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SPECIFIC BINDING OF PLATELET-ACTIVATING FACTOR (PAF) BY HUMAN PERIPHERAL BLOOD MONONUCLEAR LEUKOCYTES

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Binding of platelet-activating factor (PAF) to human peripheral blood mononuclear leukocytes was time-dependent, reversible, and saturable. [^{3}H]PAF binding to the cells was inhibited dose-dependently by unlabeled PAF and PAF receptor antagonists: L-659,989, triazolam, and alprazolam. Scatchard analysis of saturation binding data indicated one class of receptors for PAF with K_D=5.7 nM and B_{max}=18 fmol/10^6 cells (11,100 receptors/cell). PAF (10 nM) increased intracellular free calcium concentration in human lymphocytes and this effect was inhibited by L-659,989 dose-dependently. Our data suggest that human peripheral blood mononuclear leukocytes have specific receptors for PAF.

Platelet-activating factor (PAF) is a potent phospholipid released by various cell types and it may be involved in pathophysiological conditions such as anaphylaxis and inflammatory reactions (reviewed in ref.1). It appears that interactions of PAF with specific receptors on its target cells will induce biological responses. Specific binding sites for PAF have been demonstrated in platelets (2,3), neutrophils (4,5), and lung membranes (6). Recently some studies have shown that phytohemagglutinin-induced lymphocyte proliferation (7,8) and interleukin 2 production by lymphocytes (7) were inhibited by PAF. Thus it was suggested that PAF may have immunoregulatory functions in the immune system. However, the existence of PAF specific receptors in human lymphocytes has not been clearly defined yet. In this study, we identified specific receptor sites for PAF in human

Abbreviations: PAF, platelet-activating factor, 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine; PBML, peripheral blood mononuclear leukocytes; L-659,989, (±)-*trans*-2-(3-methoxy-5-methylsulfonyl-4-propoxyphenyl)-5-(3,4,5-trimethoxyphenyl)-tetrahydrofuran; HBSS, Hanks' balanced salt solution.

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peripheral blood mononuclear leukocytes and showed that PAF binding to the receptors induced intracellular calcium mobilization.

MATERIALS AND METHODS

Materials

[^{3}H]PAF (specific activity=40.1 Ci/mmol) was purchased from New England Nuclear (Boston, MA), unlabeled PAF and Fura-2/AM from Calbiochem (La Jolla, CA), N-formyl-methionyl-leucyl-phenylalanine and prostaglandin E_1 from Sigma (St. Louis, MO), L-659,989 from Merck Sharp & Dohme Research Laboratories, (Rahway, NJ), triazolam and alprazolam from Upjohn Company (Kalamazoo, MI). All other chemicals were of reagent grade.

[^{3}H]PAF Binding Assay

Peripheral blood mononuclear leukocytes (PBML) were isolated from healthy donors by centrifugation on a Ficoll-Hypaque gradient and contained 85-90% lymphocytes and 10-15% monocytes. They were washed 3X with Hanks' balanced salt solution (HBSS) and resuspended in HBSS containing 0.25% bovine serum albumin (HBSS/BSA). [^{3}H]PAF (1 nM) binding to PBML ($5x10^6$), $\pm$ 1 uM unlabeled PAF, in 1 ml of HBSS/BSA was conducted at 4^0C for 1 hr in a procedure as described for human neutrophils (4). All binding assays were performed in duplicates. In competition binding assays, the cells were preincubated at 4^0C for 15 min with solvent vehicle (control) or PAF antagonists: L-659,989, triazolam, or alprazolam which were all dissolved in DMSO. Final DMSO concentration in incubation was less than 0.2% and did not affect cell viability as determined by trypan blue exclusion technique. Scatchard analysis of data from saturation binding experiments was conducted using the LIGAND-PC program originally developed by Munson and Rodbard (9).

Intracellular Calcium Measurement

Intracellular free calcium levels were monitored by use of the fluorescent calcium chelator, Fura-2 (10). Isolated PBML were loaded with Fura-2 by incubating the cells in HBSS (10^7/ml) with Fura-2/AM (5 uM) at 37^0C for 30 min. The cells were then washed once with HBSS and resuspended in HBSS at $5x10^6$/ml. The cells were equilibrated at 37^0C for 10 min before stimulation. Ten ul of PAF (final concentration=10 nM) was added to two ml of cells in a 4 ml polystyrene curvet and cell fluorescence was monitored in a Perkin-Elmer model MPF-4 fluorescence spectrophotometer, thermostatically controlled at 37^0C. Excitation and emission wavelengths were 340 and 510 nm with 5 nm and 20 nm slits, respectively.

