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Radioligand binding of antagonists of platelet-activating factor to intact human platelets

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Two new antagonists of platelet-activating factor (PAF), the pyrrolothiazole derivative 52770 RP and the triazolodiazepine WEB 2086, have been studied as radioligands in intact human platelets. [^{3}H]52770 RP and [^{3}H]WEB 2086 bound specifically to high-affinity sites with dissociation constants (K_d) of 14.8 and 6.1 nM, respectively. The maximal number of sites for [^{3}H]52770 RP binding was approx. 15-fold higher than for [^{3}H]PAF and [^{3}H]WEB 2086. In addition, C_{16}-PAF, lyso-PAF, WEB 2086 and 52770 RP had K_i values which were nearly identical for both [^{3}H]PAF and [^{3}H]WEB 2086, whereas only 52770 RP competed for [^{3}H]52770 RP-binding sites. These results demonstrate that in human platelets the sites of [^{3}H]WEB 2086 binding are identical to [^{3}H]PAF-binding sites, whereas those of [^{3}H]52770 RP are not. [^{3}H]WEB 2086 appears, therefore, to be a suitable antagonist radioligand for labelling PAF receptors.

Platelet-activating factor; PAF antagonist; PAF receptor; Platelet

1. INTRODUCTION

Platelet-activating factor [PAF, 1-*O*-alkyl-2(*R*)-acetyl-*sn*-glycero-3-phosphorylcholine] has been implicated in several pathophysiological states including allergic inflammation, anaphylactic shock and asthma [1]. PAF induces platelet and neutrophil aggregation, bronchoconstriction, hypotension and increased vascular permeability. Of particular interest is the unique ability of PAF to cause a sustained increase in bronchial responsiveness, which is a hallmark of asthma.

Evidence is accumulating that PAF produces its various effects on target cells through activation of specific membrane receptors. By using [^{3}H]PAF as a radioligand it has been possible to demonstrate high-affinity specific binding sites on human and rabbit platelets, human neutrophils and human lung membranes [2]. Binding studies with [^{3}H]PAF have often proved difficult because of the high level of nonspecific binding and the metabolism and uptake of the radioligand. Therefore, the development of specific PAF antagonists is necessary for further elucidation of the physiological role and mode of action of this mediator. [^{3}H]Dihydrokadsurenone was the first radiolabelled antagonist used to characterise PAF receptors in rabbit platelet membranes [3]. Recently, two new radiolabelled antagonists have been introduced: the pyrrolothiazole derivative 52770 RP, which has been shown to label PAF receptors on rabbit platelets [4], and the triazolodiazepine, WEB 2086 [5]. Here, we have investigated the binding of [^{3}H]PAF and of the antagonist radioligands [^{3}H]WEB 2086 and [^{3}H]52770 RP to intact human platelets.

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

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2. MATERIALS AND METHODS

Human venous blood was collected into a tube containing 1/8 vol. citrate anticoagulant and centrifuged at 160 × *g* for 20 min at 22°C. The platelets were isolated by gel filtration on Sepharose CL-2B as described [6] and resuspended in Hepes buffer (10 mM Hepes, 145 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 0.5 mM Na_2HPO_4, 6 mM glucose, 0.1% BSA; pH 7.4).

Binding of the radioligands was measured in a final volume of 1 ml containing Hepes buffer, the radioligands at the indicated concentrations and 0.4–1.6 × 10^8 platelets. In competition experiments the final concentrations of the radioligands were: [^{3}H]PAF, 0.03 nM; [^{3}H]WEB 2086, 10 nM; [^{3}H]52770 RP, 2.5 nM. Other substances were added as indicated. Triplicate incubations were carried out at 25°C for 90 min. Bound and free radioligand were separated by rapid filtration through Whatman GF/C glass fibre filters, pre-soaked in 1% BSA for 60 min. Filters were then washed twice with 4 ml ice-cold incubation buffer.

