Expression of human platelet-activating factor receptor gene in EoL-1 cells following butyrate-induced differentiation

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Platelet-activating factor (PAF) is a potent lipid mediator of allergic inflammation through its interaction with eosinophils. Expression of the PAF receptor is modulated by many agents, including those responsible for cell differentiation. We report here that differentiation of a human eosinophilic leukaemia cell line, EoL-1, by sodium n-butyrate is associated with induction of PAF receptor gene expression, as indicated by: PAF receptor mRNA accumulation; increases in the binding of [³H]WEB 2086, a PAF antagonist; analysis of cell-surface expression of PAF receptor protein using a monoclonal anti-(PAF receptor) antibody; and augmentation of PAF-induced increase in the intracellular concentration of calcium. Using cDNA cloning, the

receptor expressed in EoL-1 cells was identified as 'Transcript 1', one of two transcripts which was previously reported from human genomic analysis (Mutoh, Bito, Minami, Nakamura, Honda, Izumi, Nakata, Kurachi, Terano and Shimizu (1993) *FEBS Lett.* 322, 129–134). The PAF-induced calcium response and phosphoinositide turnover were decreased by pertussis toxin (PTX) treatment, suggesting that these signals are coupled largely with PTX-sensitive G-protein(s) in EoL-1 cells. These systems may provide a useful experimental model with which to investigate the relationship between eosinophilic differentiation and PAF receptor induction, and the role of eosinophils in allergic responses.

INTRODUCTION

Platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF) is a potent proinflammatory mediator, which causes microvascular leakage, vasodilatation, contraction of smooth muscle, and activation of neutrophils, macrophages and eosinophils [1–4]. The accumulation of eosinophils in affected tissues characterizes many allergic disorders, including bronchial asthma and atopic dermatitis. Eosinophil chemotactic factors such as PAF, a complement fragment C5a, and formyl-methionyl-leucyl-phenylalanine (fMLP) not only induce chemotaxis [5] but also enhance many functions of eosinophils. PAF releases eosinophil peroxidase and other granular contents from human eosinophils [6,7]. Although specific binding of [³H]WEB 2086 to PAF receptor was identified in eosinophils [8], no direct data are available concerning the structure and the regulation of PAF receptor at the molecular level. Thus, mechanisms related to the induction of PAF receptor and the responses of eosinophils to PAF are important when considering the roles of eosinophils in allergic disorders.

In the present study, using a human eosinophilic leukaemia cell line, EoL-1 [9], we describe the regulation, structure and signal transduction of PAF receptor during eosinophilic differentiation.

MATERIALS AND METHODS

Materials

[methyl-³H]WEB 2086 (521.7 GBq/mmol), *myo*-D-[1,2-³H]ino-

sitol (1.7 TBq/mmol), [α -³²P]dATP (29.6 TBq/mmol) and [α -³²P]dCTP (111 TBq/mmol) were obtained from Du Pont. PAF antagonists were generous gifts from the following companies: WEB 2086 from Boehringer Ingelheim (Ingelheim), Y24180 from Yoshitomi Pharmaceutical Industries (Osaka), and CV 6209 from Takeda Chemical Industries (Osaka). PAF and pertussis toxin (PTX) were from Funakoshi (Tokyo). Other materials and reagents were of analytical grade.

Cells

EoL-1 cells were a kind gift from Drs. H. Saito (Yamagata Prefectural Central Hospital, Yamagata, Japan) and H. Saito (National Sagami Hospital, Kanagawa, Japan). EoL-1 cells were maintained in suspension culture at 37 °C and 5% CO₂ in humidified atmosphere in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 0.03% (w/v) L-glutamine, 100 i.u./ml penicillin and 100 µg/ml streptomycin. For differentiation, EoL-1 cells were diluted to 5 × 10⁵ cells/ml and 0.5 mM sodium n-butyrate was added. When EoL-1 cells were incubated with 0.5 mM sodium n-butyrate for 5 days, the percentage of cells responding to Luxol Fast Blue staining increased from 1.8 ± 0.4 to 9.4 ± 2.6% (mean ± S.D., *n* = 5).

