Regulation of the receptor for platelet-activating factor on human platelets

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Human platelets possess about 300 receptors for platelet-activating factor (PAF) per cell with a K_d of about 0.2 nM. In the present study we investigated whether these receptors are subject to intracellular control mechanisms. Preincubation with the protein kinase C inhibitor staurosporine had no effect, and also agents that increase cyclic AMP failed to change the binding of [^{3}H]PAF. The Ca^{2+}–calmodulin inhibitors W-7 and sphingosine decreased PAF binding by 50–80%. Inhibition of energy metabolism induced a fall in adenylate energy charge {AEC = ([ATP] + ½[ADP])([ATP + ADP + AMP])} and an almost parallel decrease in specific [^{3}H]PAF binding without changing the K_d. Restoration of the AEC restored the [^{3}H]PAF binding. Abrupt arrest of energy metabolism during binding of [^{3}H]PAF left the binding unchanged until the metabolic ATP level had decreased by about 90%. These data indicate that PAF receptors on human platelets are under close intracellular control, possibly via a Ca^{2+}–calmodulin-dependent phosphorylation/dephosphorylation process.

INTRODUCTION

Platelet-activating factor (PAF) is a phospholipid with a wide spectrum of biological activities involving many different cell types. The biological responses to PAF are thought to be mediated via specific receptors on the plasma membrane. A PAF receptor has recently been cloned from guinea-pig lung preparations and was identified as a 39 kDa protein with seven putative transmembrane segments [1] that are characteristic of G-protein-coupled receptors [2]. Previously, the PAF receptor on rabbit platelets was characterized as a 52 kDa protein by using a photoaffinity probe [3]. Numerous other cell types, such as monocytes [4], neutrophils [5] and eosinophils [6], are thought to have PAF receptors, on the basis of binding studies with radiolabelled PAF and PAF antagonists.

The PAF receptor on human platelets is characterized by a high-affinity binding site for PAF (K_d 0.2 nM) and a maximum binding capacity of about 300 molecules per platelet [7,8]. Interaction between PAF and its receptor results in phospholipase C-mediated production of diacylglycerol and InsP_3 from phosphatidylinositol 4,5-bisphosphate [9,10]. Apart from the InsP_3-mediated mobilization of Ca^{2+} from intracellular stores, PAF also stimulates the influx of extracellular Ca^{2+} [11]. Diacylglycerol facilitates the activation of protein kinase C (PKC), which in turn phosphorylates several proteins such as the 47000-kDa protein pleckstrin [12]. In addition, activation of the phospholipase A_2 pathway induces arachidonic acid release and thromboxane production. The PAF receptor is coupled to phospholipase C via a G-protein [13,14]. Coupling between the PAF receptor and G_i, which inhibits adenylate cyclase, could only be demonstrated in platelet membrane preparations [15], but not in intact cells [13].

Several observations point to the existence of different types of PAF receptors. The rank order of potency for several PAF-receptor antagonists is different between human neutrophils and platelets [16] and between guinea-pig macrophages and platelets [17]. Evidence for the existence of two classes of PAF receptors or different conformational states of a single type of receptor comes from studies with the PAF antagonist WEB 2086, which inhibited PAF-induced degranulation of guinea-pig eosinophils far more potently than it inhibited $O_2^{-\cdot}$ generation in these cells [18]. Binding studies with [^{3}H]PAF showed two specific binding sites on human neutrophils [5], a high-affinity site (K_d 0.2 nM) that was functionally active, and a low-affinity site (K_d 500 nM) that did not affect cell functions [19].

The PAF receptor might be subject to intracellular control mechanisms. Homologous desensitization of rabbit platelets to PAF is at least in part due to uncoupling of the PAF receptor from GTP-binding proteins [20,21], a process that could be mimicked by stimulation of PKC. cAMP decreased the response of rabbit platelets to PAF by direct inhibition of the PAF binding [20]. Activation of PKC in human neutrophils down-regulated the high-affinity receptor [19,22] and suppressed PAF-induced Ca^{2+} transients. In Kupffer cells, in which the PAF receptor is subject to a continuous turnover [23], a rise in PKC activity [24] or cAMP [25] also suppressed the number of PAF receptors.

In the present study we have investigated whether intracellular mechanisms may affect the properties of the PAF receptor on human platelets.