RESULTS AND DISCUSSIONS

The time dependency of the specific binding of [^{3}H]PAF to PBML was shown in figure 1. Specific binding of [^{3}H]PAF (1 nM) to PBML increased progressively over a 30 minute incubation period at 4^0C. Apparent equilibrium of the specific binding was reached after 30 minute. Furthermore, the specific binding was reversible since the addition of excess unlabeled PAF (final concentration=1 uM) to the incubation mixture at t=61 min dissociated bound [^{3}H]PAF from the cells (figure 1). The reversibility of PAF binding to specific binding sites at low temperature have been noted in other cells such as platelets (3) and neutrophils (4).

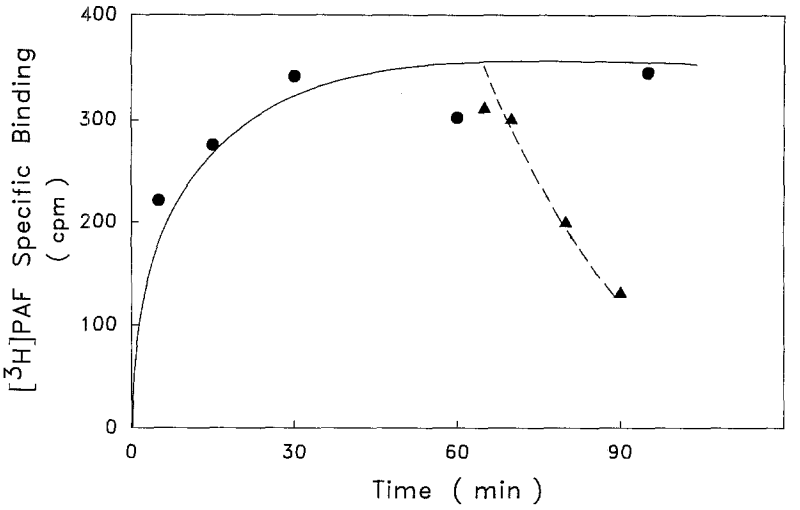

Figure 1 Time course of [^{3}H]PAF (1 nM) binding to human PBML. Broken line indicates [^{3}H]PAF specific binding measured after the addition of unlabeled PAF (final concentration=1 uM) to incubation at t=61 min. The data are representative of 3 separate experiments.

To establish the specificity of PAF binding to PBML, different reagents were tested for their abilities to compete with [^{3}H]PAF for binding to the cells. Prostaglandin E_1 and the chemotactic agent, N-formyl-methionyl-leucyl-phenylalanine, did not affect [^{3}H]PAF binding at concentrations up to 1 uM. However, unlabeled PAF displaced [^{3}H]PAF binding to PBML in a dose-dependent manner (figure 2). In addition, L-659,989, a new PAF receptor antagonist which

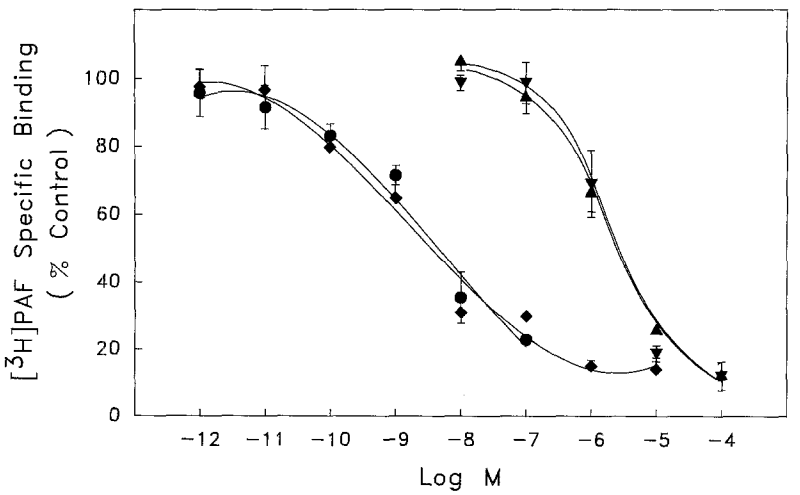

Figure 2 Inhibition of [^{3}H]PAF (1 nM) binding to human PBML by PAF (●), L-659,989 (◆), triazolam (▼), and alprazolam (▲). Results are means ±S.E. from 3 to 4 experiments.