In some experiments, PTX (0.1–100 ng/ml) or its vehicle was added to the culture medium and the cells were incubated for an additional 24 h. Under the current conditions, when EoL-1 cells were treated with PTX (100 ng/ml) for 24 h, no additional ADP-ribosylated band was detected (results not shown). This treatment did not affect the cell viability, the basal concentration of

Abbreviations used: [Ca²⁺]_i, intracellular free Ca²⁺ concentration; CHO, Chinese hamster ovary; FITC, fluorescein isothiocyanate; IP₃, *myo*-D-inositol 1,4,5-trisphosphate; KLH, keyhole limpet hemocyanin; LT, leukotriene; PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); PLC, phospholipase C; PTX, pertussis toxin; TEAB, triethylammonium hydrogen carbonate buffer.

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The nucleotide sequence data reported in this paper will appear in the EMBL, DDBJ, GSDB, and NCBI Nucleotide Sequence Databases under accession no. D31736.

intracellular calcium or the incorporation of *myo*-D-[3 H]inositol into the cells.

Assays for leukotriene (LT) $_4$ hydrolase and LTC $_4$ synthase

Eosinophils have been reported to produce LTC $_4$ preferentially to LTB $_4$ [10]. Two enzyme activities (LTA $_4$ hydrolase and LTC $_4$ synthase) of cell homogenates were assayed as described previously [11], to study the ability of EoL-1 cells to produce LTs. Cells at a different stage of differentiation were seeded (1×10^7 cells/ml) in Tyrode's buffer and sonicated for 3 s at 60 W using a Model 5202 sonicator (Ohtake Works, Tokyo). The cell homogenates were centrifuged at 10000 *g* for 10 min at 4 °C. The resulting supernatant was incubated with 70 μ M LTA $_4$ and 5 mM glutathione for 5 min. The amount of LTC $_4$ and LTB $_4$ in the products was analysed by a reversed-phase h.p.l.c. The activity was expressed as m-units/ 10^6 cells, where one unit was defined as the activity required to produce one μ mol of product/5 min.

Construction and screening of an λ ZAP II cDNA library

RNA was prepared from EoL-1 cells treated with 0.5 mM sodium *n*-butyrate for 3 days by the acid guanidium thiocyanate/phenol/chloroform method [12]. The cDNA synthesis was carried out with oligo(dT)-primed poly(A) $^+$ RNA, using a cDNA Synthesis Kit (Pharmacia). The cDNA was inserted into the *Eco*RI site of λ ZAP II vector (Stratagene). Clones (3×10^6) were first screened by hybridization with the *Pst*I-*Eco*RV fragment (~ 1.2 kbp, including the full coding region) of the human PAF receptor cDNA [13]. Hybridization was carried out in solution containing $6 \times$ SCC (0.15 M NaCl, 0.015 M sodium citrate), $5 \times$ Denhardt's solution [0.1% (v/v) BSA, 0.1% (v/v) poly(vinylpyrrolidone), 0.1% (v/v) Ficoll 400], 0.5% (w/v) SDS, 400 μ g/ml heat-denatured salmon sperm DNA, and the radiolabelled probe (2×10^6 c.p.m./ml) at 55 °C for 18 h. Filters were washed with $2 \times$ SCC, 0.1% SDS at 55 °C for 20 min and then with $0.5 \times$ SCC, 0.1% SDS at 55 °C for 15 min. After tertiary screening, six independent clones were isolated. These phages containing the cDNA inserts were converted to plasmids by rescue excision, according to the manufacturer's instructions. The restriction mapping of the inserted cDNAs was examined. DNA sequencing was performed by the method of Sanger et al. [14] with [α - 32 P]dATP and T7 DNA polymerase (Pharmacia) on both strands, using synthetic oligonucleotides as sequencing primers.

