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Characterization of PAF receptors on human neutrophils using the specific antagonist, WEB 2086

Correlation between receptor binding and function

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The antagonism of PAF effects by WEB 2086 and the receptor binding of [^{3}H]WEB 2086 were investigated in isolated human neutrophils. WEB 2086 inhibited PAF-induced β-glucuronidase release and [^{3}H]WEB 2086 bound specifically to high-affinity sites on the cells. Close concordance between affinity constants for WEB 2086 from functional and radioligand-binding studies suggests that WEB 2086 interacts with the neutrophil PAF receptors and that [^{3}H]WEB 2086 may be a useful ligand in investigation of these receptors.

Platelet-activating factor; Platelet-activating factor antagonist; Platelet-activating factor receptor; (Neutrophil)

1. INTRODUCTION

Platelet-activating factor (PAF) is a potent mediator of inflammation generated by platelets, neutrophils, eosinophils, macrophages and vascular endothelial cells. It shows several pharmacological actions such as aggregation of platelets and neutrophils, bronchoconstriction, hypotension, increase in vascular permeability and chemotaxis of inflammatory cells [1,2].

There is much evidence to suggest the existence of specific receptors for PAF on the plasma membranes of target cells [3]. Specific binding of PAF has been reported in membrane preparations [4] as well as in various tissues and cells, including human neutrophils [5]. Binding studies with [^{3}H]PAF have often proved difficult, especially with leukocytes, due to the binding of the radioligand to low-affinity, high-capacity binding sites and the uptake and metabolism of the radioligand [5]. It has been hoped that the development of specific PAF antagonists may overcome these difficulties and a number of antagonists have been developed recently.

The thieno-triazolodiazepine WEB 2086 has been shown to be a potent and specific PAF antagonist both in vitro and in vivo [6]. Using the tritiated form of this compound we have successfully identified PAF receptors in human platelets [7]. In the presence study we have investigated WEB 2086 as an antagonist of PAF-induced degranulation of human neutrophils (measured as β-glucuronidase release) and as a radioligand to label PAF receptors in the same cells.

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Abbreviations: BSA, bovine serum albumin; PAF, platelet-activating factor

2. MATERIALS AND METHODS

Leukocytes were obtained from acid citrate dextrose anticoagulated blood of normal healthy volunteers as previously described [8] and resuspended in Hepes-BSA buffer consisting

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of Hank's balanced salt solution supplemented with 20 mM Hepes and 3.5 g/l bovine serum albumin (BSA), pH 7.2. For radioligand-binding studies cells were suspended in the binding assay buffer described previously [7].

Neutrophil suspensions (7.5×10^6 cells/ml, 97 ± 0.4% pure), pre-incubated at 37°C for 10 min in the presence of 5 μg/ml cytochalasin B, were incubated in a final volume of 1 ml Hepes-BSA buffer containing PAF, or alternative stimuli, at the indicated concentrations, with or without WEB 2086 or the PAF-antagonist BN 52021 [9] at 37°C for 20 min. Reactions were halted by centrifugation at 9000 × g for 2 min. β-Glucuronidase was assayed in the cell supernatants by a modification of the method previously described [8] using p-nitrophenyl-β-D-glucuronide as substrate. Absorbance was measured at 399 nm and β-glucuronidase activity was expressed as a percentage of the total cell content of the enzyme, defined as activity released from cells treated with 0.1% Triton X-100 (v/v) for 20 min at 37°C. The cytoplasmic enzyme lactate dehydrogenase (LDH) was assayed in supernatants as previously described [8].

Binding of [^{3}H]WEB 2086 to human neutrophils was measured by a procedure similar to that described for human platelets [7]. 5×10^6–1.5×10^7 cells were added to a final volume of 1 ml of binding assay buffer containing the radioligand at the indicated concentrations and incubated with continuous shaking at 25°C for 90 min. In competition experiments the final concentration of [^{3}H]WEB 2086 was 20 nM. Other substances were added as indicated. Nonspecific binding was determined in the presence of 10 μM WEB 2086 or 1 μM PAF. All incubations were conducted in triplicate. Bound and free radioligands were separated by rapid filtration through Whatman GF/C glass fibre filters pre-soaked in 1% BSA for 1 h. Filters were then washed twice with 4 ml of ice-cold binding assay buffer.

