Volume 314, number 2, 125–129 FEBS 11845 December 1992

Endotoxin transduces Ca^{2+} signaling via platelet-activating factor receptor

Motonao Nakamura[a,*], Zen-ichiro Honda[a], Iwao Waga[a], Takashi Matsumoto[b], Masana Noma[b] and Takao Shimizu[a]

[a]*Department of Biochemistry, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan* and [b]*Life Science Research Laboratory, Japan Tobacco Inc., 6-2 Umegaoka, Midori-ku, Yokohama, Kanagawa 227, Japan*

Received 2 October 1992

Lipopolysaccharide (LPS) is a pathogenic substance causing severe multiple organ failures and high mortality. Although several LPS binding proteins have been identified, the molecular mechanism underlying the LPS signaling pathway still remains obscure. We have found that the LPS-induced Ca^{2+} increase in platelets and platelet aggregation is blocked by selective platelet-activating factor (PAF) receptor antagonists, thus suggesting a cross-talk between LPS and the PAF receptor. Next, we confirmed this hypothesis using the cloned PAF receptors [(1991) Nature 349, 342–346; (1991) J. Biol. Chem. 266, 20400–20405] expressed in *Xenopus* oocytes and Chinese hamster ovary (CHO) cells. In both systems, cells responded to LPS only when PAF receptors were expressed, and specific PAF binding was successfully displaced and reversibly dissociated by LPS. PAF receptor activation by LPS may represent a novel important pathway in the pathogenesis of circulatory collapse and systemic thrombosis caused by endotoxin.

Platelet-activating factor receptor; Endotoxin; Lipid A; Calcium signaling; Platelet

1. INTRODUCTION

Lipopolysaccharide (LPS), a major lipid constituent of the outer membrane of Gram-negative bacteria, is a potent pathological agent which causes circulatory collapse, systemic thrombosis, multiple organ failures and high mortality [1,2]. Several lines of evidence indicate that an LPS–LPS binding protein (LBP) complex bind to the cell surface CD14 molecule [3,4]. It was also reported that the macrophage scavenger receptor binds and degrades endotoxic lipids [5]. However, little is known of the functional receptor of LPS and its signaling pathway. Several investigators reported that PAF antagonists, such as WEB 2086, CV-3988, BN 50739, and SRI 63-675, are likely to have considerable utility as specific agents for treating cases of endotoxemic shock or disseminated intravascular coagulation [6]. Although there may exist a specific LPS receptor in certain cells [7,8], we propose here that a part of the pathological actions of LPS, such as platelet aggregation [9–12], are due to direct activation of the PAF receptor by LPS.

Correspondence address: T. Shimizu, Department of Biochemistry, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan. Fax: (81) (3) 3813-8732.

*On leave from Life Science Research Laboratory, Japan Tobacco Inc., Japan.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{3}H]PAF (4.23 TBq/mmol) was obtained from Amersham (Tokyo, Japan). The PAF antagonist, Y-24180, was donated from Yoshitomi Pharmaceutical Industries Ltd. (Osaka). WEB 2086 was from Boehringer-Ingelheim (Ingelheim, Germany). Other materials and reagents were of analytical grade.

2.2. *Preparation of rabbit platelets*

Rabbit blood was mixed with a 1/9 vol of 3.8% (w/v) trisodium citrate and centrifuged at 300 × *g* for 15 min to obtain platelet-rich plasma. Platelets were isolated by centrifugation at 1,500 × *g* for 15 min and suspended in HEPES/Tyrode buffer (140 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.49 mM $MgCl_2$, 0.37 mM NaH_2PO_4, 5.6 mM glucose, 25 mM HEPES, pH 7.4).

2.3. *Measurement of platelet aggregation*

Platelet aggregation was measured at 37°C with stirring according to the method of Born [13] and monitored in a CAF-100 spectrofluorometer (Japan Spectroscopy Inc.). Antagonists were added 5 min before the measurement.

