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CHARACTERIZATION OF SPECIFIC BINDING SITES FOR PAF IN THE IRIS AND CILIARY BODY OF RABBIT

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The protective effect exerted by BN 52021 a specific PAF-receptor antagonist in experimentally induced ocular inflammatory disorders led us to investigate the possible presence of specific receptors for PAF in rabbit iris and ciliary body. Two classes of PAF binding sites were found in isolated iris and ciliary process of pigmented rabbit eyes : a high affinity site $Kd_1 \simeq 4.9 \pm 0.47$ nM, $Bmax_1 \simeq 3.17 \pm 0.50$ pmoles/mg protein, a low affinity sites $Kd_2 \simeq 11.6 \pm 0.33$ nM, $Bmax_2 \simeq 12.46 \pm 2.3$ pmoles/mg protein for iris. The specific binding was not affected by lyso-PAF the biologically inactive precursor and metabolite of PAF, up to 10^{-6}M ; inhibition by unlabelled PAF demonstrated a biphasic curve partially antagonized by BN 52021. The present results demonstrate the presence of specific binding sites for PAF in rabbit eyes which could mediate the action of this mediator in eye inflammatory processes and explain the protective effect observed with BN 52021.

Platelet activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycerol-phosphorylcholine) is an ether-linked phospholipid involved in various inflammatory, cardiovascular and respiratory disorders (1, 2, 3). PAF is synthetized and released from platelets (4), neutrophils (5), macrophages (6), (7) and endothelials cells (8) when appropriately stimulated. It induces aggregation and exocytosis in platelets and neutrophils, leukopenia and thrombocytopenia. In vivo PAF also increases vascular permeability, smooth muscle contraction and induces hypotension, bronchoconstriction and renal failure (9,10).

The availability of specific PAF antagonists has allowed the investigation of its proposed role in pathophysiological models of inflammation.

One such compound, the ginkgolide BN 52021, has been described as a potent and selective PAF antagonist for PAF induced platelet and neutrophil aggregation (11) and has been demonstrated to exert a protective effect in

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various experimental models including endotoxin shock (12,13) pulmonary and cardiac anaphylaxis (14,15), brain ischemia (16,17).

Recently in rabbit eye, BN 52021 has been shown to block leukocyte infiltration and edema formation in the cornea, induced by immune keratitis (18), and to prevent the acute rise of intraocular pressure (IOP) and breakdown of the blood aqueous barrier after laser irradiation (19).

Furthermore, in alloxan-induced diabetic rats the dramatic and deleterious ex vivo decrease in the amplitude of the beta-wave of the electroretinogram (ERG) is also inhibited by BN 52021 (20).

These observations led us to examine the possible presence of specific PAF recognition sites in rabbit eye. The present study characterized PAF binding sites using ^{3}H-PAF and BN 52021 in two of the main areas implicated in eye inflammation, the iris and ciliary body.

MATERIALS AND METHODS

Materials

Synthetic tritiated PAF (^{3}H-PAF) with a specific activity of 59.5 Ci per mmole was purchased from New England Nuclear (Dupont de Nemours, France). Unlabelled PAF and lyso-PAF were purchased from Calbiochem (France), solubilized in ethanol and stored at - 20° C. BN 52021 was solubilized in DMSO. Pentobarbital (Clin-Midy), heparin (Roche) were purchased from Coger Industries (France).

Tissue preparation

Pigmented rabbits (either sex) were injected with heparin (1 000 UI per rabbit) 5 minutes prior to sacrifice by an overdose of pentobarbital. Eyes were enucleated and placed into ice cold 50 mM tris-HCl buffer pH 7.4, containing 10 mM $MgCl_2$, 2 mM EDTA, 50 UI per ml trasylol and 10^{-4}M PMSF. The eyes were arterially perfused prior to dissection in order to remove the blood of anterior segment. The iris and ciliary body were carefully dissected out and homogenized using a dounce homogenizer. The homogenates were filtered through 2 layers of gauze and centrifugated twice at 40 000 g for 20 minutes.

The pellets were resuspended in the homogenization buffer and assayed for protein content using BIORAD protein assay reagent and bovine serum albumin as the standard.