3-[4-(Chlorophenyl)-9-methyl-H-thieno[3,2-f][1,2,4]triazolo-[4,3-a][1,4]diazepin-2-yl](4-morpholinyl)-1-propanone (WEB 2086) and [^{3}H]WEB 2086 solution (15 Ci/mmol) were kindly donated by Boehringer Ingelheim (Ingelheim am Rhein, FRG). PAF and lyso-PAF (both hexadecyl form) were purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). BN 52021 was a gift from Dr P. Braquet (Ipsen Institute for Therapeutic Research, Le Plessis Robinson, France). Ficoll-Paque was purchased from Pharmacia Laboratory Separation Division, Uppsala, Sweden. All other chemicals were from standard commercial sources.

3. RESULTS

PAF induced a concentration-dependent release of β-glucuronidase from human neutrophils with an EC_{50} of 138 nM (geometric mean, 95% confidence limits 55–346 nM, n = 6) whilst the inactive precursor and metabolite lyso-PAF caused no significant release at concentrations up to 10 μM. LDH release was in no case greater than 10%, indicating no significant cytolysis. WEB 2086 and BN 52021 caused parallel rightward shifts of the PAF concentration-response curve (fig.1); Schild analysis of the shifts gave a K_B value for WEB 2086 of 11.9 nM (6.7–21 nM, n = 3) and for BN 52021 of 359 nM (n = 2). WEB 2086, at a concentration of 10 μM, did not inhibit degranulation induced by PMA or fMLP.

Specific binding of [^{3}H]WEB 2086 to human neutrophils was maximal at 25°C and declined at higher as well as lower temperatures. In the absence of any competing drug the isotherm con-

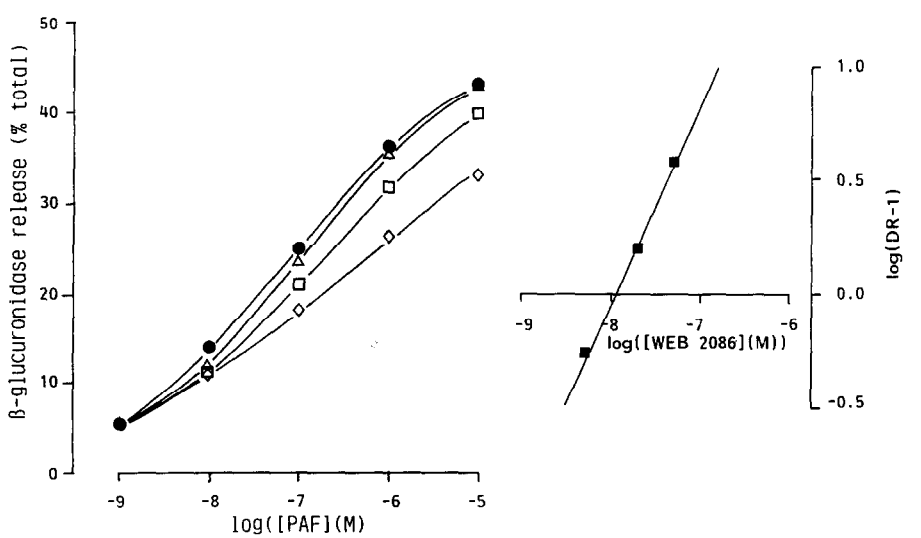

Fig.1. PAF-induced β-glucuronidase release from human neutrophils in the absence of antagonist (●) and in the presence of WEB 2086: 5 nM (△), 20 nM (□) and 50 nM (◇). Inset: Schild plot of the same data. Data are from one representative experiment (mean results given in text).

