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Regulation of platelet-activating-factor receptors and the desensitization response in polymorphonuclear neutrophils

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Platelet-activating factor (PAF) desensitizes as well as stimulates its various target cells, We find that human polymorphonuclear neutrophils (PMN) exposed to PAF became maximally unresponsive to a second PAF challenge within 15–90 s in assays of Ca^{2+} mobilization and degranulation. The cells regained full PAF-sensitivity over the ensuing 20–40 min. These effects correlated with changes in PAF receptor availability. PMN treated with PAF, washed in regular buffer and assayed for PAF binding exhibited falls (maximal in 15 s), followed by rises (reaching control levels by 60 min), in the number of high-affinity PAF receptors. However, tracking studies showed that [^{3}H]PAF accumulated on the cell surface for ~ 2 min before being internalized. Regular-buffer washes did not remove this superficial PAF, whereas a washing regimen using excess albumin to adsorb PAF removed 99 % of the surface compound. PMN washed by the latter regimen after PAF exposure lost PAF receptors relatively slowly (maximal at ~ 5 min), but the ultimate extent of this loss and the rate at which receptor expression normalized were similar to those of cells washed in regular buffer. Neither cycloheximide nor actinomycin D influenced the course of the receptor changes, but two protein kinase C (PKC) blockers, staurosporine and 1-(5-isoquinolinesulphonyl)piperazine, inhibited the receptor-receptor-depleting actions of PAF. Indeed, a phorbol diester activator of PKC also caused PMN to decrease high-affinity PAF receptor numbers, and the two PKC blockers antagonized this action at concentrations that inhibited PAF-induced PAF receptor losses. We conclude that: (*a*) PAF induces PMN to down-regulate and then to re-express PAF receptors independently of protein synthesis; (*b*) these changes are likely to underlie the later stages and reversal of desensitization; (*c*) the onset ($t \leqslant 2$ min) of desensitization, however, precedes receptor down-regulation and must be due to receptor uncoupling from transductional elements; and (*d*) down-regulation of receptors for PAF appears to be mediated by PKC and/or elements inhibited by PKC blockers.

INTRODUCTION

The pluripotent autocoid platelet-activating factor (PAF) operates through plasma-membrane receptors to initiate a standard transductional mechanism. It stimulates cells to activate G-proteins, hydrolyse endogenous lipids, raise $[Ca^{2+}]_i$ and mobilize key effector enzymes such as PKC. As consequences of these events, the cells respond functionally and then pass through a temporary state of PAF insensitivity [1–3]. The same cells also internalize PAF (1-*O*-alkyl-2-acetyl-GPC), serially convert it into *sn*-2-lyso and acylated metabolites, and store the final 1-*O*-alkyl-2-acyl-GPC product in Golgi and/or granules [4–6]. Like other similarly acting agonists, then, PAF may bind its plasmalemmal receptors to become endocytosed in vesicles that uncouple ligands from receptors, transport ligand to digestive organelles, and cycle receptors back to the cell surface [7]. In effect, this pathway shuttles receptors off and on the plasmalemma to depress and restore ligand sensitivity while clearing the microenvironment of stimulus. As a hydrophobic phospholipid, however, PAF preferentially solvates in membranes [8,9]. It will move spontaneously from carrier albumin to, e.g., red blood cells. At first, such movements are fully reversible. For example, addition of excess of albumin removes this PAF from the erythrocytes. Over time, however, red-cell PAF internalizes, thereby becoming resistant to albumin extraction [10,11]. PAF likewise enters rabbit platelets, guinea-pig PMN and rat Kupffer cells by BSA-extractable and -unextractable steps. The latter cells, unlike erythrocytes, have PAF receptors as well as PAF metabolizing activity. Nevertheless, their ability to process PAF is only moderately and indirectly blocked by PAF-receptor antagonists [12–14]. Moreover, HL-60 promyelocytes lack PAF receptors, yet metabolize the ligand even more rapidly than do their mature PAF-receptor-bearing counterparts, human PMN [15,16]. Human PMN, indeed, can be clipped of PAF receptors by Pronase without altering their ability to metabolize PAF [15]. PAF processing therefore more closely resembles the receptor-independent processing of exogenous lysophospholipids [17] than the receptor-mediated endocytotic processing of hydrophilic peptides [18]. The following model has evolved [6,10–15]. Thermodynamic forces drive PAF from BSA to the outer leaf of the plasma membrane. At this site, PAF can return to BSA, bind with its plasma-membrane receptors, or flip internally to sequester from extracellular BSA and, depending on the availability of degradative enzymes, become metabolized.

The peculiarities of PAF processing complicate receptor analysis during desensitization. Rat Kupffer cells, when incubated with PAF and then buffer-washed, show decreased ability to bind PAF. The effect develops within 4 min, reverses only when PAF is removed from the culture media and new protein synthesis is allowed to occur (i.e. cycloheximide inhibits it), and generally persists through serial BSA extractions. Cells exposed to PAF for short periods (e.g. < 4 min), however, bind PAF normally after

Abbreviations used: PMN, polymorphonuclear neutrophils; GPC, *sn*-glycero-3-phosphocholine; PAF, platelet-activating factor (1-*O*-hexadecyl-2-acetyl-GPC); [^{3}H]PAF, 1-*O*-[9,10-$^3H_2$]hexadecyl-2-acetyl-GPC; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; O_2^-, superoxide anion; CI, 1-(5-isoquinolinesulphonyl)piperazine; Me_2SO, dimethyl sulphoxide; fMLP, *N*-formylmethionyl-leucyl-phenylalanine.

