Factors controlling prostaglandin production by guinea-pig endometrial cells

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The main cell type in the guinea-pig endometrium responsible for prostaglandin (PG) $F_{2\alpha}$ production and some of the intracellular mechanisms responsible for $PGF_{2\alpha}$ synthesis in this cell type have been investigated. The glandular epithelial cells and not the stromal cells were found to be the main prostaglandin forming cells in the endometrium. A23187 (a calcium ionophore), phospholipase A_2 and melittin (an activator of endogenous phospholipase A_2) increased $PGF_{2\alpha}$ output from the glandular epithelial cells, although only the action of melittin was specific to this cell type. Phospholipase A_2 also increased the intracellular free calcium concentration in both cell types by an action largely dependent upon the presence of extracellular calcium. Protein synthesis inhibitors (actinomycin D, cycloheximide and puromycin), oestradiol, progesterone and aristolochic acid (an inhibitor of phospholipase A_2) reduced $PGF_{2\alpha}$ output from the glandular epithelial cells, although only the inhibitory action of aristolochic acid was specific. Protein synthesis inhibitors also reduced $PGF_{2\alpha}$ output from stromal cells, and the outputs of PGE_2 and 6-keto- $PGF_{1\alpha}$ from both cell types. The steroid hormones also reduced PGE_2 output from stromal cells. The findings indicate that $PGF_{2\alpha}$ production by the glandular epithelial cells is dependent upon fresh protein synthesis and the activity of endogenous phospholipase A_2 (which is a calcium-requiring enzyme).

Introduction

Prostaglandin (PG) $F_{2\alpha}$ produced by the uterus is responsible for regression of corpora lutea in the guinea-pig and, therefore, the regulation of oestrous cycle duration (see Poyser, 1995). It is clear that the endometrium in the uterus is the source of $PGF_{2\alpha}$ (Butcher *et al.*, 1962; Leaver and Seawright, 1982; Poyser, 1983a; Riley and Poyser, 1987a). Various factors (for example, regulators of phospholipase A_2 activity, ovarian steroid hormones, protein synthesis inhibitors) modulate endometrial $PGF_{2\alpha}$ synthesis in the guinea-pig (Leaver and Seawright, 1982; Riley and Poyser, 1987a, b, 1989; Johnson and Poyser, 1991). The endometrium consists of two main cell types, namely epithelial cells and stromal cells. This study has investigated which is the main endometrial cell type for PG synthesis, and whether PG production by the two cell types in the guinea-pig is under different control.

Materials and Methods

Materials

Nutrient Mixture F-10 Ham (with Hepes), Hank's Balanced Salt Solution, streptomycin, trypan blue, amphotericin B,

actinomycin D, collagenase Type 1, aristolochic acid, cycloheximide, PLA_2 (from *Naja naja* venom), A23187, melittin, puromycin, oestradiol and progesterone were purchased from Sigma Chemical Co., Poole, Dorset. Newborn calf serum and L-glutamine were purchased from Flow Laboratories, Irvine. Penicillin G potassium salt was purchased from ICN Pharmaceuticals Ltd, Thame, Oxfordshire.

Methods

Guinea-pigs (600–900 g) were examined daily and a vaginal smear was taken when the vagina was open. Day 1 of the oestrous cycle was taken as the day preceding the post-ovulatory influx of leucocytes when cornification was at a maximum. All guinea-pigs had exhibited three ovarian cycles of normal duration before being used on day 7 or day 15 of the cycle. Four guinea-pigs on day 7 were used in each of Expts 1 to 3 (i.e. $n = 4$), and three guinea-pigs on each of days 7 and 15 were used in Expt 4 (i.e. $n = 3$). Each guinea-pig was killed by stunning and excising the neck. The uterus was removed and divided into its two uterine horns. Each horn was 'opened' by cutting longitudinally, and the endometrium was carefully cut away in small pieces from the myometrium by using a pair of fine scissors. This technique results in > 85% separation of the two tissues (Leaver and Poyser, 1981). The endometrial cells were dissociated by the method of Stayaswaroop *et al.* (1979) as modified by Chaminadas *et al.* (1986). In summary, the pieces of endometrium were chopped into small fragments and placed

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in 10 ml Nutrient Mixture F-10 Ham containing Hepes (20 mmol l⁻¹), 30% heat-treated new born calf serum, collagenase type 1 (2 mg ml⁻¹), L-glutamine (10 mmol l⁻¹), amphotericin B (2.5 µg ml⁻¹), penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹). The tissue was incubated at 37°C with shaking, and the tissue fragments produced were disrupted further by gentle, repeated pipetting every 15 min. The extent of digestion was monitored periodically by examining a small volume of the suspension under an inverted microscope. After adequate cell dispersion had been obtained, the remaining tissue fragments were collected by sedimentation and discarded. The digestion process took approximately 2 h. The supernatant cell suspension was centrifuged at 100 g for 5 min. The supernatant (containing stromal cells) was removed, and the pellet containing glandular epithelial cells was twice washed and centrifuged at 100 g for 5 min. The final pellet obtained contained approximately 95% glandular epithelial cells and 5% stromal cells. The supernatant containing most of the stromal cells was centrifuged at 400 g for 10 min, and a pellet of stromal cells was obtained. There was no detectable contamination of the stromal cells with glandular epithelial cells.