Northern blot analysis

Poly(A) $^+$ RNAs obtained from different stages of EoL-1 cells were resolved on a 0.7% (w/v) agarose gel containing 6.7% (v/v) formaldehyde and blotted onto a Hybond-N $^+$ nylon membrane (Amersham). The blot was hybridized with the 32 P-labelled *Pst*I-*Eco*RV fragment of human PAF receptor cDNA at 65 °C for 18 h. The membrane was successively washed at 65 °C in $2 \times$ SCC, 0.1% SDS for 20 min and then in $1 \times$ SSC for 20 min before being exposed for 7 days to a X-ray film at -70 °C with an intensifying screen. The membrane was washed in 95 °C distilled water for 30 min and was then rehybridized with the 32 P-labelled human β -actin probe (Funakoshi, Tokyo). After being washed as described above, the membrane was exposed to a X-ray film for 20 h. The FUJIX BAS-2000 bioimage analyser (Fuji Photo Film Co., Tokyo) was used to quantify hybridized signals.

Expression of guinea pig and human PAF receptor on Chinese hamster ovary (CHO) cells

The cDNAs encoding guinea pig or human PAF receptor [13,15] were inserted into a vector, pcDNA1/NEO (Invitrogen, San Diego, CA, U.S.A.), and transfected to CHO cells by electroporation. A mock-transfection was carried out with the pcDNA1/NEO vector only. Geneticin-resistant cells were selected in F-12 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 i.u./ml penicillin, 100 μ g/ml streptomycin and 0.3 mg/ml Geneticin. After limiting dilution, stable transformants were examined by [3 H]WEB 2086 binding. For the guinea pig PAF receptor cDNA, two transformant cell lines were selected. One was a low-expression cell line, designated WT-12, that showed a binding activity of 28 fmol of [3 H]WEB 2086/ 10^6 cells, and the other was a high-expression cell line, WT-6, with a binding activity of 432 fmol of [3 H]WEB 2086/ 10^6 cells. For the human PAF receptor cDNA, one cell line, hPAFR 2, which showed a binding activity of 85 fmol of [3 H]WEB 2086/ 10^6 cells was selected. A mock-transfected cell line without inserted cDNA, termed V-2, exhibited a binding activity of < 2 fmol of [3 H]WEB 2086/ 10^6 cells.

Preparation of monoclonal antipeptide antibody against guinea pig PAF receptor

A synthetic peptide, corresponding to the guinea pig PAF receptor residues 158–173, was linked to keyhole limpet hemocyanin (KLH, Pierce) for immunization. Balb/c mice were immunized subcutaneously with 15 μ g of the peptide-KLH emulsified with complete Freund's adjuvant. The animals were boosted by intraperitoneal injection of the peptide-KLH (15 μ g) in poly[d(A-U)] (200 μ g)/PBS several times at 7 day intervals. After the last immunization, mouse spleen cells were hybridized with P3U1 myeloma cells using PEG-4000 (Merck). The fused cells were selected in a hypoxanthine/aminopterin/thymidine medium. The antibody in the culture supernatant was detected by e.l.i.s.a. Positive clones were further analysed by FACScan (flow cytometry analysis) to examine whether their supernatant can react with the guinea pig PAF receptor expressed on the CHO cells, and four clones were selected. All four clones produced IgM(κ) subtype immunoglobulin. Among the four monoclonal antibodies, one antibody, anti-(gpPAF-R $^{158-173}$) Ab-IV (Ab-IV), reacted most strongly with the surface antigen on CHO cells expressing the guinea pig PAF receptor. IgM fractions were purified using a ImmunoPure IgM Purification Kit (Pierce).

FACScan analysis of PAF receptor protein on the cell surface

Cell-surface expression of PAF receptor protein on the CHO cells, Raji cells, Molt 4 cells and EoL-1 cells was assayed by flow cytometry on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, U.S.A.). Staining of cells was performed as follows: 2×10^5 cells were washed and resuspended in Ca $^{2+}$ /Mg $^{2+}$ -free Dulbecco's PBS containing 2% (v/v) BSA and 0.1% (w/v) NaN $_3$. They were then incubated for 1 h at 4 °C with hybridoma supernatant or purified IgM fraction (1 μ g/ml) in a final volume of 0.1 ml. Cells were washed and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (Funakoshi, Tokyo) for 1 h at 4 °C. Background fluorescence was determined by staining the cells directly with FITC-conjugated antibodies.

