Influence of inhibitors on increase in intracellular free calcium and proliferation induced by platelet-activating factor in bovine oviductal cells

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Oviductal endosalpingeal cells were isolated mechanically from heifers and cultured until there was 100% confluency. The cells were loaded with the Ca^{2+} -sensitive fluorochrome, fura-2/acetoxymethylester, and cytosolic free calcium ($[\text{Ca}^{2+}]_i$) was monitored by spectrofluorimetry. Platelet-activating factor, at a concentration of 30 nmol l^{-1} , induced an intracellular Ca^{2+} increase in cultured bovine oviductal cells, mainly via influx from the extracellular space. In fura-2-loaded oviductal cells, different Ca^{2+} channel blockers were investigated to characterize the pathways responsible for the Ca^{2+} influx. The negative effects of Ni^{2+} -, La^{3+} - and Ca^{2+} -activated K^+ channel blockers, such as apamin and charybdotoxin, and Ca^{2+} channel blockers, such as dotarizine, on the platelet-activating factor-induced $[\text{Ca}^{2+}]_i$ increase indicate the minor participation of the voltage-gated Ca^{2+} channels. TMB-8 and flufenamic acid blocked the platelet-activating factor-induced Ca^{2+} increase directly on non-selective cationic channels or acted via a Ca^{2+} release-triggered Ca^{2+} influx. Platelet-activating factor, at concentrations of $1.25 \text{ } \mu\text{mol l}^{-1}$ and $2.5 \text{ } \mu\text{mol l}^{-1}$, significantly stimulated the proliferation and depolarization of oviductal cells, but $10 \text{ } \mu\text{mol l}^{-1}$ significantly decreased both parameters and exerted a cytotoxic effect on cells. After incubation with TMB-8 or flufenamic acid, the cell proliferation was inhibited in a concentration-dependent manner, with IC_{50} values of $26.57 \text{ } \mu\text{mol l}^{-1}$ and $95.29 \text{ } \mu\text{mol l}^{-1}$, respectively. The depolarization was significantly inhibited at $50 \text{ } \mu\text{mol l}^{-1}$ for both TMB-8 and flufenamic acid. The results of the present study may contribute to further understanding of the mechanism behind the actions of platelet-activating factor on oviductal cells.

Introduction

The oviduct is the site of many events involved in mammalian reproduction, including gamete transport, fertilization, early embryonic development and embryo transport. Morphological and functional changes occur in oviductal ciliary activity, which plays a central role in ampullary transport of the ova or early embryo and is under the control of steroids (Nozaki and Ito, 1987) and humoral factors, such as platelet-activating factor (PAF). Studies by Velasquez *et al.* (1995) indicate that the administration of PAF to cyclic hamsters hastens transport of their oocytes. Furthermore, Hermoso and Villalon (1995) reported that the ciliary beat of hamster oviductal ciliated cells *in vitro* is increased by the addition of PAF at concentrations from 20 to 200 nmol l^{-1} , indicating that the earlier passage of embryos to the uterus may be mediated by PAF. Yang *et al.* (1992) detected the presence of PAF receptors in the oviductal membranes of rabbits during early pregnancy using a

radioligand binding assay. PAF plays a significant paracrine role in stromal-epithelial cell interactions in the uterus during the implantation process (Harper, 1989), but its physiological role in oviduct function is unclear. Tiemann *et al.* (1996) reported that PAF induces a transient increase in intracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_i$, which may have a significant effect on cell function. PAF had a stimulatory effect on the proliferation of bovine endothelial cells (Lin and Rui, 1994), human endometrial HEC-1B cells (Ahmed *et al.*, 1994), and bovine endometrial stromal cells (Tiemann *et al.*, 1995). Previous studies showed that a PAF-induced increase in $[\text{Ca}^{2+}]_i$ in oviductal cells was, for the most part, suppressed by a Ca^{2+} -free solution, indicating that the response is mostly dependent on Ca^{2+} influx from extracellular sources (Tiemann *et al.*, 1996). The mechanism by which PAF increases calcium influx in oviductal cells is not yet clear. Extracellular calcium is thought to enter cells through calcium-specific plasma membrane channels sensitive to receptor or voltage gating (Olsen *et al.*, 1989; Triggle, 1990; Tsien, 1990). PAF seems to play a role in this process since exposure of oviductal cells to PAF leads to rapid and marked