EXPERIMENTAL

Materials

[^{3}H] PAF (1-*O*-[^{3}H]octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; sp. radioactivity 91 Ci/mmol) and [^{3}H]adenine (sp. radioactivity 22 Ci/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.). [^{32}P]P_i (sp. radioactivity 8500 Ci/mmol, carrier-free) was bought from New England Nuclear (Boston, MA, U.S.A.). Non-radiolabelled PAF was from Calbiochem–Behring Corp. (La Jolla, CA, U.S.A.). Gelatin and indomethacin were from Merck, Sharpe & Dohme (Philadelphia, PA, U.S.A.) and prostaglandin I_2 (PGI_2) was from Cayman (Boston, MA, U.S.A.). Staurosporine and antimycin A were from Boehringer (Mannheim, Germany). Sepharose 2B was bought from Pharmacia (Uppsala, Sweden), KCN from Baker Chemicals (Deventer, The Netherlands) and 2-deoxy-D-glucose from Merck (Darmstadt, Germany). D-Gluconic acid 1,5-lactone

Abbreviations used: PAF, platelet-activating factor; PKC, protein kinase C; cAMP, cyclic AMP; AEC, adenylate energy change; PGI_2, prostaglandin I_2; PMA, phorbol 12-myristate 13-acetate; diC_8, 1,2-dioctanoyl-*sn*-glycerol.

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(gluconolactone), amiloride, dibutyryl cAMP, phorbol 12-myristate 13-acetate (PMA) and 1,2-dioctanoyl-*sn*-glycerol (diC_8) were purchased from Sigma (St. Louis, MO, U.S.A.). Sphingosine was obtained from Serva (Heidelberg, Germany).

Platelet isolation

Freshly drawn venous blood was collected from healthy volunteers (with informed consent) into 0.1 vol. of trisodium citrate (130 mM). The donors claimed not to have taken any medicine during the 10 days before blood collection. The blood was centrifuged (200 *g*, 10 min, 22 °C) and the platelet-rich plasma was collected. Platelet-rich plasma was layered on a Sepharose 2B column and eluted with Ca^{2+}-free Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.7 mM $MgCl_2$, 11.9 mM $NaHCO_3$ and 0.42 mM NaH_2PO_4) containing 0.2 % BSA and 5 mM D-glucose (pH 7.25), unless stated otherwise. The final platelet concentration was 2×10^{11} cells/l.

Binding of [^{3}H]PAF

Samples of gel-filtered platelets were incubated in duplicate with different concentrations of [^{3}H]PAF (22 °C) without stirring [7]. After incubation, the samples were layered on 20 % (w/v) sucrose in Tyrode's solution and centrifuged (12000 *g*, 2 min, 22 °C). Platelet pellets were lysed in 1 % Triton X-100, and the radioactivity was measured by standard methods. The intracellular mechanisms that may control the expression of the PAF receptor were investigated by incubating gel-filtered platelets with different agents known to interfere with signal generation or energy metabolism as indicated in the Results section. The subsequent binding study was performed at 0.5 nM [^{3}H]PAF, which is close to the K_d and is sensitive to both changes in receptor number and affinity. Incubation with 0.5 nM [^{3}H]PAF (30 min, 22 °C) resulted in a receptor occupancy of 63 ± 11 % of the total number of specific binding sites (254 ± 89 sites/platelet, $n = 15$), with a non-specific binding of 32 ± 15 % of the total binding. The non-specific binding was determined in the presence of a 200-fold excess of non-radiolabelled PAF and did not change under the different conditions tested.

The total number of specific binding sites and dissociation constants were calculated from binding experiments using different concentrations of [^{3}H]PAF (0.05–2.5 nM, incubation time 75 min, 22 °C). The data were analysed with a computer-assisted iterative curve-fit program (LIGAND; coefficient of variation for curve fitting in a single assay 9 ± 3 %). In all cases the one-ligand/one-binding-site model fitted statistically better to the binding data than the two-binding-sites model. The intra-assay variance was 7 ± 4 %, as assessed by three quadruplicate binding assays.

