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Binding kinetics of PAF-acether (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) to intact human platelets

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The binding of [^{3}H]PAF-acether (1-*O*-alkyl-2-acetyl-*sn*-glycero-3- phosphocholine) to intact human gel-filtered platelets was measured at 22°C. Specific binding reached saturation within 15min at high doses of [^{3}H]PAF-acether (0.5–0.9nM), whereas about 90min were required when low doses (0.02–0.5nM) were used. Above 1nM, [^{3}H]PAF-acether non-specific binding increased progressively, which together with the demonstration of a ^{3}H-labelled metabolite suggested uptake and metabolism of [^{3}H]PAF-acether. Equilibrium analysis revealed one class of specific receptors with a K_a of $18.86 \pm 4.82 \times 10^9 M^{-1}$ and 242 ± 64 binding sites per platelet. Non-equilibrium binding revealed a similar K_a ($16.87 \times 10^9 M^{-1}$). Specific binding became irreversible after prolonged incubation, a process that was enhanced at increasing concentrations of [^{3}H]PAF-acether. Platelets made desensitized to PAF-acether by prior incubation with unlabelled PAF-acether failed to bind a second dose of PAF-acether (^{3}H-labelled), suggesting that desensitization resulted from loss of available binding sites. Under the conditions of the binding studies, PAF-acether induced exposure of the fibrinogen receptor, aggregation (in a stirred suspension) and alterations in (poly)-phosphatidylinositides. These results suggest that PAF-acether initiates platelet responses via receptor-mediated processes.

Platelet activating factor or PAF-acether, which was identified as a 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (Demopoulos *et al.*, 1979; Benveniste *et al.*, 1979), is a mediator in IgE-induced systemic anaphylaxis in rabbits (Benveniste *et al.*, 1972; Henson & Pinckard, 1977). During allergic and inflammatory reactions PAF-acether is released by leucocytes and macrophages (Siraganian & Ostler, 1971; Polonsky *et al.*, 1980). It has been shown to induce chemotaxis (Shaw *et al.*, 1981) and degranulation of polymorphonuclear leucocytes (O'Flaherty *et al.*, 1981), hypotension (McManus *et al.*, 1980) and smooth-muscle contraction (Findlay *et al.*, 1981), but its most striking biological activity is its potency as a platelet stimulator, inducing aggregation and secretion responses in the nanomolar range (Vargaftig *et al.* 1982*a*; Chesney *et al.*, 1982; Kloprogge *et al.*, 1983).

Abbreviations used: PAF-acether, platelet-activating factor; IgE, immunoglobulin E; SDS, sodium dodecyl sulphate.

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Platelet agonists generally activate the cells via receptor-mediated processes. The identification of a specific receptor for PAF-acether is complicated by its hydrophobic nature, making it a component that easily penetrates biological membranes (Lumb *et al.*, 1983). Furthermore, exogenous PAF-acether is metabolized by platelets, which probably implies uptake into the platelet and incorporation in still largely obscure metabolic pathways (Alam *et al.*, 1983; Pieroni & Hanahan, 1983; Touqui *et al.*, 1982).

Early studies by Shaw & Henson (1980) demonstrated that PAF-acether lost its activity as a secretagogue after preincubation with rabbit platelets. This was interpreted as the first indication that PAF-acether could bind to platelets and that it activated the cells via a receptor-mediated process. Valone *et al.* (1982), using ^{3}H-labelled PAF-acether, confirmed that human platelets bound PAF-acether and found two distinct types of binding sites differing about 37-fold in their affinity for PAF-acether.

The present report provides a detailed study of the kinetics of [^{3}H]PAF-acether binding to

human platelets. The results substantially deviate from the early findings by Valone *et al.* (1982) and, in addition, provide evidence for coupling between receptor occupancy and platelet activation, suggesting that platelets are activated by PAF-acether via receptor-mediated processes.

Materials and methods

Chemicals

[^{3}H]PAF-acether {1-*O*-[1,2(n)-^{3}H]hexadecyl/octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; 91.2Ci/mmol} and lyso-[^{3}H]PAF-acether (1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine; 129Ci/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.). Non-radiolabelled PAF-acether and lyso-PAF-acether were from Calbiochem–Behring Corp. (La Jolla, CA, U.S.A.) and Bachem (Bubendorf, Switzerland) respectively. All ether phospholipids were stored at −20°C in toluene/ethanol (1:1, v/v) under N_2. Before use, stock solutions were prepared by evaporating the solvent with N_2 and dissolving the ether phospholipids in 0.15M-NaCl, containing bovine serum albumin (2.5mg/ml) (Organon Technika, Oss, The Netherlands). Yohimbine was obtained from Sigma, St. Louis, MO, U.S.A.; phentolamine from Ciba–Geigy, Basel, Switzerland, and prasozine from Pfizer, Brussels, Belgium.