intracellular increase in free calcium. However, the voltage-sensitive Ca^{2+} channel classical antagonist, verapamil, had no effect on the PAF-induced increase in $[\text{Ca}^{2+}]_i$ (Tiemann *et al.*, 1996). Since a Ca^{2+} -activated K^+ -channel is present in the apical membrane of rabbit oviduct, as reported by James and Okada (1994), it is hypothesized that PAF exerts its effect on changes in $[\text{Ca}^{2+}]_i$ and, as a corollary, on the proliferation of $[\text{Ca}^{2+}]_i$ via verapamil non-sensitive channels. This hypothesis was investigated using inhibitors and the measurement of their effects on the change of $[\text{Ca}^{2+}]_i$ in oviductal cells. Furthermore, PAF may act via ion channels responsible for the total transmembrane potential difference and proliferation in intact cells. The transmembrane potential difference and proliferation in reaction to PAF and inhibitors that influenced the PAF effect were examined to obtain information about these processes. In certain cell types, the increase in $[\text{Ca}^{2+}]_i$ leads to specific responses, such as DNA synthesis and proliferation (Gregory and Kern, 1978; Popper and Batra, 1988; Humes and Cielinski, 1991). PAF is mitogenic for guinea-pig bone marrow cells (Kato *et al.*, 1988), rat mesangial cells (Montero *et al.*, 1993), mouse embryos (Roberts *et al.*, 1993) and bovine endometrial stromal cells (Tiemann *et al.*, 1995).

Oviductal cells were pretreated with the following to elucidate the pathway responsible for the Ca^{2+} influx: (i) Ni^{2+} and La^{3+} , which are known to block the influx of extracellular calcium through plasma membrane calcium channels (Triggle, 1990; Tsien, 1990; Lester, 1991); (ii) dotarizine (1-(diphenylmethyl)-4-1-3-(phenyl-1,3-dioxalen-2-yl)propyl piperazine), which has the potency to block Ca^{2+} entry, Ca^{2+} channel receptors, Ca^{2+} signals and Ca^{2+} movements (Tejerina *et al.*, 1993; Czernicki *et al.*, 1996); (iii) flufenamic acid, which is known to block Cl^- and non-selective ion channels (Wangemann *et al.*, 1986; Gögelein *et al.*, 1990); (iv) charybdotoxin (CTX), a peptide neurotoxin isolated from scorpion venom, which inhibits Ca^{2+} -activated 'maxi K^+ ' channels (Di Berardino *et al.*, 1993), and apamin, a peptide present in bee venom, which can block Ca^{2+} -activated 'small K^+ ' channels (Blatz and Magleby, 1986); and (v) TMB-8 [8-(diethylamino)octyl-3,4,5-trimethoxybenzoate], which has inhibitory effects on both calcium influx and calcium release from intracellular stores (Yue *et al.*, 1991a,b).

Materials and Methods

Materials

Tissue culture supplies, platelet-activating factor, EGTA, Triton X-100, trypsin-EDTA solution, Trypan blue solution, propidium iodide (PI), TMB-8 [8-(*N,N*-diethylamino)octyl-3,4,5-trimethoxybenzoate], flufenamic acid, LaCl_3 and NiCl_2 were obtained from Sigma Chemie GmbH (Deisenhofen). Dotarizine was from Alexis (Läufelfinger). Fura-2/acetoxymethylester (fura-2/AM) was obtained from Boehringer Mannheim Biochemicals (Mannheim). Bis-(1,3-dibutylbarbituric acid)trimethine-oxonol [DiBaC₄(3)], apamin and charybdotoxin were obtained from Molecular Probes (Eugene). All other materials were from Sigma.

Animals

Dairy heifers from a commercial abattoir were used. The reproductive tract was removed immediately after slaughter and transported on ice to the laboratory.