2.4. *Measurement of intracellular calcium concentration*

The cells were incubated in HEPES/Tyrode buffer containing 3 μM Fura-2-pentaacetoxymethyl ester (Dojin) for 1.5 h at room temperature. The Fura-2-loaded cells were washed with and resuspended in HEPES/Tyrode buffer (10^8 cells/ml), and the fluorescence of the cells was measured with a CAF-100 spectrofluorometer (Japan Spectroscopy Inc.) with excitation at 340 nm and 380 nm, and emission at 500 nm at 37°C. 1 mM of $CaCl_2$ and test substances were added with microsyringes. Antagonists were pre-incubated for 5 min. The free Ca^{2+} concentration was calculated from the equation

$$[Ca^{2+}]_i = K_d[(F-F_{min})/(F_{max}-F)]$$

where K_d represents the Ca^{2+} binding dissociation constant (224 nM for Fura-2), F is the 500 nm fluorescence ratio, F_{max} is the maximal

fluorescence ratio determined after addition of 0.1% Triton X-100 to permeabilize the cells in the presence of 1 mM Ca^{2+}, and F_{min} is the minimal fluorescence ratio determined after the addition of 5 mM EGTA.

2.5 *Expression of PAF receptor in* Xenopus *oocytes*

Human leukocyte PAF receptor cRNA was synthesized as described [14] and injected into defolliculated oocytes (about 50 ng in 50 nl per oocyte), which were then incubated at 20°C for 3 days. The ligand-induced Cl^- current was recorded as described [15].

2.6 *Expression of PAF receptor in CHO-K1 cells*

The full-length guinea-pig lung PAF receptor cDNA was subcloned into the mammalian expression vector, pRc/CMV (Invitrogen), and stably transfected in CHO-K1 cells (CHO[gpPAFR]). These cells were grown, at 37°C under 5% CO_2 in a humidified atmosphere, in Ham's F-12 medium supplemented with 10% fetal bovine serum. The cells were collected by scraping, and washed once with HEPES/Tyrode buffer.

2.7. *Binding assay*

Preparation of cell membranes and the binding assay were performed as described [14].

3. RESULTS AND DISCUSSION

It has been reported that lipid A, an active moiety of LPS, stimulates human platelets, inducing aggregation and intracellular Ca^{2+} increase [16,17]. We examined the effects of LPS or lipid A on intracellular Ca^{2+} mobilization in rabbit platelets. As shown in Fig. 1a, upon addition of LPS (5×10^{-5} g/ml) or lipid A (2×10^{-6} M) to the rabbit platelets, intracellular Ca^{2+} was elevated, and this response was completely inhibited by the PAF antagonist, Y-24180 [18,19] (10^{-6} M), in a similar manner as for PAF-elicited responses. Y-24180 at 10^{-6} M also inhibited the LPS- or lipid A-induced Ca^{2+} response in human platelets. Moreover, the effect of PAF antagonist was tested in vitro on aggregation of rabbit platelets. As shown in Fig. 1b, upon addition of PAF (10^{-10} M) or LPS (5×10^{-5} g/ml) to platelets, irreversible aggregation was observed which reached a maximum within 5 min and 7 min, respectively. Y-24180 at 10^{-6} M completely inhibited either PAF- or LPS-induced platelet aggregation. These results raised the possibility that a part of the LPS signaling pathway utilizes the signal transduction system of the PAF receptor. We previously cloned and characterized the cDNAs for guinea pig lung and human leukocyte PAF receptors using the gene expression system in *Xenopus laevis* oocytes [14,15]. To obtain direct evidence that LPS activates the PAF receptor, we carried out the following experiments using the cloned receptors.

In the *Xenopus* oocytes expression system, PAF-evoked electrophysiological responses were oscillatory and long-lasting, thus being characteristic of the response mediated through activation of the inositol trisphosphate/calcium second messenger system [20]. The oocytes injected with guinea pig or human PAF receptor cRNA showed a prominent electrophysiological response to the application of LPS (5×10^{-5} g/ml) or lipid A (2×10^{-6} M) (Fig. 2b), whereas the water-injected oocytes elicited no detectable response to these agents (n = 75, Fig. 2a). Various PAF antagonists (Y-24180 and WEB 2086) inhibited LPS- or lipid A-induced responses (data not shown). These results were reproducible in different lots and sources of LPSs (*E. coli* 0111:B4 from Difco; *E. coli* 0127:B8 from Difco and Sigma). As shown in Fig. 2c, the lipid A-evoked response was readily desensitized, as was the LPS-induced response (data not shown). In addition, oocytes which