Phospholipids used as references in t.l.c. studies were: phosphatidic acid (egg; Serdary Research Laboratories, London, Ontario, Canada), lysophosphatidylcholine (Applied Science Laboratories, Riviera Beach, PA, U.S.A.), phosphatidylserine, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol and phosphatidylcholine (Supelco, Bellefonte, PA, U.S.A.), phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-monophosphate (Sigma).

Fibrinogen (KABI, Stockholm, Sweden) was made fibronectin-free by passage through a gelatin–Sepharose 4B column (Pharmacia, Uppsala, Sweden) (Engvall *et al.*, 1978) in the presence of 10nM-6-aminohexanoic acid (Baker, Deventer, The Netherlands) and 1mM-benzamidine hydrochloride (Sigma). After four dialyses against 40vol. of Tyrode's buffer (Walsh, 1972) (12h, 4°C), the fibrinogen solution was concentrated by dehydration with Sephadex G-200 (Pharmacia). Contamination with Factor VIII/von Willebrand factor was less than 0.015% on molar basis, as measured by enzyme immunoassay (Cejka, 1982). Electrophoretic identification on SDS/(7.5%, w/v) polyacrylamide gels (Laemmli, 1970) under reducing conditions revealed a pure preparation consisting of Aα-chains (M_r 68000), Bβ-chains (57000) and γ-chains (49000).

Platelet isolation

Venous blood from healthy volunteers was collected (with informed consent) directly into 0.1vol. of 130mM-trisodium citrate. The donors claimed not to have taken any medicine during the 10 days before blood collection. After centrifugation (10min, 200*g*, 22°C) the platelets were isolated by gel filtration (Sepharose 2B; Pharmacia) (Tangen *et al.*, 1971) at room temperature in Ca^{2+}-free Tyrode solution (Walsh, 1972), pH7.25, containing 0.2% bovine serum albumin (Organon Technika) and 5mM-glucose. The final concentration of the gel-filtered platelets was adjusted to 2.0×10^8 cells/ml by dilution in Ca^{2+}-free Tyrode's buffer.

Binding studies with [^{3}H]PAF-acether

Gel-filtered platelets were incubated with different concentrations of ^{3}H-labelled PAF-acether under conditions in which aggregation and secretion were prevented. These were: firstly, the absence of stirring, in order to avoid cell–cell contact; secondly, the absence of fibrinogen, which is a cofactor of platelet aggregation (Kloprogge *et al.*, 1983); and thirdly, the use of [^{3}H]PAF-acether concentrations below 10nM, which is the threshold for optical aggregation and secretion under optimal conditions (37°C, stirring, 0.3–0.7mg of fibrinogen/ml) (Kloprogge *et al.*, 1983). Most of the studies were performed at room temperature. The specific activity of [^{3}H]PAF-acether was kept constant at all concentrations, except in the dissociation studies (see the Results section). Non-specific binding was measured by adding [^{3}H]PAF-acether in the presence of a 200-fold molar excess of non-radiolabelled PAF-acether.

After the cells had been incubated for various periods, the platelets were separated from the suspension medium by dilution in 10vol. of cold (0°C) Tyrode's solution, immediately followed by vacuum filtration through 0.8μM-pore-size Millipore filters (Millipore, Bedford, MA, U.S.A.) with an Amicon filtration manifold (Amicon Corp., Lexington, MA, U.S.A.). The filters were washed twice with 5ml of cold Tyrode solution, dried and dissolved in 1ml of 2-ethoxyethanol (Merck, Darmstadt, Germany) and the radioactivity was counted by standard procedures. The same results were obtained when the platelets were isolated by centrifugation in an Eppendorf Microfuge (10000*g*, 30s) followed by washing twice with cold (0°C) Tyrode's solution and resuspension in 1% Triton X-100 in Tyrode's solution. The recovery from the centrifugation technique was 103.4±4.1% (mean±S.D.; $n=25$); trapping was

less than 0.08% [on the basis of the presence of [^{14}C]sucrose (Amersham International) in the pellet].