[3 H]WEB 2086 binding assay

The ligand binding was assayed as described previously [13]. The membrane fractions of EoL-1 cells were incubated with 20 nM

[³H]WEB 2086 (a PAF antagonist) [16] at 25 °C for 60 min in the presence (for the non-specific binding) or absence (for the total binding) of 10 μ M WEB 2086, and the radioactivity on a Whatman GF/C glass-fibre filter was measured. For the displacement study, the binding with 10 nM [³H]WEB 2086 was inhibited by various concentrations (10^{-12} – 10^{-6} M) of PAF, Y24180, CV 6209, or WEB 2086, and IC₅₀ values for each drug were determined by three independent experiments.

Determination of cytosolic free Ca²⁺ and inositol trisphosphates

Changes in intracellular free Ca²⁺ concentration ([Ca²⁺]_i) induced by PAF were measured by fluorimetry, as described previously [17]. EoL-1 cells (3×10^5 cells) loaded with 3 μ M Fura 2-AM were incubated at 37 °C with 1 mM CaCl₂ for 10 min, and PAF solutions were added.

The production of *myo*-D-inositol 1,4,5-trisphosphate (IP₃) was measured as described previously [18] with some modifications. EoL-1 cells were labelled with 1.85 MBq/ml of *myo*-[³H]inositol for 4 h, and incubated with 10 mM LiCl for 1 h. The cells were stimulated with various concentrations of PAF for the indicated time. The reaction was stopped with perchloric acid (6% v/v final concentration) and neutralized with 10 M KOH, then centrifuged. The final supernatant was diluted with distilled water and separated on a Sep-Pak Accell Light QMA cartridge column (Waters) by the stepwise elution using 3 ml each of 0.02, 0.1, 0.23, 0.4 and 0.6 M triethylammonium hydrogen carbonate buffer (TEAB) (pH 8.4, adjusted with CO₂). The radioactivity of each fraction was determined in a liquid scintillation counter. The standards *myo*-D-[³H]inositol 1-monophosphate, *myo*-D-[³H]inositol 1,4-bisphosphate, [³H]IP₃ and *myo*-D-[³H]inositol 1,3,4,5-tetrakisphosphate were recovered in the 0.1, 0.23, 0.4 and 0.6 M TEAB fractions respectively.

RESULTS

Effects of sodium n-butyrate on cell differentiation

The increasing percentage of the Luxol Fast Blue positive cells (from 1.8 to 9.4% after culturing for 5 days with sodium n-butyrate) indicated that this treatment committed EoL-1 cells to the eosinophilic lineage, as Luxol Fast Blue staining is a specific method for the detection of eosinophilic granules. But the proportion of the eosinophilic-granule-positive cells was not as large, probably because the granules of the differentiated EoL-1 cells were immature compared with those of normal eosinophils. It was reported that the granules of the differentiated EoL-1 cells lacked the crystalloid core and matrix usually observed in mature eosinophil granules [9]. The enzyme activities of LTA₄ hydrolase and LTC₄ synthase increased time-dependently, and reached approx. 3- and 8-fold higher values after culturing for 7 days respectively (Figure 1a). The LTC₄ synthase activity was much higher than the LTA₄ hydrolase activity. This predominant production of LTC₄ was thought to be characteristic of eosinophils [10]. Thus, EoL-1 cells revealed the eosinophilic phenotype when treated with sodium n-butyrate.

