Cell type specificity and protein kinase C dependency on the stimulation of prostaglandin E₂ and prostaglandin F_{2α} production by oxytocin and platelet-activating factor in bovine endometrial cells

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The regulation of prostaglandin E₂ and prostaglandin F_{2α} in primary cultures of epithelial and stromal cells of bovine endometrium was investigated using two physiological agents, oxytocin and platelet-activating factor, and the protein kinase C activator, 4β-phorbol 12-myristate 13-acetate. At the basal level, epithelial cells produced more prostaglandin F_{2α} than did stromal cells ($P \leq 0.0001$) and stromal cells produced more prostaglandin E₂ than did epithelial cells ($P \leq 0.0001$). In the presence of oxytocin, production of prostaglandin E₂ and F_{2α} increased in a dose-dependent manner, only in epithelial cells. Stromal cells did not respond to oxytocin, suggesting that the oxytocin response is cell type specific, acting preferentially on the cell type in which prostaglandin F_{2α} is the major prostaglandin produced. Platelet-activating factor increased prostaglandin E₂ and prostaglandin F_{2α} production in epithelial cells at 1 and 10 pmol l⁻¹ (PGE₂: 10 pmol l⁻¹, $P \leq 0.01$; PGF_{2α}: 1 pmol l⁻¹, $P \leq 0.02$). Stromal cells also responded to platelet-activating factor, but only at high concentrations (PGE₂: 0.1 μmol l⁻¹, $P \leq 0.001$; PGF_{2α}: 0.1 μmol l⁻¹, $P \leq 0.0001$). These results further demonstrate the differences in epithelial and stromal cells; epithelial cells are more sensitive to platelet-activating factor than are stromal cells. Phorbol 12-myristate 13-acetate increased prostaglandin production in a dose-dependent manner in both epithelial and stromal cells, indicating that protein kinase C activation can increase prostaglandin production in these cells. Higher concentrations of phorbol 12-myristate 13-acetate down-modulated protein kinase C activity. The role of protein kinase C in oxytocin- and platelet-activating factor-induced increase in prostaglandin was investigated by down-modulating protein kinase C in epithelial cells and subsequently treating the cells with oxytocin and platelet activating factor. Oxytocin- and platelet-activating factor-induced increases in prostaglandin production were not found in protein kinase C-down-modulated cells, suggesting that protein kinase C activation is necessary for this response.

Introduction

The importance of prostaglandins in early reproductive processes of various species is well established (Kennedy, 1980; Kennedy, 1983; Smith and Kelly, 1988a). Prostaglandin E₂ (PGE₂) is required for changes in vascular permeability in the endometrium that occur before implantation. Inhibitors of prostaglandin synthesis, such as indomethacin, delay or inhibit these changes, and prevent implantation (reviewed by Smith and Kelly, 1988a). Prostaglandin F_{2α} (PGF_{2α}) is the luteolytic agent in nonpregnant ruminants (Horton and Poyser, 1976; Thatcher *et al.*, 1984). It is thought that oxytocin, released in a pulsatile fashion from the posterior pituitary gland, causes a release of uterine PGF_{2α}. The small amount of PGF_{2α} released triggers the release of additional oxytocin from the corpus luteum. Oxytocin then binds to endometrial oxytocin receptors

to stimulate PGF_{2α} secretion (Roberts *et al.*, 1976; Silvia *et al.*, 1991). In cattle, PGF_{2α} can terminate pregnancy at any time. Thus, the suppression of uterine luteolytic signals and the maintenance of corpus luteum function are important for the establishment and maintenance of pregnancy.

In addition to oxytocin, other agents regulate prostaglandins in reproductive processes. Among these, platelet-activating factor (PAF) is known to be a potent biological mediator that can stimulate reproductive processes such as fertilization of eggs, implantation and growth of embryos (O'Neill, 1991). PAF is also secreted by preimplantation mouse (O'Neill, 1988) and human (O'Neill *et al.*, 1987; Collier *et al.*, 1988) embryos and thus could be an embryonic signal for maternal recognition of pregnancy. PAF can also increase prostaglandin release in bovine (Gross *et al.*, 1990), ovine (Salamonsen, 1991), human (Smith and Kelly, 1988b) and rabbit (Kasamo *et al.*, 1992) endometrial cells, and thus could mediate processes by regulating prostaglandin production.

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Both oxytocin and PAF activate the inositol-specific phospholipase C in various cell systems (Flint and Sheldrick, 1986; Moore *et al.*, 1988; Shukla, 1991), generating two second messengers: inositol triphosphate, which increases intracellular calcium, and diacylglycerol, which can activate protein kinase C (Nishizuka, 1988). Calcium in human decidual cells (Mitchell, 1991) or the activation of protein kinase C in human amnion cells (Moore *et al.*, 1988; Zakar and Olson, 1988) increase prostaglandin production.

