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EVIDENCE FOR TWO PLATELET ACTIVATING FACTOR RECEPTORS ON EOSINOPHILS: DISSOCIATION BETWEEN PAF-INDUCED INTRACELLULAR CALCIUM MOBILIZATION DEGRANULATION AND SUPEROXIDES ANION GENERATION IN EOSINOPHILS

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Summary. We have compared platelet activating factor (PAF)-induced eosinophil peroxidase (EPO) release and intracellular calcium mobilization with superoxide anion ($\cdot O_2^-$) generation from guinea pig eosinophils. EPO release and Ca^{2+} mobilization occurred at lower concentrations of PAF (EC_{50} values of 1.3 nM and 11.5 nM, respectively) while $\cdot O_2^-$ production was observed at higher concentrations (EC_{50} of 31.7 uM). Receptor characterization with the competitive PAF antagonist, WEB 2086, gave pA_2 values of 8.5 and 8.3 for EPO enzyme release and rise in $[Ca^{2+}]_i$, respectively, and 5.8 for the $\cdot O_2^-$ production. In addition, PAF-induced degranulation and elevation of $[Ca^{2+}]_i$ were dependent on extracellular Ca^{2+} whereas PAF-stimulated $\cdot O_2^-$ generation was dependent on the presence of extracellular Mg^{2+} ions. These results suggest the existence either of two subtypes of the PAF receptor or a single receptor that can exist in one of two affinity states on guinea pig eosinophils.

Platelet activating factor (PAF) is a biologically active phospholipid with a wide spectrum of effects on tissues and cells (1). These responses are stereoselective and occur at low concentrations of PAF suggesting that specific receptors are involved. Binding experiments using [^{3}H]PAF or radiolabeled PAF antagonists have further confirmed the existence of specific

Abbrevations: PAF: platelet activating factor; fMLP: N-formyl-methionyl-leucyl-phenylalanine; PMA: phorbol 12-myristate 13-acetate; EPO: eosinophil peroxidase; $\cdot O_2^-$: superoxide anion; $[Ca^{2+}]_i$: intracellular Ca^{2+} concentration.

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binding sites for PAF (1). High affinity receptors have been found on a number of inflammatory cell types including platelets (2-4), neutrophils (5,6), eosinophils (7), and lung membranes (8,9). Recently, the existence of a second PAF receptor subtype has been suggested on the basis of the relative potencies of PAF antagonists (10,11,12).

The interaction between PAF and eosinophils may be of particular relevance to allergic disease since PAF is one of the most potent activators of eosinophils. In the present study, we have compared eosinophil enzyme secretion (eosinophil peroxidase), elevation of intracellular free calcium ion concentration ($[Ca^{2+}]_i$) and superoxide anion ($\cdot O_2^-$) release induced by PAF. In addition, we have used the specific PAF receptor antagonist WEB 2086, to determine receptor affinity.

MATERIAL AND METHODS

Materials. Hanks balanced salt solution (HBSS) was purchased from Flow Laboratories Ltd.(Richmansworth, U.K.), Percoll was purchased from Pharmacia Fine Chemicals AB, (Uppsala, Sweden) and C_{18}-PAF and C_{18}-lyso-PAF were obtained from Bachem (Switzerland). WEB 2086 was kindly donated by Boehringer Ingelheim (Ingelheim, W.Germany). PMA, fMLP, bovine serum albumin, polymyxin B sulfate, EGTA and digitonin were from Sigma Chemicals Ltd.(Poole, U.K.) and fura-2 acetoxymethyl ester from Molecular Probes (Eugene, Oregon, U.S.A.).

Preparation of eosinophils. Eosinophils were obtained from polymyxin B (1 mg/ml i.p./week) treated male guinea pigs (650-1000 g) by weekly peritoneal lavage. The cells were purified using a discontinuous density gradient of isomolar Percoll solutions (1.100, 1.090, 1.085, 1.080, and 1.070 g/ml) as previously described (13). Eosinophils with a purity of > 95% and a viability > 99% were found in fractions 3, 4, and 5. The fractions were pooled and washed three times in HBSS buffer.