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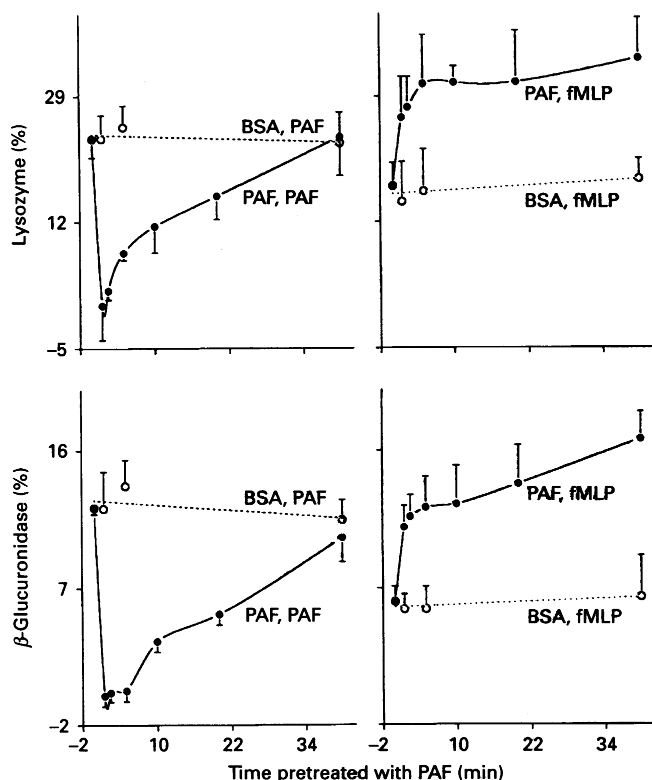

Fig. 1. Degranulation responses by PAF-pretreated PMN

PMN were incubated with BSA (broken lines) or 10 nM-PAF (continuous lines) for 0.5–39 min, exposed to cytochalasin B for 1 min, and challenged with 10 nM-PAF (left panels) or 3.2 nM-fMLP (right panels). Release of lysozyme (upper panels) and β-glucuronidase (lower panels) was corrected for the amount of enzyme released by BSA-challenged but otherwise identically handled (including BSA or PAF preincubation) cells. Data are given as the mean net percentage of total cellular enzyme released ± S.E.M. for five experiments. PAF-pretreated BSA-challenged PMN released ~ 11% of lysozyme and ~ 6% of β-glucuronidase. For BSA-pretreated BSA-challenged PMN these values were ~ 9% and 4% respectively.

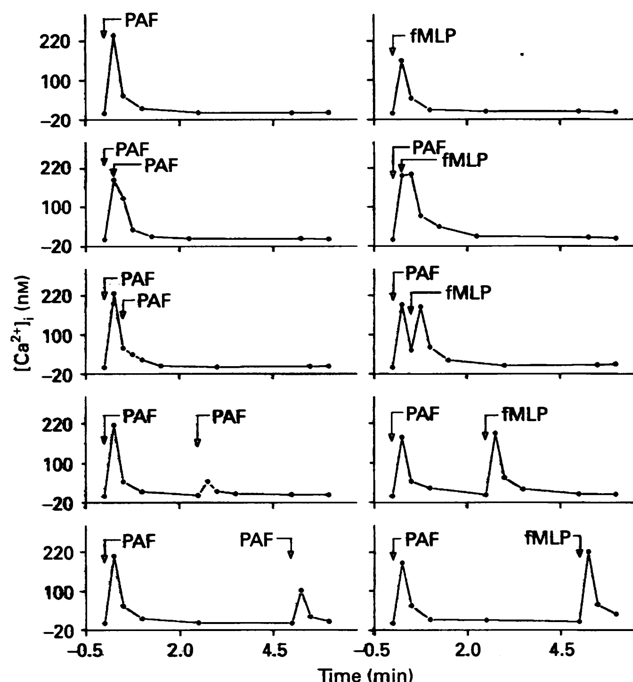

Fig. 2. $[Ca^{2+}]_i$ in serially stimulated PMN

Fura-2-loaded PMN were challenged with 1 nM-PAF (the upper right panel gives results for cells challenged only with 1 nM-fMLP). At 0.25, 0.5, 2.5 and 5 min thereafter, PMN were re-challenged with 1 nM-PAF (left panels) or 1 nM-fMLP (right panels). Results are representative of 4–8 experiments.

BSA extraction. Evidently, PAF-treated buffer-washed Kupffer cells have surface PAF that interferes with binding assays. BSA extraction removes this contaminant to reveal that PAF-treated cells continue to express a full complement of PAF receptors for at least 4 min. The slower, non-reversing loss of PAF receptors appears responsible for Kupffer-cell desensitization [14]. On the other hand, PAF-desensitized rabbit platelets do not decrease their PAF receptors [12]. Available studies thus indicate that PAF can slowly and irreversibly down-regulate its receptors or rapidly uncouple its receptors from effector elements. Neither action, however, has any obvious relation to the rapid, fully reversible, desensitization effects which PAF exhibits *in vivo* (e.g. anaphylaxis) or on many cells *in vitro*. We show here that PAF briefly desensitized and down-regulated its receptors in human PMN. Down-regulation resisted BSA extraction, reversed rapidly even in PMN treated with protein-synthesis inhibitors, and was sharply decreased by PKC blockers. The data support a three-stage model of desensitization that may be applicable to many of the rapidly reversing bioactions of PAF *in vivo* as well as *in vitro*.

MATERIALS AND METHODS

Reagents and buffers

We prepared [^{3}H]PAF (56 Ci/mmol), PAF and CI [6,19] and purchased staurosporine (from Fluka Chemical Corp., Ronkonkoma, NY, U.S.A.), cytochalasin B, cytochrome *c*, cycloheximide, PMA and delipidated BSA (from Sigma Chemical Corp., St. Louis, MO, U.S.A.), fMLP, actinomycin D and superoxide dismutase (from Calbiochem, La Jolla, CA, U.S.A.), Fura-2 AM (from Molecular Probes, Mountain View, CA, U.S.A.), and dipalmitoyl-GPC (from Serdary Research, London, Ont., Canada). PMN were suspended in Hanks' buffer, pH 7.4, containing 1.4 mM-Ca^{2+} [20] or BSA extraction buffer (Ca^{2+}-free Hanks', 50 g of BSA/100 ml, 10 mM-EDTA, pH 9.3). PAF analogues and fMLP were taken up in Hanks' buffer containing 250 mg of BSA/100 ml and added to PMN so that the final BSA concentration was 6.25 mg/100 ml; PMA and staurosporine were dissolved in Me_2SO and diluted 1:100 with PMN; CI, cycloheximide and actinomycin D were dissolved in water.