[^{3}H]Phospholipid analysis

In a few experiments, the incubation of gel-filtered platelets with [^{3}H]PAF-acether was terminated by adding 3.6ml of methanol/chloroform (2:1, v/v) to 1ml of platelet suspension. The lipids were extracted as described by Bligh & Dyer (1959), subjected to t.l.c. and separated on high-performance t.l.c. plates (Merck; no. 5641) using a basic solvent system of chloroform/methanol/aq. 20% (v/v) methylamine (30:18:5, by vol.) (Pieroni & Hanahan, 1983). Lipid spots were located with I_2 vapour. The material contained in the spots was collected and extracted in chloroform/methanol (2:1, v/v) and the radioactivity was measured by standard methods. Recovery of the ^{3}H was 60±7% (mean±S.D., $n=12$).

Analysis of platelet responses

Aggregation of gel-filtered platelets induced by PAF-acether was measured by two techniques: firstly, by recording the change in light transmission at 22°C in a Payton dual-channel aggregation module (Payton Associates, Scarborough, Ontario, Canada) and secondly, by counting the disappearance of single platelets (Frojmovic *et al.*, 1982). For the latter technique, platelets were incubated with PAF-acether with or without fibrinogen (0.3mg/ml) under stirring and non-stirring conditions (22°C). The incubation was stopped by fixing the platelets in 9vol. of a mixture of cold (0°C) 0.5% glutaraldehyde (Fluka, Buchs, Switzerland). The platelets were counted in a Platelet Analyzer 810 (Baker Instruments, Allentown, PA, U.S.A.) with apertures set at 3.2 and 16μm^3. In unstimulated platelet suspensions 94.5±1.1% (mean±S.D., $n=11$) of the total platelets were counted within these settings.

PAF-acether-induced fibrinogen binding was measured using ^{125}I-labelled fibrinogen obtained with the Iodogen-labelling procedure in Iodogen-coated conical 3ml glass Mini Vials (Chrompack, Middelburg, The Netherlands) (Nieuwenhuizen *et al.*, 1980) in three successive incubations of 20min each (0°C). ^{125}I was obtained from Amersham International. The ^{125}I-fibrinogen was dialysed three times against 500vol. of non-radioactive KI (10g/litre in 0.15M-NaCl) at 4°C for 12h, followed by dialysis against 500vol. of Tyrode's buffer (12h, 4°C). Insoluble contaminants were removed by centrifugation (10000*g*, 10min). Clottability, initiated with 5 units of thrombin (Roche, Basel, Switzerland)/ml, was >99% and the content of free ^{125}I was never more than 4.2% of the total ^{125}I radioactivity. The specific activity of ^{125}I-fibrinogen varied between 0.5 and 3.0Ci/mmol. ^{125}I-labelled fibrinogen was stored at −70°C and used within 3 weeks after preparation.

Changes in phosphoinositides and phosphatidic acid in platelets on stimulation with PAF-acether were measured according to a modification of the procedure described by Jolles *et al.* (1981) for rat brain cells. In short, platelet-rich plasma was labelled with $^{32}P_i$ (0.1mCi/ml, carrier-free, type NEX-054; New England Nuclear, Boston, MA, U.S.A.) for 60min at 37°C. The platelets were gel-filtered in Ca^{2+}-free Tyrode's solution, without NaH_2PO_4, at 22°C and incubated with 0.5nM-PAF acether. The reaction was terminated after 20min by adding 2ml of chloroform/methanol/13M-HCl (100:50:1, by vol.; 0°C). The lipids were extracted as described by Bligh & Dyer (1959). Phospholipids were separated by high-performance t.l.c. and the radioactivity was measured as described by Jolles *et al.* (1981).

Results

PAF-acether binding

A time course of [^{3}H]PAF-acether binding to intact human platelets at concentrations ≤0.1nM showed an increase of radioactivity associated with the platelets during 60–80min before equilibrium was reached (Fig. 1). At higher concentrations (0.5–1.0nM) this level was reached within 15min and remained unchanged during 2h incubation.

Equilibrium kinetics

Saturation experiments during 90min incubation periods with different concentrations of [^{3}H]-PAF-acether (0.02–0.9nM) showed two phases

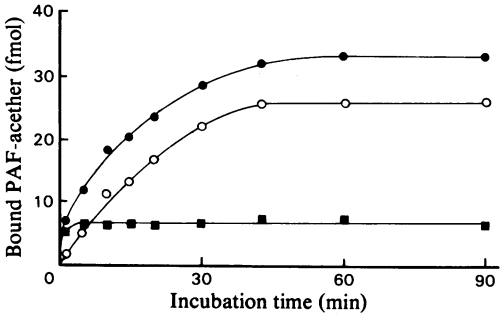

Fig. 1. *Time course of [^{3}H]PAF-acether binding to gel-filtered human platelets*

The [^{3}H]PAF-acether concentration was 0.1nM. Means of duplicate measurements are shown for total binding (●), non-specific binding (■) and specific binding (○); the latter was obtained from the difference between total and non-specific binding.

