Functional platelet-activating factor receptors linked to inositol lipid hydrolysis, calcium mobilization and tyrosine kinase activity in the human endometrial HEC-1B cell line

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Platelet-activating factor (PAF; sn-1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is thought to be an important mediator of embryo–endometrial interactions in early pregnancy, and an understanding of its role in the establishment of early human pregnancy can only follow an understanding of its mechanism of action. In a human endometrial epithelial cell line, HEC-1B, the presence of mRNA encoding the platelet-activating factor receptor was demonstrated by reverse transcription–polymerase chain reaction. The presence of functional receptors was shown by inositol trisphosphate accumulation and a rise in the concentration of intracellular free calcium evoked by platelet-activating factor in myo-[2,3H]inositol-labelled and fura-2-loaded cells, respectively. Platelet-activating factor evoked rapid and concentration-dependent increases in the concentration of intracellular free calcium and inositol trisphosphate that were inhibited by the platelet-activating factor receptor antagonist WEB 2086, indicating that the responses are receptor mediated. Inositol trisphosphate accumulation evoked by platelet-activating factor was unaffected by pretreatment with pertussis toxin. Platelet-activating factor also stimulated the tyrosine phosphorylation of at least two major proteins of 80 kDa and 44 kDa; the smaller protein is an isoform of mitogen-activated protein kinase. These results show that functional platelet-activating factor receptors are located on the endometrial epithelial cell line HEC-1B and are linked to inositol lipid hydrolysis, calcium mobilization and tyrosine kinase activity.

Introduction

Disorders of implantation cause infertility and may be the cause of life-threatening complications in pregnancy (Reginald et al., 1987). Impaired implantation is a major cause of peri-implantation embryo loss (Yovich and Matson, 1988), and Wilcox et al. (1988) reported that about 30% of all pregnancies end before they are detected clinically. Abnormalities might arise in the preparation of the endometrium, which results in failed adhesion or abnormal development of pre- or post-implanted embryos.

Successful mammalian pregnancy requires the preparation of a receptive endometrium to which the embryo attaches and invades. Implantation is characterized by an inflammatory-type response, that is, expansion of extracellular fluid volume, increased vascular permeability and vasodilatation (Hertig, 1964; McRae and Heap, 1988). Morphological and animal studies show that the concentrations of the prostaglandins PGE₂, PGF₂α and 6-keto-PGF₁α are high at implantation sites (Kennedy and Zamecnik, 1978). PGE₂ induces changes in local vascular permeability and stromal oedema that accompany implantation (Kennedy, 1983). Indomethacin, an inhibitor of prostaglandin synthesis, prevents implantation in mice (Kinoshita et al., 1985), rabbits (Snabes and Harper, 1984) and rats (Acker et al., 1988).

Platelet-activating factor (PAF; sn-1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is an inflammatory mediator, antagonists of which inhibit implantation in mice (Spinks and O’Neill, 1988; Ando et al., 1990; Spinks et al., 1990) and rats (Acker et al., 1988). The failure of one study (Milligan and Finn, 1990) to confirm these findings might be explained by the choice of antagonists and the mode of drug administration (Harper, 1992). PAF increases vascular permeability, as PAF antagonists result in significantly fewer dye bands in the uterus on day 4 of pregnancy in mice (Spinks and O’Neill, 1988). PAF is not only produced by stromal cells of human endometrium,
where its concentration is hormonally regulated (Alecozay et al., 1991), but also by mouse (Ryan et al., 1989), sheep (Battye et al., 1991) and human embryos (Collier et al., 1990); addition of PAF to culture medium increases the metabolic rate, cleavage rate and implantation potential of mouse embryos (Ryan et al., 1990a, b). Studies in women confirm these findings (O'Neill et al., 1989, 1992).

The cellular mechanism(s) whereby platelet-activating factor (PAF) enhances prostaglandin biosynthesis may play a pivotal role in local cellular events at the time of implantation in the human endometrium (Ahmed and Smith, 1992a). In human endometrial explants, PAF-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) was suggested to be under ovarian steroid regulation (Ahmed and Smith, 1992b). The hydrolysis of PtdIns(4,5)P_2 by phosphoinositidase generates inositol trisphosphate (Ins(1,4,5)P_3) and 1,2-diacylglycerol (DAG). Ins(1,4,5)P_3 stimulates the release of stored calcium (Ca^{2+}), while DAG is the main physiological activator of phosphatidylinerine-dependent protein kinase C (Berridge and Irvine, 1989).