Bioassays

Leucocyte preparations (> 95% PMN, < 5 platelets/100 PMN, no red cells) were isolated from human blood [6]. For assay of O_2^-, 10^7 PMN were incubated in 1 ml of Hanks' buffer containing 50 nmol of cytochrome *c*, with or without 50 μg of superoxide dismutase, for 20 min at 37 °C and challenged while being monitored at 550 nm. Results are given as maximal rates (nmol/min) of superoxide dismutase-inhibitable O_2^- formation [20]. For degranulation, 1.3×10^6 PMN in 0.5 ml of Hanks' buffer were incubated at 37 °C for 20 min, treated with 2.5 μg of cytochalasin B for 1 min, challenged for 5 min, placed on ice, and centrifuged (200 *g*, 4 min, 4 °C) to obtain supernatants which were assayed for lysozyme, β-glucuronidase and lactate dehydrogenase. Results are given as net enzyme release, i.e. the percentage of total cell enzyme released by challenged PMN minus that released by unchallenged but otherwise identically handled (including, where indicated, PAF-desensitization) PMN

[20]. None of the stimuli, drugs, buffers, or reaction conditions used here caused net release of lactate dehydrogenase. For $[Ca^{2+}]_i$ assays, 10^7 PMN were loaded with 1 μM Fura-2 AM, incubated in 1 ml of Hanks' buffer for 20 min at 37 °C, and challenged while being excited alternately at 340 and 380 nm and monitored at 510 nm. Results are given as the nM rise in $[Ca^{2+}]_i$ above the levels of resting PMN [20].

[³H]PAF extraction and metabolism

For this, 10^7 PMN were incubated in 1 ml of Hanks' buffer for 20 min at 4 or 37 °C, exposed to [³H]PAF with or without unlabelled PAF for 0.25–90 min, and centrifuged (12000 *g*, 5 s, 4 °C). To extract radiolabel, 10^7 PMN were incubated in 1.5 ml of BSA extraction buffer for 10 min, centrifuged (12000 *g*, 60 s, 4 °C), washed and incubated in extraction buffer twice again, and washed in Ca^{2+}-free Hanks' buffer three more times. PMN pellets and the seven supernatants were counted for radioactivity [20]. For metabolic studies, [³H]PAF-pretreated PMN were washed twice in Hanks' buffer or extracted with BSA. The final PMN were mixed with 1 ml of buffer and 1.6 ml of methanol/chloroform (1:1, v/v). After isolation of chloroform layers, methanol/water layers were mixed with 2 × 0.8 ml of chloroform. Pooled chloroform layers were dried under a stream of N_2, applied to pre-activated (180 °C, 3 h) silica-gel G t.l.c. plates, and developed to 15 cm with chloroform/methanol/acetic acid/water (50:25:8:4, by vol.). Sections (5 mm) of these plates were assayed for radioactivity and identified as co-migrating with PAF, lyso-PAF and diacyl-GPC standards [6].

PAF binding

For this, 10^7 PMN were incubated in 1 ml of Hanks' buffer at 37 °C for 20 min, treated with PAF for 0.25–90 min (37 °C), and centrifuged (12000 *g*, 5 s, 4 °C). Pellets were suspended and washed in 2 × 1.5 vol. of Ca^{2+}-free Hanks' buffer (4 °C) or processed in the BSA extraction regimen, resuspended at 5×10^6 PMN/ml in Hanks' buffer (4 °C), incubated for 60 min on ice with [³H]PAF with or without unlabelled PAF, and centrifuged (12000 *g*, 1 min, 4 °C) through 400 μl of silicone oil. Supernatants and pellets were counted for radiolabel. Results are given as specific binding, i.e. the fraction of label bound by PMN exposed to 10 pM-[³H]PAF minus that of PMN exposed to 10 pM-[³H]PAF × 100 nM-PAF. Receptor quantification used the LIGAND SCAPRE program on data from PMN incubated with 10 pM-[³H]PAF plus 0, 0.03, 0.09, 0.31, 0.99, 3.15, 10, 31.6, 100 or 150 nM-PAF.

RESULTS

Desensitization

To define the kinetics and possible reversibility of desensitization, PMN were treated with 10 nM-PAF for 0.5–40 min, incubated for 1 min with cytochalasin B (which is required for PMN degranulation [6]), stimulated, and assayed for lysozyme and β-glucuronidase release. The PMN released little of the granule enzymes in response to the first PAF challenge and, moreover, degranulated weakly when re-stimulated with 10 nM-PAF. PAF sensitivity was minimal at the first tested time (1.5 min), but recovered by 40 min (Fig. 1, left panels). Note that the same PMN had enhanced responses to fMLP (Fig. 1, right panels). We next turned to a different assay, Ca^{2+} transients, which, unlike exocytosis, rapidly reverse, do not require cytochalasin B, and therefore are capable of resolving PMN sensitivity at early times after PAF stimulation. PMN challenged with 1 nM-PAF raised $[Ca^{2+}]_i$ to peak levels by 15 s. $[Ca^{2+}]_i$ quickly normalized even in PMN re-stimulated with PAF within 15 or 30 s (Fig. 2, left panels). fMLP challenge of these PMN, in contrast, markedly broadened the PAF-initiated $[Ca^{2+}]_i$ peak or evoked a separate Ca^{2+} transient (Fig. 2, right panels). In fact, fMLP induced 20–40% greater $[Ca^{2+}]_i$ rises in PMN incubated with PAF for ⩾ 2.5 min, relative to BSA-pretreated cells. Fig. 3 (upper panel) shows that PMN treated with 0.1–10 nM-PAF had concentration-dependent, time-dependent, and fully reversible PAF-desensitization responses. The same cells, however, always responded to 1–32 nM-fMLP with normal or enhanced $[Ca^{2+}]_i$ rises (results not shown). Thus PAF desensitizes PMN to itself, but not to an unrelated agonist. Its desensitizing effects develop in 15 s, but reverse thereafter.