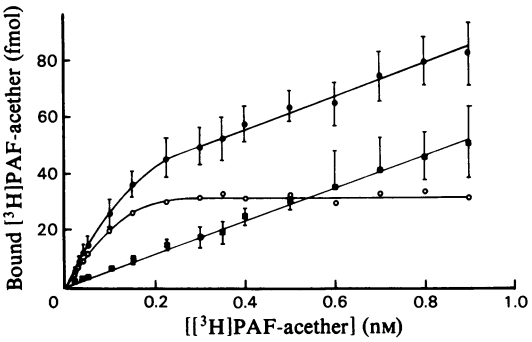

Fig. 2. *Saturation kinetics of [3H]PAF-acether binding to gel-filtered human platelets*
Duplicate samples (0.5ml), after 90min of incubation, were analysed for total binding (●), non-specific binding (■) and specific binding (○). Data are means ± S.D. for nine experiments.

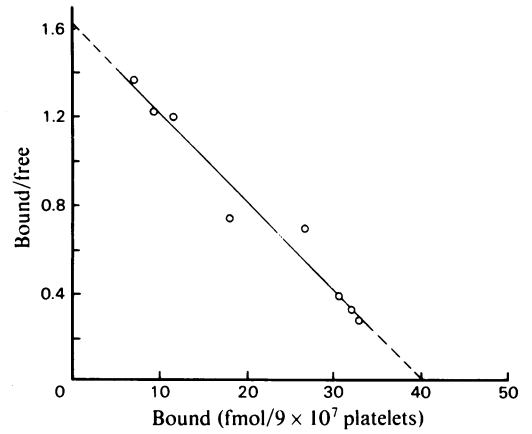

Fig. 3. *Scatchard plot of the specific binding of [3H]PAF-acether to gel-filtered human platelets*
'Free' means the free concentration of [3H]PAF-acether after 90min of incubation. $K_a = 20.05 \times 10^9 M^{-1}$; 269 binding sites per platelet.

(Fig. 2): a first phase (0.02–0.30nM) in which [3H]-PAF-acether binding increased progressively with [3H]PAF-acether concentration, and a second phase (0.3–0.9nM) with a moderate, linear increase in [3H]PAF-acether binding. Above 1nM-[3H]-PAF-acether, total binding increased progressively, which was entirely due to non-specific binding, suggesting uptake of [3H]PAF-acether in the platelets. In accordance with this assumption, we found, in this range, degradation of [3H]PAF-acether to a 3H-labelled metabolite (see below), whereas below 1nM, no derivatives of [3H]PAF-acether could be detected. Scatchard analysis (Fig. 3) of the data between 0.02 and 0.9nM-[3H]PAF-acether demonstrated one class of specific binding sites with an association constant, K_a, of 18.86 ($\pm$4.82) $\times 10^9 M^{-1}$ and 242 $\pm$ 64 binding sites per platelet (mean $\pm$ S.D., $n = 9$). Experiments with lyso-[3H]PAF-acether performed under similar conditions revealed only non-specific binding, to an extent similar to the non-specific binding in the [3H]PAF-acether studies (results not shown).

The temperature-dependence of [3H]PAF-acether binding was evaluated by repeating the experiments at 4 and 37°C. The amount of non-specific binding increased at increasing temperature. In contrast, specific binding was similar at 4 and 37°C and showed the kinetics found at room temperature (results not shown).