Primary cultures of separated epithelial and stromal cells of the bovine endometrium are well established (Fortier *et al.*, 1988). These cells possess physiological characteristics; β -adrenergic agonists and PGE_2 increase cAMP concentrations and the cells produce PGE_2 and $\text{PGF}_{2\alpha}$. Thus, it is possible to study co-culture systems of epithelial or stromal cells with bovine embryos (Fortier *et al.*, 1994) or with *Ureaplasma diversum*, which is associated with infertility (Kim *et al.*, 1994). Since these factors influence the prostaglandin production of epithelial and stromal cells, it is important to characterize further the biochemical activities of these cells. This study investigates the regulation of prostaglandin secretion by cultured epithelial and stromal cells of the bovine endometrium in response to oxytocin and PAF. Furthermore, the role of protein kinase C as a mechanism by which these agents regulate prostaglandin secretion is investigated.

Materials and Methods

Isolation of epithelial and stromal cells

Bovine uteri were obtained from an abattoir and placed on ice until further processing at the laboratory. The day of the oestrous cycle of the tissue was estimated by examining the morphology of the ovary (Ireland *et al.*, 1979). Tissues of early stages of oestrus (days 1–4) were used to eliminate possible variations in cellular physiology that may occur in tissues at more advanced stages in the oestrous cycle. The cell cultures were prepared aseptically as described by Fortier *et al.* (1988). From one uterus (two horns) the digestion of epithelial cells normally yielded 75×10^6 cells diluted to $0.5 \times 10^6 \text{ ml}^{-1}$ in a final volume of 150 ml RPMI-1640 (Flow Laboratories, McLean, VA) containing 50 mg gentamycin ml^{-1} (Sigma, St Louis, MO) and 10% (w/v) fetal bovine serum (Flow Laboratories) depleted of steroids by dextran-charcoal extraction (FBS-DC). Stromal cells recovered from the second digestion include a heterogeneous population of cells composed of stromal fibroblast-like cells, erythrocytes and residual epithelial cells. Only stromal cells were counted and diluted to a final concentration of $0.5 \times 10^6 \text{ cells ml}^{-1}$ in RPMI supplemented with gentamycin. The purity of the stromal preparation was improved by changing the medium 18 h after plating during which time selective attachment of stromal cells occurred. Cells were plated in 24-well culture plates (Becton Dickinson, Lincoln Park, NJ), incubated at 37°C with 5% CO_2 :95% air. Epithelial and stromal cells isolated from early cycle tissues reached confluence after approximately 6–7 days in culture. The medium was changed every 4–5 days.

Prostaglandin production

At early confluence, the culture medium was replaced with fresh RPMI-1640 containing 40 mmol Hepes l^{-1} (Sigma) and 1% (v/v) FBS-DC. The pH of the medium was adjusted to 7.4. Cells were incubated at 37°C with 5% CO_2 for the various times shown. The culture medium was collected in glass tubes and stored at -20°C until further processing. The protein content of cells for time course studies were measured by the protein-dye binding method described by Bradford (1976).

For the stimulation studies, 0.1 nmol–10 μmol oxytocin l^{-1} (Sigma) or 1 pmol–1 μmol 1- α -phosphatidylcholine l^{-1} , β -acetyl- γ -o-hexadecyl (PAF) (Sigma), was added to the culture medium. PAF was first diluted in culture medium supplemented with 3 mg bovine albumin l^{-1} , essentially fatty-acid free (Sigma), to prevent degradation of PAF (Kudolo and Harper, 1990). This preparation of PAF was used to stimulate prostaglandin production. Culture medium with bovine albumin was added to corresponding control wells. For the protein kinase C activator response study, 0.16 nmol–1.6 μmol 4 β -phorbol 12-myristate 13-acetate (Sigma) was added to the culture medium. Cells were incubated at 37°C with 5% CO_2 :95% air for 24 h. The accumulation of prostaglandins secreted into the culture medium during this time was measured. The culture medium was collected in glass tubes and stored at -20°C until further processing.

Protein kinase C tachyphylaxis studies

At early confluence, the culture medium was replaced with fresh RPMI-1640 containing 40 mmol Hepes l^{-1} (Sigma), 1% (v/v) FBS-DC, and 1.6 μmol 4 β -phorbol 12-myristate 13-acetate l^{-1} . Cells were incubated at 37°C with 5% CO_2 :95% air for 24 h to allow tachyphylaxis of protein kinase C. Tachyphylaxis is defined here as a down-modulation of protein kinase C activity in viable cells by prior exposure to 4 β -phorbol 12-myristate 13-acetate. The culture medium was changed and 1.6 μmol 4 β -phorbol 12-myristate 13-acetate l^{-1} was added with various concentrations of oxytocin or PAF. Cells were incubated for an additional 24 h. The culture medium was collected in glass tubes and stored at -20°C until further processing.

Measurement of PGE_2 and $\text{PGF}_{2\alpha}$

PGE_2 and $\text{PGF}_{2\alpha}$ were measured by the enzyme-linked immunosorbent assay (ELISA), using commercial kits (Cayman Chemical Co., Ann Arbor, MI). Briefly, competition binding occurred between a PGE_2 or $\text{PGF}_{2\alpha}$ acetylcholinesterase tracer (Cayman Chemical Co.) and the prostaglandins in the culture sample in the presence of a fully characterized PGE_2 antiserum (donated by T. G. Kennedy) or $\text{PGF}_{2\alpha}$ antiserum (BioQuant, Ann Arbor, MI). Crossreactivity of the antiserum with $\text{PGF}_{2\alpha}$ and PGE_2 was 1% and 2.5%, respectively, and the inter- and intra-assay coefficients of variation were, respectively, 15% and 11.5% for $\text{PGF}_{2\alpha}$ and 18.3% and 9.2% for PGE_2 .