was shown to competitively inhibit PAF binding to its receptors in rabbit and human platelet membranes (11), was a potent inhibitor of PAF specific binding to PBML in our assays. Figure 2 shows that the inhibition curves of PAF and L-659,989 were almost identical. For comparison, two benzodiazepines, triazolam and alprazolam, which have been reported to possess PAF receptor antagonist properties (12,13), were evaluated in the competition binding assays. They were about 1000-fold less potent than L-659,989 as the inhibitor since the calculated equilibrium inhibition constants (K_i) of L-659,989, triazolam, and alprazolam for [^{3}H]PAF binding to PBML are 10.7 nM, 12.7 uM, and 11 uM, respectively. Our data indicate that L-659,989 is indeed a potent antagonist of PAF binding to its specific sites. No effect on cell viability was observed with all the antagonists tested.

The affinity and number of binding sites for PAF in PBML were determined by saturation binding assay. Figure 3 (inset) shows that PAF specific binding to PBML increased with concentration of the labeled ligand and the binding was saturable. Computerized analysis of the binding data from saturation experiments revealed a linear Scatchard plot (figure 3), indicating the presence of a single population of binding sites. The equilibrium dissociation constant (K_D) and the number of binding sites (B_{max}) of this population of PAF binding sites

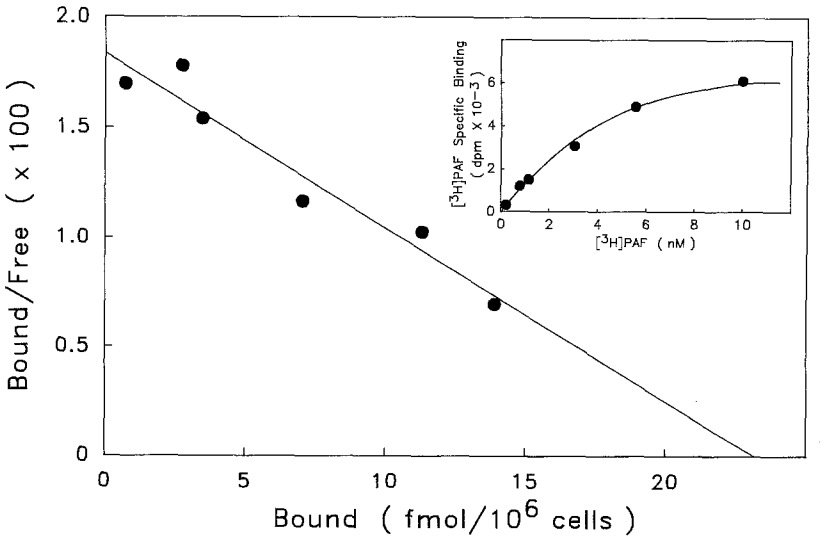

Figure 3 Scatchard plot of [^{3}H]PAF binding to human PBML. (Inset) Saturation binding of [^{3}H]PAF (0.2 to 10 nM) to human PBML. The data are representative of 3 separate experiments.

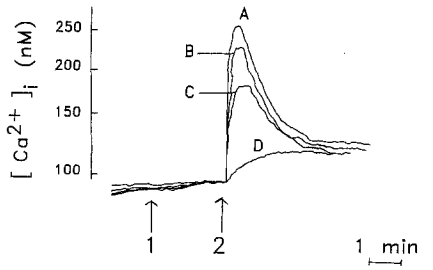

Figure 4 Changes in intracellular free calcium induced by PAF (10 nM) in human PBML pretreated with solvent vehicle (control, A), L-659,989: 1 nM (B), 10 nM (C), and 100 nM (D) 2 min before PAF stimulation. Arrows 1 and 2 indicate the addition of L-659,989 (or vehicle) and PAF, respectively. Results are representative of 3 separate experiments.

were 5.7±0.7 nM (n=5) and 18.5±3.1 fmol/10^6 cells, respectively. In addition, the Hill plot is linear with a Hill coefficient=0.97±0.02 (n=5), suggesting that there is no positive or negative cooperative binding. Assuming an equimolar ligand-receptor complex, a B_{max} of 18.5 fmol/10^6 cells corresponds to about 11,100 receptors/cell. This number of receptors is approximately 5- and 8-fold greater than that found for PAF in human neutrophils (14) and human platelets (2), respectively.