Measurements of $[Ca^{2+}]_i$. Cells (10^7 ml^{-1}) were incubated for 30 min at 37°C with 2.5 uM fura-2 as previously described (14). After incubation, eosinophils were washed three times in calcium-free, HEPES buffered Tyrode solution and resuspended to a final concentration of 1.5 x 10^6 cells/ml. 2ml of the cell suspension were dispensed into disposable cuvettes and the external Ca^{2+}-concentration ($[Ca^{2+}]_o$) adjusted to 1 mM with $CaCl_2$. Cuvettes were transferred to an Bowman spectrophotofluorimeter (excitation 339nm, emission 500 nm - 4 nm slit width), fitted with a stirring attachment for fluorescence reading at 37°C. Leakage of fura-2 from eosinophils was shown to be small as determined by the degree of quench in fluorescence signal produced by the addition of 1 mM Ni^{2+}. The intracellular calcium concentration ($[Ca^{2+}]_i$) was determined by the chelation method and formula (15). F_{max} was measured by

lysing with 40 uM digitonin in the presence of 1 mM Ca^{2+}. F_{min} was determined by adjusting the pH of the lysed cells to 8.5 with 20 ul 2 M NaOH followed by the addition of 10 mM EGTA. Cells were allowed to equilibrate at 37°C for 2 min before the agonist or vehicle was added directly to the cell containing cuvettes. WEB 2086 and the vehicle were added 1 min before the agonist.

Functional assays. Eosinophil peroxidase (EPO), lactate dehydrogenase and $\cdot O_2^-$ generation were measured as described elsewhere (16).

Statistics. Negative log EC_{50} values from each concentration-response curve were derived by linear regression analysis of percentage of the maximum $[Ca^{2+}]_i$ for the agonist versus log concentration at concentrations immediately above and below the 50% response level. The apparent dissociation constant (K_B) of WEB 2086 was calculated by the method of Furchott (17) using the equation

$$K_B = [\text{antagonist}]/\text{dose-ratio} - 1.$$

Dose-ratio was calculated according to the following equation: antilog [(-log molar EC_{50} for PAF in the absence of antagonist) - (-log molar EC_{50} for PAF in the presence of antagonist). Schild plot was obtained according to Arunalakshana and Schild (18); pA_2 values were obtained with the aid of a computer and graphics plotter. For each stimulus, experiments were performed at least three times in duplicate or triplicate using cell preparations from different animals on different days. Results were expressed as mean ± SEM and were analyzed by Student's t-test.

RESULTS

PAF-induced elevation of $[Ca^{2+}]_i$, EPO release and $\cdot O_2^-$ generation. Both the elevation in $[Ca^{2+}]_i$ and the release of EPO occurred at a similar PAF concentration range between 0.1 nM and 10 uM (Fig. 1). The responses were dose-dependent with a maximum seen at 100 nM and 1 uM, respectively, and declined at higher concentrations. Mean EC_{50} of values the responses obtained from 9 independent experiments were 11.5 nM and 1.23 nM for the $[Ca^{2+}]_i$ and EPO release, respectively. By contrast, $\cdot O_2^-$ generation by eosinophils was only observed at PAF concentrations above 1 uM with a maximum effect at 30 uM and a mean EC_{50} of 6.2 uM (n=5). No $\cdot O_2^-$ production occurred with lyso-PAF (100 nM to 1 mM).

In a separate series of experiments, the protein kinase C activator, phorbol myristate acetate (PMA), was examined on