Protein phosphorylation

Platelet-rich plasma was acidified with acid–citrate–dextrose to a final pH of 6.5 and incubated with 0.1 mCi/ml [^{32}P]P_i (60 min, 37 °C). ^{32}P-labelled platelets were obtained by centrifugation (700 *g*, 20 min, 22 °C) and resuspended in Tyrode's solution. After stimulation, samples were collected into 0.5 vol. of 3 × concentrated Laemmli sample buffer and incubated at 100 °C for 5 min. Samples were then separated by SDS/PAGE in 11 % gels [26]. The protein fractions were stained with Coomassie Brilliant Blue; the distribution of radioactivity was determined by autoradiography of the dried gels. The areas containing the 20- and 47-kDa proteins were collected and heated in H_2O_2 (30 %; 2 h, 80 °C), and the radioactivity was determined by liquid-scintillation counting. The ^{32}P content was 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).

Measurement of metabolic ATP and ADP

Platelet-rich plasma was incubated with 20 μCi/ml [^{3}H]adenine (45 min, 37 °C). The radiolabelled platelets were isolated by gel-filtration on a Sepharose 2B column in Tyrode's solution with 0.2 % gelatin instead of BSA. Samples of [^{3}H]adenine-labelled platelets were collected in 2 vol. of freshly prepared EDTA/ethanol (10 mM EDTA in 86 % ethanol, pH 7.4, 0 °C). After centrifugation (12000 *g*, 2 min), the supernatants were analysed for ^{3}H-labelled ATP, ADP, AMP, IMP and hypoxanthine/inosine by high-voltage paper electrophoresis, and the radioactivity was determined by liquid-scintillation counting. The adenylate energy charge (AEC), $([ATP] + \frac{1}{2}[ADP])/([ATP] + [ADP] + [AMP])$, was calculated from the distribution of the ^{3}H radioactivity in the corresponding fractions [27].

Analysis of functional responses

During the preincubation with different effectors and the [^{3}H]PAF-binding assay no aggregation occurred, as measured by counting the number of single platelets after fixation in 9 vol. of 0.5 % glutaraldehyde (0 °C). The platelets were counted electronically in a Platelet Analyzer 810 (Baker Instruments, Allentown, PA, U.S.A.) with the apertures set at 3.2 and 16 μm^3. The number of single platelets was never below 94 % of the total platelet count. Secretion of α-granule contents was less than 5 % of total, based on the liberation of β-thromboglobulin (radioimmunoassay kit from Amersham International). The thromboxane B_2 concentration of the gelfiltered platelets was 0.6 ± 0.2 nmol/10^{11} platelets, as measured by radioimmunoassay (New England Nuclear).

Statistics

The results are expressed as means ± S.D. The numbers of binding sites per platelet were compared by using Student's *t* test for paired values. A difference was considered significant at $P < 0.05$.

RESULTS

Modulation of the specific [^{3}H]PAF binding

In order to investigate whether the PAF receptor on platelets was regulated by intracellular mechanisms, platelets were incubated with a number of effectors known to interfere with various steps in signal-generating sequences, as well as with inhibitors of energy metabolism (Table 1). Subsequently, the binding of PAF was analysed by incubating the cells with 0.5 nM [^{3}H]PAF for 30 min in the absence or presence of an excess non-radiolabelled PAF. Inhibition of the PKC activity with staurosporine did not change PAF binding, and also an increase in cAMP content, either directly with dibutyryl cAMP or indirectly with PGI_2, failed to change the binding characteristics of [^{3}H]PAF. Also, inhibition of formation of prostaglandin endoperoxides/thromboxane A_2 with indomethacin (30 μM, 45 min; results not shown), or blockade of the Na^+/H^+ exchanger, which controls cytosolic pH, with amiloride, left the specific [^{3}H]PAF binding

Table 1 Modulation of specific [^{3}H]PAF binding

Gel-filtered platelets were incubated with the indicated effectors at 22 °C before addition of 0.5 nM [^{3}H]PAF. The specific PAF binding was determined and expressed as a percentage of the control binding (160±18 occupied sites/platelet). The concentrations and preincubation times of the effectors were: staurosporine 1 μM, 10 min; dibutyryl cAMP 250 μM, 1 min; PGI_2 30 nM, 5 min; amiloride 0.2 mM, 30 min; W-7 25 μM, 30 min; 2-deoxy-D-glucose 30 mM, 30 min; antimycin A 15 μM, 30 min. Data are means±S.D. of 5–10 duplicate experiments; asterisks denote a significant difference from the control.