To determine whether PAF specific binding sites on PBML are functionally active, we measure intracellular calcium mobilization in response to PAF using the fluorescent probe Fura-2/AM. Figure 4 shows that PAF at 10 nM induced a rapid rise in intracellular free calcium in PBML. Furthermore, addition of the PAF antagonist, L-659,989, (1 to 100 nM) to the cell suspension 2 min before the PAF stimulation resulted in a dose-related attenuation of the response, indicating that PAF specific receptors in PBML are functionally active and mediate the change in intracellular free calcium concentration. Our results are in accord with the study by Valone who showed that PAF increased intracellular free calcium in $P388D_1$ murine macrophages (14).

In summary, our radioligand binding studies demonstrate the presence of specific receptors for PAF that are funtionally active in human PBML. Our data support previous findings which suggest that PAF may have a potential role in the regulation of immune responses. However, the exact mechanism by which PAF inhibits lymphocyte proliferative response is still unclear. It is interesting to note

that mitogen such as phytohemagglutinin is known to induce phosphatidylinositol 4,5-bisphosphate hydrolysis, inositol 1,4,5-trisphosphate formation and calcium mobilization in lymphocytes (16,17). Present results suggest that PAF may modulate steps distal to early signalling processes. The nature and identity of a second messenger for PAF that may be involved in these regulatory processes remains to be determined.

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REFERENCES

1. Braquet, P., Touqui, L., Shen, T.Y., and Vargaftig, B.B. (1987) Pharmacol. Rev. 39, 98-145.
2. Valone, F.H., Coles, E., Reinhold, V.R., and Goetzl, E.J. (1982) J. Immunol. 129, 1637-1641.
3. Hwang, S.-B., Lee, C.-S.C., Cheah, M.J., and Shen, T.Y. (1983) Biochemistry 22, 4756-4763.
4. O'Flaherty, J.T., Surles, J.R., Redman, J., Jacobson, D., Piantadosi, C., and Wykle, R.L. (1986) J. Clin. Invest. 78, 381-388.
5. Hwang, S.-B. (1988) J. Biol. Chem. 263, 3225-3233.
6. Hwang, S.-B., Lam, M.-H., and Shen, T.Y. Biochem. Biophys. Res. Commun. 128, 972-979.
7. Rola-Pleszczynski, M., Pignol, B., Pouliot, C., and Braquet, P. (1987) Biochem. Biophys. Res. Commun. 142, 754-760.
8. Dulioust, A., Vivier, E., Salem, P., Benveniste, J., and Thomas, Y. (1988) J. Immunol. 140, 240-245.
9. Munson, P.J. and Rodbard, D. (1980) Anal. Biochem. 107, 220-239.
10. Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985) J Biol. Chem. 260, 3440-3450.
11. Ponpipom, M.M., Hwang, S.-B., Doebber, T.W., Acton, J.J., Alberts, A.W., Biftu, T., Brooker, D.R., Bugianesi, R.L., Chabala, J.C., Gamble, N.L., Graham, D.W., Lam, M.-H., and Wu, M.S. (1988) Biochem. Biophys. Res. Commun. 150, 1213-1220.
12. Kornecki, E., Ehrlich, Y.H., and Lenox, R.H. (1984) Science, 226, 1454-1456.
13. Chesney, C.M., Pifer, D.D., and Cagen, L.M. (1987) Biochem. Biophys. Res. Commun. 144, 359-366.
14. O'Flaherty, J.T., Redman Jr., J.F., Schmitt, J.D., Ellis, J.M., Surles, J.R., Marx, M.H., Piantadosi, C., and Wykle, R.L. (1987) Biochem. Biophys. Res. Commun. 147, 18-24.
15. Valone, F.H. (1988) J. Immunol. 140, 2389-2384.
16. Tsien, R.Y., Pozzan, T., and Rink, T.J. (1982) Nature 295, 68-71.
17. Weiss, A., Imboden, J., Hardy, K., Manger, B., Terhorst, C., and Stobo, J. (1986) Ann. Rev. Immunol. 4, 593-619.