Non-equilibrium kinetics

The binding of PAF-acether to platelets can be represented by the equation:

$$L + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RL \qquad (1)$$

where L stands for free PAF-acether as the ligand and R for the putative receptor for PAF-acether; k_1 and k_2 are the respective rate constants of the association and dissociation reactions (Williams & Lefkowitz, 1978). The binding kinetics follow the equation:

$$\frac{d[LR]}{dt} = (k_1 + k_2)([LR_{eq.}] - [LR]) \qquad (2)$$

where LR is the ligand–receptor complex before, and $LR_{eq.}$, after, equilibrium is reached. Platelets were incubated with different concentrations of [3H]PAF-acether (0.055–0.33nM) and the initial specific binding was measured by collecting samples at early time points after addition of [3H]PAF-acether. Under these conditions the free concentration of [3H]PAF-acether did not change significantly ($[L] \gg [LR]$). Hence $k_1[L] = k_1'$ and eqn. (2) can be rearranged in the form:

$$\ln\left(\frac{[LR_{eq.}]}{[LR_{eq.}] - [LR]}\right) = k_{obs.} \cdot t \qquad (3)$$

in which $k_{obs.}$ (the observed rate constant) stands for $k_1' + k_2$. Fig. 4(*a*) illustrates the graphic notation of eqn. (3) for different concentrations of [3H]PAF-acether. From the slopes of these lines, the observed rate constant, $k_{obs.}$, at different concentrations of PAF-acether are derived. A plot of $k_{obs.}$ versus the corresponding concentrations of free [3H]PAF-acether (Fig. 4*b*) follows the equation:

$$k_{obs.} = [L]k_1 + k_2$$

and yields a k_1 of $0.3914 \times 10^9 M^{-1} \cdot s^{-1}$ and a k_2 of $0.0232 s^{-1}$. On the basis of these data, an associ-

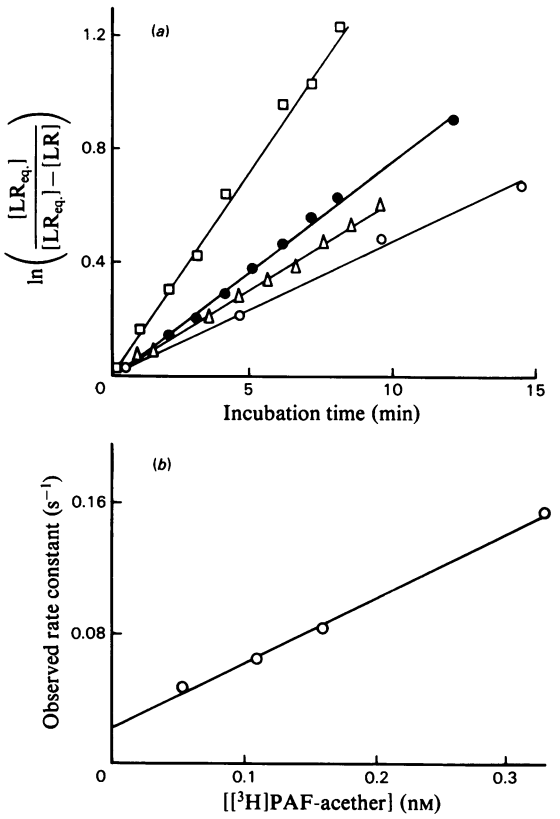

Fig. 4. *Kinetics of initial specific binding of [^{3}H]PAF-acether to gel-filtered human platelets*

(*a*) This represents eqn. (3) (see the text) for 0.33 mM-(□), 0.16 nM- (●), 0.11 nM- (△) and 0.055 mM- (○) [^{3}H]PAF-acether, where $LR_{eq.}$ is the ligand–receptor complex at equilibrium and LR the ligand–receptor complex of specific bound [^{3}H]PAF-acether. The slopes of the lines stand for the observed rate constant, $k_{obs.}$. (*b*) The observed rate constants, $k_{obs.} = [L]k_1 + k_2$, measured from the slope (*a*), versus the corresponding [^{3}H]PAF-acether concentration. The slope is k_1 and the intercept of the *y*-co-ordinate is k_2. $K_a = k_1/k_2 = 16.87 \times 10^9\ M^{-1}$.

ation equilibrium constant $[K_a = (k_1/k_2)]$ of $16.87 \times 10^9\ M^{-1}$ is derived. This value is close to the values measured under equilibrium conditions.

Role of Ca^{2+} ions in [^{3}H]PAF-acether binding and effect of α_1- and α_2-adrenergic antagonists

Equilibrium binding studies (22°C) revealed no effect of Ca^{2+} ions present in concentrations up to 3 mM. Similarly, the presence of 3 mM-EDTA did not effect the binding kinetics. Under similar conditions we found no effect of the α_1- and/or α_2-adrenergic antagonists yohimbine, phentolamine and prasozine in concentrations up to 10 μM.

Table 1. *Dissociation kinetics of specific binding of [^{3}H]PAF-acether*

Gel-filtered platelets were incubated with different concentrations of [^{3}H]PAF-acether. After 30 min, a 200-fold molar excess of non-radiolabelled PAF-acether was added and the percentages of specific binding were measured 5 and 30 min later. Results are means ± S.D. ($n = 7$).