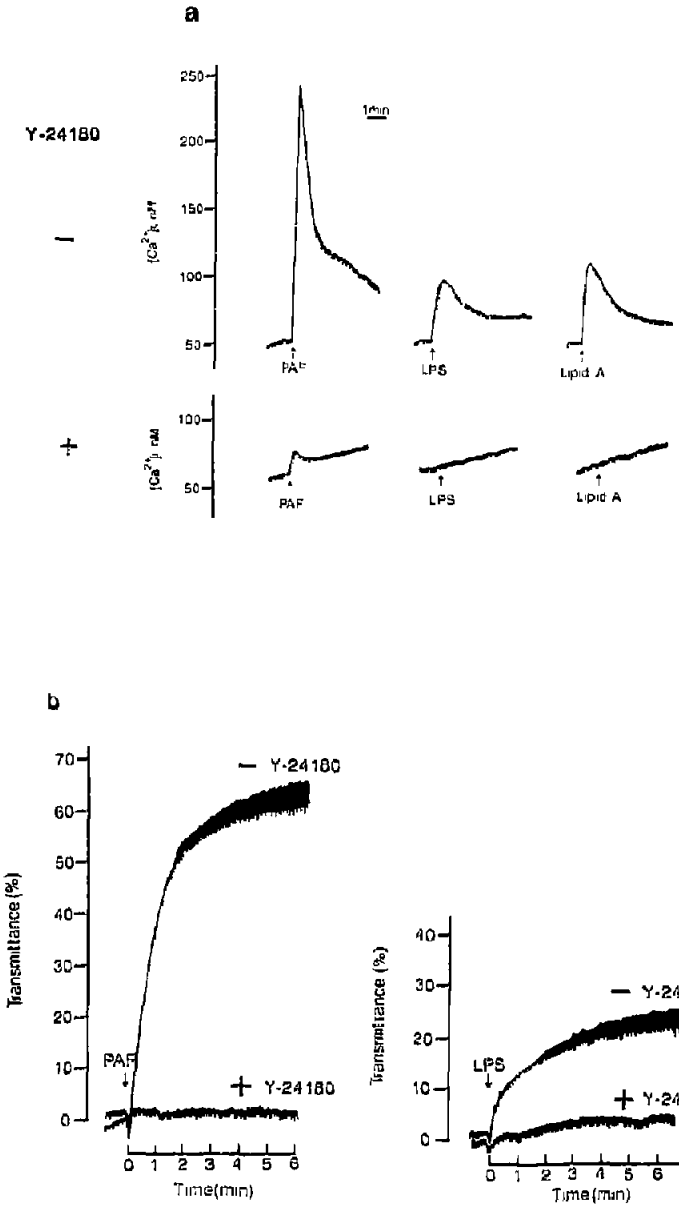


Fig. 1. Effect of PAF antagonists on PAF-, LPS- or lipid A-induced calcium responses in Fura-2 loaded rabbit platelets. (a) Representative tracings of intracellular calcium mobilization by PAF (10^{-8} M), LPS (5×10^{-5} g/ml) or lipid A (2×10^{-6} M) in rabbit platelets in the presence or absence of Y-24180 (10^{-6} M). (b) Aggregation of platelets (2×10^8 cells/ml) induced by PAF (10^{-10} M) (left panel) or LPS (5×10^{-5} g/ml) (right panel) in the presence or absence of Y-24180 (10^{-6} M).