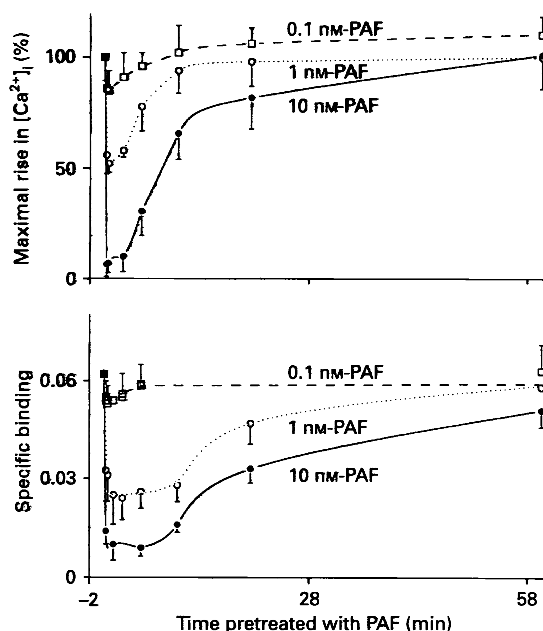

Fig. 3. Ca^{2+} transients and [³H]PAF binding by PAF-pretreated PMN

For the upper panel, Fura-2-loaded PMN were incubated with 0.1–10 nM-PAF for 0.25–60 min and then challenged with 10 nM-PAF. Data report the rise in $[Ca^{2+}]_i$ 15 s after PAF challenge as percentages of the values in PMN pretreated with BSA for 0.25–60 min and then challenged with PAF. For the lower panel, PMN were incubated with 0.1–10 nM-PAF (37 °C) for 0.25–60 min, quickly buffer-washed (4 °C), and assayed for PAF binding. Data report the mean fractions of [³H]PAF specifically bound by 5×10^6 PMN. Non-specific binding averaged ~ 0.02 and showed no significant changes with the various PAF pretreatments. Results are the means ± S.E.M. for 4 (upper panel) or 9–11 (lower panel) experiments.

PAF binding

To examine PAF receptors during desensitization, PMN were incubated with 0.1–100 nM-PAF, washed twice in Hanks' buffer (4 °C) to remove > 99.5% of the original suspending medium, and assayed for binding at 4 °C. These PMN lost [³H]PAF specific binding capacity. The effect was maximal at 15 s, persisted for ~ 10 min, and then reversed (Fig. 3, lower panel). Scatchard plots showed that control PMN had 7200 ± 1400 (mean ± S.E.M., $n = 11$) high-affinity receptors; this value fell to 340 ± 120 in PMN treated with 1 nM-PAF for 5 min at 37 °C. The K_a for high-affinity binding as well as the parameters of low-affinity binding were not significantly changed (Table 1; also see Fig. 8). PAF

Table 1. High- and low-affinity PAF binding parameters for PMN treated in various ways

In these experiments 10^7 PMN were incubated (37 °C) in 1 ml with BSA or 1–10 nM-PAF for ¼–40 min, buffer-washed or BSA-extracted (see the Materials and methods section) at 4 °C, and assayed for the binding of 10 pM–150 nM-PAF. Where indicated, PMN were pretreated with 1 μM-staurosporine or 200 μM-CI for 30 min (37 °C) before challenge. Data are affinity constants and receptor numbers per PMN (means ± S.E.M. for 8–12 experiments), as determined by the LIGAND program. LIGAND detected non-specific binding fractions of 0.017–0.020 with the various treatments, and two receptor types ($P < 0.05$, F-distribution) in all treatment groups except for PMN treated with 10 nM-PAF and then buffer-washed. In the last group, LIGAND found only one receptor type, which we report under 'Low-affinity' receptors. *Values significantly lower than those for BSA-challenged PMN ($P < 0.05$, Student's unpaired t-test); **values significantly higher than for PMN challenged with 1 nM-PAF in the absence of staurosporine or CI; ***values significantly higher than for buffer-washed PMN; ****values significantly higher than the corresponding values for buffer-washed PMN and significantly lower than for PMN challenged with BSA before BSA extraction.

Receptors...	High affinity		Low affinity	
	$10^{-8} \times K_a$ (M^{-1})	$10^{-3} \times R_t$ (per PMN)	$10^{-6} \times K_a$ (M^{-1})	$10^{-5} \times R_t$ (per PMN)
Buffer-washed				
BSA, 5 min	8±2	7±1	10±8	2±2
1 nM-PAF, ¼ min	7±1	0.5±0.4*	9±4	2±3
1 nM-PAF, 5 min	9±3	0.3±0.1*	10±3	3±3
1 nM-PAF, 40 min	8±3	6±3	8±5	3±1
10 nM-PAF, ¼ min	0	0	12±10	5±4
10 nM-PAF, 5 min	0	0	11±4	4±4
BSA, 40 min	6±2	6±2	9±5	3±1
Staurosporine, buffer-washed				
BSA, 5 min	6±2	8±1	10±6	3±3
1 nM-PAF, 5 min	7±1	6±2**	12±9	2±3
CI, buffer-washed				
BSA, 5 min	6±2	7±1	8±6	3±1
1 nM-PAF, 5 min	9±4	2±0.2**	8±5	3±2
BSA-extracted				
BSA, 5 min	5±2	6±2	8±6	4±4
10 nM-PAF, ¼ min	7±3	4±2***	5±4	3±2
10 nM-PAF, 5 min	6±1	1±2****	10±11	3±4
10 nM-PAF, 40 min	4±2	5±2	8±9	2±3

thus decreased the apparent availability of its high-affinity receptors in concert with its desensitizing actions.