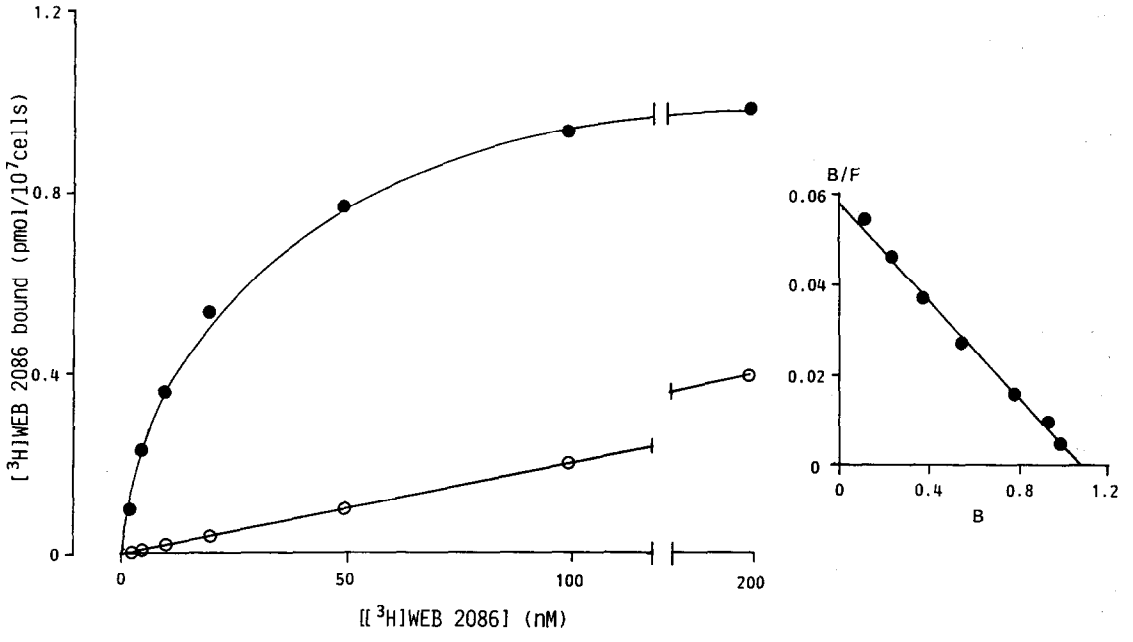

Fig.2. Specific (●) and nonspecific (○) binding of [^{3}H]WEB 2086 to human neutrophils. Inset: Scatchard plot of the same data. Data are from one representative experiment (mean results given in text).

formed to a simple hyperbola (fig.2). The derived Scatchard plot was linear, indicating a homogeneous population of non-interacting binding sites. The K_D was 18.9 ± 0.6 nM (arithmetic mean ± SE, n = 5) and the B_{max} was 670 ± 34 fmol/10^7 cells, corresponding to 40000 ± 2000 sites/cell. The ratio of the calculated rate constants for dissociation (K_2 = 1.39 min^{-1} at 25°C) and association ($K_1 = 9.3 \times 10^7$ $min^{-1} \cdot M^{-1}$) of [^{3}H]WEB 2086 with human neutrophils gave a kinetically derived estimate of K_D of 15 nM. From competition experiments the K_I of WEB 2086 for inhibition of [^{3}H]WEB 2086 binding was 16.3 nM (11.3–23.7 nM, n = 3) and the slope factor (n_H) of the competition curves was 0.98 ± 0.03, indicating again homogeneity of the binding sites. BN 52021 competed for [^{3}H]WEB 2086-binding sites with a K_I of 300 nM. The K_I value of PAF was 170 pM (110–260 pM, n = 3), whereas lyso-PAF did not compete for [^{3}H]WEB 2086-binding sites at concentrations up to 10 μM.

4. DISCUSSION

In the present report we have shown that WEB 2086 is a specific and competitive antagonist of PAF-induced azurophil granule enzyme release from human neutrophils with an antagonist affinity constant (K_B) of 11.9 nM against PAF ad shows no antagonistic action against two other stimuli of neutrophil degranulation (PMA and fMLP). We have also described direct radioligand binding of [^{3}H]WEB 2086 to specific sites on these cells: the dissociation constant (K_D) calculated from [^{3}H]WEB 2086 saturation isotherms was 18.9 nM and the kinetically derived K_D was 15 nM. The inhibition constant (K_I) for displacement of [^{3}H]WEB 2086 from its binding sites by WEB 2086 was 16.3 nM, which is close to both the K_B and K_D values. The K_B of 359 nM for BN 52021 is also similar to the K_I of 300 nM. The measures of WEB 2086 affinity from functional and radioligand-binding experiments, therefore, are in close agreement.

Direct radioligand-binding studies using [^{3}H]-PAF have identified a high-affinity PAF-binding site with a K_D of 220 pM and B_{max} corresponding to 1100 sites/cell [5]. We have demonstrated a PAF antagonist-binding site for which PAF has a K_I of 170 pM which is in close agreement with the K_D for [^{3}H]PAF. However, our B_{max} of 40000 sites per cell is markedly different from the reported density of [^{3}H]PAF-binding sites. It is possible that

the much lower density of binding sites detected with [^{3}H]PAF may reflect a down-regulation of receptors after incubation with the radiolabelled agonist.

The selectivity of the PAF antagonism by WEB 2086, the specificity of the binding of [^{3}H]WEB 2086 and the concordance of the indices of receptor affinity of WEB 2086 obtained from functional and radioligand-binding studies lead us to conclude that, in human neutrophils, WEB 2086 exerts its PAF antagonistic actions through direct interaction with the PAF receptor. WEB 2086 and the specific antagonist radioligand [^{3}H]WEB 2086 will prove useful in efforts to elucidate the physiological role and mechanism of action of PAF.

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