Statistical analyses

Statistical analysis was performed by analysis of variance. Data were analysed by the least squares analysis of variance

using the SuperANOVA software package. Sources of variation included experiment, cell type (Figs 1, 2 and 3) or treatment (oxytocin for Fig. 3; 4 β -phorbol 12-myristate 13-acetate for Fig. 6), time (Figs 1 and 3) or dose (Figs 2, 4, 5, and 6) and their interactions. All data were transformed logarithmically before analysis to reduce heterogeneity of regression errors. Values in the text are presented as antilogs of the least-squares means of logarithmically transformed data. For Fig. 6, anti-log values of the least-squares means are expressed as a percentage of the control (percentage change = amount of prostaglandins released with oxytocin or PAF $\times$ 100/amount of PG released without oxytocin or PAF). This procedure improves the graphical presentation of the effects of oxytocin and PAF with or without 4 β -phorbol 12-myristate 13-acetate pretreatment. Orthogonal contrasts were used to compare cell types or treatments. Regression analysis of the least-squares means was done for Figs 2 and 6; this determined the slope of the line used to compare the responses for each cell type or treatment. For Figs 4 and 5, the value for each dose was compared with the control value to demonstrate which doses had an effect on prostaglandin production.

Results

Prostaglandin production by epithelial and stromal cells of the bovine endometrium

Basal PGE₂ and PGF_{2 α} production by epithelial and stromal cells were measured at various times over 48 h. These cells continually produced both prostaglandins. Epithelial cells produced more PGF_{2 α} than did stromal cells ($P \leq 0.0001$) and stromal cells produced more PGE₂ than did epithelial cells ($P \leq 0.0001$) (Fig. 1). These results confirm previous observations (Fortier *et al.*, 1988) that epithelial and stromal cells actively produce prostaglandins, which accumulate in culture, and that the major prostaglandin produced by each cell type is different.

Prostaglandin production by endometrial cells in response to oxytocin and PAF

The influence of oxytocin on prostaglandin production in epithelial and stromal cells is shown (Fig. 2). The significant cell type $\times$ dose interaction ($P \leq 0.0001$) indicated that the increase in prostaglandin production with oxytocin dose differed between cell types. This interaction was investigated by comparing the slope (β) of the regression line. PGE₂ ($\beta = 0.120$, $P \leq 0.001$) and PGF_{2 α} ($\beta = 0.144$, $P \leq 0.001$) production by epithelial cells increased in a dose-dependent manner in response to oxytocin. Stromal cells did not respond to oxytocin, even at high doses (PGE₂: $\beta = 0.003$, $P \leq 0.832$; PGF_{2 α} : $\beta = 0.021$, $P \leq 0.0650$). This difference was further demonstrated by oxytocin time course studies (Fig. 3). The significant treatment (presence or absence of oxytocin) $\times$ dose interaction ($P \leq 0.001$) for epithelial cells indicated that the increase in prostaglandins with time was different in oxytocin-treated cells compared with the control cells. The oxytocin-treated epithelial cells produced much higher amounts of PGE₂ and PGF_{2 α}

Fig. 1. Time course of basal prostaglandin production by epithelial and stromal cells. At confluence, the culture medium was replaced with RPMI + 40 mmol Hepes l^{-1} + 1% (w/v) FBS depleted of steroids by dextran-charcoal extraction. Cells were incubated at 37°C; 5% CO₂ and at various times shown, the culture medium was collected. Prostaglandins E₂ (a) and F_{2 α} (b) were measured in epithelial cells (□) and stromal cells (○). Data are expressed as the anti-log of the least-squares means of log transformed values of three experiments. SEM, PGE₂ = 1.109; SEM, PGF_{2 α} = 1.074 (SEM values are anti-logs of log transformed standard errors of the least-squares means).

than did the control cells ($P \leq 0.001$). Oxytocin-treated values measured at 2 h were already significantly higher than the control value ($P \leq 0.001$) and the PGF_{2 α} response was more sustained with time than was the PGE₂ response. However, for stromal cells there was no significant treatment $\times$ dose interaction. The oxytocin-treated stromal cells produced the same amounts of PGE₂ and PGF_{2 α} as the control cells.

PGE₂ and PGF_{2 α} contents were measured in epithelial and stromal cells in the presence of increasing doses of PAF (Fig. 4). Again there was a significant cell type $\times$ dose interaction ($P \leq 0.0001$). In epithelial cells, prostaglandin concentrations increased in the presence of PAF at low concentrations (PGE₂: 10 pmol l^{-1} , $P \leq 0.01$; PGF_{2 α} : 1 pmol l^{-1} , $P \leq 0.02$), demonstrating that these cells are highly sensitive to PAF. However, stromal cells responded to PAF only at high concentrations, with an increase in both PGE₂ (0.1 μ mol l^{-1} , $P \leq 0.001$) and PGF_{2 α} (0.1 μ mol l^{-1} , $P \leq 0.0001$). These results indicate that PAF stimulates the production of both prostaglandins in epithelial and stromal cells, and that epithelial cells are more sensitive to PAF than are stromal cells.