[³H]PAF extractions and metabolism

PMN incubated with [³H]PAF at 4 or 37 °C for 0.25–40 min released < 2 % of bound label with two Hanks'-buffer washes. Washes with neutral pH buffers containing 0.5–2 g of BSA/100 ml can remove 80–90 % of the non-internalized [³H]PAF from cells [10–14]. We improved these results, using a pH 9.3 buffer plus 50 g of BSA/100 ml and 10 mM-EDTA. Efficacy was determined by incubating 10^7 PMN in 1 ml of buffer containing sub-stimulatory (32 pM-[³H]PAF) or stimulatory (100 pM-[³H]PAF + 9.9 nM-PAF) doses of ligand PAF at 4 °C. Cells incubated with 32 pM-[³H]PAF for 40 min took up 17.1 ± 2.7 % (mean ± S.E.M., $n = 4$) of added label. After BSA extractions (see the Materials and methods section), these PMN retained only 0.4 ± 0.1 % of initially added radioactivity. PMN incubated with 100 pM-[³H]PAF plus 9.9 nM-PAF at 4 °C for 40 min had 5.3 ± 0.5 % and 0.06 ± 0.06 % of radioactivity before and after BSA extraction. In general, the regimen released ~ 99 % of the [³H]PAF bound to PMN at 4 °C over 0.5–40 min (Fig. 4, left panels). However, PMN treated with 32 pM-[³H]PAF for even 10 min at 37 °C incorporated 72 ± 3 % of the [³H]PAF and retained 61 ± 0.3 % of label after BSA extraction. PMN similarly incubated with 100 pM-[³H]PAF + 9.9 nM-PAF contained 97 ± 0.3 % and 88 ± 4 % of label before and after extraction. Hence, BSA removed only modest amounts of ³H from PMN incubated with [³H]PAF at 37 °C for 10 min. The regimen's effectiveness at 37 °C decreased with time: PMN exposed to 32 pM-[³H]PAF for 0.25, 0.5, 1.25, 5, 10 or 20 min released respectively 41, 33, 22, 21, 19, 17 and 14 % of initially bound label to the extraction regimen; for PMN incubated with 100 pM-[³H]PAF + 9.9 nM-PAF these values were 78, 71, 42, 22, 18, 12 and 9 % (Fig. 4, right panels). T.l.c. analyses determined the nature of the released radiolabel. ³H in the extracellular fluid from all initial incubation (4 and 37 °C) or in PMN incubated with [³H]PAF at 4 °C co-migrated (> 95 %) with PAF (results not shown). Label (> 95 % recovery) in PMN exposed at 37 °C to 32 pM-[³H]PAF or 100 pM-[³H]PAF + 9.9 nM-PAF and then washed in buffer contained species co-migrating with PAF and diacyl-GPC. Material co-migrating with PAF reached a peak at 2.5 min and declined thereafter, whereas material co-migrating with diacyl-GPC progressively accumulated (Fig. 5, left panels). BSA extraction released 50–80 % of the PAF-co-migrating label that bound to PMN during the initial 0.5–2.5 min of incubation. It released only 20–40 % of PAF label from 5–20 min incubations and never had significant effects on the diacyl-GPC-co-migrating compound (Fig. 5, right panels). The data support five conclusions. First, at 4 °C, PAF attaches to PMN at superficial sites (e.g. plasmalemma receptors and outer leaf) that are fully accessible to BSA. Second, PAF associates with these same sites at 37 °C, but then sequesters from BSA via an apparent internalization reaction. Third, PMN rapidly deacetylate and acylate internalized PAF. Fourth, acylated metabolite, like its internalized precursor PAF, is sequestered from extracellular BSA. And, fifth, PMN treated with PAF at 37 °C for ⩽ 2.5 min retain appreciable amounts of superficially localized structurally intact PAF even after two buffer-washes. For example, 10^7 PMN incubated (37 °C) with 10 nM-PAF for 2.5 min, and then buffer-washed, contained 2.1 pmol (120000 molecules/cell) of BSA-extractable PAF.

[³H]PAF binding to BSA-extracted PMN

After BSA extraction, control PMN had ~ 20 % declines in [³H]PAF specific binding. The regimen itself had a small suppressive effect on high-affinity PAF receptors (Table 1). Never-

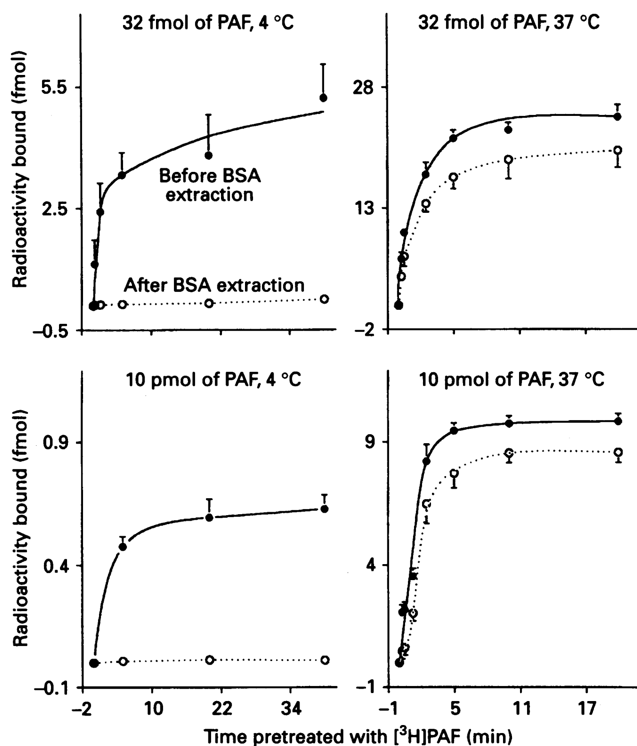

Fig. 4. Cellular content of radiolabel after BSA extraction of [^{3}H]PAF-pretreated PMN

PMN (10^7 in 1 ml of buffer) were incubated with 32 fmol of [^{3}H]PAF (upper panels) or 0.1 pmol of [^{3}H]PAF+9.9 pmol of PAF (lower panels) at 4 °C (left panels) or 37 °C (right panels). At indicated times thereafter, cells were processed (4 °C) in the BSA-extraction regimen (see the Materials and methods section). Data are expressed as radioactivity associated with 10^7 PMN before (continuous lines) or after (broken lines) BSA extraction and represent the means±S.E.M. of 4–8 experiments. Note the different ordinate and abscissa scales.

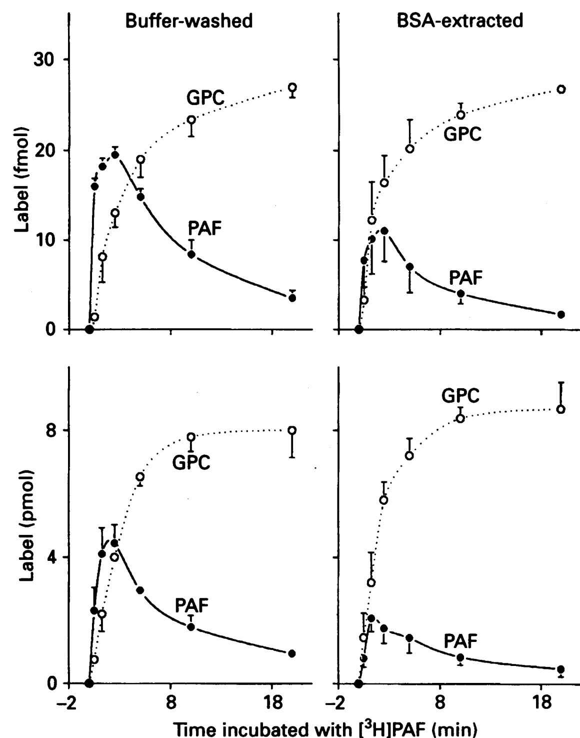

Fig. 5. Composition of radiolabel in PMN pretreated with [^{3}H]PAF

PMN (10^7 in 1 ml of buffer) were incubated (37 °C) with 32 fmol of [^{3}H]PAF (upper panels) or 0.1 pmol of [^{3}H]PAF+9.9 pmol of PAF (lower panels) for the indicated times. Cells were then washed twice in regular Hanks' buffer (left panels) or processed by the BSA-extraction regimen (right panels). Data are expressed as radioactivity from 10^7 PMN that co-migrated on t.l.c. with PAF or diacyl-GPC standards, and are the means±S.E.M. of 3–8 experiments. Note the different ordinate scales.