Molecular cloning of the PAF receptor from a human leucocyte cDNA library implies that it is a G-protein-linked receptor with seven transmembrane loops (Nakamura et al., 1991). By using the reverse transcription–polymerase chain reaction (RT–PCR) this study for the first time demonstrates mRNA encoding the PAF receptor in proliferative and secretory human endometrium and also in a human endometrial epithelial cell line (HEC-1B). This cell line is derived from uterine epithelium, and retains many of the characteristics of endometrial epithelial cells. Evidence is presented to show that PAF receptors on HEC-1B cells are functionally linked to inositol lipid hydrolysis, the mobilization of intracellular free Ca^{2+} ([Ca^{2+}]) and activation of a tyrosine kinase pathway.

Materials and Methods

Materials

All radiolabelled compounds were from Amersham International (Amersham) and fura-2/AM was from Calbiochem (Nottingham). PAF-acether (De-hydro-PAF [C_{16}],) phorbol myristate acetate (PMA), epidermal growth factor (EGF) EGTA, DMSO digitonin and BSA were from Sigma Chemical Co. (Poole, Dorset). HEC-1B cells were purchased from the American Type Culture Collection (Rockville, MD). Components for the culture medium were obtained from Flow Laboratories (High Wycombe).

Preparation and stimulation of cells

HEC-1B cells were maintained in 175 cm² flasks in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal calf serum, 1% L-glutamine and 1% antibiotic–antimycotic mixture (10 000 U penicillin, 10 µg streptomycin ml⁻¹ and 25 µg fungizone ml⁻¹) at 37°C in 95% O₂ and 5% CO₂ at 95% humidity. Subconfluent monolayers were plated at a density of 10⁶ cells ml⁻¹ in 12-well culture plates and labelled for 48 h at 37°C with ^3H]inositol (5 µCi ml⁻¹) in inositol-free DMEM without fetal calf serum. Before agonist stimulation, labelling medium was removed and the cells washed by incubation with 0.5 ml DMEM, pH 7.4, containing 0.2% (w/v) BSA for 30 min followed by two consecutive incubations for 15 min in this buffer. After the steps to remove the unincorporated label, stimulations were initiated by the addition of PAF-acether in buffer containing 0.2% (w/v) BSA or PAF-acether in the presence of the PAF antagonist WEB2086 or PMA or EGF or vehicle.

Preparation of RNA and RT–PCR

RNA was prepared from frozen tissue by the method of Chomsinziski and Sarchi (1987). Briefly, tissue was homogenized in a buffer containing guanidine thiocyanate (Gibco, Uxbridge) and total RNA purified by acid–phenol extraction and ethanol precipitation. cDNA was synthesized using avian myoblastic virus (AMV) reverse transcriptase (Super RT, HT Biotechnology, Cambridge). RNA (3–5 µg) was primed with oligo dT (Pharmacia, Milton Keynes), according to the manufacturers’ instructions for 60 min at 42°C. For initial amplification by PCR, 1 µl of the RT products were amplified with AmpliTaq (Cetus, Emeryville) in the buffer recommended by the manufacturers. The reaction mixture was amplified with primers 1 and 4 for 30 cycles as follows: 95°C, 60 s; 56°C, 50 s; 72°C, 60 s. A fiftieth of the product was transferred to a fresh reaction mix (50 µl) containing primers 2 and 3, and reamplified using the above conditions for 25 cycles. The products (10 µl) were then analysed by 2% agarose gel electrophoresis. The primers used in this study that are specific for the PAF receptor are shown in Table 1.