[^{3}H]PAF (spec. act. 120 Ci/mmol) was purchased from Amersham International, *N*-(3-[2-^{3}H]chlorophenyl)-3-(3-pyrridinyl)-1H,3H-pyrrolo[1,2-*c*]thiazole 7-carboxamide ([^{3}H]52770 RP, spec. act. 29 Ci/mmol) was kindly donated by Rhône-Poulenc Santé (Vitry-sur-Seine, France). 3-[4-([2-^{3}H]Chlorophenyl)-9-methyl-H-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-2-yl] (4-morpholinyl)-1-propanone ([^{3}H]WEB 2086, spec. act. 14 Ci/mmol) was kindly provided by Boehringer Ingelheim (FRG). C_{16}-PAF and lyso-PAF were obtained from Bachem (Torrance, CA). Other substances were from standard commercial sources.

3. RESULTS

A representative saturation isotherm for [^{3}H]52770 RP binding at 25°C is shown in fig.1. Non-specific binding increased linearly with [^{3}H]52770 RP concentration whilst specific [^{3}H]52770 RP binding was saturable with increasing concentrations of the radioligand. Nonlinear curve fitting of the data gave a K_d of 14.8 nM for [^{3}H]52770 RP binding. The derived Scatchard plot of the data was linear, indicating a homogeneous population of non-interacting binding sites with a binding capacity (B_{max}) of 3750 binding sites per cell (table 1). In kinetic experiments, both association and dissociation appeared to be monophasic,

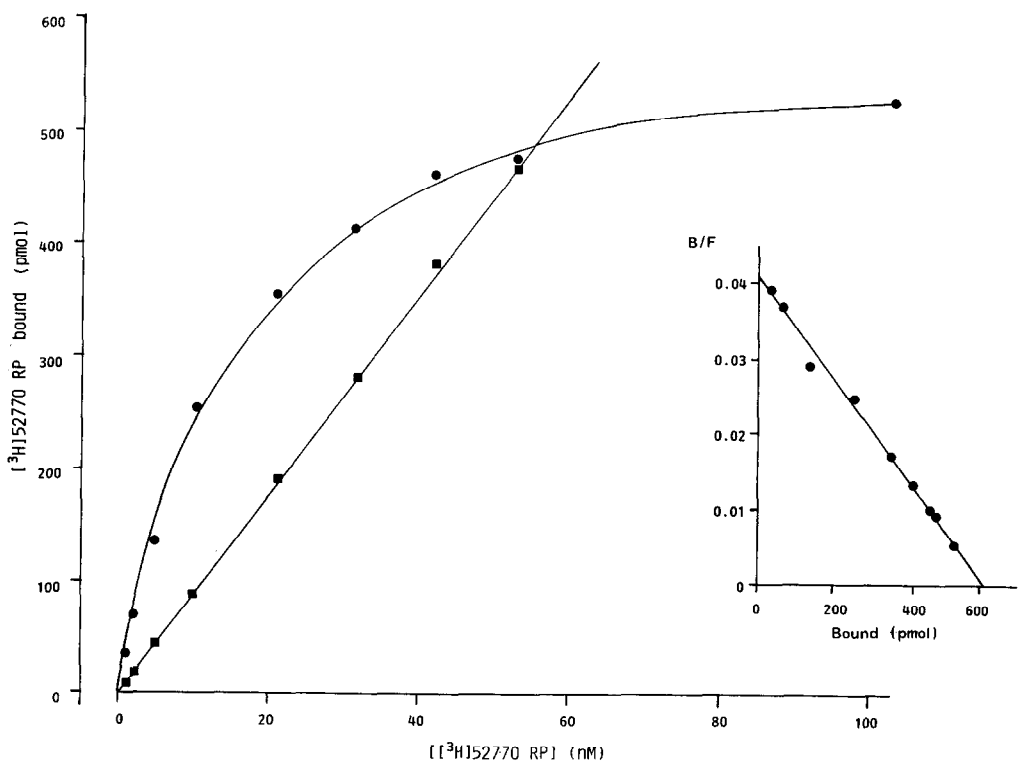

Fig.1. Saturation of specific (●) and nonspecific (■) [^{3}H]52770 RP binding to intact human platelets (9.76 × 10^7 per assay). Nonspecific binding was determined in the presence of 10 μM 52770 RP. (Inset) Scatchard plot of the same data. Data are means from triplicate experiments.

