Vol. 190, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1993 Pages 162-166

EVIDENCE FOR THE PRESENCE OF SPECIFIC HIGH AFFINITY CYTOSOLIC BINDING SITES FOR PLATELET-ACTIVATING FACTOR IN HUMAN NEUTROPHILS

Stanislav Svetlov[+] and Santosh Nigam[*]

Department of Gynecology, Klinikum Steglitz, Free University Berlin, Hindenburgdamm 30, D-1000 Berlin 45, Germany

Received November 17, 1992

In this study evidence for the presence of specific cytosolic platelet-activating factor (PAF) binding sites has been presented. The equilibrium dissociation constant (K_d) as determined by Scatchard analysis was 4.29×10^{-9} M for the cytosolic and 3.71×10^{-9} M for the membrane fraction. The maximal number of binding sites estimated were 219 fmol/100 μg protein for the cytosol and 154 fmol/100 μg protein for the membrane, respectively. The specific receptor binding of [^{3}H]PAF to cytosol could be displaced by two potent specific PAF receptor antagonists, BN 50739 and WEB 2086, the equilibration inhibition constants (K_i) being 1.27×10^{-7} M and 5.7×10^{-6} M, respectively. These data demonstrate clearly the role of intracellularly synthesized PAF as a second messenger in the receptor-mediated neutrophil activation.

Platelet-activating factor (PAF), structurally identified as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine [1-3], is a biologically active phospholipid and involved in a variety of pathophysiological processes [4]. PAF is produced by and acts on the different cell populations such as platelets, endothelial cells, neutrophils, monocytes/macrophages including mesangial cells and Kupffer cells [4-9]. The formation of PAF in human neutrophils stimulated by chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) is apparently regulated by protein kinase(s) C, which has been found to be involved in the activation of phospholipase A_2 [10]. The FMLP-activated neutrophils secrete only a part of the PAF synthesized, whereas a major part of it is retained within the cell [11].

It has been generally accepted that the extracellular PAF activates the human neutrophils via binding to its putative receptor. The specific surface receptors for PAF have been characterized on the human neutrophils as well as on the isolated membrane fractions from intact cells by radioligand technique [12-14]. High and low affinity binding sites for [^{3}H]PAF were found in both cases [12,13,15]. Whereas the role of extracellular PAF produced in the neutrophil

+ *On leave from Institute of Pediatric Surgery, Moscow.*

* To whom correspondence should be addressed.

0006-291X/93 $4.00

activation has been studied extensively [7,16,17]. very little attention has been paid to the potential role of endogeneous PAF as intracellular messenger during the cell activation [18-20].

In a recent study, we reported that intracellular PAF can potentiate the activation of phospholipase A_2 via specific intracellular receptor binding sites [11]. This paper demonstrates for the first time the presence of high affinity PAF binding sites in the cytosol of human neutrophils. These cytosolic binding sites are specific and can be competitively displaced with specific PAF receptor antagonists BN 50739 and WEB 2086.

MATERIALS AND METHODS

Materials:

[^{3}H]PAF (1-*O*-[1',2'-^{3}H]alkyl-2-acetyl-*sn*-glycero-3-phospho-choline; sp. act. 40.1 Ci/mmol) was purchased from NEN, FRG. Unlabeled C_{16}-PAF was obtained from Nova Biochem, Switzerland. Ficoll-Paque and Dextran 500 were supplied by Pharmacia, Sweden, and Aprotinin, Pepstatin A, Leupeptin, PMSF and FMLP by Sigma, FRG. BN 50739 was a kind gift from Dr. P. Braquet, IHB Research Laboratories, France and WEB 2086 from Boehringer Ingelheim, FRG. The working solutions of unlabeled PAF and [^{3}H]PAF were prepared by dilution of aliquotes in PBS containing 0.25 % BSA as a carrier. WEB 2086 was dissolved in PBS. BN 50739 was prepared as a stock solution in DMSO and diluted with 50 % DMSO in PBS. Final concentration of DMSO in the samples (< 0.25%) did not affect [^{3}H]PAF binding.