theless, PMN stimulated with 10 nM-PAF (37 °C) and then BSA-extracted had > 90% falls in [^{3}H]PAF binding and high-affinity receptors (Table 1) compared with unstimulated cells. The effect began more slowly than that in buffer-washed PMN (Fig. 6). Extracted PMN nevertheless regained [^{3}H]PAF binding and receptors as quickly as did PMN washed in Hanks' buffer (Table 1). Hence the rapid declines in [^{3}H]PAF binding and high-affinity receptors observed in PAF-treated buffer-washed cells probably reflect PAF carry-over from desensitization to binding assays. Virtually all of the losses occurring after 90–150 s, in contrast, are unexplained by such contamination.

Inhibitor studies

PMN were incubated with 20 μg of cycloheximide/ml or 10 μg of actinomycin D/ml for up to 3 h under conditions that completely block their capacity to synthesize proteins [21]. The cells maintained normal [^{3}H]PAF specific binding capacity and responded to 10 nM-PAF with typical falls and rises in [^{3}H]PAF binding (results not shown). On the other hand, PMN incubated with staurosporine or, to a lesser extent, CI had significantly decreased responses to PAF in [^{3}H]PAF specific-binding (Fig. 7) and receptor (Fig. 8, Table 1) assays. The two PKC blockers acted at the same concentrations that inhibited the ability of a direct PKC activator, PMA, to stimulate PMN production of O_2 or to depress [^{3}H]PAF specific binding (Fig. 9). Evidently, then, protein synthesis is not required for maintaining PAF receptors in resting PMN or for re-expressing PAF receptors in desensitized PMN. However, an element(s) sensitive to staurosporine and CI appears involved in the receptor-depressing actions of PAF.

DISCUSSION

PAF stimulates PMN to hydrolyse phosphatidylinositols, raise $[Ca^{2+}]_i$, mobilize PKC, and activate Ca^{2+}- and PKC-sensitive elements that presumably proceed to elicit, e.g., degranulation [1–3]. However, accumulation of phosphatidylinositol cleavage products declines after 10 s [22], $[Ca^{2+}]_i$ begins to fall within 15 s (Fig. 2), degranulation ceases at ~30 s [23], and PKC mobilization reverses after 120 s [24]. At these times, PAF remains mostly outside the PMN (Fig. 4, right panels), and the extracellular fluid of PMN suspensions treated with 100 nM-PAF for 3 min is fully capable of stimulating fresh PMN [25]. PMN thus seem exhausted or unperturbable within 10 s of a single PAF exposure. Indeed, we found that PAF-treated PMN were insensitive to a second PAF challenge by 15 s in $[Ca^{2+}]_i$ assays (Fig. 2), and at the first time tested, 1.5 min, in degranulation assays (Fig. 1). Nevertheless, the cells had full or even primed responses to fMLP (Figs. 1 and 2). PAF thus induced an almost immediate stimulus-selective state of desensitization. The cells were not globally dysfunctional, but rather had a restricted defect in responding to PAF. Our studies examined causes for this.

PMN exposed to PAF at 37 °C and then buffer-washed, rapidly ($t_{\frac{1}{2}}$ < 15 s) and reversibly ($t_{\frac{1}{2}}$ ~ 20 min) lost [^{3}H]PAF specific binding capacity (Fig. 3, lower panel) and high-affinity receptors (Table 1). Previous studies have demonstrated these

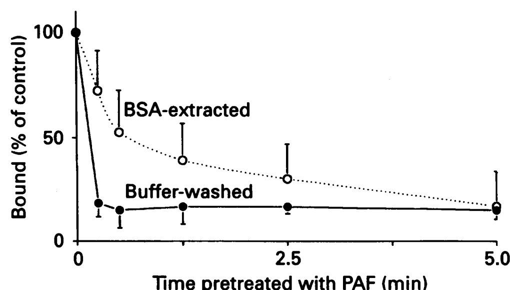

Fig. 6. [³H]PAF binding to PAF-pretreated PMN

PMN (10^7 in 1 ml of buffer) were incubated with BSA or 10 nM-PAF for 0.25–5 min and then washed twice in Hanks' buffer (4 °C) or processed by the BSA-extraction regimen before assaying [³H]PAF specific binding. Non-specific binding for the treatment groups did not vary significantly from 0.02. Data are reported for specific binding as percentages of control PMN (i.e. PMN treated was BSA in place of PAF for the indicated times) for 8–11 experiments. Binding values for PAF-pretreated PMN at 0.25, 0.5 and 1.25 min were significantly higher ($P < 0.05$, Student's paired t-test) in BSA-extracted PMN than in buffer-washed PMN.

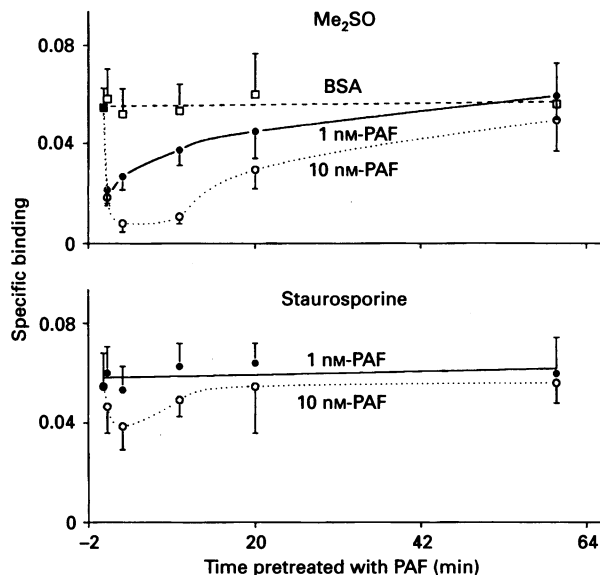

Fig. 7. Effects of staurosporine on [³H]PAF binding to PAF-pretreated PMN

PMN were incubated with Me_2SO (upper panel) or 1 μM-staurosporine (lower panel) for 30 min at 37 °C, challenged with BSA (dashed line), 1 nM-PAF (continuous line) or 10 nM-PAF (dotted line) for 2.5–60 min, and assayed for PAF binding. Staurosporine by itself did not significantly alter PAF binding (results not shown). Results are the mean fractions (±S.E.M.) of [³H]PAF specifically bound by 5×10^6 PMN for 8 experiments. Non-specific binding (~ 0.02) did not vary significantly between treatment groups.