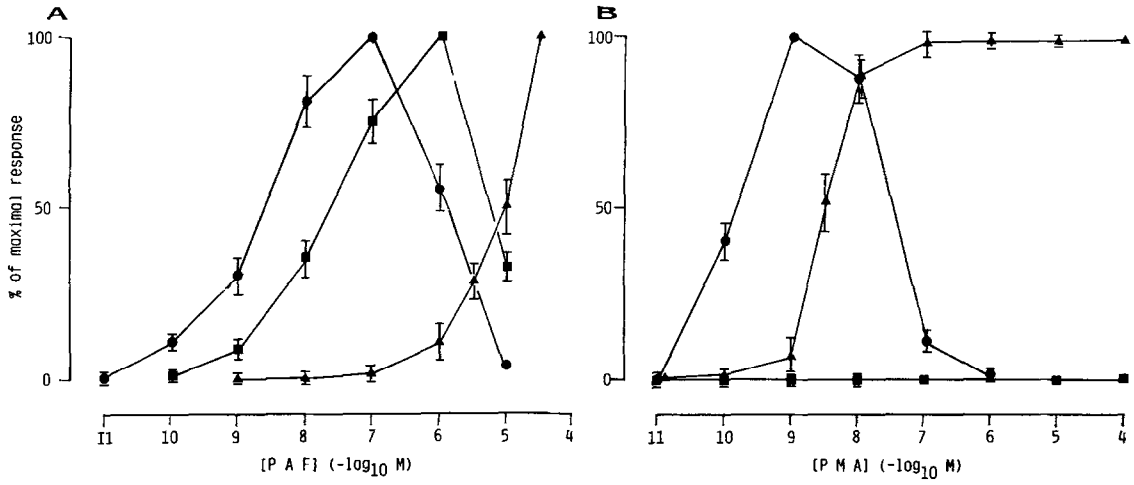

Figure 1. Dose-response curves of eosinophil peroxidase release (●), elevation of intracellular free calcium ion concentration (■), and superoxide anion generation (▲) by PAF (Panel A) and PMA (Panel B). Mean ± SEM of three experiments are shown.

$[Ca^{2+}]_i$, EPO release and $\cdot O_2^-$ production. PMA caused a dose-dependent release of EPO (EC_{50} of 0.6 nM) and generation of $\cdot O_2^-$ (EC_{50} of 31.7 nM). By contrast, PMA had no effect on $[Ca^{2+}]_i$ at concentrations between 0.1 nM and 100 uM (Fig.1B). The effects of PAF and PMA were not due to cell damage since the lactate dehydrogenase release was < 5% in either the presence or the absence of these stimuli.

Effect of WEB 2086. Eosinophils were incubated with various concentrations of PAF in the absence and presence of 30 nM, 100 nM and 300 nM WEB 2086 for 5 min. As shown in Fig. 2A, WEB 2086 gave parallel rightward shifts in the PAF concentration-response curve. The K_B of 12.6 ± 3.3 nM was independent of antagonist concentration. Schild analysis (Fig.2A, Inset) of the data yielded a pA_2 of 8.27 with a slope not significantly different from unity ($p > 0.05$). WEB 2086 had no effect on leukotriene B_4 or fMLP elicited change in $[Ca^{2+}]_i$.

Similar results were obtained for inhibition of PAF-stimulated EPO release by WEB 2086 (Fig.2B), which produced