[^{3}H]PAF-acether] (nM)	Dissociation (%) after: 5 min	30 min
0.11	21.1 ± 13.1	49.8 ± 6.4
0.27	19.2 ± 7.8	53.7 ± 5.2
0.53	12.0 ± 6.6	35.4 ± 7.8
1.10	11.3 ± 3.0	27.3 ± 10.6

Dissociation kinetics

The displacement of specifically bound [^{3}H]PAF-acether was investigated by incubating platelets with 0.55 nM-[^{3}H]PAF-acether. At various times after equilibrium had been established, 120 nM non-radiolabelled PAF-acether was added and the displacement of [^{3}H]PAF-acether was measured. The longer platelets were incubated with [^{3}H]PAF-acether the smaller was the fraction that could be displaced by excess of non-radiolabelled PAF-acether. The decrease in reversible PAF-acether binding was approximately linear with time. At 0.5 nM-PAF-acether, all [^{3}H]PAF-acether had bound irreversibly after about 90 min. Formation of an irreversible PAF-acether–receptor complex occurred more quickly at higher concentrations (Table 1).

In agreement with these observations were the results of a second type of experiments. This consisted of a first incubation (30 min) with non-radiolabelled PAF-acether followed by gel-filtration through Sepharose 2B, and a second incubation with PAF-acether (30 min), which was now radiolabelled. A high dose of PAF-acether (1 μM) in the first step prevented all specific binding in the second step, indicating that all receptors had been irreversibly changed during the first incubation. A low dose of [^{3}H]PAF-acether (0.4 nM) in the first step reduced the binding in the second step (Fig. 5), suggesting that less binding sites were available in these platelets.

Metabolism of [^{3}H]PAF-acether

As illustrated in Table 2, incubation of platelets with high concentrations of [^{3}H]PAF-acether resulted in the formation of a ^{3}H-labelled fraction with the chromatographic properties, on high-performance t.l.c., of an alkyl(long-chain)acyl-phosphocholine (Pieroni & Hanahan, 1983). At lower doses of [^{3}H]PAF-acether its formation was

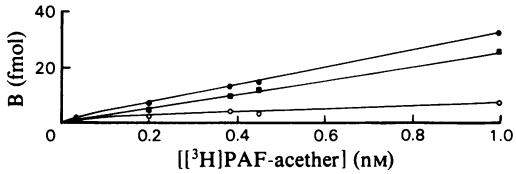

Fig. 5. *Preincubated human gel-filtered platelets with PAF-acether: saturation kinetics of [3H]PAF-acether binding to these platelets*

Gel-filtered platelets were preincubated with 0.4nM-PAF-acether and gel-filtered for a second time. After 90min incubation with [3H]PAF-acether (0.05–0.9nM), duplicate samples (0.5ml) were analysed for total binding (●), non-specific binding (■) and specific binding (○). In order to facilitate comparison with binding to normal platelets, the scale has been made similar to that in Fig. 2.

Table 2. *Metabolic conversion of [3H]PAF-acether by human platelets*

Gel-filtered platelets were incubated for 30min with [3H]PAF-acether at 22°C and duplicate samples were analysed for 3H-labelled degradation products. Abbreviation used: PC, phosphatidylcholine. Data are expressed as percentages of total 3H radioactivity and recalculated in absolute amounts. Data are ranges ($n = 2$) or means ± S.D. ($n = 4$).

	Formation of [3H]alkylacyl-PC:	
[3H]PAF-acether] (nM)	[3H]PAF-acether] (%)	Molecules/h per cell
0.02–0.50	<0.1	<3
0.9	0.1 – 0.2	4 – 8
3.5	0.6 ± 0.1	85 ± 20
47	3.9 – 4.1	7830 – 8190

greatly diminished, and was virtually undetectable below 1nM-[3H]PAF-acether, the concentration at which the receptor studies were carried out. Under all conditions tested, no formation of lyso-[3H]PAF-acether could be demonstrated. These data indicate that, under the conditions of the receptor studies, the PAF-acether molecule remained intact and that involvement in metabolic sequences was negligible.