Preparation of subcellular fractions:

Human polymorphnuclear neutrophils were isolated from 40 ml venous blood of healthy volunteers by a discontinuous percoll gradient as described [21]. Washed cells were resuspended in 10 ml HBSS free from Ca^{2+} and Mg^{2+} to a density of 10^7 cells/ml. To the cell suspension (5 ml) were added 1.3 mM Ca^{2+} and 1.0 mM Mg^{2+}. Following equilibration for 10 min at 37^{o}C were added 5 ml of ice-chilled 10 mM HEPES, pH 7.4. The suspension was then quickly transferred into a precooled Parr Bomb (Parr Instruments, USA). Neutrophils were cavitated for 30 min in ice under high pressure of nitrogen. The lysate was centrifuged at 1000 x g for 15 min at 4^{o}C. The so obtained supernatant was further centrifuged at 100.000 x g for 1 h at 4^{o}C. The resulting pellet containing membrane fraction was resuspended in cold PBS solution. The supernatant containing cytosolic fraction was stored at -80^{o}C till analysis. Protein concentration and 5'-nucleotidase activity in the fractions were determined by using commercial kits from Sigma, FRG.

Binding experiments:

The binding of [^{3}H]PAF to isolated membrane and cytosolic fractions was performed in a 24-well immunological plate containing 0.025% bovine serum albumin (BSA). Plates were equilibrated with incubation buffer for 5 min at room temperature before starting the experiment. For saturation experiments binding was initiated by addition of 0.125 - 5.0 nM of [^{3}H]PAF with or without 1000-fold excess of unlabeled PAF as tandem samples and plates were incubated for 60 min. The time-course study was performed with 2 nM [^{3}H]PAF applied in a similar way and incubations were carried out for various times as indicated. For inhibition studies PAF receptor antagonists BN 50739 or WEB 2086 were added at various concentrations 5 min prior to addition of 2 nM [^{3}H]PAF. All experiments were performed in duplicate. The binding was stopped by rapid aspiration of the medium and the wells washed 3 times with 0.5 ml PBS containing 0.025% BSA. The plates were then dried under reduced pressure for 10 min. The bound [^{3}H]PAF was extracted by addition of 0.5 ml of methanol and keeping for 30 min at room temperature. An aliquot was transferred to scintillation vials, mixed with 10 ml of cocktail (Ultima Gold, Packard, FRG) and the radioactivity was counted in a Beckman Counter LS 1801, USA. The amounts of [^{3}H]PAF bound were corrected for volumes and calculated per 100 μg protein.

The non-specific binding was defined as the total binding in the presence of 1000-fold excess unlabeled PAF. The specific binding was calculated as difference between total and non-specific binding. All results obtained in the saturation, inhibition and time-course experiments were analyzed by using a non-linear regression programm Inplot 4 (GraphPad Software, U.S.A.). The

apparent dissociation constant K_d and the maximal binding B_{max} were determined from binding isotherms by non-linear regression analysis of untransformed data and by using Scatchard analysis of experimental data. The percentage of inhibition was defined as 100 x (total binding - total binding with antagonist)/specific binding as described previously [22]. The IC_{50} values were determined from direct Hill plot. Equilibrium inhibition constant K_i was calculated by using the Cheng & Prusoff equation [23]: $K_i = IC_{50}/(1+L/K_d)$, where L is the concentration of labelled ligand and K_d the apparent dissociation constant of [^{3}H]PAF binding by the neutrophil fractions.

RESULTS AND DISCUSSION

This report is the first demonstration of specific binding sites for [^{3}H]PAF in cytosol of human neutrophils. Fig. 1A and 1B show that a substantial non-specific binding for [^{3}H]PAF, which accounted 40-60 % of the total binding, was obtained for both cytosolic as well as membrane fractions from human neutrophils. The specific binding was not fully saturated up to a concentration of 5 nM [^{3}H]PAF. The time-courses for the specific binding of [^{3}H]PAF fit in the exponential association curves with $t_{1/2}$ = 14.3 min for the membrane and 15.4 min for the cytosolic fraction (Figs. 1A & 1B: Insets). The Scatchard analysis shown in Fig. 2 estimated the dissociation constant K_d 3.71 x 10^{-9} M for the membrane and 4.29 x 10^{-9} M for the cytosolic fraction, respectively, which are indicative of high affinity PAF binding sites. The maximal number of binding sites B_{max} was 154 fmol/100 μg protein for the membrane and 219 fmol/100 μg protein for the cytosol, respectively. The competition experiments revealed that BN 50739 and