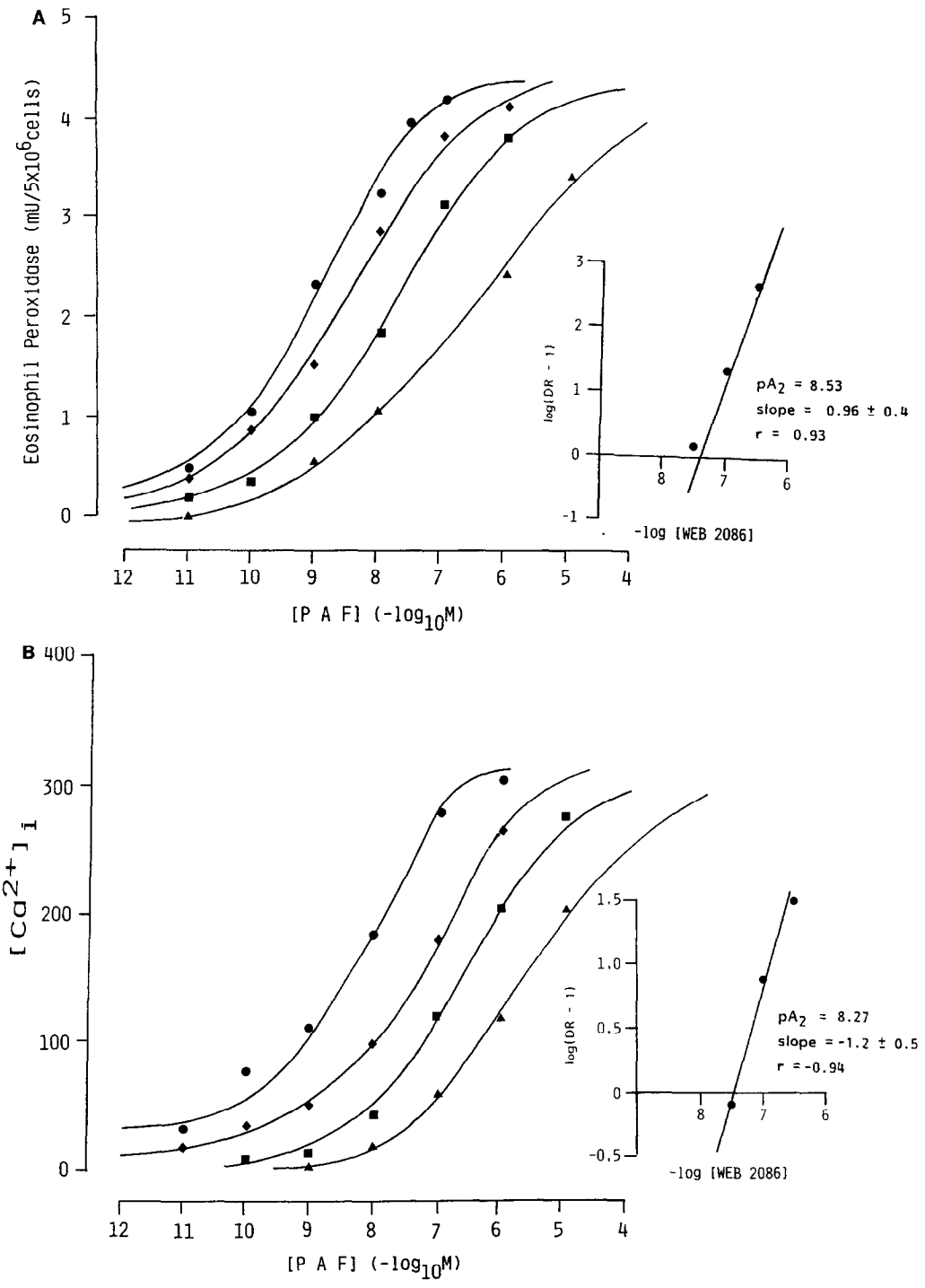

Figure 2. Effect of the PAF antagonist WEB 2086 on PAF-induced eosinophil peroxidase release (Panel A) and rise in intracellular free calcium concentration (Panel B). Guinea pig eosinophils were preincubated with buffer (●), 30 nM WEB 2086 (◆), 100 nM WEB 2086 (■), and 300 nM (WEB 2086 (▲) before addition of increasing concentrations of PAF. Mean values of three experiments are shown. Insets: Schild analysis of the antagonism of WEB 2086 on eosinophils.