Oviductal cell preparation and culture

The isolation of oviductal endosalpingeal cells was carried out mechanically as described by Tiemann and Hansen (1995). Briefly, oviducts collected from heifers were stored in Dulbecco's PBS until processing. Oviducts were separated from connective tissue and the fimbria was removed. Each oviduct was then stripped of endosalpingeal cells by moving the forceps from the utero-tubal junction towards the infundibulum. The endosalpingeal cells were extruded by opening the infundibulum and then were washed three times in TCM-199 supplemented with 1% (v/v) antibiotic-antimycotic (ABAM). Cells from each heifer were cultured separately. Viability, determined by Trypan blue exclusion, was 95%. Single and aggregated oviductal cells (approximately 2×10^6 cells ml^{-1} , as determined by haemocytometer counting) were cultured in 80 cm^2 tissue culture flasks (Greiner, Frickenhausen). The cells were cultured until 100% confluence in TCM-199 with 10% (v/v) FCS and 1% (v/v) ABAM at 37°C in humidified air (5% CO_2). The medium was changed every 2 days. After 6–8 days of culture, the adherent cells were gently washed twice with incomplete Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} . Ten millilitres of trypsin-EDTA (0.02–0.05%) in incomplete HBSS was then added and kept at 37°C for 20–30 min. FCS (final concentration 10%, v/v) was added to the cell suspension. The cells were washed twice with PBS and resuspended in RPMI 1640 medium for the determination of $[\text{Ca}^{2+}]_i$ or in TCM-199 for the detection of the transmembrane potential difference changes and in cell proliferation assays.

Measurements of intracellular calcium

The fluorescence of the fura-2-loaded oviductal cells was measured with a spectrofluorometer (Shimadzu RF-5001 PC). Briefly, the cells were loaded with fura-2/AM (final concentration, $2 \mu\text{mol l}^{-1}$) in RPMI 1640 medium under gentle shaking in the dark at room temperature for 45 min. The cells were centrifuged (500 g for 3 min at 4°C) and washed three times in sodium-Hepes buffer, pH 7.4. Fura-loaded cells (2×10^6) were transferred to a thermostatic quartz cuvette and stirred continuously at 37°C. After equilibration (5 min, 37°C), each agonist was pipetted directly into the cell suspension before addition of PAF. Fura-2-loaded cells were preincubated with apamin for 50 s, TMB-8, LaCl_3 or NiCl_2 for 2 min, flufenamic acid for 5 min, charybdotoxin for 15 min, and dotarizine for 90 min before the addition of PAF. PAF was added at a final concentration of 30 nmol l^{-1} . Fluorescence was excited at 340 and 380 nm using a rotating filter wheel, and the emission was measured at 490 nm. The fluorescence intensities of the ratio 340:380

were used for calculating the concentrations of $[Ca^{2+}]_i$ as described by Tiemann *et al.* (1996).

Detection of transmembrane potential difference

Changes in transmembrane potential differences were described using the oxonol dye DiBaC₄(3) as an indicator (Löhrke *et al.*, 1995). The negatively charged oxonol is repelled from intact cells with high transmembrane potential (Löhrke *et al.*, 1997). In depolarizing the cells, the fluorescence intensity increases proportionally to the decrease of the transmembrane potential difference, as has been described for a variety of cell types (Rink *et al.*, 1980).

The cells in monolayer were treated without and with different concentrations of PAF (0, 1.25, 2.5, 5 and 10 $\mu\text{mol l}^{-1}$), TMB-8 (0, 2.5, 5, 10, 20, 50 and 100 $\mu\text{mol l}^{-1}$) or flufenamic acid (0, 5, 10, 20, 50 and 100 $\mu\text{mol l}^{-1}$) in serum-free TCM-199 medium for 24 h. The adherent cells were washed gently twice with HBSS without Ca^{2+} or Mg^{2+} . Then trypsin-EDTA in HBSS was added. After 20–30 min, the cells were washed twice with PBS and resuspended in PBS. The cells were incubated with DiBaC₄(3) in PBS at a final concentration of 0.25 $\mu\text{mol l}^{-1}$ at 37°C for 30 min, and then the cells were washed twice with PBS. In cells with inside-negative potentials, the negatively charged oxonol dye is excluded from cells as their transmembrane potential becomes more negative. In a calibration experiment, the oviductal cells were equilibrated by buffer solution (156 mmol l^{-1} NaCl, 5 mmol l^{-1} Hepes). A depolarization effect of cells was detected by iso-osmotically varying the extracellular K^+ concentration (60 and 100 mmol l^{-1} KCl). In comparison with the control, the depolarization increased to 117% at 60 mmol KCl l^{-1} and to 146% at 100 mmol KCl l^{-1} . The viability of cells was assayed by the propidium iodide (PI) exclusion test. PI was added to the cell suspension at a final concentration of 40 $\mu\text{mol l}^{-1}$ for 5 min at room temperature (Löhrke *et al.*, 1995), and then the cells were washed twice with PBS.