The binding assay

40 to 70 µg of protein was added to a final volume of 1 ml of 10 mM tris HCl buffer, containing $MgCl_2$ 5 mM, BSA 0.025 %, trasylol 50 UI per ml, PMSF 10^{-4}M, pH 7 in plastic tubes containing ^{3}H-PAF (10^{-9}M) with or without 10^{-6}M unlabelled PAF (for nonspecific binding), lyso-PAF or the PAF-receptor antagonist BN 52021.

The incubation was carried out for 40 minutes at 25° C. The unbound ligand was separated from the bound by immediate filtration through Whatman GF/B fiber-Filters presoaked 24 hrs in the binding buffer and using the BRANDEL vacuum system (Brandel, Biomedical Research and Development Laboratories Gathersburg MD/USA). The assay tubes and filters were washed 3 times with 3 ml of precooled binding buffer.

The filters were placed into polyethylene vials containing 10 ml of liquid scintillation fluid (instagel, Packard). The radioactivity was measured using a 45 % efficiency LKB counter.

The specific binding was calculated as previously described (21). Binding data were analyzed by Scatchard method using the computerized programm of VINDIMIAN et al. (22).

In some experiments the stability of the tritiated ligand was analysed after 40 minutes incubation and extraction from the reaction mixture using the solvent system, chloroform : methanol, 2 : 1 and separation on T.L.C. silica plates (Prolabo SIL G_{25} UV 250) using a chloroform : methanol : ammonia, 70 : 35 : 7. system.

In our binding conditions intact ^{3}H-PAF represented 93 % of the total radioactivity recovered from the plates.

RESULTS

Binding of ^{3}H-PAF

- **Saturation study** : ^{3}H-PAF specific binding was found in cornea, iris and ciliary body and was characterized in the latter two. The specific binding as a function of protein concentration was linear in the range of 10 to 90 µg of protein both in iris and ciliary body, and was about 20 % of the total binding. Study of the specific binding as a function of increasing concentrations of ^{3}H-PAF (from 8 10^{-10} to 1,4 x 10^{-8}M) demonstrated a biphasic saturation isotherm (Figure 1). The first step reached a maximum at 4-5 nM and a second step was observed at 10.8 nM.

- **Competition study** : The specificity of the binding to iris and ciliary body was assessed by determining the ability of unlabelled PAF, lyso-PAF and the PAF receptor antagonist BN 52021, to compete with ^{3}H-PAF binding. As

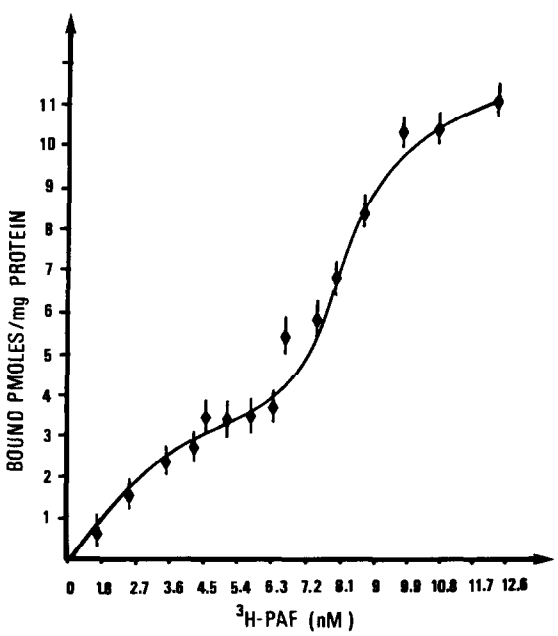

Figure 1 - Typical saturation isotherm for ^{3}H-PAF binding in iris at 25°C. The binding was performed over radioligand concentration range of 1 nM to 13.5 nM. Each point is the average of triplicate and n = 4. The assay was performed in tris 10 mM, $MgCl_2$ 5 mM, PMSF 10^{-4}M, trasylol 50 UI per ml and BSA 0.025 % pH 7 buffer.

