fibrinogen binding capacity (Table 1). Maximal fibrinogen binding requires 100 nM PAF-acether or more, which probably leads to a higher degree of receptor occupancy during the early periods of PAF-acether-platelet interaction.

Scatchard analysis of the fibrinogen binding data reveals two types of binding characteristics, a high- and a low-affinity binding type differing about 10-fold in dissociation constants and number of binding sites. Initial binding studies in the range where binding was completely reversible were consistent with two types of binding. Dual binding characteristics were also reported for platelets stimulated with ADP (DiMinno *et al.*, 1983; Kornecki *et al.*, 1981; Peerschke *et al.*, 1980), collagen and arachidonate (DiMinno *et al.*, 1983) and the kinetic properties described in these studies are in the range found with PAF-acether-stimulated platelets. The possibility that different binding affinities for native and iodinated fibrinogen caused the complex Scatchard plots has been refuted by isotope dilution experiments. In contrast, Bennett & Vilaire (1979) and Marguerie *et al.* (1980, 1983) found only a single class of binding sites with characteristics close to our data for low-affinity binding, but these authors left the high-affinity binding range unexplored. Hence, the kinetics of fibrinogen binding induced by PAF-acether seem similar to those provoked by other platelet-stimulating agents, suggesting that despite the diversity among agonists and the mechanisms by which they activate the platelets, all lead to a common step that enables fibrinogen to bind. Studies with intact platelets (Kornecki *et al.*, 1981) as well as with plasma membranes (Fujimura & Phillips, 1983) have revealed the glycoprotein IIb-IIIa complex as the receptor to which fibrinogen binds. This complex is deficient in thrombasthenic platelets and those platelets failed to show specific fibrinogen binding both in the high- and low-affinity range. Hence, both types of fibrinogen binding appear properties of the same glycoprotein complex and it is therefore surprising that the two types of binding are mediated via different, mutually independent, mechanisms. One mechanism, mediated via secreted ADP, is responsible for the low-affinity binding of fibrinogen, as evidenced by its complete absence in the presence of ADP scavengers and in storage pool deficient platelets; the other, mediated via arachidonate metabolites, is responsible for the high-affinity binding of fibrinogen, as illustrated by its complete abolishment by inhibitors of endoperoxide and thromboxane formation.

From these two, the formation of thromboxane A_2 is probably crucial since the thromboxane synthase inhibitor UK 38,485 alone is fully capable of preventing high-affinity binding.

There have been conflicting reports regarding the role of ADP and arachidonate metabolites in fibrinogen binding to platelets. The strong interference of ADP scavengers led Marguerie & Plow (1983) and Morinelli *et al.* (1983) to attribute a central role to secreted ADP, irrespective of the agonist used for stimulation. This concept was weakened by the observation that degranulated platelets bound fibrinogen upon stimulation with arachidonate, an effect that could be completely abolished with indomethacin (Harfenist *et al.*, 1982). Furthermore, Bennet *et al.* (1981) found that normal, ADP-stimulated platelets bound fibrinogen via mechanisms sensitive to aspirin and indomethacin. Hence, in retrospect a role for both ADP and arachidonate metabolites was implicated in most of these reports. Our present findings explain the discrepancies found in these studies. First, the fact that ADP and arachidonate metabolites induce different binding affinities makes the effect of inhibitors dependent of the chosen fibrinogen concentrations. Second, since various agonists activate secretion and arachidonate metabolism to different extents, interference with these pathways will depend on the type of agonist.

The effect of ADP scavengers and inhibitors of arachidonate metabolism is somewhat unexpected since the conditions of our binding experiments (22 °C, non-stirred suspensions) are not thought to result in extensive secretion or prostanoid synthesis. Careful analysis of these phenomena confirmed that only very little secretion and arachidonate metabolism occurred, totalling only a few percent of the extents found with maximal stimulation by thrombin (5 units of thrombin/ml, 5 min stirring at 37 °C). However, even in this low range both processes could be inhibited further by ADP scavengers and indomethacin. Probably these minute amounts are sufficient for occupying ADP and thromboxane A_2 receptors in the same or adjacent platelets.

The fact that high- and low-affinity fibrinogen binding could be specifically inhibited opened the possibility to compare the binding kinetics with the role of fibrinogen in platelet aggregation. At 22 °C aggregation was completely abolished by ADP scavengers both at high and low concentrations of fibrinogen. Apparently, single platelet disappearance then depended completely on

low-affinity binding. This accords with earlier work (Kornecki *et al.*, 1981), that demonstrated a similar correlation with optical aggregation. Although at low fibrinogen levels a role for high-affinity binding would be expected, indomethacin treatment had no effect. Probably the high number of low-affinity binding sites compensated for the relative low binding affinity, enabling platelet-platelet interaction even at low levels of fibrinogen. At 37 °C, again a major role for low-affinity binding was evident when the fibrinogen concentration was high (> 200 nM). At low fibrinogen levels, however, a role for high-affinity binding became apparent and below 10 nM-fibrinogen aggregation solely depended on high-affinity binding. At this temperature the number of low-affinity binding sites was about 6-fold lower than at 22 °C and closer to the range of high-affinity binding sites. Consequently, less compensation for lower binding affinity was possible, enabling high-affinity binding to become involved.

Apart from binding to its receptor, PAF-acether is taken up and metabolized, but it is uncertain whether these processes contribute to platelet activation. Two observations argue against such a role. First, the kinetics of fibrinogen binding found with 0.5 nM and 500 nM-PAF-acether are similar, although only above 1 nM-PAF-acether is receptor occupancy accompanied by uptake and metabolism (Klopogge & Akkerman, 1984). Second, the increase in fibrinogen binding found when the PAF-acether concentration is raised from 0.5 to 1.0 nM (where uptake is negligible) is of the same magnitude as found when the dose of PAF-acether is increased from 1 to 100 nM (where uptake and metabolism exceeds receptor binding several-fold; Klopogge & Akkerman, 1984). Thus, fibrinogen binding initiated by PAF-acether appears primarily the result of receptor-mediated processes.

In conclusion, the early interaction between PAF-acether and platelets triggers exposure of high-affinity fibrinogen binding via arachidonate metabolites, probably thromboxane A₂, and low-affinity binding via secreted ADP. Both properties are apparent under equilibrium conditions and during initial binding and appear associated with the glycoprotein IIb-IIIa complex. Whether this reflects two properties of the same binding sites or heterogeneity of sites is at present uncertain. Preliminary studies show that a number of monoclonal antibodies against glycoprotein IIb-IIIa, as well as the tetrapeptide Gly-Pro-Arg-Pro, interfere with both types of binding, suggesting the involvement of a single type of binding site.

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