The fluorescence was imaged by an Diaphot 200 (Nikon) microscope using an 100 W mercury lamp and an epi-fluorescence filter block with 450–490 nm (excitation)–510 nm (dichroic mirror)–520 nm long pass (emission). Images (512 $\times$ 512 $\times$ 24 bit) were grabbed and analysed using a chilled colour CCD-camera (C5310, Hamamatsu), a RGB-frame grabber (MVP, Matrox), a PC (Pentium®, Windows 95®) and image analysis software (analysis 2.01®, SIS). The total area of cells was measured for each fluorescence image using a phase-contrast picture of the same frame and automatic detection of objects with analySIS®. The corresponding fluorescence image was separated into two grey value pictures using the red and green intensities. In these two images, the stained areas and the mean values of intensities in the stained areas were monitored automatically.

Proliferation

Cells were plated at $2 \times 10^4 \text{ ml}^{-1}$ (24-well plate) in TCM-199 and 1% (v/v) ABAM with different concentrations of PAF

Fig. 1. Platelet-activating factor (PAF)-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) increase in fura-2-loaded bovine oviductal cells. Each value is the mean $\pm$ SD of two experiments.

(0, 1.25, 2.5, 5 and 10 $\mu\text{mol l}^{-1}$), TMB-8 (0, 2.5, 5, 10, 20, 50 and 100 $\mu\text{mol l}^{-1}$) or flufenamic acid (0, 5, 10, 20, 50, 100 and 200 $\mu\text{mol l}^{-1}$) in duplicate. After 48 h, the monolayers were washed with HBSS and then harvested by trypsin-EDTA as described above and dispersed into 0.9% (v/v) NaCl solution. Proliferative cell growth was determined by electronic particle counting (Coulter Counter Multisizer II).

The permeability of cells exposed to different concentrations of PAF, TMB-8 or flufenamic acid was assayed by Trypan blue exclusion test. Vital cells exclude the stain.

Statistical analysis

The experiment was run in sets of three or four for each oviductal cell preparation. Data are expressed as mean $\pm$ SD. Pairwise comparisons between treated and the corresponding controls were performed, using one-factor analysis of variance (ANOVA) and a Student-Newman-Keuls test to determine significant differences ($P < 0.05$). The relative data were transformed with SAS statistical software for estimation of IC_{50} values. The transforming model was the 'three parametric log transforming model'.

Results

Ca^{2+} response

Addition of PAF to the fura-2-loaded bovine oviductal cells resulted in an increase in fluorescence, indicative of a $[Ca^{2+}]_i$ increase. PAF at concentrations of 30 or 90 nmol l^{-1} induced increases in $[Ca^{2+}]_i$ in approximately the same range (Fig. 1). At PAF concentrations of 10 nmol l^{-1} and 20 nmol l^{-1} , the $[Ca^{2+}]_i$ increase was markedly reduced. The original trace of $[Ca^{2+}]_i$ for 10, 30 and 90 nmol l^{-1} PAF is shown (Fig. 2). The

Fig. 2. Original trace of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in fura-2-loaded bovine oviductal cells exposed to different concentrations of platelet-activating factor (PAF): (a) 90 nmol l^{-1} , (b) 30 nmol l^{-1} and (c) 10 nmol l^{-1} .

concentration of $30 \text{ nmol PAF l}^{-1}$ (final concentration) was used in the experiments with oviductal cells. Preincubation of the fura-2-loaded cells for 2 min with La^{3+} ($10 \mu\text{mol l}^{-1}$ final concentration) or Ni^{2+} ($20 \mu\text{mol l}^{-1}$ final concentration) did not inhibit the PAF-induced Ca^{2+} response (Fig. 3; representative picture for Ni^{2+}). Preincubation of the cells