Induction of [³H]WEB 2086 binding and Ca²⁺ mobilization during differentiation

The [³H]WEB 2086 binding increased time-dependently during 3 days after addition of butyrate and reached a plateau at about a 4-fold higher level (Figure 1b). Scatchard plots indicated a K_d value of 14.4 nM for undifferentiated cell membranes and 13.2 nM for the day-5 differentiated sample. The displacement study showed that this receptor binding was most strongly

Figure 1 Differentiation to eosinophilic phenotype and PAF receptor expression in EoL-1 cells by sodium n-butyrate

EoL-1 cells were cultured with 0.5 mM sodium n-butyrate during the indicated periods. Two enzyme activities, the PAF receptor binding, and the PAF-induced Ca²⁺ response after each period were determined. Data represent means $\pm$ S.D. ($n = 3$). **, $P < 0.01$ versus undifferentiated cells. (a) Enzyme activities of LTA₄ hydrolase (□) and LTC₄ synthase (○) in the cell homogenates were determined. (b) The PAF receptor binding activity of cell membranes was determined, using 20 nM [³H]WEB 2086 as a ligand. Scatchard plots of the undifferentiated cells (○) and day-5 differentiated cells (●) are shown (inset). K_d and B_{max} values were 14.4 nM and 49 fmol/mg of protein in undifferentiated cell membranes, and 13.2 nM and 166 fmol/mg of protein in differentiated cell membranes respectively. (c) The maximum increase of [Ca²⁺]_i with 3 nM PAF was measured. The basal level of [Ca²⁺]_i was 108.5 ± 8.3 nM ($n = 5$) in undifferentiated cells and did not reveal any significant change during differentiation.

inhibited by PAF (IC₅₀ 1.3 nM), followed by Y24180 (1.6 nM), CV 6209 (4.0 nM) and WEB 2086 (50.1 nM) in this order; the potency of inhibition was the same as that for the cloned human PAF receptor transfected in COS-7 cells [13].

To test the biological response through PAF receptor, mobilization of [Ca²⁺]_i was examined. When Fura-2 AM-loaded EoL-1 cells were stimulated with PAF, a rapid and transient increase of [Ca²⁺]_i was observed in a concentration-dependent manner (EC₅₀ 0.5 nM). Lyso-PAF (up to 10 μ M) was ineffective, and 3 nM PAF did not induce Ca²⁺ mobilization in the presence of 1 μ M WEB 2086, suggesting that PAF acts on EoL-1 cells through a specific, receptor-mediated mechanism. This PAF-induced Ca²⁺ response was enhanced during the differentiation (Figure 1c).

Figure 2 Induction of PAF receptor mRNA with sodium n-butyrate in EoL-1 cells

(a) Poly(A)⁺RNAs (5 µg) from undifferentiated (time = 0) and differentiated EoL-1 cells (0.5 mM sodium n-butyrate present during the indicated period) were separated on an agarose gel and hybridized with a human PAF receptor probe (upper panel). Human rRNAs were used as size markers. Hybridization to the β-actin probe as an internal control is shown (lower panel). The arrows indicate the major band for PAF receptor mRNA (PAF-R, 3.8 kbp), and the possible minor band (*, 4.2 kbp). (b) The relative intensity of PAF receptor mRNA signal standardized with β-actin signal was determined with a FUJIX BAS-2000 bioimage analyser. Data represent means ± S.D. (n = 3). The basal value of the signal ratio was 1.0.

The response doubled in day-1 differentiated cells, and after treatment for 3 days the response reached a plateau at a level 3-fold higher than that for undifferentiated cells. The increase of [³H]WEB 2086 binding and the enhancement of Ca²⁺ mobilization paralleled the induction of the LT synthetic activities, hence these phenomena may reflect an increase in the receptor protein during differentiation.

Induction of PAF receptor mRNA during differentiation

The expression of the human PAF receptor gene in undifferentiated and differentiated EoL-1 cells was investigated. Poly(A)⁺ RNAs isolated from these cells were hybridized with the human PAF receptor cDNA. Two bands were detected, a major band of size 3.8 kbp and a minor band of size 4.2 kbp, whose intensity was about one sixth of the major band, as determined by a FUJIX BAS-2000 bioimage analyser (Figure 2a). The size of the major band was the same as that reported previously in human leukocytes and EoL-1 cells [13], and the 4.2 kbp band may represent a minor species of PAF receptor mRNA (discussed below). Undifferentiated cells expressed a relatively low level of PAF receptor mRNA. After a 1 h exposure to butyrate, the mRNA level was slightly increased, and at 6 h the signal doubled. After a 3 day exposure, the amount of PAF receptor mRNA increased 3.5-fold and reached a plateau (Figure 2b). These data indicate that the increase of PAF receptor during cell differentiation is regulated at the transcriptional level.