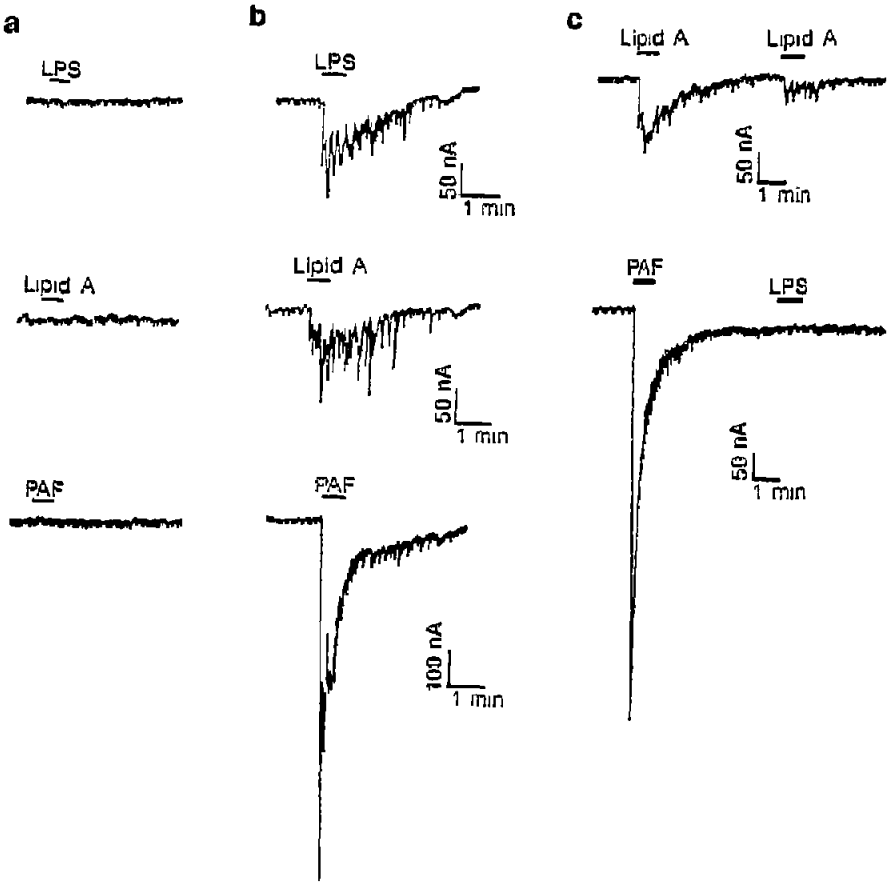


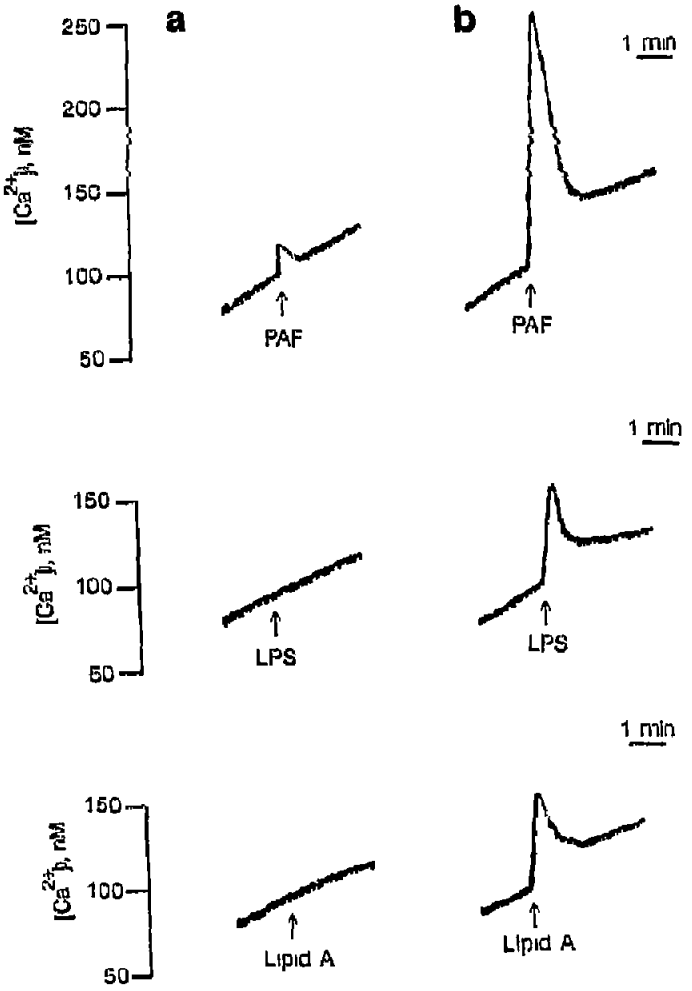
Fig 2. PAF-, LPS- and lipid A-induced Cl^- currents in *Xenopus* oocytes. (a) The electrophysiological responses to LPS, lipid A and PAF in oocytes injected with sterile water (b) The responses to LPS, lipid A and PAF in oocytes injected with the transcript of the human leukocyte PAF receptor cDNA. (c) Desensitization of the lipid A response, and the response to LPS after application of PAF in oocytes injected with human leukocyte PAF receptor cRNA. The concentrations used were: LPS 5×10^{-5} g/ml; lipid A (Daiichi Pure Chem) 2×10^{-6} M, and PAF 10^{-8} M. The downward deflection indicates the inward current The bar above the current trace shows the duration of drug application

had shown the nearly maximal response (>1 μA) after application of PAF showed no detectable response to LPS. These results strongly indicate that LPS and lipid A both act as agonists for the cloned PAF receptors.