Fig. 2. Prostaglandin production in response to oxytocin. At confluence, the culture medium was replaced with RPMI + 40 mmol Hepes l⁻¹ + 1% (w/v) FBS depleted of steroids by dextran-charcoal extraction + 0.1 nmol–10 μmol oxytocin l⁻¹. Cells were incubated at 37°C; 5% CO₂ for 24 h. (a) PGE₂ and (b) PGF_{2α} produced by epithelial cells and (c) PGE₂ and (d) PGF_{2α} produced by stromal cells were measured. Data are expressed as the anti-log of the least-squares means of log transformed values of three experiments. SEM, PGE₂ = 1.094; SEM, PGF_{2α} = 1.084 (SEM values are anti-logs of log transformed standard errors of the least-squares means). Slopes (β) of the regression lines for epithelial cells (PGE₂: β = 0.120, *P* ≤ 0.001; PGF_{2α}: β = 0.144, *P* ≤ 0.001) and stromal cells (PGE₂: β = 0.003, *P* ≤ 0.832; PGF_{2α}: β = 0.021, *P* ≤ 0.0650) were measured.

The effect of protein kinase C tachyphylaxis on prostaglandin production by epithelial cells in response to oxytocin and PAF

The effect of protein kinase C activation on the stimulation of prostaglandins by oxytocin and PAF was investigated by exposing cells to various concentrations of 4β-phorbol 12-myristate 13-acetate to ensure that protein kinase C activation does stimulate prostaglandin production in these cells. 4β-phorbol 12-myristate 13-acetate did increase both prostaglandins in both cell types in a dose-dependent manner (Fig. 5). Concentrations as low as 0.1 nmol l⁻¹ and 1 nmol l⁻¹ significantly increased prostaglandin production in epithelial (PGE₂: 1.0 nmol l⁻¹, *P* ≤ 0.001; PGF_{2α}: 0.1 nmol l⁻¹, *P* ≤ 0.0001) and stromal cells (PGE₂ and PGF_{2α}: 0.1 nmol l⁻¹, *P* ≤ 0.0001). However, after a maximum response was attained, higher concentrations of 4β-phorbol 12-myristate 13-acetate appeared to decrease this stimulation until basal concentrations of prostaglandin were observed, which indicates protein kinase C tachyphylaxis.

The role of protein kinase C in the oxytocin- and PAF-induced increases in prostaglandin production in epithelial cells was investigated in a second series of experiments in which

protein kinase C of epithelial cells was down-modulated, after which the cells were exposed to oxytocin or PAF (Fig. 6). There was a significant treatment (pretreatment with 4β-phorbol 12-myristate 13-acetate) × dose interaction (*P* ≤ 0.0001), indicating that the changes in prostaglandins with different doses differed for 4β-phorbol 12-myristate 13-acetate pretreated cells compared with control cells. In cells in which protein kinase C activity was unaffected, prostaglandin production increased in response to oxytocin (PGE₂: β = 0.110, *P* ≤ 0.001; PGF_{2α}: β = 0.143, *P* ≤ 0.001) and PAF (PGE₂: β = 0.035, *P* ≤ 0.001; PGF_{2α}: β = 0.033, *P* ≤ 0.01). When protein kinase C tachyphylaxis occurred, no significant increases due to oxytocin (PGE₂: β = 0.023, *P* ≤ 0.148; PGF_{2α}: β = 0.016, *P* ≤ 0.294) or PAF (PGE₂: β = -0.0004, *P* ≤ 0.954; PGF_{2α}: β = -0.001, *P* ≤ 0.386) were observed.

Discussion

Epithelial and stromal cells of the bovine endometrium possess distinct morphological and physiological properties in culture (Fortier, 1988). Growth patterns of cells vary in culture

Fig. 3. Prostaglandin production by endometrial cells in the presence (Δ) and absence ($\square$; control) of $0.1 \mu\text{mol oxytocin l}^{-1}$ as a function of time. At confluence, the culture medium was replaced with RPMI + 40 mmol l^{-1} Hepes + 1% (w/v) FBS depleted of steroids by dextran-charcoal extraction + $0.1 \mu\text{mol oxytocin l}^{-1}$. Cells were incubated at 37°C ; $5\% \text{ CO}_2$ for the various times shown. (a) PGE_2 and (b) $\text{PGF}_{2\alpha}$ produced by epithelial cells and (c) PGE_2 and (d) $\text{PGF}_{2\alpha}$ produced by stromal cells were measured. Data are expressed as the anti-log of the least-squares means of log-transformed values of three experiments. SEM, epithelial $\text{PGE}_2 = 1.064$; SEM, $\text{PGF}_{2\alpha} = 1.064$; SEM, stromal $\text{PGE}_2 = 1.138$; SEM, $\text{PGF}_{2\alpha} = 1.081$ (SEM values are anti-log of log transformed standard errors of the least-squares means).