<table>
<thead>
<tr>
<th>Primer number</th>
<th>Bases</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-96 to -78</td>
<td>CCA GGA CCC AGA CAG AGA</td>
</tr>
<tr>
<td>2</td>
<td>-76 to -57</td>
<td>ACA CGG TCA CTG CAT GTG AA</td>
</tr>
<tr>
<td>3</td>
<td>1189 to 1171</td>
<td>CTG GCT CTG CAT CAT CAG T</td>
</tr>
<tr>
<td>4</td>
<td>1212 to 1190</td>
<td>GAG TTC TGG ATT TTC CAA CAG C</td>
</tr>
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*Numbering is according to Nakamura et al. (1991).

Measurement of inositol phosphate accumulation

For experiments with inositol phosphate, the cells were incubated for 10 min with 10 mmol LiCl 1⁻¹ and stimulation was initiated for the times indicated, in a final volume of 0.5 ml at 37°C. The reaction was terminated by aspirating the medium and replacing it with 0.5 ml of 15% (w/v) trichloroacetic acid at 4°C. The multwell plates were kept at 4°C for 15 min to extract cellular [^3H]labelled inositol phosphates. The ether-extracted and neutralized inositol phosphate fractions were analysed by anion-exchange chromatography on small columns.
of Dowex 1-X8, as described by Ahmed and Smith (1992b). The inositol trisphosphate ([3H]InsP$_3$) fraction was collected directly into scintillation vials, to which was added 10 ml of scintillation fluid, and was then counted in a scintillation analyser (Canberra Packard Instrument Co., Zurich).

**Intracellular Ca$^{2+}$ measurements**

For [Ca$^{2+}$]$_i$ measurement, cells were resuspended in a medium containing (in mmol l$^{-1}$) 145 NaCl, 5 KCl, 1 MgSO$_4$, 10 sodium Hepes, 1 CaCl$_2$, 0.2% BSA, pH 7.4, at 37°C, at a density of about $1.5 	imes 10^6$ cells ml$^{-1}$. Cells were loaded with fura-2 by incubating them with 2 µmol fura-2/AM l$^{-1}$ for 60 min at room temperature (18–22°C). The cells were then collected by centrifugation for 5 min at 350 g and washed once in medium as above. Cells were resuspended at a final density of approximately $2 	imes 10^6$ cells ml$^{-1}$ in medium as above but without CaCl$_2$ or BSA.

Fura-2 fluorescence was measured from 2 ml aliquots of suspension in a stirred cuvette that was maintained at 37°C, using a Cairn Research Spectrophotometer (Cairn Research, Sittingbourne). CaCl$_2$ (1 mmol l$^{-1}$) or EGTA (1 mmol l$^{-1}$) were added as required. Excitation wavelengths of 340 and 380 nm were provided by a rotating filter wheel and fluorescence was measured at 500 nm. [Ca$^{2+}$]$_i$ was calculated from the 340:380 nm fluorescence ratio, according to the method of Grynkiewicz et al. (1985). $R_{\text{max}}$ was determined after addition of 50 nmol digitonin l$^{-1}$ in the presence of 1 mmol CaCl$_2$ l$^{-1}$ and $R_{\text{min}}$ was determined after the subsequent addition of 20 mmol EGTA l$^{-1}$ and 20 mmol Tris base l$^{-1}$.

In some experiments manganese ions were used as a tracer for divalent cation entry. Cells were stimulated in the presence of 1 mmol CaCl$_2$ l$^{-1}$ and 0.5 mmol MnCl$_2$ l$^{-1}$. The quench of fura-2 fluorescence by Mn$^{2+}$ was determined at the isobestic (Ca$^{2+}$-insensitive) excitation wavelength of 360 nm (Sage et al., 1989).