findings, but did not consider that some of the desensitizing dose of PAF may adhere with PMN to contaminate subsequent binding assays [15,26]. Standard BSA extraction methods release 80–90 % of this contamination [10–14]. We used a buffer containing very high BSA concentrations (50 mg/100 ml), 10 mM-EDTA, and alkaline pH (9.3) to release 99 % of the PAF taken up by PMN at 4 °C. This washing regimen therefore stripped PMN of virtually all PAF that associated with high-affinity receptors, low-affinity receptors and non-specific adsorption sites. Never-

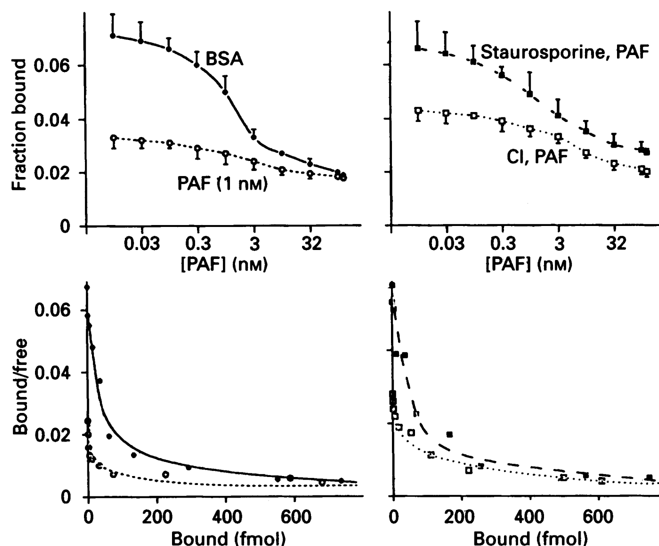

Fig. 8. Displacement curves (upper panels) and Scatchard plots (lower panels) for PAF binding to PMN treated in various ways

PMN (10^7 in 1 ml of buffer) were incubated (37 °C) for 30 min and then challenged with BSA (——) or 1 nM-PAF (----) for 5 min. Alternatively, the cells were pretreated with 1 μM-staurosporine (– – –) or 200 μM-Cl (······) for 30 min before a 5 min challenge with 1 mM-PAF. Cells were then quickly buffer-washed, resuspended at 5×10^6 cells/ml, and assayed for PAF binding at 4 °C. Results give the binding (±S.E.M.) by 5×10^6 PMN for 8–11 experiments. Non-specific binding, as determined by LIGAND, did not vary significantly from 0.019.

theless, BSA extractions were very much less effective in releasing PAF bound to PMN at 37 °C (Fig. 4). At 37 °C, PAF initially accumulated in PMN in a BSA-extractable form. Within 2.5 min, however, it was converted progressively into a BSA-unextractable form and then rapidly accumulated as acylated metabolite (Fig. 5). Hence, PMN incubated with PAF at 37 °C for < 2.5 min and then buffer-washed retain significant amounts of intact PAF in a superficial (i.e. BSA-extractable) site. This contamination probably contributed to the loss of [³H]PAF specific binding capacity and high-affinity PAF receptors observed in PMN that were buffer-washed after exposure to PAF for < 2.5 min; it cannot explain later-developing changes in PAF binding. At 37 °C, then, PAF acts relatively slowly to decrease the availability of, or down-regulate, its high-affinity receptors. These receptors may have assumed a low-affinity configuration, formed a non-dissociating complex with PAF, been internalized, or been shed from the cell. Although our experiments do not discriminate among these possibilities, we note that desensitized PMN soon regained PAF receptors (Table 1) even when pretreated with cycloheximide or actinomycin D. Hence, the PAF receptor changes observed here are unlikely to be due to irreversible processes (such as receptor shedding) that are followed by a compensatory expression of newly synthesized receptors.

Homma *et al.* [12] found that PAF did not alter rabbit platelet PAF receptors, whereas Chao *et al.* [14] reported that PAF caused a true (i.e. resistant to BSA extraction) down-regulation of its Kupffer-cell receptors. The latter effect endured for hours and required protein synthesis for reversal. PMN responses to PAF had some characteristics of both these cell types. Like Kupffer cells, PMN exposed to PAF down-regulated their high-affinity PAF receptors. This occurred in PMN that were BSA-extracted between desensitization and binding assays (Fig. 6, Table 1). Nevertheless, down-regulation reversed in ~ 60 min

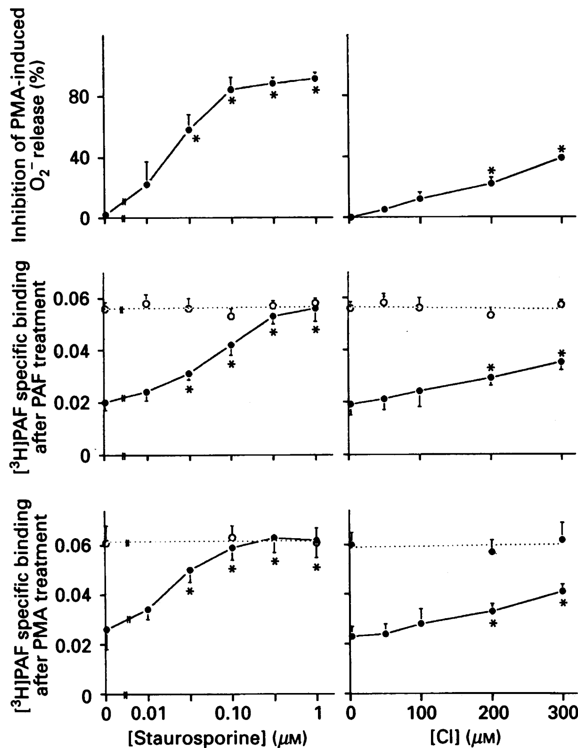

Fig. 9. Comparative effects of staurosporine and CI on PMN responses to PMA or PAF