Figure 3 Molecular cloning of the PAF receptor cDNA from EoL-1 cells

(a) Restriction maps of the isolated clones. Using human PAF receptor cDNA 'phPAF' [13] as a probe, six independent clones (EP-1 to -6) were screened from a cDNA library of butyrate-differentiated EoL-1 cells. 'Exon 1' shows one of the exons for the human PAF receptor genome [24], which encodes the upstream region of the exon-intron junction (between -39 and -38). The protein coding region is shown by a thick line. The direction and extent of sequence determination are indicated by arrows. (b) Nucleotide sequence of the 5'-non-coding region of PAF receptor cDNA. Arrows indicate the start of each cDNA and the end of 'exon 1'. The major transcription start site with the possible initiator sequence is marked by an asterisk and an underline. The initiation codon ATG is also underlined.

Cloning and sequencing of the PAF receptor cDNA

The molecular heterogeneity of PAF receptor has been suggested in many cells and tissues [19–21], including eosinophils [22]. To investigate whether EoL-1 cells have the same type of receptor as leukocytes, we cloned the PAF receptor from a cDNA library of day-3 differentiated EoL-1 cells. Six independent clones (EP-1 to -6) were isolated. These clones were sequenced on both strands, and they have nucleotide sequences identical with that of the human leukocyte cDNA (Figure 3). Thus, the PAF receptor protein of EoL-1 cells is the same as that of leukocytes, except possibly for post-translational modifications. We reported previously two types of mRNA (Transcript 1 and 2) for the human PAF receptor, the difference being divergent 5'-non-coding sequences [23,24]. Human leukocytes and EoL-1 cells have only Transcript 1, whereas other cells and tissues possess both Transcript 1 and Transcript 2 [24]. All isolated clones from the EoL-1 cDNA library had nucleotide sequences corresponding to that of Transcript 1. These data indicate that the expression of PAF receptor gene in EoL-1 cell is regulated by the same promoters as in leukocytes.

The 5' ends of the five isolated clones were located downstream of the putative transcription start sites identified by our previous study [24], but EP-6 had a longer 5'-non-coding region. EP-6 encoded some of the identical sequence to a genomic clone 'exon 1' and it contained the major transcriptional start site (TCCTCTTTCTCAC) which included a highly homologous sequence to the 'initiator' (CCTCATTTCT) found in the murine terminal deoxynucleotidyltransferase gene. As the 'initiator' is

Figure 4 FACS analysis of PAF receptor expression

Different types of cells were incubated with or without (background) anti-(gpPAF-R¹⁵⁸⁻¹⁷³) Ab-IV, and labelled with FITC-conjugated anti-(mouse IgM). (a) CHO cells expressing guinea pig PAF receptor. V-2, mock-transfected cells; WT-12, low-expression transformant; and WT-6, high-expression transformant. (b) CHO cells expressing human PAF receptor (hPAFR-2). (c) Human lymphocyte cell lines; Molt 4 (T cell) and Raji (B cell). (d) Undifferentiated and differentiated (day-1 and day-3) EoL-1 cells. The background histograms did not show any significant change during the differentiation.

reported to represent the functional promoter related to cellular differentiation and development [25,26], this start site may play an important role in the induction of PAF receptor during differentiation of EoL-1 cells. Considering the structure of EP-6 and the 4.2 kbp band detected by Northern blot analysis (Figure 2a), there might be another transcription start site at the upper 5'-non-coding region in exon 1.