according to cell type; stromal cells are abundantly harvested from the endometrium and they attach and grow quite quickly in culture, whereas there are fewer epithelial cells and they are more fragile (M. A. Fortier *et al.*, unpublished). Epithelial cells differ considerably in the rate of cell attachment and growth in culture depending on the day of the oestrous cycle on which the tissue was harvested. Cells harvested from uteri at early cycle (days 1–4) attach quickly, in about 24 h, and grow well, reaching confluence after approximately 7 days, whereas cells from mid-cycle tissues (days 5–12) attach after 2–3 days, and reach confluence after 10–14 days. In the latter case, an opportunity arises for any stromal cells that have entered the epithelial preparation to grow and monopolize the space, thus decreasing the degree of purity of epithelial cells. One method used in our laboratory to measure the degree of purity of epithelial and stromal cell preparations is by the $\text{PGE}_2:\text{PGF}_{2\alpha}$ ratio, given that epithelial cells produce much more $\text{PGF}_{2\alpha}$ than PGE_2 and stromal cells produce more PGE_2 than $\text{PGF}_{2\alpha}$. For example, a ratio greater than 1 in an epithelial cell preparation indicates considerable contamination with stromal cells. The degree of purity of cell preparations becomes a crucial factor when their biochemical response to various physiological agents is characterized, given that epithelial and stromal cells

behave quite differently in the presence of these agents, as shown in this study. For example, when epithelial cells with a $\text{PGE}_2:\text{PGF}_{2\alpha}$ ratio ≥ 1 , were treated with oxytocin, the dose-dependent PGE_2 response to oxytocin was not seen (results not shown). This finding can be explained by the presence of stromal cells in the epithelial cell preparation, since stromal cells do not respond to oxytocin and the basal PGE_2 production is high enough to mask any dose-dependent effect of oxytocin on PGE_2 production by epithelial cells.

The ability of oxytocin to stimulate $\text{PGF}_{2\alpha}$ release is higher at the time of luteolysis (Lafrance and Goff, 1985; Flint and Sheldrick, 1986). This may be due to the rising number of oxytocin receptors (McCracken *et al.*, 1984; Miranda *et al.*, 1993). Oestradiol enhances responsiveness to oxytocin (Lafrance and Goff, 1988) and increases oxytocin receptor concentrations (Hixon and Flint, 1987). However, the oestradiol dependency of the oxytocin response is controversial (see Silvia *et al.*, 1991 for review). It may not be required for the development of the responsiveness to oxytocin needed to initiate luteolysis but may be required to ensure that luteolysis proceeds to completion. Vallet *et al.* (1990) demonstrated that oestrogen and oxytocin receptors are abundant in long-term ovariectomized ewes and can be downregulated by hormones.

Fig. 4. Prostaglandin production in response to platelet-activating factor (PAF). At confluence, the culture medium was replaced with RPMI + 40 mmol Hepes l⁻¹ + 1% (w/v) FBS depleted of steroids by dextran-charcoal extraction + 1 pmol–1 μmol PAF l⁻¹. Cells were incubated at 37°C; 5% CO₂ for 24 h. (a) PGE₂ and (b) PGF_{2α} produced by epithelial cells and (c) PGE₂ and (d) PGF_{2α} produced by stromal cells were measured. PAF-treated values are compared with the control value in which no PAF was present to determine which doses had a significant effect on prostaglandin production (**P* < 0.01). Data are expressed as the anti-log of the least-squares means of log transformed values of three experiments. SEM, PGE₂ = 1.072; SEM, PGF_{2α} = 1.081 (SEM values are anti-logs of log transformed standard errors of the least-squares means).

McCracken *et al.* (1984) proposed that progesterone blocks the formation of endometrial oxytocin receptors in ruminants. Concentrations of oxytocin receptors increased after withdrawal of exogenous progesterone in ovariectomized ewes (Zhang *et al.*, 1992). It is also possible that long-term exposure to progesterone increases the oxytocin receptor concentration as a result of stimulation that takes time to manifest itself or owing to a slow desensitization of the uterus to the inhibitory effects of progesterone (Silvia *et al.*, 1991). In the study reported here, we showed that oxytocin stimulates PGE₂ and PGF_{2α} production in epithelial cells in a dose-dependent manner, indicating that oxytocin receptors are present and, when occupied by their ligand, can increase prostaglandin synthesis. Hormone concentrations in culture are at a minimum and yet epithelial cells of the bovine endometrium continue to express oxytocin receptors throughout the time they are grown in culture. Thus, the continual presence of oestradiol and progesterone does not appear to be obligatory for the induction of oxytocin receptors *in vitro*. It is possible that *in vivo* inhibitory factors (progesterone or other factors) are present that suppress oxytocin receptor concentrations but that these

are absent in culture. The increases observed in both PGF_{2α} and PGE₂, with a tendency to favour the production of PGF_{2α} at later times, suggest that oxytocin may regulate prostaglandin production at two different levels: one, common to PGE₂ and PGF_{2α}, early in its action whether it is on the enzymes involved in arachidonic acid liberation or cyclooxygenase; and another on mechanisms involving enzymes such as the endoperoxide F reductase, or 9-keto-reductase for long-term preferential stimulation of PGF_{2α}. This preferential production in oxytocin-stimulated cells may be simply an amplification of the effect that occurs at the basal level.