Effector	[^{3}H]PAF binding (%)
Control	(100)
Staurosporine	97±13
Dibutyryl cAMP	102±9
PGI_2	107±5
Amiloride	94±5
W-7	36±11*
2-Deoxy-D-glucose	98±21
Antimycin A	102±15
Deoxyglucose + antimycin A	57±10*

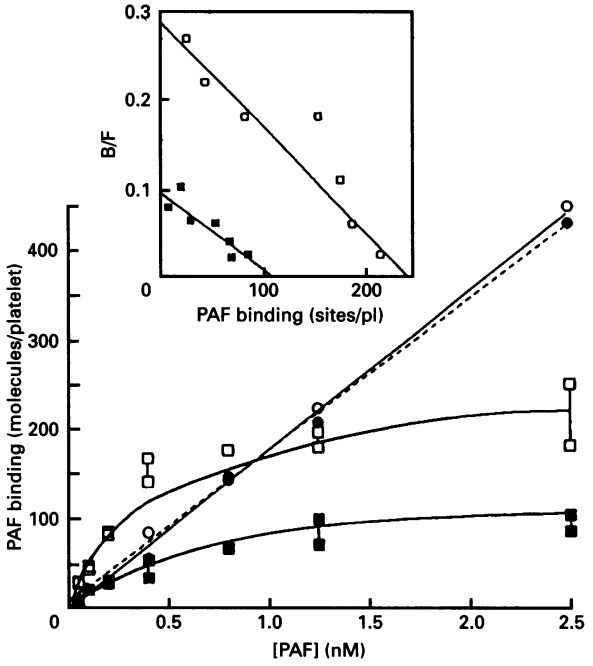

Figure 1 Inhibition of energy metabolism and [^{3}H]PAF binding

The Figure shows the specific binding of [^{3}H]PAF (□, ■) after a 30 min preincubation with a combination of 30 mM 2-deoxy-D-glucose and 15 μM antimycin A (■) and with buffer (□). The Scatchard plots are given in the insert. For this particular experiment LIGAND analysis revealed 95±9 sites/platelet (K_d 0.32±0.07 nM) after arrest of ATP resynthesis and 220±47 sites/platelet (K_d 0.53±0.20 nM) for the control platelets. The non-specific binding (○, control; ●, + inhibitors) remained constant. The Figure is representative of four similar experiments.

undisturbed. In contrast, preincubation with the calmodulin inhibitor W-7 at a concentration at which non-specific effects are kept to a minimum [28] induced a significant fall in specific [^{3}H]PAF binding of about 60%. The effect of metabolic inhibitors was investigated in platelets gel-filtered in Tyrode's solution containing 0.2% gelatin instead of BSA and 1 mM glucose. The

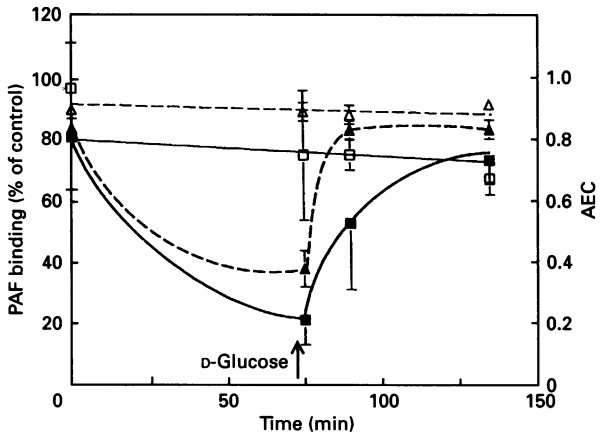

Figure 2 Correlation between AEC and [^{3}H]PAF binding

[^{3}H]Adenine-labelled platelets were incubated in the presence of 1 mM KCN and without D-glucose at 37 °C. After 75 min (arrow), energy generation was restored by addition of 1 mM D-glucose (final concn.). At the indicated times samples were withdrawn to measure the AEC (▲). Concurrently, unlabelled platelets from the same donor were incubated under the same conditions. Platelet samples were allowed to reach room temperature for 5 min, [^{3}H]PAF was added and the specific binding was determined after 30 min incubation (■). Controls were the same suspensions to which 1 mM D-glucose was added at the beginning of the incubation period (AEC, △; [^{3}H]PAF binding, □). The data are means±S.D. of four experiments.

pretreatment with 2-deoxy-D-glucose, an inhibitor of glycolytic energy generation, and antimycin A, an inhibitor of mitochondrial energy generation, did not change the specific binding of [^{3}H]PAF, but a combination of these inhibitors decreased the specific binding to about 50%. Under all conditions tested, the non-specific binding of [^{3}H]PAF was not affected by pretreatment with the various effectors. Taken together, these data indicate that the PAF receptor on platelets is sensitive to modulation of calmodulin and the availability of metabolic energy.