In Expts 1 to 3, the glandular epithelial cells and stromal cells were each resuspended in 30–40 ml Nutrient Mixture F-10 Ham containing Hepes (20 mmol l⁻¹), L-glutamine (10 mmol l⁻¹), amphotericin B (2.5 µg ml⁻¹), penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹). Aliquots (2.5 ml) of the cell suspension were dispersed into an adequate number of the wells of a cell culture plate. The glandular epithelial cell suspensions contained 2.3×10^3 to 4.5×10^3 cells ml⁻¹, and the stromal cell suspension contained 2.2×10^5 to 5.6×10^5 cells ml⁻¹. The cell culture plates were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. After 24 h of culture, the glandular epithelial cells had lost their shapes and had become flattened to form a monolayer. Stromal cells became attached to the plastic surface and, after 48 h of culture, a confluent layer of cells was formed. The medium in each well was changed every 72 h, and the cells were cultured for 6 days before being used in Expts 1 to 3.

In each experiment, the cells were cultured for a further 24 h in the absence and presence of treatments as outlined below. The culture medium was changed after 2, 8 and 24 h, and the samples of medium obtained were stored at -20°C. The amounts of PGF_{2α}, PGE₂ and 6-keto-PGF_{1α} in each sample were measured, without extraction, by radioimmunoassay. Cell viability at the time of plating and at the end of culture was determined using a trypan blue exclusion method. To 0.1 ml of a cell suspension prepared in Hank's Balanced Salt Solution was added 0.4 ml of 0.4% (w/v) Trypan blue solution. After 10 min, the cells were washed and then monitored in a haemocytometer. Cell viability at the time of plating was >98% and after culture was >95%.

In Expts 1 to 3, two wells containing each cell type from each animal were untreated (controls). In Expt 1, the remaining cells of each type from each animal were treated in duplicate with phospholipase (PL) A₂ (2 U ml⁻¹) or the calcium ionophore, A23187 (1 µg ml⁻¹). In Expt 2, the remaining cells of each type were treated in duplicate with oestradiol (3.7 µmol l⁻¹), progesterone (3.2 µmol l⁻¹), melittin (2 µg ml⁻¹) or aristolochic acid (50 µmol l⁻¹). In Expt 3, the remaining cells

were treated in duplicate with actinomycin D (10 and 50 µg ml⁻¹), cycloheximide (10 and 50 µg ml⁻¹) and puromycin (10 and 50 µg ml⁻¹). The concentration of aristolochic acid used inhibits PLA₂ activity in human neutrophils (Rosenthal *et al.*, 1989), and the concentrations of the other compounds used increase or inhibit PG output from the guinea-pig superfused uterus or cultured endometrium (Poyser and Bryden, 1983; Poyser, 1987; Riley and Poyser, 1987b, 1989; Johnson and Poyser, 1991). The average of each set of duplicate results was used for each animal.

In Expt 4, the suspensions of glandular epithelial cells and stromal cells were not incubated. After preparation, the cells were washed twice in Krebs solution (for composition: see Mitchell *et al.*, 1977) or in calcium-depleted Krebs solution (from which the calcium chloride had been omitted) both buffered with Hepes (20 mmol l⁻¹) at pH 7.4. After centrifugation (at 100 or 400 g for 5 or 10 min, as appropriate), the cells were resuspended in the appropriate Krebs solution and loaded with fura-2 acetoxymethyl ester (fura-2AM) by incubating for 30 min at room temperature. Cells were then washed and centrifuged, resuspended in the appropriate Krebs solution, and left to stand for a further 30 min to allow the intracellular fura-2AM to hydrolyse to fura-2. The cells were then washed, resuspended in calcium-containing or calcium-depleted Krebs solution, and transferred to a quartz cuvette maintained at 37°C. Fluorescence was measured in the absence and presence of PLA₂ (1–10 U ml⁻¹ added cumulatively) in a Shimadzu spectrofluorophotometer (Type RF-5000) at 505 nm with alternate excitation at 340 nm and 380 nm. The intracellular free calcium concentration ([Ca²⁺]_i) was calculated using the equation of Grynkiewicz *et al.* (1985):

$$[\text{Ca}^{2+}]_i = k_d \times b \times (R - R_{\min}) / (R_{\max} - R)$$

where, k_d is the dissociation constant for the fura-2-Ca²⁺ complex, and was assumed to be 225 nmol l⁻¹ at 37°C (Grynkiewicz *et al.*, 1985; Buchan and Martin, 1991); b is the ratio of the 380 nm signals in Ca²⁺-depleted and Ca²⁺-containing Krebs solution; R is the ratio of the fluorescence at 340 nm to that at 380 nm; and $R_{\max}$ and $R_{\min}$ are the maximum and minimum fluorescence ratios obtained in the presence of ionomycin (10 µmol l⁻¹; a calcium ionophore) and EGTA (30 µmol l⁻¹; a calcium chelator), respectively.