FACS analysis of PAF receptor protein

We developed monoclonal antibodies against a synthetic peptide, corresponding to the guinea pig PAF receptor residues 158–173 in the second extracellular loop of the putative tertiary structure. Ab-IV recognized the guinea pig PAF receptor expressed in CHO cells (Figure 4a). Right-ward shifts of FITC signals were observed in V-2 cells (mock-transfected CHO cells) and parental CHO cells. However, when V-2 cells were incubated with mouse myeloma IgM (product no. 02-6800, Zymed Laboratories, San Francisco, CA, U.S.A.), which was not related to PAF receptor, the FITC signal was the same as the background. Ab-IV may recognize some other epitope(s) on the surface of CHO cells besides PAF receptor protein, as any significant [³H]WEB 2086 binding was not observed on parental CHO cells or V-2 cells. WT-12 cells (the low-expression level transformant) gave a stronger signal than V-2 cells, and WT-6 cells (the high-expression level transformant) gave a much stronger signal (Figure 4a). Thus, these additional right-ward shifts of the histogram from that of V-2 cells reflect the expression of guinea-pig PAF receptor protein.

We checked whether this antibody can recognize human PAF receptor protein, because the corresponding residues of human receptor (TNTVPDSAGSGNVTRC) are not identical to those of the guinea pig (TNVVS NKAGSGNITRC). A stable transformant cell line, hPAFR-2, expressing human PAF receptor cDNA was analysed. As shown in Figure 4(b), significant labelling of hPAFR-2 cells was observed. Two human lymphocyte

cell lines were subjected to FACS analysis. A B-cell line, Raji, reported to possess PAF receptor [27], was strongly labelled, but a T-cell line MOLT 4 was labelled at a low level (Figure 4c). One previous report demonstrated that T lymphocytes do not express PAF receptor without proper activation [28], whereas another report suggested that PAF represents a potential cellular mediator in MOLT 4 [29]. Partial activation could be the reason for the low level of surface labelling detected in MOLT 4 cells.

Although undifferentiated EoL-1 cells transcribed only very low levels of PAF receptor mRNA (Figure 2), they expressed some receptor protein at their cell surface (Figure 4d). During the differentiation with sodium n-butyrate, day-1 EoL-1 cells were labelled more strongly than undifferentiated cells and day-3 cells were highly labelled.

Effects of PTX on Ca²⁺ mobilization and IP₃ production

PAF induced intracellular Ca²⁺ mobilization in EoL-1 cells. The type of G-protein(s) involved in the PAF responses may differ from cell to cell [30,31]. In CHO cells expressing the guinea pig PAF receptor, PTX is not inhibitory to PAF-induced IP₃ production [18]. To investigate the roles of G_i protein in PAF-receptor-induced intracellular signal transduction, day-7 differentiated EoL-1 cells were treated with PTX. The increases in [Ca²⁺]_i were inhibited dose-dependently (results not shown). When cells were incubated with 100 ng/ml of PTX (all G_i was ADP-ribosylated at this concentration), the increase of [Ca²⁺]_i was inhibited by 50% (Figure 5a). Figure 5(b) shows a rapid production of IP₃ induced by 10 nM PAF in the differentiated cells. The peak response occurred at 20 s after the addition of PAF. The IP₃ production was inhibited by PTX in a dose-dependent manner and the maximum inhibition was ~75% (Figure 5c). These data indicate that the phospholipase C (PLC) activation by PAF in EoL-1 cells occurs largely through PTX-sensitive G protein(s).

Figure 5 Effects of PTX on PAF-induced signals in EoL-1 cells

(a) $[Ca^{2+}]_i$ responses to 3 nM PAF in undifferentiated cells (i), or in day-7 differentiated cells treated without (ii) or with (iii) PTX (100 ng/ml). PAF (3 nM) was added to Fura-2 AM-loaded cells, and $[Ca^{2+}]_i$ was measured by fluorometry. (b) PAF-induced rapid synthesis of IP_3 in day-7 differentiated EoL-1 cells. EoL-1 cells labelled with $myo-D-[^3H]$ inositol were treated with 10 nM PAF in the presence of 10 mM LiCl for 0–120 s. The inositol phosphates were extracted, and the IP_3 fractions were separated on an anion-exchange cartridge (see the Materials and methods section). Data represent means $\pm$ S.D. ($n = 3$). **, $P < 0.01$ versus basal levels. (c) The effects of PTX (0–100 ng/ml) on the IP_3 production over 20 s in day-7 differentiated EoL-1 cells. Data represent means $\pm$ S.D. of ($n = 3$). **, $P < 0.01$ versus toxin-free cells.