Role of metabolic energy in [^{3}H]PAF binding

This was investigated in platelets suspended in Tyrode's solution in the presence of 1 mM glucose. In experiments with antimycin A, BSA was replaced with gelatin.

As shown in Figure 1, pretreatment with a combination of 30 mM 2-deoxy-D-glucose and 15 μM antimycin A decreased the specific binding considerably without changing the non-specific binding. Scatchard analysis (Figure 1, insert) revealed an average decrease in binding sites from 268±101 to 112±32 sites/platelet ($n = 4$; $P = 0.03$), whereas no significant changes in the binding affinity were seen (K_d 0.45±0.13 and 0.48±0.10 nM with and without inhibitors respectively; $P = 0.75$). Concurrent measurement of the AEC showed a decrease from 0.94±0.04 to 0.72±0.02 upon treatment with the deoxyglucose/antimycin A mixture, supporting the concept that the decrease in binding sites was due to a decrease in energy availability.

In order to investigate whether the decrease in specific binding was reversible, the experiments were repeated under conditions that permitted reversible changes in energy availability. Platelets were incubated at 37 °C in Tyrode's solution containing 1 mM KCN to inhibit mitochondrial ATP resynthesis and in the absence of glucose, which abolishes glycolytic ATP resynthesis, since the cells contain little glucose. Under these conditions the platelets consume glycogen, but are unable to meet the demands of energy-consuming processes [29]. As a result, the AEC gradually decreases. Figure 2 illustrates that the decrease in AEC was accompanied by a sharp fall in the specific binding measured

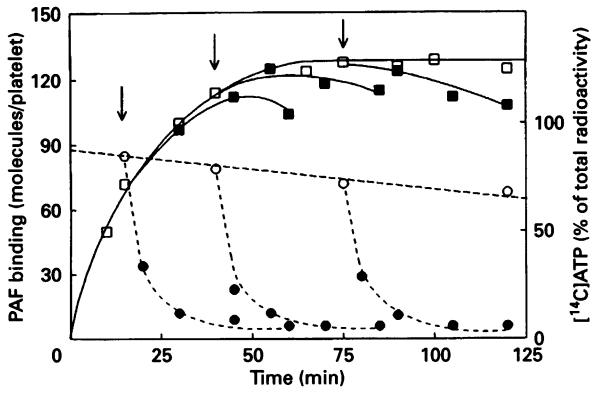

Figure 3 Binding of PAF during metabolic blockade

[^{3}H]Adenine-labelled platelets were incubated in the presence of 1 mM glucose and 1 mM KCN at 22 °C. The specific binding of [^{3}H]PAF (□) and the level of [^{14}C]ATP (○) were measured. At the times indicated by the arrows, energy generation was abruptly blocked by a combination of 2-deoxy-D-glucose/gluconolactone. The effect of this treatment on the specific binding of [^{3}H]PAF (■) and the level of [^{14}C]ATP (●) was evaluated.

Table 2 Comparison between [^{3}H]PAF binding and protein phosphorylation

Gel-filtered platelets were incubated with the indicated effectors at 22 °C before addition of 0.5 nM [^{3}H]PAF. Phosphorylation of the 47 and 20 kDa proteins was measured at the beginning of the 30 min incubation with [^{3}H]PAF in separate but identically treated suspensions. The concentrations and incubation times of the effectors were: PMA 50 nM, 5 min; diC_8 5 μM, 5 min; staurosporine 1 μM, 5 min; sphingosine 25 μM, 5 and 30 min. Binding and phosphorylation data (percentages of controls) are means ± S.D. of four experiments; asterisks indicate a significant difference compared with controls ($P < 0.05$).