Oxytocin did not induce PGE₂ or PGF_{2α} release in stromal cells, indicating that the oxytocin response is cell type specific. Chan *et al.* (1993) reported that oxytocin stimulates prostaglandin release in endometrial tissue but not in myometrial tissue of the rat uterus, owing to the presence of two distinct subtypes of the oxytocin receptor. They propose that these receptors are different or that the receptors at the two sites are similar but that the transduction mechanism is different. This may also be true for epithelial and stromal cells of the bovine endometrium. It is possible that stromal cells do possess

Fig. 5. Prostaglandin production in response to 4β-phorbol 12-myristate 13-acetate. At confluence, the culture medium was replaced with RPMI + 40 mmol Hepes l⁻¹ + 1% (w/v) FBS depleted of steroids by dextran-charcoal extraction + 0.16 nmol–1.6 μmol 4β-phorbol 12-myristate 13-acetate l⁻¹. Cells were incubated at 37°C; 5% CO₂ for 24 h. (a) PGE₂ and (b) PGF_{2α} produced by epithelial cells and (c) PGE₂ and (d) PGF_{2α} produced by stromal cells were measured. Treated values are compared with the control value in which no 4β-phorbol 12-myristate 13-acetate was present to determine which doses had a significant effect on prostaglandin production (**P* < 0.001). Data are expressed as the anti-log of the least-squares means of log transformed values of two experiments. SEM, PGE₂ = 1.119; SEM, PGF_{2α} = 1.140 (SEM values are anti-logs of log-transformed standard errors of the least-squares means).

oxytocin receptors but they are regulated in a different way from epithelial cells.

PAF is physiologically active at 10⁻¹²–10⁻⁹ mol l⁻¹ (Venable *et al.*, 1993), which makes it one of the most potent agonists known. Smith and Kelly (1988b) report increases in PGE₂ release by human gland cells with 10 or 100 ng PAF ml⁻¹, but no increase with 1 ng ml⁻¹. Alecozay *et al.* (1991) report increases in PGE₂ with 5 ng PAF ml⁻¹, but only in the presence of oestradiol. It has been shown for the first time, in the study reported here, that PAF at concentrations as low as 10 pmol l⁻¹ and 100 pmol l⁻¹, in the absence of steroid hormones, can significantly increase PGF_{2α} and PGE₂ synthesis, respectively, in epithelial cells. It is possible that the presence of bovine albumin stabilized PAF prevents enzymatic degradation (Kudolo and Harper, 1990). Stromal cells respond to PAF but only at high concentrations (0.1–1 μmol l⁻¹). Again the PAF response is cell type specific but, in this case, in its sensitivity. The production of PAF by bovine embryos is suggested by Scodras (1993); however, a large number of embryos are required to produce detectable amounts. If bovine embryos produced PAF and this increased PGF_{2α} *in vivo*, recognition and maintenance of pregnancy would not occur, since PGF_{2α} is

luteolytic. PAF is thus unlikely to be a major embryonic signal in cows. However, if bovine embryos do produce PAF chronically, down-modulation of protein kinase C activity could occur and prevent the stimulation of PGF_{2α} by oxytocin. Battye *et al.* (1992) demonstrated that PAF can suppress oxytocin-induced PGF_{2α} release in ewes.

Both oxytocin and PAF activate the inositol phospholipid cascade (Shukla, 1991; Miranda *et al.*, 1993), indicating that these hormones bind to membrane receptors and initiate the phospholipase C–inositol phosphate cascade. The two second messengers generated by this cascade are IP₃, which releases Ca²⁺ from intracellular stores, and diacylglycerol, which activates protein kinase C (Nishizuka, 1988). Activation of protein kinase C increases prostaglandin production (Kniss *et al.*, 1990; Lafrance and Goff, 1990; Moore *et al.*, 1991). It has been shown in the study reported here that activation of protein kinase C increases prostaglandin production and is independent of cell type. High concentrations of 4β-phorbol 12-myristate 13-acetate resulted in tachyphylaxis of protein kinase C as demonstrated in amnion cells (Moore *et al.*, 1991) and rat thymic cells (Liu *et al.*, 1993). Furthermore, this study shows that protein kinase C plays an important role in increasing

Fig. 6. Prostaglandin production in cells in which protein kinase C tachyphylaxis has occurred in response to oxytocin or platelet-activity factor (PAF). At confluence, the culture medium was replaced with RPMI + 40 mmol Hepes l⁻¹ + 1% (w/v) FBS depleted of steroids by dextran-charcoal extraction + 1.6 μmol 4β-phorbol 12-myristate 13-acetate l⁻¹ and cells were incubated for 24 h at 37°C; 5% CO₂. The medium was changed, fresh 4β-phorbol 12-myristate 13-acetate (1.6 μmol l⁻¹) added, and cells were treated with oxytocin (0.1 nmol l⁻¹–10 μmol l⁻¹) or PAF (1 pmol l⁻¹–1 μmol l⁻¹). Cells were incubated at 37°C; 5% CO₂ for an additional 24 h. PGE₂ and PGF_{2α} produced by epithelial cells in response to (a) oxytocin (■: control PGE₂, β = 0.110, P ≤ 0.001; ▲: PGF_{2α}, β = 0.143, P ≤ 0.001; □: protein kinase C-tachyphylaxed PGE₂, β = 0.023, P ≤ 0.148; △: PGF_{2α}, β = 0.016, P ≤ 0.294) and (b) PAF (■: control PGE₂, β = 0.035, P ≤ 0.001; ▲: PGF_{2α}, β = 0.033, P ≤ 0.01; □: protein kinase C-tachyphylaxed PGE₂, β = -0.0004, P ≤ 0.954; △: PGF_{2α}, β = -0.001, P ≤ 0.386) were measured. Anti-log values of the least-squares means of log transformed values of two experiments are expressed as a percentage of the control value (percentage change = amount of prostaglandins released with oxytocin or PAF × 100/amount of prostaglandins released without oxytocin or PAF); SEM, oxytocin response: PGE₂ = 1.069; SEM, PGF_{2α} = 1.057; SEM, PAF response: PGE₂ = 1.076; SEM, PGF_{2α} = 1.062 (SEM values are anti-logs of log transformed standard errors of the least-squares means).