Fig. 3. Representative tracing from three experiments showing no effect of $20 \mu\text{mol Ni}^{2+} \text{ l}^{-1}$ on platelet-activating factor (PAF)-induced intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in fura-2-loaded cultured bovine oviductal cells. The cells were incubated (a) without or (b) with Ni^{2+} (final concentration, $20 \mu\text{mol l}^{-1}$) for 2 min before the addition of PAF (final concentration, 30 nmol l^{-1}).

with apamin (0.1 or $0.2 \mu\text{mol l}^{-1}$ final concentration) (Fig. 4) and charybdotoxin (10 nmol l^{-1} final concentration) failed to inhibit PAF-induced $[\text{Ca}^{2+}]_i$ in oviductal cells. The addition of dotarizine (100 nmol l^{-1}) before the addition of PAF did not inhibit the Ca^{2+} response (data not shown). The PAF-induced $[\text{Ca}^{2+}]_i$ increase was reduced by TMB-8 in a dose-dependent manner. The increase of $[\text{Ca}^{2+}]_i$ caused by $30 \text{ nmol PAF l}^{-1}$ alone was $73.65 \pm 17.07 \text{ nmol l}^{-1}$. After preincubation of the cells with TMB-8 for 2 min, the PAF-induced Ca^{2+} increase was reduced in the following manner. With TMB-8 at concentrations of 5 , 10 and $20 \mu\text{mol l}^{-1}$, the increase was significantly reduced to $23.3 \pm 2.8\%$, $15.5 \pm 2.5\%$ and $6.9 \pm 3.2\%$ ($P < 0.05$; $n = 3$), respectively. After 5 min preincubation with $20 \mu\text{mol TMB-8 l}^{-1}$, a cytotoxic effect on cells was not detectable using propidium iodide and DiBAC₄(3) uptake as criteria for cell damage.

The addition of $20 \mu\text{mol flufenamic acid l}^{-1}$ 5 min before the addition of PAF led to the inhibition of the Ca^{2+} response (Fig. 5c). This effect was not cytotoxic because the DiBAC₄(3) and PI staining were the same as in the control. The

Fig. 4. Representative tracing from three experiments showing no effect of apamin on platelet-activating factor (PAF)-induced intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in fura-2-loaded cultured bovine oviductal cells. The cells were incubated (a) without or (b) with apamin (final concentration, $0.2 \mu\text{mol l}^{-1}$) for 50 s before the addition of PAF (final concentration, 30 nmol l^{-1}).

inhibition also occurs at concentrations of $10 \mu\text{mol}$ flufenamic acid l^{-1} (data not shown), but preincubation of cells with 5 mol flufenamic acid l^{-1} resulted in the partial inhibition of the PAF-induced $[\text{Ca}^{2+}]_i$ increase (Fig. 5b) compared with the control (without flufenamic acid, but with 30 nmol PAF l^{-1} ; Fig. 5a).

Responses of the transmembrane potential

The changes in the fluorescence intensity of oviductal cells occur after incubation with different concentrations of PAF, TMB-8 or flufenamic acid (Fig. 6). In PAF concentrations of 2.5 and $5 \mu\text{mol l}^{-1}$, the oviductal cells were significantly depolarized as indicated by the $\text{DiBAC}_4(3)$ fluorescence increase (Fig. 7). This depolarization was not accompanied by the cytotoxicity of cells, as the number of cells was not significantly reduced. However, at $10 \mu\text{mol}$ PAF l^{-1} , the $\text{DiBAC}_4(3)$ fluorescence was significantly decreased compared with the control (cells incubated only in medium). This diminution was accompanied by a marked inhibition of the

Fig. 5. Effect of adding flufenamic acid to fura-2-loaded cultured bovine oviductal cells. The cells were incubated (a) without, (b) with $5 \mu\text{mol}$ flufenamic acid l^{-1} (final concentration), or (c) with $20 \mu\text{mol}$ flufenamic acid l^{-1} (final concentration) 5 min before the addition of 30 nmol platelet-activating factor (PAF) l^{-1} .

appearance of mitosis (Fig. 8). The concentration of $10 \mu\text{mol}$ PAF l^{-1} caused the cells to detach from the culture plate and the high PI and Trypan blue staining that occurred indicated that the plasma membrane of the cells became permeable to both substances.

At a concentration of $10 \mu\text{mol}$ TMB-8 l^{-1} , the $\text{DiBAC}_4(3)$ fluorescence of cells increased significantly as compared with the control, indicating depolarization. The depolarization was not indicative of cytotoxicity because the number of cells was approximately in the same range as the control.