Platelet responses induced by PAF-acether

In order to investigate whether the formation of a PAF-acether–receptor complex had any relevance for the initiation of platelet responses, the effect of PAF-acether binding on several cellular processes was studied. Platelets were incubated with PAF-acether under the conditions where the receptor studies had been carried out (0.5nM-PAF-acether, 22°C), except for the aggregation studies, where stirring was required. PAF-acether triggered shape-change, but no optical aggregation occurred unless fibrinogen was added (Table 3). The disappearance of single platelets in stirred suspensions was already considerable in the presence of PAF-acether alone, but further extended by the addition of fibrinogen.

Table 4 illustrates that platelet stimulation with 0.5nM-PAF-acether at 22°C triggers the exposure of the fibrinogen receptor. In accordance with other reports (Niewiarowski *et al.*, 1981), a high- and low-affinity binding could be demonstrated. Receptor occupancy also induced alterations in (poly)phosphatidylinositides and triggered a fall in [32P]phosphatidylinositol 4-phosphate and [32P]-phosphatidylinositol 4,5-bisphosphate and an accumulation of [32P]phosphatidylinositol and [32P]phosphatidic acid.

Discussion

A number of observations in the present paper favour the presence of a specific receptor for PAF-acether on human platelets. First, binding of [3H]PAF-acether is saturable and can be displaced by excess of unlabelled ligand provided that the formation of an irreversible ligand–receptor complex, which develops in time, is prevented. Second, no specific binding is observed when the molecule lacks the acetyl group, indicating a high specificity of the receptor site. Third, receptor occupancy initiates several cellular responses such as alterations in the metabolism of (poly)phosphatidylinositides, exposure of the fibrinogen receptor and a slight aggregation that is further enhanced by exogenous fibrinogen. The presence of a receptor accords with observations by Wykle *et al.* (1981) and Blank *et al.* (1982), who demonstrated that only the (*R*)-form of the molecule triggered aggregation, whereas the (*S*)-form had no effect. The role of a specific inhibitor developed by Terashita *et al.* (1983) is also best explained by disturbing receptor–agonist interaction, although so far its identification has been restricted to functional studies.

The kinetics of [3H]PAF-acether binding indicate the presence of a single class of receptor sites with about 240 receptors per platelet, assuming that each receptor binds a single molecule of PAF-acether. The values for K_a as measured under equilibrium and non-equilibrium conditions are in good agreement, indicating that potentially disturbing factors, such as the presence of albumin and the irreversibility of PAF-acether binding that develops in time, are not involved. The presence of albumin, as a lipid carrier, is a prerequisite for this type of study. Furthermore, isolated platelets are best preserved in a medium containing albumin in

Table 3. *Receptor-mediated aggregation induced by PAF-acether*

Aggregation of gel-filtered human platelets (expressed as percentage disappearance of single platelets) induced by 0.5nM-PAF-acether was measured in the presence and in the absence of fibrinogen (0.3mg/ml) at 22°C (means ± S.D., $n = 4$), with and without stirring (900rev./min).

	Optical aggregation (%, 5min, stirred)	Single platelet disappearance	
		5min, stirred	90min, non-stirred
Control	—	<5	<5
PAF-acether	0	34.2±4.4	<5
PAF-acether + fibrinogen	17.6±5.0	81.7±3.3	44.7±5.5

Table 4. *Receptor-mediated platelet responses induced by PAF-acether: fibrinogen binding and stimulation of the phosphatidylinositol cycle*

Binding of fibrinogen was studied by incubating gel-filtered platelets for 1h at 22°C with ^{125}I-fibrinogen at different concentrations between 0.27nM and 2μM in the presence of 0.5nM-PAF-acether. The amount of non-specific binding of fibrinogen was measured by a parallel incubation with ^{125}I-fibrinogen in the absence of PAF-acether. Control studies between 3nM- and 20nM-^{125}I-fibrinogen had shown that those cells bound as much ^{125}I-fibrinogen as PAF-acether-treated platelets incubated with ^{125}I-fibrinogen and a 100-fold molar excess of non-radiolabelled fibrinogen. At higher concentrations, a 100-fold molar excess of non-radiolabelled fibrinogen did not easily dissolve. Scatchard analysis showed two binding sites (HA, high affinity; LA, low affinity) (means ± S.D., $n = 3$). Platelets, prelabelled with [^{32}P]orthophosphate, were exposed to PAF-acether (0.5nM) for 20min at 22°C, and the ^{32}P radioactivity in phosphatidic acid (PtdA), phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns4*P*) and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] was determined. Data are expressed as percentages of radioactivity measured at zero time (means ± S.D., $n = 11$). $^{*}P < 0.005$; $^{**}P < 0.025$ versus controls (Student's *t* test). The decrease in PtdIns4*P* was not significant ($P > 0.05$).