Table 1

Characteristics of radioligand binding to intact human platelets

Radioligand	K_d (nM)	Number of sites per cell
[^{3}H]PAF	0.016	240
[^{3}H]WEB 2086	6.1	260
[^{3}H]52770 RP	14.8	3750

Data are taken from figs 1–3

confirming the homogeneity of the binding sites (not shown); from the data an association constant K_{+1} of 0.92×10^8 $min^{-1} \cdot M^{-1}$ and a dissociation constant K_{-1} of 0.94 min^{-1} were calculated, giving a kinetic K_d of 10 nM, which is in good agreement with K_d determined in the saturation experiments.

The saturation isotherm for [^{3}H]WEB 2086 binding to human platelets is shown in fig.2. The K_d for [^{3}H]WEB 2086 binding is 6.1 nM with a B_{max} of 260 binding sites per cell.

The saturation of [^{3}H]PAF binding to human platelets in the absence and presence of either 100 nM 52770 RP or 100 nM WEB 2086 is shown in fig.3. The presence of either PAF antagonist causes considerable flattening of the saturation curves. As determined by the Scatchard plot, the B_{max} value for [^{3}H]PAF binding (240 sites/cell) was almost identical to that determined by [^{3}H]WEB 2086 binding (260 sites/cell). The K_d and B_{max} values for [^{3}H]PAF binding are very similar to those reported in [7]. The K_d values of [^{3}H]PAF binding are 0.016 nM in the absence of any antagonist and 0.155 and 0.209 nM in the presence of 100 nM 52770 RP and 100 nM WEB 2086, respectively. These shifts in K_d values were used to calculate the affinities of the competitors. Calculation of the data according to the Schild equation ($K_B = C/(CR - 1)$, where C denotes the concentration of the competitor and CR the ratio of the apparent K_d values in the presence and absence of the competitor) gives K_B values of 11.5 nM for