Fig. 6. Typical fluorescence picture of the fluorochrome bis-oxonol (DiBAC₄(3)) staining on bovine oviductal cells. (a) Phase-contrast and (b) staining. Scale bars represent 50 μm .

At 20 $\mu\text{mol TMB-8 l}^{-1}$, the DiBAC₄(3) fluorescence of cells began to decrease compared with the control and, at 50 $\mu\text{mol TMB-8 l}^{-1}$, the DiBAC₄(3) fluorescence was significantly reduced. At 50 $\mu\text{mol TMB-8 l}^{-1}$, the cells began to detach from the culture plate and about 70% were Trypan blue stained. After incubation of oviductal cells with flufenamic acid, the DiBAC₄(3) fluorescence was significantly increased at 20 $\mu\text{mol flufenamic acid l}^{-1}$ compared with the control or to lower concentrations, whereas, at 50 $\mu\text{mol flufenamic acid l}^{-1}$, a significant decrease of DiBAC₄(3) fluorescence was observed and 40% of cells were Trypan blue stained.

Proliferation

Concentrations of 1.25 $\mu\text{mol PAF l}^{-1}$ and 2.5 $\mu\text{mol PAF l}^{-1}$ significantly stimulated the proliferation of oviductal cells to 157% and 127%, respectively, compared with the control (Fig. 8). A concentration of 10 $\mu\text{mol PAF l}^{-1}$ significantly decreased the cell proliferation compared with the control. TMB-8 and flufenamic acid had no effect other than cell death. After the 48 h incubation, TMB-8 or flufenamic acid inhibited the cell proliferation in a concentration-dependent manner, with an IC_{50} value of 26.57 $\mu\text{mol l}^{-1}$ and 95.29 $\mu\text{mol l}^{-1}$, respectively.

Fig. 7. Effect of platelet-activating factor (PAF) ($\square$), or TMB-8 (∇), or flufenamic acid ($\bullet$) on transmembrane potential assessed by DiBAC₃(3) in cultured bovine oviductal cells after 24 h treatment. Each value is the mean $\pm$ SD for three experiments performed in duplicate. The fluorescence of the control is considered to be 100%. Significant differences between the control and treatment groups are indicated: * $P < 0.05$.

Discussion

Platelet-activating factor, an inflammatory mediator with potent vascular permeability property, may be the first physiological signal produced by the embryo for the maternal recognition of pregnancy. Mouse and human preimplantation embryos produce PAF (O'Neill, 1985; Smal *et al.*, 1990) and, therefore, PAF is a potential mediator of embryonic signalling to the oviduct. The oviduct is under endocrine control and locally produced factors, such as PAF, play a decisive role in the regulation of oviduct function. Although the effect of PAF on the regulation of oviduct contractility and relaxation, as well as in the ciliary motion of hamster oviduct epithelial cells, has been observed by Hermoso and Villalon (1995), there are few functional studies of the effect of PAF on bovine oviductal cells.

Previous results showed that the increase in $[\text{Ca}^{2+}]_i$ in oviductal cells induced by PAF was reduced when extracellular calcium was depleted, indicating that the effect of PAF on $[\text{Ca}^{2+}]_i$ involves an influx from the extracellular space. The mechanism by which PAF increases calcium influx in oviductal cells is little understood. Oviductal cells were pretreated with different substances to characterize the pathway responsible for the Ca^{2+} influx. The PAF-induced $[\text{Ca}^{2+}]_i$ increase was not inhibited by pretreatment of cells with Ni^{2+} and La^{3+} , indicating that the Ca^{2+} influx is not blocked at the plasma membrane calcium channels. Neither the 'small' nor the 'maxi'- Ca^{2+} -activated K^+ channels were involved in the PAF-induced Ca^{2+} influx because charybdotoxin and apamin had no effect on this pathway. In contrast, other cell types (for example, human B lymphoblastoid cells) responded to PAF with a $[\text{Ca}^{2+}]_i$

Fig. 8. Effect of platelet-activating factor (PAF) ($\square$), TMB-8 (∇), or flufenamic acid ($\bullet$) on proliferation in cultured bovine oviductal cells after 48 h treatment. Each value is the mean $\pm$ SD for three experiments performed in duplicate. The fluorescence of the control is considered to be 100%. Significant differences between the control and treatment groups are indicated: * $P < 0.05$.

increase by voltage-dependent mechanisms via 'maxi' K^+ channels (Di Berardino *et al.*, 1993).