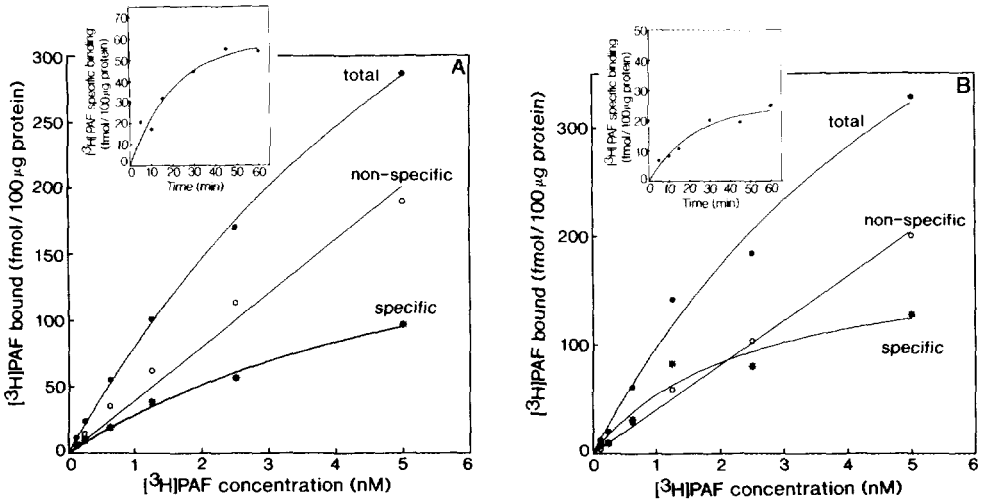
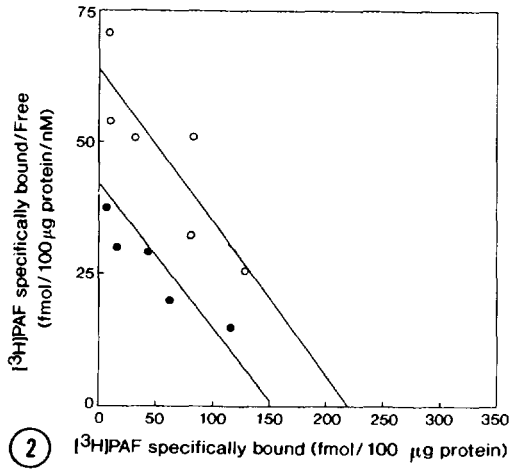


Figure 1: Binding isotherm of [^{3}H]PAF to human neutrophil membrane (A) and cytosol (B)

Membrane and cytosolic proteins (around 100 μg) were incubated in a 24-well immunological plate for 60 min at room temperature with increasing concentrations of [^{3}H]PAF (0.125-5.0 nM). Specific binding (*) is calculated as the difference between total (•) and non-specific (o) binding determined in presence of 1000-fold excess of unlabeled PAF.

Insets: Time-course of association of [^{3}H]PAF to membrane (A) and cytosol (B)

Membrane and cytosolic proteins were incubated for various periods of time with 2 nM [^{3}H]PAF at room temperature. Results represent typical binding isotherm from three separate experiments performed in duplicate.



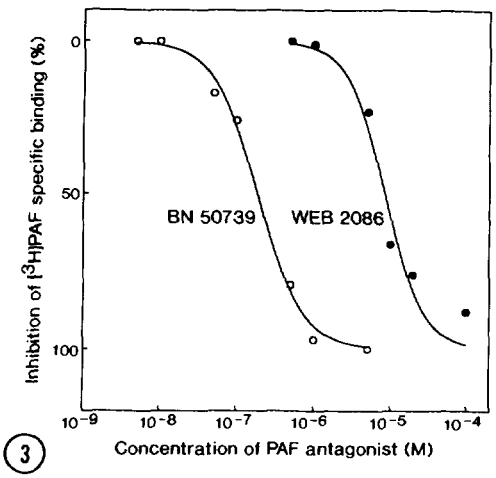


Figure 2: Scatchard analysis of the specific binding of [^{3}H]PAF to human neutrophil membranes (•) and cytosol (o) calculated from binding isotherms
Each point represents the mean of at least three separate experiments performed in duplicate.

Figure 3: Displacement curves by PAF receptor antagonists BN 50739 (o) and WEB 2086 (•) of 2 nM [^{3}H]PAF binding to cytosol of human neutrophils
Cytosolic protein was incubated with 2nM [^{3}H]PAF for 60 min at room temperature. Each point represents the mean of at least three separate experiments performed in duplicate.

WEB 2086, potent specific PAF receptor antagonists, inhibited the specific binding of [^{3}H]PAF to the cytosolic fraction in a dose-dependent manner (Fig. 3), thus demonstrating the specificity of cytosolic binding sites. The K_i values of BN 50739 and WEB 2086 were 1.27×10^{-7} M and 5.7×10^{-6} M, respectively, as calculated from the equation of Cheng and Prusoff [21].