prostaglandin release in response to oxytocin and PAF. Thus, the activation of protein kinase C is one mechanism by which oxytocin and PAF increase prostaglandin production in epithelial cells of the bovine endometrium.

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References

- Alecozay AA, Harper MJK, Schenken RS and Hanahan DJ (1991) Paracrine interactions between platelet-activating factor and prostaglandins in hormonally-treated human luteal phase endometrium *in vitro* *Journal of Reproduction and Fertility* **91** 301–312
- Battye KM, O'Neill C and Evans G (1992) Evidence that platelet-activating factor suppresses uterine oxytocin-induced 13,14-dihydro-15-keto-prostaglandin F_{2α} release and phosphatidylinositol hydrolysis in the ewe *Biology of Reproduction* **47** 213–219
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding *Analytical Biochemistry* **72** 248–254
- Chan WY, Chen DL and Manning M (1993) Oxytocin receptor subtypes in the pregnant rat myometrium and decidua: pharmacological differentiations *Endocrinology* **132** 1381–1386
- Collier M, O'Neill C, Ammit AJ and Saunders DM (1988) Biochemical and pharmacological characterization of human embryo-derived platelet activating factor *Human Reproduction* **3** 993–998
- Flint APF and Sheldrick EL (1986) Ovarian oxytocin and the maternal recognition of pregnancy *Journal of Reproduction and Fertility* **76** 831–839
- Fortier MA, Guilbault LA and Grasso F (1988) Specific properties of epithelial and stromal cells from the endometrium of cows *Journal of Reproduction and Fertility* **83** 239–248
- Fortier MA, Nadeau Y, Blouin L and Sirard MA (1994) Cultured endometrial cells as a detection system for quantitative evaluation of bovine embryo quality *Theriogenology* **41** 196 (Abstract)
- Gross TS, Thatcher WW, O'Neill C and Danet-Desnoyers G (1990) Platelet-activating factor alters the dynamics of prostaglandin and protein synthesis by endometrial explants from pregnant and cyclic cows at day 17 following estrus *Theriogenology* **34** 205–218
- Hixon JE and Flint APF (1987) Effects of a luteolytic dose of oestradiol benzoate on uterine oxytocin receptor concentrations, phosphoinositide turnover and prostaglandin F_{2α} secretion in sheep *Journal of Reproduction and Fertility* **79** 457–467
- Horton EW and Poyser NL (1976) Uterine luteolytic hormone: a physiological role for prostaglandin F_{2α} *Physiological Reviews* **56** 595–651
- Ireland JJ, Coulson PB and Murphee RL (1979) Follicular development during four stages of the estrous cycle of beef cattle *Journal of Animal Science* **49** 1261–1269
- Kasamo M, Brandt M, Ishikawa M, Shimizu T and Harper MJK (1992) *In vitro* prostaglandin release from and platelet-activating factor accumulation in isolated endometrial cells from pregnant and pseudopregnant rabbits *Biology of Reproduction* **46** 829–845
- Kennedy TG (1980) Prostaglandins and the endometrial vascular permeability changes preceding blastocyst implantation and decidualization *Progress in Reproductive Biology* **7** 234–243
- Kennedy TG (1983) Prostaglandin E₂, adenosine 3':5'-cyclic monophosphate and changes in endometrial vascular permeability in rat uteri sensitized for the decidual cell reaction *Biology of Reproduction* **29** 1069–1076
- Kim JJ, Quinn PA and Fortier MA (1994) *Ureaplasma diversum* infection *in vitro* alters prostaglandin E₂ and prostaglandin F_{2α} production by bovine endometrial cells without affecting cell viability *Infection and Immunity* **62** 1528–1533
- Kniss DA, Mershon J, Su HC, Sonek J, Fertel RH, Waxman M, Iams JD and Gabbe SG (1990) Evidence of a role for protein kinase C in epidermal growth factor-induced prostaglandin E₂ synthesis in amnion cells *American Journal of Obstetrics and Gynecology* **163** 1883–1890