After preincubation of oviductal cells with 20 $\mu\text{mol l}^{-1}$ TMB-8 for 5 min, the PAF-induced increase in $[\text{Ca}^{2+}]_i$ was completely inhibited. This inhibition was not due to a cytotoxic effect. This finding is consistent with the range of dose of TMB-8 (1.8–470.0 $\mu\text{mol l}^{-1}$) required to block responses of skeletal muscle *in vitro* (Malagoni and Chiou, 1974) and of neurohybrid cells (10–50 $\mu\text{mol l}^{-1}$) to PAF (Yue *et al.*, 1991a,b). The inhibitory effect of TMB-8 on PAF-induced increases in $[\text{Ca}^{2+}]_i$ of oviductal cells is due largely to an inhibition of plasma membrane calcium influx. This is in agreement with reports showing TMB-8 inhibition of calcium uptake in adrenal glomerulosa cells (Kojima *et al.*, 1985) and neurohybrid NG108-15 cells (Yue *et al.*, 1991b). However, the exact mechanism of the inhibitory effect of TMB-8 on the cellular response to PAF remains to be clarified. It is possible that TMB-8 displaces calcium from its binding site on the surface membrane, and acts subsequently to stabilize the membrane which prevents calcium influx (Sato, 1992).

In the present study, flufenamic acid at concentrations of 10 and 20 $\mu\text{mol l}^{-1}$ significantly reduced the PAF-mediated $[\text{Ca}^{2+}]_i$ increase in oviductal cells, whereas 5 $\mu\text{mol l}^{-1}$ flufenamic acid partially reduced the $[\text{Ca}^{2+}]_i$ increase, indicating that a non-selective ion channel may be involved in this effect (Wangemann *et al.*, 1986; Gögelein *et al.*, 1990). Flufenamic acid, a Cl^- and non-selective cation channel blocker, causes a release of Ca^{2+} from intracellular stores (Poronnik *et al.*, 1992; Lee *et al.*, 1996) in a manner similar to thapsigargin (Diserbo *et al.*, 1995). In many cell types, the intracellular increase of free Ca^{2+} is dependent on events linked to Ca^{2+} release-

triggered Ca^{2+} influx (Berridge, 1993). The respective inhibitory effect of flufenamic acid may indicate an important role for the release of intracellular Ca^{2+} stores in the PAF-induced $[\text{Ca}^{2+}]_i$ increase. This calcium release can activate calcium influx and PAF acts via a Ca^{2+} release-triggered Ca^{2+} influx. It is possible that the PAF receptor is associated with the cationic channel blocked by flufenamic acid. The results reported by Yue *et al.* (1991b) and Tiemann *et al.* (1996) have shown that the PAF-induced Ca^{2+} influx was mediated through receptor-operated Ca^{2+} channels, but was not affected by verapamil, a voltage-dependent Ca^{2+} channel inhibitor. Studies on the effect of flufenamic acid on oviductal cells have not yet been reported to our knowledge. However, in rat mesangial cells, 100 μmol flufenamic acid l^{-1} partially reduced the H_2O_2 -mediated $[\text{Ca}^{2+}]_i$ increase (Meyer *et al.*, 1996). In the present study, this concentration of flufenamic acid was cytotoxic for oviductal cells in primary cultures.

The change of transmembrane potential is caused by ion exchange and the change of ion flux modifies the transmembrane potential difference. Inward current through the putative receptor-operated channels could produce appreciable changes in membrane potential. Avdonin *et al.* (1991) suggested that activators of receptor-operated channels trigger the activation of Ca^{2+} and Na^{+} currents and, by increasing $[\text{Ca}^{2+}]_i$, the K^{+} efflux is stimulated. The balance between these currents can determine the agonist effect on membrane potential. The present results indicate that PAF causes dose-dependent depolarization in intact oviductal cells. This finding is in agreement with the report by Katruk and Nelson (1991) that membrane potential measurements using the fluorescent dye bis-oxonol revealed a transient membrane depolarization in macrophages concomitant to the increase in $[\text{Ca}^{2+}]_i$ induced by PAF. Channels that are influenced by PAF can be responsible for both the Ca^{2+} uptake and the transmembrane potential of intact oviductal cells. This fact supports the result obtained with flufenamic acid, that non-selective cationic channels can be activated by PAF, because Ca^{2+} -specific or -selective channels have only a small influence on the transmembrane potential (Wilson and Chused, 1985).