To further confirm this observation, we examined the cellular response to LPS and lipid A through the PAF receptor stably transfected into CHO cells (CHO[gpPAFR]). As shown in Fig. 3b, the intracellular Ca^{2+} concentration was increased in Fura-2 loaded CHO[gpPAFR] cells following the application of either LPS or lipid A, in a similar manner as seen after PAF application, whereas no response was detectable using 10^{-6} M lyso-PAF (data not shown). The mock-transfected control cells (CHO[Vec]) showed a minimal response to PAF but no detectable response to either LPS or lipid A (Fig. 3a). The absence of LPS binding to parental cells (CHO-K1) has already been demonstrated by other investigators [5,21]. These elevations in Ca^{2+} were dose dependent between 5×10^{-8} g/ml and 5×10^{-5} g/ml for LPS and between 2×10^{-9} M and 2×10^{-6} M for lipid A (Fig. 3c and d). Although the local concentration of LPS in endotoxemic shock remains undetermined, EC_{50} values for LPS (around 10^{-6} g/ml) and lipid A (around 10^{-7} M) correlated well with data observed previously by others with human platelets [17] and murine peritoneal macrophages [22]. Y-24180, at a concentration of 10^{-6} M, reduced the PAF (10^{-8} M)-elicited Ca^{2+} response by 90%, and completely inhibited the LPS (5×10^{-5} g/ml)- or the lipid A (2×10^{-6} M)-evoked responses (Fig. 3c and d).

To exclude the possibility that LPS- or lipid A-induced Ca^{2+} mobilization is due to a rapid production of PAF, we measured PAF production in LPS- or lipid A-stimulated CHO[gpPAFR] cells. Total lipids were extracted and separated by thin layer chromatography [23] to collect the PAF-containing fraction. PAF contents were measured using [^{125}I]PAF Radioimmunoassay Kits (Du Pont). In this system, no production of PAF was observed by either LPS (5×10^{-5} g/ml) or lipid A (2×10^{-6} M) stimulation within 2 min, whereas the LPS-induced responses occurred practically instantaneously (Fig. 3b).

To obtain direct evidence that LPS binds to the PAF receptor molecule, the displacement of [^{3}H]PAF binding



by LPS was examined using membranes prepared from CHO[gpPAFR] cells. LPS effectively blocked the specific binding of [^{3}H]PAF in a dose-dependent manner (Fig. 4a). The IC_{50} value was about 5×10^{-6} g/ml, and *90% of [^{3}H]PAF binding was inhibited by 5×10^{-5} g/ml* LPS. *This IC_{50} value was comparable with the EC_{50}* value that evoked the Ca^{2+} response in CHO[gpPAFR] cells (Fig. 3c). As shown in Fig. 4b, when LPS (1×10^{-4} g/ml) was added to the incubation mixture 30 min after [^{3}H]PAF binding, dissociation of [^{3}H]PAF was observed with a half-life of 60 min. These data provide confirmative evidence that LPS binds directly to the PAF receptor.

Other signals of LPS, however, such as synthesis of tumor necrosis factor (TNF)-α in human monocytes, was not inhibited by PAF antagonists (I. Waga et al., unpublished data). We propose, therefore, that LPS exerts a variety of biological effects via several distinct pathways.

We have shown herein the first evidence that at least a part of the LPS-signaling pathway, which is involved in platelet aggregation, utilizes the signal-transduction system mediated by direct activation of the PAF receptor. The pathophysiological significance of this cross-talk needs to be further examined in other organs involved in endotoxemic shock.