	Fibrinogen binding (mol/platelet)		PtdIns response (%)			
	HA	LA	PtdA	PtdIns	PtdIns4*P*	PtdIns(4,5)P_2
Control	—	—	106.2±4.5	107.7±2.8	113.3±5.3	104.7±4.0
PAF-acether	1790±370	21600±5600	178.8±13.9*	123.1±5.6**	98.4±4.2	85.1±4.0**

order to avoid cell activation and secretion (Fine *et al.*, 1976). Nevertheless, the characteristics of [^{3}H]PAF-acether binding described here only apply to our test conditions, in which albumin is a crucial factor.

Our data substantially deviate from the early findings by Valone *et al.* (1982), who described two types of binding to washed centrifuged human platelets. One type was saturable with a K_a of $0.003 \times 10^9 M^{-1}$, which is about 600 times lower than our association constant. This type of binding was saturated when about 1400 molecules bound per platelet, whereas we found six times less binding sites. The other type of binding was unsaturable and, in accordance with our data, probably reflected uptake of [^{3}H]PAF-acether. The report of Valone *et al.* (1982) described primarily the kinetics of total binding using a constant amount of PAF-acether with various specific activities and a 5min incubation period for equilibrium analysis. These and other technical differences might explain the discrepancy between the two studies. More in accordance with our data are the properties of [^{3}H]PAF-acether binding to isolated rabbit platelet membranes, showing a K_a of $0.7 \times 10^9 M^{-1}$ with 150–300 receptors per platelet (Hwang *et al.*, 1983). This binding is fully reversible after 15min, whereas intact platelets form an irreversible complex at a velocity that increases at higher doses of PAF-acether. Probably the formation of an irreversible complex requires an intact cellular apparatus, by analogy with the binding of fibrinogen to its receptor on activated platelets, which also becomes irreversible in time (Marguerie *et al.*, 1980). Partial saturation of the receptors with unlabelled PAF-acether followed by gel filtration and incubation with a second dose of PAF-acether (^{3}H-labelled) suggests a decrease in number of available receptors sites, whereas the remaining receptors have a normal affinity towards PAF-acether. Whether this implies irreversible alterations in the receptor or internaliza-

tion of the entire receptor–ligand complex as demonstrated for platelet-derived-growth-factor receptors (Heldin *et al.*, 1982) remains to be elucidated.

The affinity of PAF-acether binding is 10^5 times higher than that for ADP (Legrand *et al.*, 1980) and 4–20 times higher than for thrombin (Siegl *et al.*, 1979), which is one of the most potent agonists in platelet activation. The fact that PAF-acether binding does not require Ca^{2+} ions limits the role of this cation to the formation of fibrinogen bridges that link one platelet to another (Marguerie & Plow, 1982). Platelets pretreated with PAF-acether become desensitized for a second dose of PAF-acether, a property that is relieved by the inclusion of adrenaline (epinephrine) in the second activation step (Vargaftig *et al.*, 1982*b*). One might speculate that desensitization is the result of the formation of irreversible PAF-acether–receptor complexes. However, no effect of adrenaline (1 μM) was found on the binding kinetics (E. Kloprogge, unpublished work), suggesting that the role of adrenaline is not at the receptor level.

Although the present data link receptor occupancy to platelet responses, an additional role for PAF-acether uptake and metabolism cannot be ruled out. At 37°C, [^{3}H]PAF-acether uptake surpasses receptor binding severalfold, making receptor studies at this temperature quite difficult. Exogenous PAF-acether is converted by platelets into lyso-PAF-acether via a reaction catalysed by an acetyl hydrolase (Snyder *et al.*, 1983). The next enzyme in this sequence, acyl-CoA:1-acyl-*sn*-glycero-3-phosphocholine acyltransferase, is present in platelet microsomes (Snyder *et al.*, 1983; McKean *et al.*, 1982) and able to form alkylacylphosphocholine, an analogue with a long-chain acyl group at the *sn*-2 position. In our studies we found a ^{3}H-labelled phospholipid with the chromatographic properties of a phosphatidylcholine after prolonged incubation with high (>1 nM) amounts of [^{3}H]PAF-acether. A similar fraction has been found by Pieroni & Hanahan (1983), who concluded that its formation was too slow to be important in platelet activation. A definite conclusion regarding the relative roles of receptor occupancy and uptake and metabolism awaits the development of a specific inhibitor for the uptake site that leaves the receptor site unaltered.

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