**Protein tyrosine phosphorylation**

Cells were incubated with agonist or vehicle as outlined above. The reaction was terminated by rapid aspiration followed by three washes in ice-cold buffer containing 50 mmol Hepes l$^{-1}$, 10 mmol Na$_2$P$_2$O$_7$ l$^{-1}$, 100 mmol NaF l$^{-1}$, 4 mmol EDTA l$^{-1}$ and 2 mmol Na$_3$VO$_4$ l$^{-1}$. Hot sample buffer (750 µl) was added to the cells and the cell contents solubilized by passing the sample repeatedly through a syringe needle (gauge 21). The samples were boiled for 5 min and stored at −80°C until use. Samples (20 µl) were run on SDS–PAGE (10%) and electroblotted on to nitrocellulose. The blot was blocked for 3 h in Tris-buffered saline (150 mmol NaCl l$^{-1}$, 50 mmol Tris l$^{-1}$) containing 0.2% Tween (NaTT) and 3% BSA at room temperature, and then incubated overnight in NaTT containing 2% BSA and 100 ng ml$^{-1}$ of a rabbit polyclonal antiphosphotyrosine antibody. The blots were then washed repeatedly with NaTT (4 × 15 min) and incubated in NaTT containing 0.2% BSA and 1:6000 dilution of anti-rabbit immunoglobulin linked to horseradish peroxidase (Amersham International) for 60 min. The blots were rinsed in NaTT (×3) and washed for a further 2 h (8 × 15 min). The blots were then developed using enhanced chemiluminescence detection as detailed by the manufacturer (Amersham International).

**Statistical analysis**

Statistical analysis was performed using the Student’s paired $t$ test.

**Results**

The presence of mRNA encoding the PAF receptor was demonstrated using RT–PCR. cDNA from HEC-1B cells was amplified using the primers 1 and 4 followed by 2 and 3 (Table 1). This produced a single band of the predicted size. The band of 1150 bp was sequenced to confirm that it was PAF receptor cDNA (Fig. 1).

The dependency of the PAF-potentiating effect on the dose and time of [3H]InsP$_3$ accumulation was determined (Fig. 2).
Stimulation of the human endometrial epithelial cell line with 100 nmol PAF 1⁻¹ caused the rapid generation of [³H]InsP₃, which reached 73.4% ± 7.28% more than the control value after 30 s, the earliest time-point measured (n = 4, P < 0.01) and increased to 2.815 times the basal activity after 2 min (P < 0.001; Fig. 2a). Thereafter, the concentration remained high and the rate of increase in [³H]InsP₃ was less pronounced. The dose dependency of PAF-stimulated [³H]InsP₃ accumulation in HEC-1B cells over 20 min is shown (Fig. 2b). Within the concentration range of 1–1000 nmol l⁻¹, PAF produced a progressive increase in [³H]InsP₃ formation.

The cytosolic free Ca²⁺ concentration was determined by measuring the fluorescence intensity of intracellular fura-2 AM. Resting [Ca²⁺]ᵢ in HEC-1B cells was 53 ± 5 nmol l⁻¹ (n = 18) in the presence of 1 mmol external Ca²⁺ 1⁻¹ and 22 ± 3 nmol l⁻¹ (n = 15) in the absence of external Ca²⁺ (with 1 mmol EGTA 1⁻¹ and no added Ca²⁺). PAF (100 pmol l⁻¹–1 µmol l⁻¹) evoked a rise in [Ca²⁺]ᵢ in the absence of external Ca²⁺, indicating the release of Ca²⁺ from internal stores (Fig. 3a). In the presence of 1 mmol external Ca²⁺ 1⁻¹, the PAF-evoked increase of [Ca²⁺]ᵢ reached a larger peak and then declined to reach a plateau above the resting level, suggesting stimulated Ca²⁺ entry in addition to release (Fig. 3b). The peak PAF-evoked rises in [Ca²⁺]ᵢ at concentrations of 100 pmol l⁻¹, 10 nmol l⁻¹ and 1 µmol l⁻¹ were 68 ± 18, 390 ± 29 and 468 ± 43 nmol l⁻¹, respectively, in the absence of external Ca²⁺ and 112 ± 12, 690 ± 54 and 1078 ± 43 nmol l⁻¹, respectively, in the presence of 1 mmol external Ca²⁺ 1⁻¹ (n = 5). The time of onset decreased and the rate of rise in [Ca²⁺]ᵢ increased with increasing concentrations of PAF in both the presence and absence of external Ca²⁺ (Fig. 3a and b).