Acknowledgements: We are grateful to Y. Enomoto for his assistance with experiments, and Drs. K. Inoue, I. Kudo (University of Tokyo) and S. Toyoshima (Japan Tobacco Inc.) for critical suggestions. We thank Dr. H. Bito of our Department for critically reading the manuscript and M. Ohara for valuable comments. This work was supported in part by grants from the Ministry of Education, Science and Culture, the Ministry of Health and Welfare of Japan, Toray Science Founda-

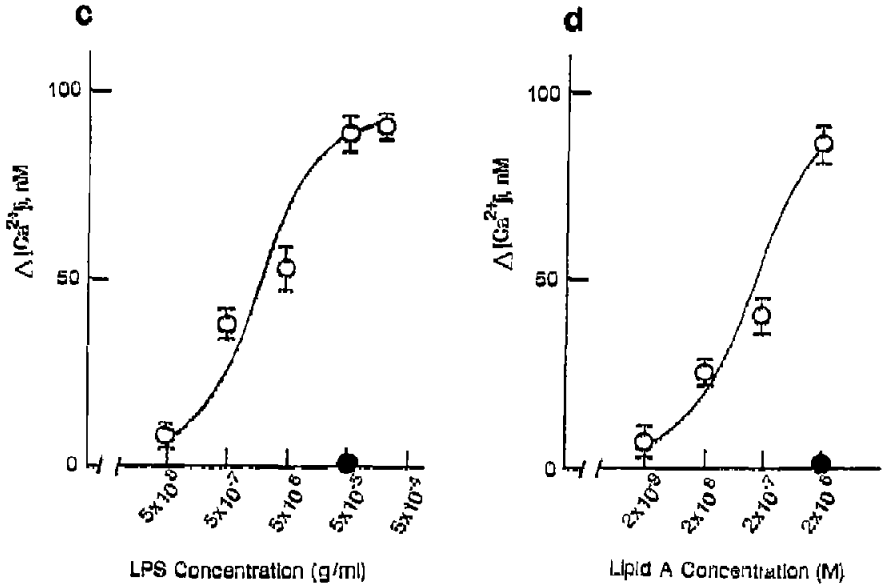


Fig. 3. PAF-, LPS- and lipid A-induced calcium mobilization in transfected CHO cells. (a,b) Representative tracings of intracellular calcium mobilization by 10^{-8} M PAF, 5×10^{-5} g/ml LPS and 2×10^{-6} M lipid A in CHO[Vec] cells (a) and CHO[gpPAFR] cells (b). (c,d) Dose–response curves of LPS (c) and lipid A (d) in eliciting a calcium increase in CHO[gpPAFR] cells. The LPS- or lipid A-induced response was inhibited by Y-24180 (10^{-6} M) (filled circles). Each point is the mean of three assays ± S.E.M.

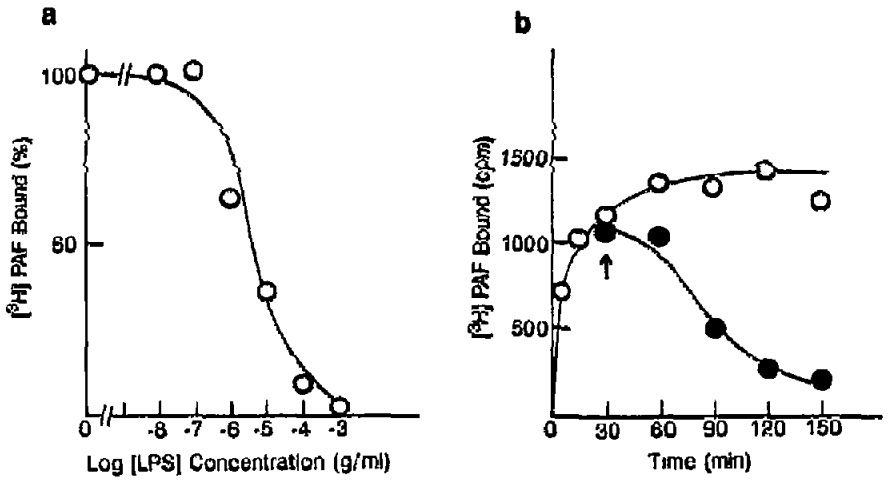


Fig. 4. Displacement (a) and dissociation (b) of [^{3}H]PAF binding to membranes of CHO[gpPAFR] cells by LPS. Each point represents the mean of duplicate determinations. Results are from one of three independent experiments. (a) [^{3}H]PAF (5×10^{-10} M) binding competition study was carried out as described [14] except with an incubation time of 150 min. Data are expressed as a percentage of the binding without LPS (100%) vs. the logarithm of the LPS concentration. (b) [^{3}H]PAF (5×10^{-10} M) was incubated with CHO[gpPAFR] cell membranes at 25°C. Dissociation of [^{3}H]PAF binding was determined by addition of LPS (10^{-4} g/ml) at 30 min (indicated by an arrow). Data are plotted as radioligand specific binding vs. incubation time.