The activation of non-selective cationic channels by PAF can be significant for the proliferation of oviductal cells. The results of the present study indicate that PAF is capable of stimulating the cell division of oviductal cells. In general, calcium uptake seems to play a role in the regulation of proliferation (Whitefield *et al.*, 1976; Hazelton *et al.*, 1979; Shultz and Raji, 1990). How the induced Ca^{2+} signal is connected with the specific cell responses, for example proliferation, is little understood. Results from other cell systems indicate an important function of Ca^{2+} -dependent activation of protein kinase C (Nishizuka, 1989). The present study shows that the proliferative effect of PAF on oviductal cells may directly involve a membrane depolarization. A consistent change in DiBAC₄(3) fluorescence occurs in a concentration range of 1.25–5.00 μmol l^{-1} , in which proliferation was also stimulated. The concentration of 10 μmol PAF l^{-1} is unphysiological because it markedly inhibits the proliferative growth of oviductal cells. Changes in bis-oxonol fluorescence at 10 μmol PAF l^{-1} may be caused by an inhibition of Na^{+} - K^{+} -ATPase. Mohr and Fewtrell (1987) reported that inhibition of the Na^{+} - K^{+} -ATPase results in a

marked depolarization of cells. Flufenamic acid and TMB-8 at concentrations of about 100 μmol l^{-1} and 20 μmol l^{-1} , respectively, are cytotoxic because they inhibit the proliferative growth of oviductal cells to 50%. Desgranges *et al.* (1991) reported an IC_{50} value of about 30 μmol TMB-8 l^{-1} in rat arterial smooth muscle cells, and that the growth of cells was totally inhibited at 80 μmol TMB-8 l^{-1} . This finding is in the range of the present results and the cytotoxicity can be caused by non-specific toxic effects, such as reduction of cellular ATP content. Brand and Felber (1984) observed that 50 μmol TMB-8 l^{-1} significantly decreased both the cellular ATP concentration and the mitochondrial membrane potential in rat thymocytes.

The different biological effects of PAF in oviductal cells ($[\text{Ca}^{2+}]_i$, transmembrane potential and proliferation) have been obtained at different concentrations of the agonist, indicating that calcium increase is upstream of the proliferation. PAF-mediated calcium signalling and mitogenesis in oviductal cells may be mediated by distinct subtypes of receptors. The present data and those of Kudolo and Harper (1989) in the pregnant rat uterus, and Baldi *et al.* (1994) and Maggi *et al.* (1994) in human endometrial cancer cell line HEC-1A, indicate that a low concentration of PAF induces $[\text{Ca}^{2+}]_i$ via the high-affinity site of the receptor and that a high concentration of PAF induces proliferation via its low-affinity site. Maggi *et al.* (1994) reported the presence of two populations of PAF receptors with affinity constants in the nanomolar and micromolar range. Preliminary data obtained in flow cytometric experiments with the analysis of double immunofluorescence on oviductal cells indicate that there are two kinds of cell populations that have different amounts of PAF receptor (data not shown). One population was antivimentin-positive (55% of total cells) and was PAF receptor bearing to about 29% of total cells. The other population was anticytokeratin-positive (20% of total cells) and 6.9% of this population was PAF-receptor bearing. It is possible that the two types of cell possess subtypes of PAF-receptors with different affinity constants, one in the nanomolar and one in the micromolar range. However, further investigation of the binding of PAF to its receptor is required.

In summary, the present study demonstrates that the PAF-induced intracellular free Ca^{2+} in oviductal cells appears to be mediated by PAF activation of non-selective cationic membrane channels and that PAF may act via a Ca^{2+} release-triggered Ca^{2+} influx. In view of the importance of Ca^{2+} homeostasis in cell functions (for example, in proliferation), the present data contribute to the further understanding of the mechanism of PAF actions on oviductal cells.

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