DISCUSSION

The present study provides evidence that when EoL-1 cells are induced to differentiate towards the eosinophilic phenotype, the expression of PAF-receptor gene is modulated to increase functionally active receptor protein. Northern blot analysis revealed that the increase of PAF receptor gene transcription during differentiation occurred from 1 h onwards. Induction of the PAF receptor gene transcription (Figure 2) paralleled the increase of functional PAF receptor protein, as indicated by an increase in radioligand binding (Figure 1b), by the enhancement of PAF-dependent Ca^{2+} mobilization (Figure 1c), and by FACS analysis (Figure 4d). Though the amount of receptor protein increased during differentiation, no significant change was observed in the K_d value for $[^3H]WEB 2086$ binding and in the EC_{50} value for Ca^{2+} mobilization. Our data suggest that the gene expression for PAF receptor is regulated at the transcriptional level in butyrate-differentiated EoL-1 cells. The induction of PAF receptor has been studied in a human promyelocytic leukaemia cell line, HL-60, by dimethylsulphoxide towards the granulocyte phenotype [32,33], and by $1\alpha,25$ -

dihydroxy vitamin D_3 towards the macrophage phenotype [34]. In EoL-1 cells, the amount of mRNA for the PAF receptor increased with the combination of interleukin-5, granulocyte/macrophage colony-stimulating factor, and n-butyrate [13], and Ca^{2+} mobilization with PAF was induced by dibutyryl cyclic AMP [35]. However, no direct evidence has been obtained on the structure and regulation of PAF receptor at the molecular level. In the present work, we demonstrate for the first time that gene expression and function of the PAF receptor are coupled to the cellular differentiation of EoL-1 cells by sodium n-butyrate.

Though various tissues and cells contain two species of mRNA for PAF receptor [24], EoL-1 cells have only one kind of mRNA as leukocytes. So, the gene expression is controlled by the promoter regions of 'exon 1', which contains transcription factors such as NF- κ B and Sp-1, and the putative initiator highly homologous to the 'initiator' found in the murine terminal deoxynucleotidyltransferase gene. This 'initiator' might also act as the functional promoter related to the cellular differentiation and to development in EoL-1 cells. Northern blot analysis and the isolation of the longer-sized clone, EP-6, indicate the existence of a minor transcription start site.

When PAF binds to its receptor, it activates G-protein, which in turn can activate either a phosphoinositide-specific PLC or can inhibit adenylate cyclase. The G-protein(s), PLC and/or adenylate cyclase constitute the 'signal-transducer proteins' for PAF receptor in various cells and tissues [31,36]. The type of G-protein(s) involved in the PAF responses may differ from cell to cell, and depend on which effector system is coupled [30,31]. PAF-stimulated IP_3 production was sensitive to PTX treatment in rabbit neutrophils, human macrophages, and human platelets, and partially sensitive in A431 cells and rabbit platelets. However, it was resistant in U937 cells and peripheral blood mononuclear leukocytes (reviewed in [31]). Thus, both PTX-sensitive and -insensitive G-protein may be involved in the PLC pathway through PAF receptor. In a previous study, PTX attenuated PAF-induced $[Ca^{2+}]_i$ changes in human eosinophils [37]. Our present results also suggest that the PLC pathway is largely coupled with PTX-sensitive G-protein(s) in EoL-1 cells. This PLC pathway through the PAF receptor is involved in Ca^{2+} -dependent cellular responses, such as degranulation in eosinophils [22,37].

We have characterized the expression of specific, biologically active receptors for PAF as a newly recognized function of eosinophilic differentiation in EoL-1 cells. This provides a well characterized system useful for studies of mechanisms of PAF-stimulated cellular signal-transduction events and for identifying regulatory factors for leukocyte-type mRNA to express PAF receptors.

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