In our study, the B_{max} in the membrane was higher (154 vs 31.3 fmol/100 μg protein), but the affinity of [^{3}H]PAF binding sites K_d was lower (3.71 nM vs 0.47 nM) than those reported by Hwang et al. [15]. The reason for this discrepancy may be due to different experimental techniques and conditions for binding employed in our laboratory. For instance, in most of the studies binding of [^{3}H]PAF was performed in liquid suspensions with subsequent separation of bound and free radioliand by using the filtration technique, which did not allow the examination of cytosolic fraction, because filters trapped only < 5% of applied cytosol [13]. In addition, those membrane preparations contained NADPH:cytochrome C reductase, which shows the inclusion of components of endoplasmatic reticulum and other particulate subcellular fractions [13]. In order to avoid these drawbacks, we investigated the [^{3}H]PAF binding in cytosol and membranes by using the technique of immobilisation on a hard background.

In conclusion, we have demonstrated the presence of specific binding sites for PAF of high affinity in cytosol as well as in the membrane of human neutrophils. Furthermore, the specific receptor binding of [^{3}H]PAF to cytosol could be displaced by two potent specific PAF receptor antagonists, BN 50739 and WEB 2086. We believe that our data added more support to the role of endogenous PAF as a second messenger in the neutrophil activation. However, it is unclear at

present whether these intracellular PAF receptors are a part of membrane associated receptors or they represent an independent cytosolic type of soluble receptors.

ACKNOWLEDGMENTS

Authors acknowledge thankfully the generous support of the Association for International Cancer Research, UK and Foreign Office of the Free University Berlin to conduct this study.

REFERENCES

1. Demopoulos, C.A., Pinckard, R.N. and Hanahan, D.J. (1979) J. Biol. Chem. 254, 9355-9358.
2. Benveniste, J., Tence, M., Bidault, J., Boullet, C. and Polonsky, J. (1979) Compt. Rend. Acad. Sci. Paris 289D, 1037-1040.
3. Blank, M.L., Snyder, F., Byer, L.W., Brooks, B. and Muirhead, E.E. (1979) Biochem. Biophys. Res. Comm. 90, 1194-1200.
4. Prescott, S.M., Zimmermann, G.A. and McIntyre, T.M. (1990) J. Biol. Chem. 265, 17381-17384.
5. Camussi, G., Aglietta, M., Coda, R., Bussolino, F., Piacibello, W. and Tetta, C. (1981) Immunology 42, 191-199.
6. Braquet, P. and Rola-Plesczynski, M. (1987) Immunology Today 8, 345-352.
7. Braquet, P., Touqui, L., Shen, T.Y. and Vargaftig, B.B. (1987) Pharmacol. Rev. 39, 97-145.
8. Hwang, S.B. (1990) J. Lipid Med. 2, 123-158.
9. Wei, C., Heling, L., Hanahan, D.J. and Olson, M.S. (1992) J. Biol. Chem. 267, 6725-6734.
10 Müller, S. and Nigam, S. (1992) Eur. J. Pharmacol. 218, 251-258.
11. Müller, S. and Nigam, S. (1992) Biochem. Biophys. Res. Comm., in press.
12. Valone, F.H. and Goetzel, E.J. (1983) Immunology 48, 141-149.
13. O'Flaherty, J.T., Surles, J.R., Redman, J., Jacobsen, D., Piantadosi, C. and Wykle, R.L. (1986) J. Clin. Invest. 78, 381-388.
14. Marquis, O., Robaut, C. and Cavero, I. (1988) J. Pharmacol. Exp. Ther. 244, 709-715.
15. Hwang, S.B. (1988) J. Biol. Chem. 263, 3225-3233.
16. Handley, D.A. (1988) Drugs of the Future 13, 137-152.
17. Braquet, P., Mencia-Huerta, J.M., Chabriet, P.E., Touqui, L. and Vargaftig, B.B. (1987a) ISI Atlas Sci. 1, 187-198.
18. Stewart, A.G., Dubbin, P.N., Harris, T., and Dusting, G.J. (1990) Proc. Natl. Acad. Sci. USA 87, 3215-3219.
19. Tool, A.T., Verhoeven, A.J., Roos, D., and Koenderman, L. (1989) FEBS-Lett. 259, 209-212.
20. Doebber, T.W., and Wu, M.S. (1987) Proc. Natl. Acad. Sci. 84, 7557-61.
21. Müller, S. and Nigam, S. (1990) J. Lipid Med. 3, 329-341.
22. Hwang, S.B., Cheah, M.J., Lee, C.-S.C. and Shen, T.Y. (1984) Thromb. Res. 34, 519-531.
23. Cheng, Y.C. and Prusoff, W.H. (1973) Biochem. Pharmacol. 22, 3099-3108.