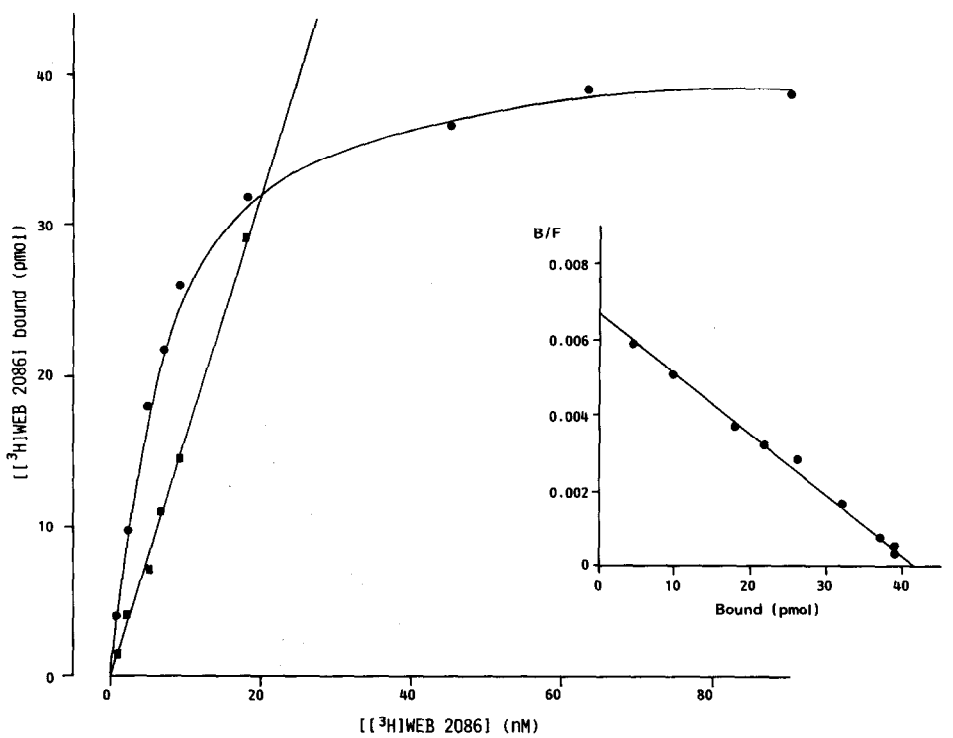

Fig.2. Saturation of specific (●) and nonspecific (■) [^{3}H]WEB 2086 binding to intact human platelets (9.6×10^7 per assay). Nonspecific binding was determined in the presence of 10 μM WEB 2086. (Inset) Scatchard plot of the same data. Data are means from triplicate experiments.

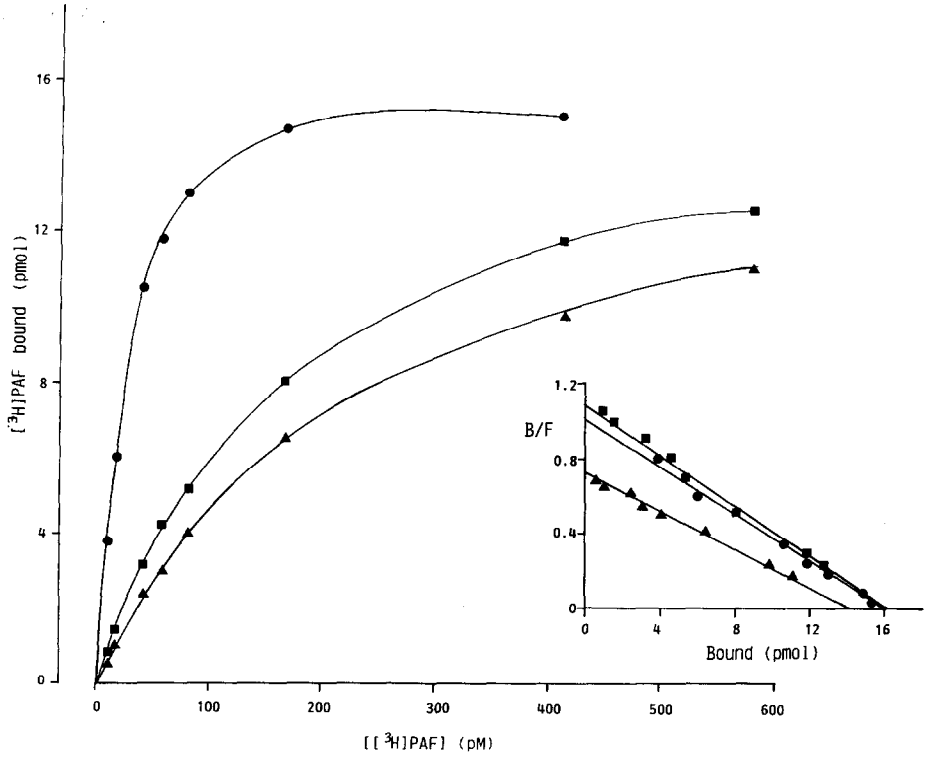

Fig.3. Saturation of [^{3}H]PAF binding to intact human platelets (4×10^7 per assay) in the absence (●) and presence of 100 nM 52770 RP (■) and 100 nM WEB 2086 (▲). (Inset) Scatchard plot of the same data. Data are means from triplicate experiments.

52770 RP and 8.3 nM for WEB 2086. These values are in very good agreement with the K_d values for the binding of the tritiated compounds derived from the saturation experiments.

In order to characterise the radioligand-binding sites, competition experiments were performed. The inhibition of [^{3}H]PAF binding by C_{16}-PAF, 52770 RP and WEB 2086 is shown in fig.4. All compounds attain the same degree of maximal inhibition of [^{3}H]PAF binding. For all compounds the Hill slope factors (n_H) are near unity, suggesting a bimolecular reaction. This also indicates that the inhibition of [^{3}H]PAF binding by these compounds is due to an interaction with the receptor itself. The K_i values of the compounds are close to the corresponding K_d values (table 2). Lyso-PAF, which is devoid of significant biological activity via PAF receptors, only weakly inhibited specific [^{3}H]PAF binding. Nearly identical K_i values were obtained for inhibition of [^{3}H]WEB 2086 binding, demonstrating the identity of [^{3}H]PAF- and [^{3}H]WEB 2086-binding sites. In contrast, only 52770 RP inhibited [^{3}H]52770 RP binding, whereas C_{16}-PAF, lyso-PAF and WEB 2086 had no effect on [^{3}H]52770 RP binding at concentrations up to 10 μM.