tion, Uehara Memorial Foundation and Yamada Science Foundation.

REFERENCES

[1] Morrison, D.C. and Ryan, J.L. (1987) Annu. Rev. Med. 38, 417–432.
[2] Raetz, C.R.H. (1990) Annu. Rev. Biochem. 59, 129–170.
[3] Schumann, R.R., Leong, S.R., Flaggs, G.W., Gray, P.W., Wright, S.D., Mathison, J.C., Tobias, P.S. and Ulevitch, R.J. (1990) Science 249, 1429–1431.
[4] Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J. and Mathison, J.C. (1990) Science 249, 1431–1433.
[5] Hampton, R.Y., Golenbock, D.T., Penman, M., Krieger, M. and Raetz, C.R.H. (1991) Nature 352, 342–344
[6] Braquet, P. and Hosford, D. (1990) in. Cellular and Molecular Aspects of Endotoxin Reactions (Nowotny, A. ed) pp. 295–303, Elsevier, Amsterdam.
[7] Morrison, D.C. (1990) in: Cellular and Molecular Aspects of Endotoxin Reactions (Nowotny, A. ed.) pp. 183–189, Elsevier, Amsterdam.
[8] Raetz, C.R.H., Ulevitch, R.J., Wright, S.D., Sibley, C.H., Ding, A. and Nathan, C.F. (1991) FASEB J. 5, 2652–2660.
[9] Hanahan, D.J. (1986) Annu. Rev. Biochem. 55, 483–509.
[10] Snyder, F. (1987) Platelet-Activating Factor and Related Lipid Mediators, Plenum Press, New York.
[11] Prescott, S.M., Zimmerman, G.A. and McIntyre, T.M (1990) J. Biol. Chem. 265, 1781–1784.
[12] Shukla, S.D. (1991) Lipids 26, 1028–1033.
[13] Born, G.V.R. (1962) Nature 194, 927–929.
[14] Nakamura, M., Honda, Z., Izumi, T., Sakanaka, C., Mutoh, H., Minami, M., Bito, H., Seyama, Y., Matsumoto, T., Noma, M. and Shimizu, T. (1991) J. Biol. Chem. 266, 20400–20405.
[15] Honda, Z., Nakamura, M., Miki, I., Minami, M., Watanabe, T., Seyama, Y., Okado, H., Toh, H., Ito, K., Miyamoto, T. and Shimizu, T. (1991) Nature 349, 342–346.
[16] Gragarek, J., Timmons, S. and Hawiger, J. (1988) J. Clin. Invest. 82, 964–971.
[17] Romano, M., Molino, M. and Cerletti, C. (1991) Biochem. J. 278, 75–80.
[18] Terasawa, M., Aratani, H., Setoguchi, M. and Tahara, T. (1990) Prostaglandins 40, 553–569.
[19] Takehara, S., Mikashima, H., Muramoto, Y., Terasawa, M., Setoguchi, M. and Tahara, T. (1990) Prostaglandins 40, 571–583.
[20] Shimizu, T. (1992) in: PAF Receptor Signal Mechanism (Shukla, S.D. ed.) in press, CRC Press, New York.
[21] Hara-Kuge, S., Amano, F., Nishijima, M. and Akamatsu, Y. (1990) J. Biol. Chem. 265, 6606–6610.
[22] Prpic, V., Weiel, J.E., Somers, S.D., DiGuiseppi, J., Gonias, S.L., Pizzo, S.V., Hamilton, T.A., Herman, B. and Adams, D.O. (1987) J. Immunol. 139, 526–533.
[23] Sugiura, T., Fukuda, T., Masuzawa, Y. and Waku, K. (1990) Biochim. Biophys. Acta 1047, 223–232.