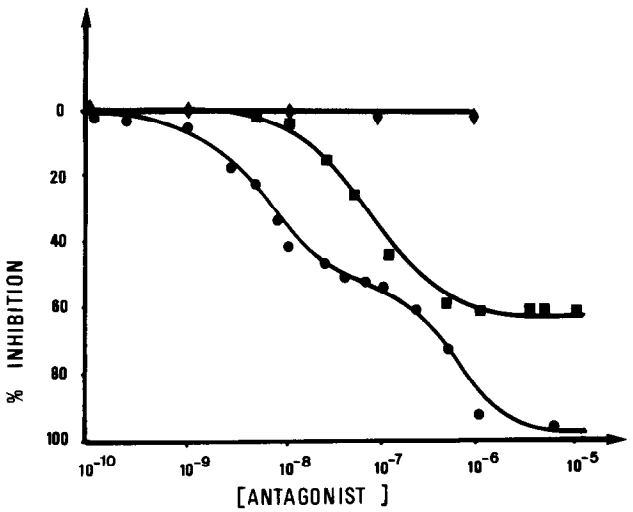

Figure 2 - Inhibition of ^{3}H-PAF (1-3 nM) binding to iris homogenate by unlabelled PAF (●—●), BN 52021 (■—■) and lyso-PAF (◆—◆). Each point is the average of triplicate determinations ; n = 4.

shown in figure 2, no displacement was observed with lyso-PAF up to 10^{-6}M. A biphasic displacement curve was observed with unlabelled PAF and ^{3}H-PAF binding was partially displaced (60 %) by BN 52021.

Scatchard plot analysis of the binding data from both saturation and competition studies demonstrated a curvilinear distribution (Figure 3). A

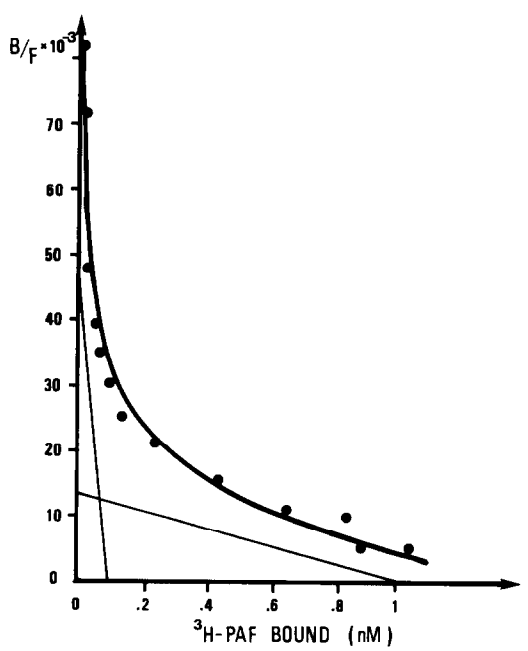

Figure 3 - Scatchard analysis of ^{3}H-PAF binding to iris homogenate in tris 10 mM, $MgCl_2$ 5 mM, PMSF 10^{-4}M, trasylol 50 UI per ml, BSA 0.025 % pH 7 buffer, at 25°C. Each point is the average of triplicate determinations ; n = 4.

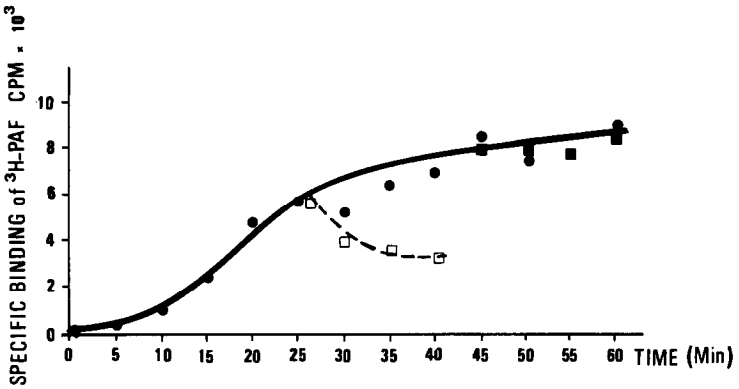

Figure 4 - Typical association and dissociation kinetic curve of ^{3}H-PAF binding in iris or ciliary body. The experiment was performed at 25°C in tris 10 mM, $MgCl_2$ 5 mM, PMSF 10-^{4}M, trasylol 50 UI per ml and bovine serum albumin 0.025 % pH 7 buffer.

high affinity site (Kd_1 = 4,9 ± 0.47 nM $Bmax_1$ = 3.17 ± 0.5 pmole/mg protein and a low affinity site (Kd_2 = 11.6 ± 0.33 nM, $Bmax_2$ = 12.46 ± 2.3 pmole/mg for iris ; n=5).