4. DISCUSSION

The pyrrolothiazole derivative [^{3}H]52770 RP and the thienotriazolodiazepine [^{3}H]WEB 2086 have been introduced recently as new antagonist radioligands for PAF receptors. From competition experiments as well as from saturation experiments for [^{3}H]PAF binding to intact human platelets reported here it is evident that both compounds act as competitive receptor antagonists. In direct radioligand-binding experiments both compounds bind specifically to high-affinity sites on human platelets. C_{16}-PAF, 52770 RP and WEB 2086 have K_i values which are nearly identical for inhibition of both [^{3}H]PAF and [^{3}H]WEB 2086 binding. In

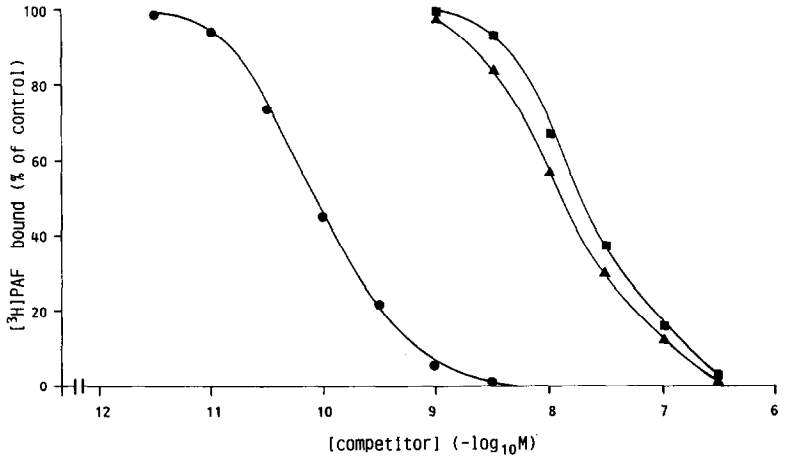

Fig.4. Inhibition of [^{3}H]PAF binding to intact human platelets by C_{16}-PAF (●), WEB 2086 (▲) and 52770 RP (■). Slope factors (n_H) are 1.0 for C_{16}-PAF, 1.02 for WEB 2086 and 1.06 for 52770 RP. Data are means from triplicate experiments.

Table 2

Inhibition of radioligand binding to intact human platelets

Compound	K_i (nM)		
	[^{3}H]PAF	[^{3}H]WEB 2086	[^{3}H]52770 RP
C_{16}-PAF	0.024 (0.010–0.053)	0.019 (0.012–0.029)	>10000
Lyso-PAF	>10000 (30%)[a]	>10000 (19%)[a]	>10000
WEB 2086	9.9 (3.1–31.2)	5.3 (4.1–7.0)	>10000
52770 RP	13.8 (6.5–29.4)	14.5 (8.0–26.4)	18.9 (12.0–29.9)

[a] % inhibition at 10 μM

Data are presented as geometric means with 95% confidence limits from three experiments

contrast, only 52770 RP competed for [^{3}H]52770 RP-binding sites, while both PAF and WEB 2086 have no affinity for [^{3}H]52770 RP-binding sites. In addition, the B_{max} value for [^{3}H]52770 RP binding is about 15-fold higher than those of [^{3}H]PAF and [^{3}H]WEB 2086 binding. These results clearly demonstrate that in human platelets the binding sites of [^{3}H]WEB 2086 are identical to those of [^{3}H]PAF binding, while those of [^{3}H]52770 RP are not. [^{3}H]WEB 2086 appears, therefore, to be a suitable antagonist radioligand for labelling PAF receptors.

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