- Kudolo GB and Harper MJK (1990) Estimation of platelet-activating factor receptors in the endometrium of the pregnant rabbit: regulation of ligand availability and catabolism by bovine serum albumin *Biology of Reproduction* **43** 368–377
- Lafrance M and Goff AK (1985) Effect of pregnancy on oxytocin-induced release of prostaglandin F_{2a} in heifers *Biology of Reproduction* **33** 1113–1119
- Lafrance M and Goff AK (1988) Effects of progesterone and oestradiol-17 β on oxytocin-induced release of prostaglandin F_{2a} in heifers *Journal of Reproduction and Fertility* **82** 429–436
- Lafrance M and Goff AK (1990) Control of bovine uterine prostaglandin F_{2a} release *in vitro* *Biology of Reproduction* **42** 288–293
- Liu P, Wen M, Sun L and Hayashi J (1993) Activation of phospholipase A_2 and stimulation of prostaglandin E_2 production by transforming growth factor- α in rat thymic epithelial cells requires influx of calcium *Biochemical Journal* **293** 109–113
- McCracken JA, Schramm W and Okulicz WC (1984) Hormone receptor control of pulsatile secretion of PGF $_{2a}$ from the ovine uterus during luteolysis and its abrogation in early pregnancy *Animal Reproduction Science* **7** 31–55
- Mirando MA, Willard CB and Whiteaker SS (1993) Relationships among endometrial oxytocin receptors, oxytocin-stimulated phosphoinositide hydrolysis and prostaglandin F_{2a} secretion *in vitro*, and plasma concentrations of ovarian steroids before and during corpus luteum regression in cyclic heifers *Biology of Reproduction* **48** 874–882
- Mitchell MD (1991) The regulation of decidual prostaglandin biosynthesis by growth factors, phorbol esters, and calcium *Biology of Reproduction* **44** 871–874
- Moore JJ, Dubyak GR, Moore RM and Vander Kooy D (1988) Oxytocin activates the inositol-phospholipid-protein kinase C system and stimulates prostaglandin production in human amnion cells *Endocrinology* **123** 1771–1777
- Moore JJ, Moore RM and Vander Kooy D (1991) Protein kinase C activation is required for oxytocin-induced prostaglandin production in human amnion cells *Journal of Clinical Endocrinology and Metabolism* **72** 1073–1080
- Nishizuka Y (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation *Nature* **334** 661–665
- O'Neill C (1988) Partial characterization of the embryo-derived platelet activating factor in mice *Journal of Reproduction and Fertility* **75** 376–389
- O'Neill C (1991) A physiological role for PAF in the stimulation of mammalian embryonic development *Trends in Pharmacological Sciences* **12** 82–84
- O'Neill C, Gidley-Baird AA, Pike IL and Saunders DM (1987) A bio-assay for embryo-derived platelet activating factor as a means of assessing quality and pregnancy potential of human embryos *Fertility and Sterility* **47** 969–975
- Roberts JS, McCracken JA, Gavagan JE and Soloff MS (1976) Oxytocin-stimulated release of prostaglandin F_{2a} from ovine endometrium *in vitro*: correlation with estrous cycle and oxytocin-receptor binding *Endocrinology* **99** 1107–1114
- Salamonsen LA (1991) Effect of platelet activating factor on prostaglandin release from ovine endometrial cells in culture *Prostaglandins Leukotrienes and Essential Fatty Acids* **44** 67–70
- Scodras JM (1993) *Assessment of Co-culture Systems and Criteria of Viability for Bovine Embryos Produced in Vitro*. PhD Thesis, University of Guelph
- Shukla SD (1991) Inositol phospholipid turnover in PAF transmembrane signalling *Lipids* **26** 1028–1033
- Silvia WJ, Lewis GS, McCracken JA, Thatcher WW and Wilson L Jr (1991) Review: hormonal regulation of uterine secretion of prostaglandin F_{2a} during luteolysis in ruminants *Biology of Reproduction* **45** 655–663
- Smith SK and Kelly RW (1988a) Prostaglandins and the establishment of pregnancy. In *Implantation, Biological and Clinical Aspects* pp 147–160 Eds M Chapman, G Grudzinskas and T Chard. Springer-Verlag, Berlin
- Smith SK and Kelly RW (1988b) Effect of platelet-activating factor on the release of PGF $_{2a}$ and PGE $_2$ by separated cells of human endometrium *Journal of Reproduction and Fertility* **82** 271–276
- Thatcher WW, Bartol FF, Knickerbocker JJ, Curl JS and Wolfenson D (1984) Maternal recognition of pregnancy in cattle *Journal of Dairy Science* **67** 2797–2811
- Vallet JL, Lamming GE and Batten M (1990) Control of endometrial oxytocin receptor and uterine response to oxytocin by progesterone and oestradiol in the ewe *Journal of Reproduction and Fertility* **90** 625–634
- Venable ME, Zimmerman GA, McIntyre TM and Prescott SM (1993) Platelet-activating factor: a phospholipid autacoid with diverse actions *Journal of Lipid Research* **34** 691–702
- Zakar T and Olsen DM (1988) Stimulation of human amnion prostaglandin E_2 production by activators of protein kinase C *Journal of Clinical Endocrinology and Metabolism* **67** 915–923
- Zhang J, Weston PG and Hixon JE (1992) Role of progesterone and oestradiol in regulation of uterine oxytocin receptors in ewes *Journal of Reproduction and Fertility* **94** 395–404