Effector	[^{3}H]PAF binding (%)	Protein phosphorylation (%)	
		47 kDa	20 kDa
Control	(100)	(100)	(100)
PMA	109 ± 23	425 ± 13*	220 ± 15*
diC_8	104 ± 31	375 ± 20*	99 ± 8
Staurosporine	97 ± 13	92 ± 8	25 ± 13*
Sphingosine, 5 min	82 ± 5*	99 ± 10	130 ± 12
Sphingosine, 30 min	24 ± 15*	73 ± 6*	50 ± 16*

during a subsequent 30 min incubation with [^{3}H]PAF. When glycolytic ATP resynthesis was restored by addition of 1 mM D-glucose (final concn.), the AEC recovered and reached normal values 15 min later. The recovery of the AEC was accompanied by complete restoration of the PAF binding, although it took about 75 min before the range of control suspensions was reached. Thus the number of accessible PAF receptors depends on the availability of metabolic energy.

In order to clarify how much energy was required for the optimal binding of [^{3}H]PAF, platelets were deprived of metabolic energy during the interaction with PAF. Figure 3 illustrates the content of metabolic ATP, measured as ^{14}C radioactivity in platelets incubated in Ca^{2+}-free Tyrode's solution containing 1 mM glucose and 1 mM KCN. Under these conditions energy production and consumption are almost in equilibrium and [^{14}C]ATP shows only a slight decline. The sudden blockade of energy generation by a mixture of 30 mM 2-deoxy-D-glucose and 10 mM gluconolactone (final concns.) led to a rapid fall in [^{14}C]ATP to below 10% of total ^{14}C radioactivity within 20 min. Concurrent measurement of the specific PAF binding showed that the binding proceeded undisturbed during the first 25–30 min and thereafter decreased compared with the binding to platelets with undisturbed energy metabolism. Thus the specific binding of PAF requires little energy and is disturbed when the level of [^{14}C]ATP decreases below 10% of total ^{14}C radioactivity. In this range, the level of [^{14}C]ADP was between 12 and 7% of total ^{14}C radioactivity, indicating almost complete exhaustion of metabolic energy.

Comparison between [^{3}H]PAF binding and protein phosphorylation

Since the energy requirement of the binding of PAF suggested a role for phosphorylation processes, [^{3}H]PAF binding was compared with the phosphorylation of the 47 kDa protein pleckstrin, a major substrate for PKC, and the 20 kDa protein myosin light chain, known to be phosphorylated by a Ca^{2+}–calmodulin-dependent kinase [30]. Treatment with PMA raised the phosphorylation patterns well above control values without affecting the binding of PAF (Table 2). Also, addition of diC_8 increased the phosphorylation of the 47 kDa protein, but left the 20 kDa protein unchanged, again without changing the binding of [^{3}H]PAF. As noted above, pretreatment with 1 μM staurosporine left the binding undisturbed, but induced a 4-fold decrease in 20 kDa-protein phosphorylation. A 5 min treatment with sphingosine slightly decreased the PAF binding without changing protein phosphorylation. A prolonged preincubation of 30 min, however, greatly decreased the specific binding of [^{3}H]PAF, to about 20%. This treatment also decreased the phosphorylation of the 47 kDa and 20 kDa proteins. Thus, modulation of PKC activity failed to affect the specific binding of [^{3}H]PAF. Also Ca^{2+}–calmodulin-dependent myosin light-chain kinase activity correlated poorly with PAF binding.

DISCUSSION

In this study we demonstrate that PAF receptors on human platelets are under control of intracellular mechanisms. The calmodulin inhibitor W-7 inhibited the binding, and it is therefore reasonable to assume that Ca^{2+}–calmodulin-dependent processes are involved. The specific binding of PAF was equally susceptible to modulation of energy metabolism. When a period of decreased energy availability was followed by complete restoration of metabolic ATP supply, the recovery of the PAF binding was complete. Metabolic blockade during [^{3}H]PAF–platelet interaction had no effect until [^{14}C]ATP had decreased below 10% of total ^{14}C radioactivity. This means that specific PAF binding is disturbed when the energy stored in metabolic ATP plus ADP had decreased with more than 80%. Thus the PAF receptor requires little energy, and only after severe depletion of energy supply is a decrease in PAF binding observed.