To assess specificity of the PAF receptor, experiments were conducted in the presence of the specific PAF receptor antagonist WEB 2086 (Casals-Stenzel et al., 1987). The increase in [Ca²⁺]ᵢ, evoked by 10 nmol PAF 1⁻¹ in the presence of external Ca²⁺ was completely blocked by the prior addition of WEB 2086 (10 nmol l⁻¹), indicating that the response is mediated by the PAF receptor (Fig. 4). WEB 2086 inhibited PAF-evoked [³H]InsP₃ production in a similar way (results not shown).

PAF (10 nmol l⁻¹) evoked an increase in the rate of entry of Mn²⁺, although this was rather small and showed a delay in onset that was longer than that observed for the increases in [Ca²⁺]ᵢ, in both the presence and absence of external Ca²⁺.
Fig. 4. Effect of the platelet-activating factor (PAF) antagonist WEB 2086 on the PAF-evoked rise in the intracellular calcium concentration ([Ca$^{2+}$]) in human HEC-1B cells. Fura-2-loaded cells were stimulated with 10 nmol PAF l$^{-1}$ in the presence of 1 mmol external Ca$^{2+}$ l$^{-1}$ after preincubation with 10 µmol WEB 2086 l$^{-1}$ for 2 min (WEB 2086), or the vehicle alone (control).

Fig. 5. Entry of Mn$^{2+}$ into fura-2-loaded human HEC-1B cells evoked by platelet-activating factor. Cells were suspended in 1 mmol external calcium l$^{-1}$ and 0.5 mmol MnCl$_2$ l$^{-1}$ was added just before the experiment was performed. Traces show fura-2 fluorescence recorded at the isosbestic wavelength (360 nm) in arbitrary units. The upper trace shows the basal level of Mn$^{2+}$ quench (control); the lower trace shows the effect of adding 10 nmol PAF l$^{-1}$. Digitonin (50 nmol l$^{-1}$) was added to confirm the availability of unquenched indicator at the end of the experiment.

The study reported here shows that functional PAF receptors are expressed by the transformed endometrial cell line HEC-1B. This was demonstrated by the expression of mRNA encoding the PAF receptor and also by the fact that the PAF receptor antagonist WEB 2086 attenuated the PAF-evoked inositol trisphosphate accumulation and rise in [Ca$^{2+}$]. Although autoradiography studies have indicated binding sites for PAF in the rabbit endometrium (Kudolo et al., 1991), the location of PAF receptors in specific cells of the human endometrium has not previously been determined (Van der Weiden et al., 1991). The expression of PAF receptor mRNA in both the epithelial cell line and normal endometrium suggests that PAF receptors may be located on the epithelial cells in normal human endometrium.

The observations that PAF-stimulated PtdIns(4,5)P$_2$ hydrolysis and [Ca$^{2+}$] mobilization are inhibited by WEB 2086 suggests that the effect is mediated by a specific PAF receptor.

Discussion

The study reported here shows that functional PAF receptors are expressed by the transformed endometrial cell line HEC-1B. This was demonstrated by the expression of mRNA encoding the PAF receptor and also by the fact that the PAF receptor antagonist WEB 2086 attenuated the PAF-evoked inositol trisphosphate accumulation and rise in [Ca$^{2+}$]. Although autoradiography studies have indicated binding sites for PAF in the rabbit endometrium (Kudolo et al., 1991), the location of PAF receptors in specific cells of the human endometrium has not previously been determined (Van der Weiden et al., 1991). The expression of PAF receptor mRNA in both the epithelial cell line and normal endometrium suggests that PAF receptors may be located on the epithelial cells in normal human endometrium.

The observations that PAF-stimulated PtdIns(4,5)P$_2$ hydrolysis and [Ca$^{2+}$] mobilization are inhibited by WEB 2086 suggests that the effect is mediated by a specific PAF receptor.
The response to PAF resulted in the rapid accumulation of the $[^{3}H]$InsP$_3$ fraction, which was inhibited by WEB 2086 — indicating a receptor-mediated activation of endometrial PtdIns(4,5)P$_2$ specific phospholipase C. The present study and the observation that PAF production is confined to stromal cells (Alecozay et al., 1991) suggest a paracrine role for PAF in human endometrium.