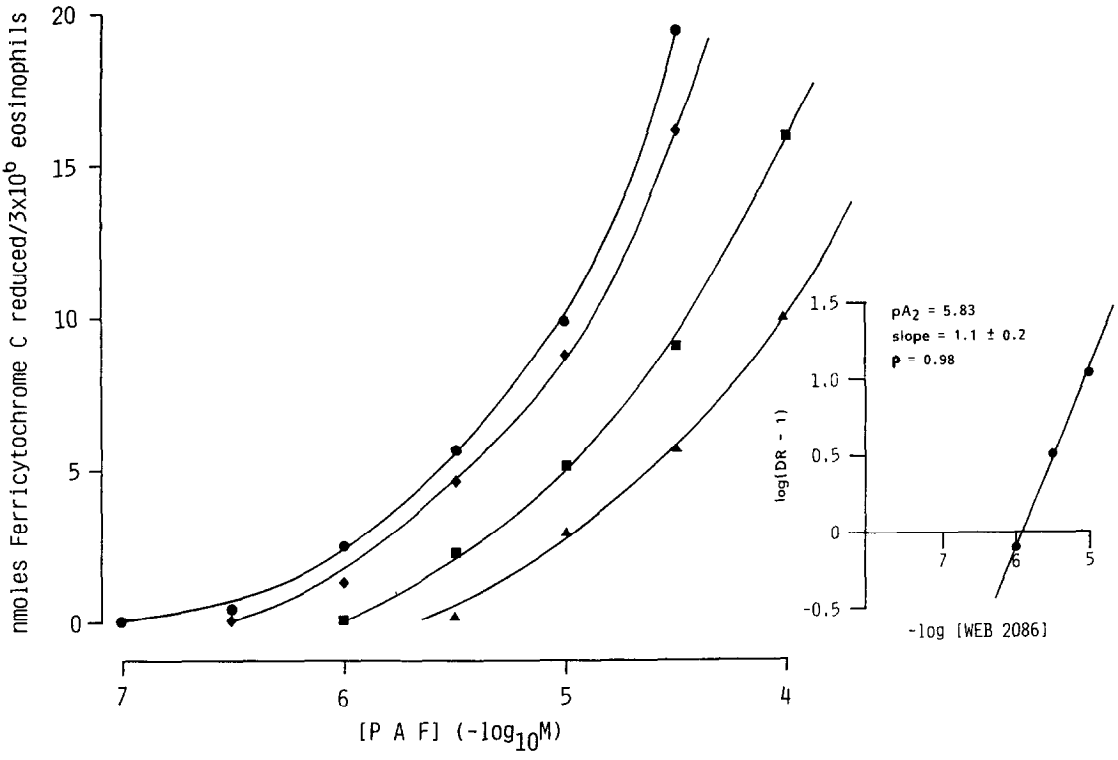

Figure 3. Effect of WEB 2086 on PAF-induced superoxide anion generation. Purified eosinophils were preincubated for 10 min with buffer (●), 1 uM WEB 2086 (◆), 3 uM WEB 2086 (■), and 10 uM WEB 2086 (▲) before adding increasing concentrations of PAF. Mean values of three experiments are shown. Inset: Schild analysis of the data.

parallel rightward shifts in the PAF concentration-response curves. The respective K_B value of 6.6 was independent of antagonist concentration as expected for a competitive antagonist. Schild analysis of the data produced a pA_2 of 8.53 (Fig.2B, Inset). The slope of the regression line defining the pA_2 was not significantly different ($P > 0.05$) from unity. Again, WEB 2086 did not affect the EPO release induced by PMA or by calcium ionophore A23187 (data not shown).

WEB 2086 also produced parallel rightward shifts in the PAF-induced $\cdot O_2^-$ generation (Fig. 3). Schild analysis of the data yielded a pA_2 of 5.83 (Fig.3, Inset) with a slope of the regression line not different from unity ($P > 0.05$).

Table 1. Effect of extracellular Ca^{2+} and Mg^{2+} ions on PAF-induced eosinophil peroxidase (EPO) release, rise in intracellular free calium concentration ($[Ca^{2+}]_i$), and superoxide anion ($\cdot O_2^-$) generation

	Rise in $[Ca^{2+}]_i$ (nM)	EPO release (uU/4x10^6cells)	$.O_2^-$ generation (nmoles Cytochrome C/ reduced/10^6 cells)
0 mM Ca^{2+}/Mg^{2+}	73 + 12	1190 + 454	47 + 13
1 mM Ca^{2+}/Mg^{2+}	342 + 22	7148 + 950	4965 + 381
1 mM Ca^{2+}	305 + 29	5206 + 785	75 + 39
1 mM Mg^{2+}	102 + 18	2851 + 537	4340 + 472

Mean ± SEM of five experiments are shown.

Influence of divalent cations. We also studied the effect of extracellular Ca^{2+} and Mg^{2+} ions on PAF-induced stimulation of eosinophils. Cells were washed three times in Ca^{2+} and Mg^{2+}-free buffer. Twenty min prior to the addition of agonist or vehicle either $CaCl_2$ or $MgCl_2$ were added to give a final external ion concentration of 1 mM. The absence of Mg^{2+} neither effected EPO release nor the rise in $[Ca^{2+}]_i$ stimulated by PAF (100 nM). By contrast, the absence of Ca^{2+} in the extracellular medium significantly reduced these responses (Table 1). Treatment of eosinophils with PAF (3 uM) in the absence of both divalent cations, however, almost completely inhibited the $\cdot O_2^-$ generation (Table 1). Furthermore, addition of 1mM Ca^{2+} failed to increase $\cdot O_2^-$ production induced by PAF whereas 1 mM Mg^{2+} restored the full response. However, the effect of PMA (100 nM) on $\cdot O_2^-$ production was independent of the extracellular divalent cation concentration (data not shown).

D I S C U S S I O N

The principle conclusion to be drawn from these data is that guinea pig eosinophils may mediate the effects of PAF through two transduction mechanisms, based on the following