The changes in detectable receptors seen during modulation of energy supply are best explained by assuming that PAF receptors on platelets can change from a high-affinity conformation to a low-affinity conformation and back. The low-affinity state with K_d values possibly in the micromolar range is beyond the range of [^{3}H]PAF concentrations tested in the present study. Unfortunately, at higher concentrations PAF is rapidly taken up and metabolized, making binding studies virtually impossible [7].

Ca^{2+}-channel blockers such as verapamil inhibit the binding of PAF to PAF receptors on platelets [31,32] and neutrophils [33], suggesting that these receptors are closely linked to Ca^{2+} channels in the plasma membrane. The inhibition is complex, showing competitive as well as non-competitive kinetics. The non-competitive effect of verapamil has been explained by inhibition of calmodulin [28], which might be in concert with our present

findings. The cloned PAF receptor from guinea-pig lung does not contain Ca^{2+}-binding sites [1]. Therefore, if the receptor on platelets has a similar structure, a role for Ca^{2+}–calmodulin in the regulation of PAF binding must be sought elsewhere.

The cytoplasmic tail of the cloned PAF receptor contains four serine and five threonine residues as possible phosphate acceptors [1], and direct regulation of the receptor by phosphorylation is therefore feasible. A similar mechanism has been demonstrated for other receptors involved in signal transduction, e.g. the epidermal-growth-factor receptor and the β-adrenergic receptor [34,35]. Also, the glucocorticoid receptor on mouse pituitary cells requires a normal ATP level for optimal binding [36].

A central role in phosphorylation processes in platelets is played by PKC, which upon activation enhances the phosphorylation of several proteins such as pleckstrin and myosin light chain. In unstimulated platelets the activity of PKC is low and ^{32}P incorporation into the 47 and 20 kDa proteins is minor. Not surprisingly, inhibition of PKC activity by staurosporine had no effect on the binding of [^{3}H]PAF, although in pituitary cells the PKC inhibitor sphingosine inhibited the binding of thyrotropin-releasing hormone [37]. The binding of PAF to platelets was only inhibited by sphingosine after prolonged incubation, possibly because sphingosine is also inhibitor of Ca^{2+}–calmodulin-dependent processes [38].

Direct activation of PKC with phorbol ester or diC_8 failed to affect the binding of PAF. In contrast, PKC-dependent phosphorylation is an important regulatory mechanism for the β-adrenergic receptor [34], the epidermal-growth-factor receptor [39] and the leukotriene-B4 receptor [22], where activation of PKC leads to desensitization of the receptor. PAF receptors on human neutrophils [19,22] and rat Kupffer cells [24] are also under control of PKC. The mechanisms by which PKC activators down-regulate these receptors is not fully understood, and may involve receptor internalization, degradation and inactivation. Thus human platelets differ in this respect from neutrophils and Kupffer cells, but not from rabbit platelets, where PMA treatment left the binding of PAF intact [20].

Feedback regulation of receptors via phosphorylation by cAMP-dependent protein kinase A is a major mechanism for desensitization of the β-adrenergic and other receptors with an hepathelical structure [2]. In platelets, high concentrations of cAMP decrease the binding of thrombin by 60%, mainly by decreasing the number of receptors [40]. Also, the PAF receptors on rabbit platelets [20] and rat Kupffer cells [25] are down-regulated by a high cAMP concentration. Whether this is mediated via protein kinase A or via other mechanisms is not yet clear. The sequence of the receptor for PAF shows two basic residues N-terminal to Thr-401, thereby forming a possible protein kinase A-phosphorylation site [35]. Surprisingly, neither a direct increase in cAMP by dibutyryl cAMP nor an indirect increase by PGI_2 had any effect on the PAF binding to human platelets. Nevertheless, the need for optimal energy availability suggests that PAF receptors on human platelets are regulated by phosphorylation processes, although PKC and PKA are probably not involved.

Our present data provide new insights into the way human platelets regulate their PAF receptors. Obviously, the availability of metabolic energy does not vary *in vivo* as extremely as under our experimental conditions. Most platelet functions are accompanied by a fall in metabolic ATP, but only the rapid and complete secretion induced by high agonist concentrations brings the energy availability into the range of the present studies [27]. On the other hand, the loss of PAF-responsiveness seen in platelets that are stored for transfusion purposes [41] may well result from a shortage of metabolic ATP, since those conditions are known to impair the cell's energy metabolism [42].

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