PMN were incubated with the indicated concentrations of staurosporine (left panels) or CI (right panels) for 30 min at 37 °C. Cells were then challenged with 1 nM-PMA and assayed for O_2^- release (upper panels; PMN incubated without an inhibitor released 5.3 ± 0.9 nmol of O_2^-/min per 10^7 cells; results are given as percentages of this value). Alternatively, inhibitor-pretreated PMN were challenged with 1 nM-PAF or BSA (centre panels: continuous and broken lines respectively) for 2.5 min, or with 1 nM-PMA or Me_2SO (lower panels: continuous and broken lines respectively) for 5 min. The PMN were then washed twice and assayed for [^{3}H]PAF binding at 4 °C. Binding results are the fraction of [^{3}H]PAF specifically bound by 5×10^6 PMN. Non-specific binding did not significantly vary from ~ 0.02 in the various treatment groups. Data are the means of ($\pm$S.E.M.) of 5 (upper two panels) or 9–11 (lower four panels) experiments: * values significantly ($P < 0.05$, Student's paired t test) lower (upper two panels) or higher (lower four panels) than the corresponding values for the responses of PMN not treated with staurosporine or CI.

regardless of the presence of cycloheximide or actinomycin D. PAF thus induced a prolonged, irreversible (i.e. possibly requiring new receptor synthesis to reverse), down-regulation of Kupffer-cell PAF receptors, yet had only evanascent, protein-synthesis-independent, actions in PMN. On the other hand, BSA-extraction experiments detected differences between the onset of declines in PAF receptor binding ($t_{\frac{1}{2}}$ ~ 30 s, Fig. 6) and PAF insensitivity ($t_{\frac{1}{2}}$ < 15 s, Figs. 1 and 2, and the upper panel of Fig. 3). PMN desensitization that developed shortly after PAF challenge must therefore reflect an uncoupling of PAF receptors from transducer elements similar to that occurring in rabbit platelets [12]. Additionally, PMN regained PAF sensitivity ($t_{\frac{1}{2}}$ ~ 10–20 min) more quickly than [^{3}H]PAF specific binding ($t_{\frac{1}{2}}$ ~ 40 min). We note that PAF enhances PMN responses to diverse stimuli [27–29], including fMLP (Fig. 1, right panels). Since priming may reflect the excitation of post-receptor events [30], PAF-desensitized PMN could be primed even to PAF. That is, the receptors expressed during the recovery stage of desensitization may have an enhanced ability to elicit function. High-affinity PAF receptor losses obviously contribute to the desensitized state, but events such as receptor uncoupling and priming may modulate the onset and reversal, respectively, of PAF insensitivity.

PAF induces PMN to mobilize PKC [23,24,31–33]; PKC blockers inhibit PMN responses to PAF [23]; and PKC activators, e.g. PMA, share with PAF the ability to stimulate PMN to produce O_2^-, degranulate, and, of particular interest here, down-regulate high-affinity PAF receptors [33,34]. These data suggest that PKC may mediate not only the stimulating, but also the receptor-down-regulating, actions of PAF. Our studies add support to this notion. Two PKC blockers, staurosporine and CI, inhibited the effects of PAF on its receptors (Figs. 7 and 8, Table 1). Both drugs acted at concentrations paralleling their respective potencies in inhibiting PMA-induced O_2^- production and PMA-induced decreases in [^{3}H]PAF specific binding (Fig. 9). Control experiments indicated that 1 μM-staurosporine and 200 μM-CI did not interfere with [^{3}H]PAF specific binding or alter the ability of 1 nM-PAF to raise $[Ca^{2+}]_i$ (results not shown). Moreover, recent studies provide evidence implicating PKC in the bioactions of PAF on diverse cell types [35–39]. The data, when taken together, suggest that PKC has a general role in down-regulating PAF receptors. By analogy with other systems [40,41], the phosphorylating enzyme may achieve this effect by acting directly on PAF receptors or on systems that support PAF receptors (e.g. G-proteins). Since PKC blockers show non-selective inhibitory effects [42,43], however, our results seem best interpreted conservatively: they implicate a staurosporine- and CI-sensitive element in PAF-induced receptor down-regulation, but further studies are needed to define this element's identity and its role, if any, in PAF-desensitization responses exhibited by other cell types.

In conclusion, our studies afford an alternative model for PAF desensitization that may be generally applicable to cells which, when exposed to PAF, mobilize PKC and pass through a brief period of PAF-insensitivity. The model divides desensitization into three stages. Stage I involves almost immediate losses in PAF-sensitivity that are not accompanied by comparable changes in PAF receptors. These receptors must have uncoupled from transductional elements while retaining their PAF-binding capacity. PMN stage I occurs for $1\frac{1}{2}$–$2\frac{1}{2}$ min after challenge. Stage II cells continue in a maximally desensitized state and have maximally down-regulated their high-affinity PAF receptors. PKC or an element inhibited by PKC blockers may mediate receptor down-regulation. PMN stage II begins by ~ 1.25–2.5 min and ends after ~ 5–10 min of PAF exposure. During this stage, PMN also became primed, as indicated by their hypersensitivity to fMLP. In stage III, cells recover PAF-sensitivity and regain high-affinity PAF receptors. Although receptor recovery is essential for reversing desensitization, an underlying non-selective state of priming may explain imbalances between PAF-sensitivity and a slower recovery of PAF receptors. PMN stage III proceeds for ~ 40 min and may reflect the recycling of previously expressed receptors, receptor interconversions between low- and high-affinity states, and/or the uncovering of cryptic receptors. It does not involve any appreciable synthesis of new receptors. As the three stages proceed, cells internalize PAF relying principally on a direct pathway rather than a receptor-mediated endocytotic route. In stage I, PAF accumulates mostly in BSA-extractable superficial sites, such as on plasma-membrane receptors and in the outer leaflet of the plasmalemma. This PAF interferes with standard PAF-binding assays. However, surface-attached PAF quickly flips internally and thereafter becomes progressively metabolized to its acylated bio-inactive storage product. PAF is thereby cleared from the environment around newly expressed PAF

receptors and the cell reconstitutes its ability to detect and respond to fresh PAF challenges.

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