PAF bound to a specific membrane receptor activates the hydrolysis of PtdIns(4,5)P$_2$ by phosphoinositidase via a G protein to yield DAG and Ins(1,4,5)P$_3$ (Barzaghi et al., 1989). Ins(1,4,5)P$_3$ induces an increase in [Ca$^{2+}$], which is required for Ca$^{2+}$-dependent processes. High [Ca$^{2+}$], increases the activity of phospholipases. In many tissues, including human (Bonney, 1985) and guinea-pig (Downing and Poyser, 1983) endometrium, phospholipase A$_2$ activity is dependent on Ca$^{2+}$, which is therefore influenced by the release of Ca$^{2+}$ from intracellular stores. Stimulation of phospholipase A$_2$ activity by Ca$^{2+}$ after exposure to PAF represents an additional potential source of arachidonic acid for prostaglandin synthesis. Smith and Kelly (1988) have shown that PAF stimulates PGE$_2$ production only from secretory-phase endometrium, which is consistent with the pattern of inositol phosphate accumulation (Ahmed and Smith, 1992b). The stimulation of endometrial phospholipase C by PAF may in part account for PAF-induced secretion of PGE$_2$. Thus, PAF-induced prostaglandin synthesis may arise either by the release of arachidonic acid from DAG or by increased phospholipase A$_2$ activity.

PTX has been used in several tissues and cell types to test whether a G protein is involved in the coupling of receptors to phospholipases by a second messenger. In HEC-1B cells, the formation of $[^{3}H]$InsP$_3$ in response to PAF appears to be mediated by a PTX-insensitive G protein, as does PAF-evoked [Ca$^{2+}$], and inositol phosphate synthesis in U-937 cells (Barzaghi et al., 1989).

A recent study has shown that PAF stimulates the activation of the tyrosine kinase pp60$^{c-src}$ in rabbit platelets and causes its rapid translocation from cytosol to the membranes (Dhar and Shukla, 1991). We found that in HEC-1B cells PAF stimulated the tyrosine phosphorylation of two major proteins of approximately 80 kDa and 42–44 kDa. Although these proteins have not been identified, recent studies suggest that pp44 is an isoform of MAP kinase (Ray and Sturgill, 1987). The finding that both PMA and EGF stimulate the phosphorylation of pp44 support this suggestion as MAP kinase can be activated by tyrosine kinase receptor activation and by PKC-mediated activation (Wood et al., 1992). This enzyme family has been shown to play a pivotal role in the regulation of cell division in a number of cell types (Pulvener et al., 1991; Wood et al., 1992).

Both PMA and PAF also stimulate the tyrosine phosphorylation of pp80. In other cells this protein has been shown to be strongly activated by classical G-protein-linked agonists and PMA (Zachary et al., 1990). It is unclear whether the activation of pp80 is controlled by a PKC-dependent kinase upstream of pp80 or if pp80 itself contains sites that are regulated by phosphorylation of serine residues (Saville et al., in press). Taken together, these results suggest that functional PAF receptors may play a role in the regulation of growth and...
division of endometrial cells by coupling to a tyrosine kinase pathway.

In addition to MAP kinase, a number of tyrosine kinase substrates, such as focal adhesion kinase and cytoskeletal proteins, may also be regulated by phosphorylation of tyrosine residues (Saville et al., in press). As PAF stimulates tyrosine phosphorylation of a number of proteins, it is reasonable to speculate that it may also regulate endometrial cell motility and adhesion by activating this pathway. PAF antagonists have been shown to inhibit trophoblast outgrowth in vitro in a dose-dependent manner (Spinks et al., 1990), suggesting that PAF may be involved in the activation of blastocyst attachment and the resulting differentiation of the trophectoderm into invasive trophoblast. These events may be mediated by tyrosine kinase substrates. Hence, HEC-1B cells provide a convenient model to study PAF-mediated intracellular signal-transduction mechanisms in uterine epithelium, and will help to increase our understanding of the cellular mechanism(s) of implantation, which may lead to new methods of pharmacological intervention for infertility and contraception.

This work was supported by grant No. 030060/1.5 from the Wellcome Trust. S. O. Sage was a Royal Society University Research Fellow of 1983. The authors thank C. H. Weber, Boehringer Ingelheim Ltd, Ingelheim, for the generous gift of WEB 2086.

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