observations. Firstly, enzyme secretion (degranulation) and elevation in intracellular [Ca^{2+}] proved to be more sensitive to PAF than generation of $.O_2^-$. EPO release and Ca^{2+} mobilization occurred in the concentration range of 0.1 nM to 1 uM with respective EC_{50} values of 1.2 nM and 11.5 nM, in agreement with previously published data (19). Characterization of these responses with the competitive PAF receptor antagonist, WEB 2086, by Schild analysis yielded pA_2 values for EPO release and calcium mobilization of 8.5 and 8.3. By contrast, PAF-induced $\cdot O_2^-$ generation occurred at approximately 1000 fold higher concentrations (1 uM to 30 uM) with a EC_{50} of 6.8 uM and a pA_2 value for WEB 2086 of 5.8. Adopting the guidelines suggested by Furchgott (17), a 3-fold difference in the affinity constants for antagonist can be considered as evidence for two distinct receptors. Secondly, enzyme secretion and elevation in intracellular [Ca^{2+}] and $\cdot O_2^-$ generation in response to PAF are dependent on different extracellular divalent cations. EPO secretion as well as elevation of $[Ca^{2+}]_i$ in PAF-stimulated eosinophils were significantly reduced in the absence of extracellular Ca^{2+} but were independent of extracellular Mg^{2+} concentration. By contrast, $\cdot O_2^-$ production, was dependent on extracellular Mg^{2+}, while extracellular Ca^{2+} did not affect the response.

In contrast to the differential ion-dependencies of PAF-induced cell responses, stimulation of both EPO release and $\cdot O_2^-$ generation by PMA was not dependent on the presence of either extracellular Ca^{2+} and Mg^{2+} as has been observed for other cell types (20). This does not completely exclude the possibility that intracellular Ca^{2+} movements occur, however. Tauber et el. (21) demonstrated that activation of human neutrophils by PMA is Ca^{2+}-dependent, even though PMA does not

produce a measurable change in $[Ca^{2+}]_i$ as measured with the fluorescent Ca^{2+} indicator dye fura-2. When cells are depleted of intracellular Ca^{2+} or when intracellular Ca^{2+} mobilization is blocked, no $\cdot O_2^-$ generation or depolarization can be elicited. The effective extracellular Ca^{2+} concentration that restores 50% activity is as low as 100 uM for depolarization compared to 1 uM for $\cdot O_2^-$ production, suggesting different ion requirements for these functions. Thus, our results do not preclude the possibility that low extracellular Ca^{2+} concentrations are required for PMA-induced $\cdot O_2^-$ production.

Taken together, our results indicate that PAF mediates its effects on guinea pig eosinophils by interacting either with two different PAF binding sites or through one receptor which exists in both high and low affinity states. Binding of PAF to the 'high affinity' receptor results in an increase in permeability of the plasma membrane to Ca^{2+}, leading to influx of Ca^{2+} and to a subsequent rise in $[Ca^{2+}]_i$ which in turn is linked to degranulation and release of EPO. A 'low affinity' PAF receptor, by contrast, may be linked through a Mg^{2+}-dependent mechanism to oxidative metabolism and $\cdot O_2^-$ release. Since PMA is equally effective in stimulating degranulation and $\cdot O_2^-$ release and is independent of divalent cations, a protein kinase C may be involved in both of these transduction pathways.

The existence of PAF receptor subtypes on rabbit neutrophils was first suggested based on their sensitivity to pertussis toxin (11). Later, Lambrecht and Parnham (10) described different potencies of kadsurenone in inhibiting PAF-induced chemiluminescence of guinea pig peritoneal macrophages and pig peripheral blood leukocyte aggregation. More recently, Hwang

(12) found that the relative potencies of PAF agonists and PAF antagonists on human platelets differ from those on neutrophils, and that the cellular responses to PAF in these cells were differentially affected by pertussis toxin, cholera toxin and the monovalent cations Na^+ and Li^+ ions.

Our results support the idea that PAF receptors are not homogeneous. They provide indirect evidence that PAF can stimulate eosinophil activation through a 'high' affinity receptor leading to eosinophil enzyme release and intracellular calcium mobilization. In addition, PAF is also able to activate a 'low affinity' receptor which is coupled to the oxidative response of the cell. Whether these two receptors are different or are the same existing in both high and low affinity states remains to be determined. It is interesting to note that PAF-induced eosinophil chemotaxis (22) and eosinophil adherence to vascular endothelium cells (23) occur at concentrations that we have shown to stimulate enzyme release and Ca^{2+} mobilization indicating the involvement of high affinity receptors in these cellular functions. In pathogenic terms, the 'high affinity' receptor may account for the chemotactic attraction and degranulation of eosinophils over a longer distance. By contrast, for the production of the short-lived toxic oxygen metabolites, which can only act locally, higher PAF concentrations are needed to elicit the response. Such concentrations of PAF may only be achieved locally at the site of inflammation in close proximity to PAF producing cells.

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