- **Kinetic study** : As shown by the kinetic curve (Figure 4), the specific binding of ^{3}H-PAF (1-3 nM) to iris and ciliary body reached a steady state within the first 25 minutes of reaction and remained stable up to 60 min at 25° C. An excess of unlabelled PAF (1 000 fold) added to the reaction mixture at 30 min and 45 min of the time course showed a partial reversibility of the binding (about 50 %) at 30 min, whereas no reversibility was observed after 45 min.

DISCUSSION

The ^{3}H-PAF binding studies on rabbit iris and ciliary body reported here demonstrate the presence of PAF recognition sites.

The percent of specific binding in these tissues is low compared to the 70-80 % of specific binding in rabbit platelet membrane in the same buffer conditions, but is comparable to the low specific binding (30-40 %) described for human lung tissue (24) or to the 20-25 % specific binding described for brain tissue (21). It is also possible that melanin, which is present in high concentrations in the crude preparation used for these tissues, might have increased the nonspecific binding.

Specific binding as a function of increasing concentrations of labelled PAF, over the range of 8.10^{-10} to 14.10^{-9}M have shown from replicated experiments, a two step saturation isotherm, suggesting heteregenous binding characteristics. Results of saturation experiments were confirmed by

displacement studies where the two binding steps were easier to describe. Displacement by unlabelled PAF presented a biphasic inhibition curve. The scatchard analysis revealed a curvilinear distribution, which was better described by a two component computerized analysis.

In the iris, the binding characteristics of the first component were Kd_1 = 4.9 ± 0.47 nM and $Bmax_1$ = 3.17 pmol/mg protein and for the second component Kd_2 = 11.6 ± 0.33 nM associated with $Bmax_2$ = 12.46 pmol/mg protein. Similar results were obtained for ciliary body with Kd_1 = 5.7 ± 0.09 nM $Bmax_1$ = 3.41 ± 1 pmol/mg and Kd_2 = 24.4 ± 0.91 nM $Bmax_2$ = 16.6 ± 0.51 pmol/mg.

In our study, the specificity of the binding was assessed by competition experiments using lyso-PAF, the biologically inactive analog of PAF, and BN 52021, a specific PAF receptor antagonist. Lyso-PAF failed to inhibit ^{3}H-PAF binding up to 10^{-6}M. The inhibition curve obtained with BN 52021 noticeably differed from that of PAF since it was monophasic and only partial. In fact, BN 52021 did not inhibit completely ^{3}H-PAF binding in these tissues. The maximum inhibition obtained was about 60 % and appeared to be associated only with the first step of the competition curve. In kinetic experiments, a partial dissociation of the binding (50 %) was observed at 35 min of the time course ; after 45 min incubation the ^{3}H-PAF binding was resistant to dissociation. This property may be attributed to disapearence of PAF binding site associated with a captation of the bound ligand since ^{3}H-PAF in the medium was not significantly degradated in our assay.

The presence of PAF specific binding sites in eyes was expected since several recent studies have demonstrated the participation of PAF in many inflammatory processes in the eye. Local low dose injections of PAF in rat conjunctiva elicitated a marked inflammatory response antagonized by a PAF-receptor antagonist (23). BN 52021 was also reported to inhibit leukocyte infiltration and corneal edema in immune keratitis (18).

Given preventively, the drug also inhibits the rise of intraocular pressure and the subsequent leakage of serum proteins into the aqueous humor due to the disruption of the blood aqueous barrier by laser irradiation (19). PAF is also produced by embryo chick retina upon stimulation with neurotransmitters (25). These results implicate PAF in eye function and pathology and suggest that its effect may be receptor mediated.

Our study shows the coexistence of two subclasses of PAF binding site in rabbit iris and ciliary body, two of the main areas involved in ocular inflammation. How these two classes of binding sites interact with PAF during inflammation processes, and whether these two subclasses represent two distinct types of receptor or two different conformational states of the same receptor site remains however to be clarified.

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