Details of radioimmunoassay

PGF_{2α}, PGE₂ and 6-keto-PGF_{1α} were measured using antibodies raised in this laboratory. The crossreactivities have been reported by Poyser (1987). The standard PG solutions used in the assay contained an amount of culture medium equivalent to that used for the samples. The intra-assay and inter-assay coefficients of variation were <12% for each assay. The detection limit was 30 pg PG per assay tube for the three assays.

Statistical analysis

The results were analysed by one-way analysis of variance (ANOVA), the paired *t* test or Student's *t* test as appropriate.

Table 1. Effects of phospholipase A₂ (PLA₂; 2 U ml⁻¹) and A23187 (1 µg ml⁻¹) on mean ± SEM (*n* = 4) outputs of prostaglandin (PG) F_{2α}, PGE₂ and 6-keto-PGF_{1α} from glandular epithelial cells and stromal cells of guinea-pig endometrium during three consecutive periods of cell culture

Treatment	PG output (pg (1000 cell) ⁻¹ h ⁻¹)					
	0–2 h		Culture period 2–8 h		8–24 h	
	Glandular	Stromal	Glandular	Stromal	Glandular	Stromal
PGF_{2α}						
Control	103.1 ± 7.0	0.42 ± 0.10	51.2 ± 16.3	0.20 ± 0.07	28.9 ± 4.1	0.10 ± 0.02
PLA ₂	100.4 ± 4.1	0.47 ± 0.09	52.4 ± 13.2	0.22 ± 0.04	50.2 ± 8.7 [†]	0.17 ± 0.04 [†]
A23187	180.6 ± 7.3 [†]	0.55 ± 0.09	29.1 ± 7.6	0.12 ± 0.02	9.4 ± 1.8*	0.04 ± 0.01*
PGE₂						
Control	112.4 ± 21.9	1.02 ± 0.11	34.2 ± 4.8	0.37 ± 0.02	13.2 ± 1.1	0.16 ± 0.01
PLA ₂	176.5 ± 35.2 [†]	1.63 ± 0.11 [†]	63.9 ± 13.8 [†]	0.56 ± 0.04 [†]	34.4 ± 9.6 [†]	0.24 ± 0.01 [†]
A23187	134.2 ± 51.1	1.03 ± 0.07	30.2 ± 3.9	0.32 ± 0.02	20.2 ± 6.1	0.16 ± 0.01
6-keto-PGF_{1α}						
Control	57.7 ± 5.2	0.49 ± 0.09	18.9 ± 2.8	0.17 ± 0.02	9.4 ± 2.1	0.06 ± 0.01
PLA ₂	72.2 ± 11.3	0.51 ± 0.07	22.1 ± 2.7	0.18 ± 0.02	13.2 ± 1.8 [†]	0.09 ± 0.01 [†]
A23187	62.3 ± 10.7	0.42 ± 0.05	13.2 ± 1.6	0.10 ± 0.01*	7.2 ± 0.7	0.05 ± 0.01*

*Significantly (*P* < 0.05) lower than corresponding control value.[†]Significantly (*P* < 0.05) higher than corresponding control value.

Results

In Expts 1 to 3 the basal, initial outputs of PGF_{2α}, PGE₂ and 6-keto-PGF_{1α} were between 95- and 450 times higher from glandular epithelial cells than from stromal cells (Tables 1–3). The basal outputs of the three prostaglandins significantly (*P* < 0.05) declined with time over the 24 h culture period.

Experiment 1: effects of PLA₂ and A23187 on PG output

On the glandular cells, PLA₂ significantly (*P* < 0.05) increased PGF_{2α} output 1.7-fold after 24 h, PGE₂ output 1.6- to 2.6-fold after 2, 8 and 24 h, and 6-keto-PGF_{1α} 1.2-fold after 24 h of culture (Table 1). A23187 significantly (*P* < 0.05) increased the output of PGF_{2α} 1.7-fold after 2 h but significantly (*P* < 0.05) decreased PGF_{2α} output by 69% after 24 h of culture. A23187 had no significant effect on the outputs of PGE₂ and 6-keto-PGF_{1α} from glandular epithelial cells (Table 1).

On stromal cells, PLA₂ significantly (*P* < 0.05) increased the outputs of PGF_{2α} and 6-keto-PGF_{1α} 1.7-fold and 1.4-fold, respectively, after 24 h, and the output of PGE₂ 1.5- to 1.6-fold after 2, 8 and 24 h of culture (Table 1). A23187 significantly (*P* < 0.05) increased PGF_{2α} output 1.3-fold after 2 h, but significantly (*P* < 0.05) decreased PGF_{2α} output by 58% after 24 h of culture. A23187 also significantly (*P* < 0.05) decreased the output of 6-keto-PGF_{1α} by 22 to 39% after 8 and 24 h. A23187 had no effect on PGE₂ output from stromal cells (Table 1).

Experiment 2: effects of oestradiol, progesterone, melittin and aristolochic acid on PG output

Oestradiol and progesterone significantly (*P* < 0.05) decreased PGF_{2α} output by 28 to 41% from glandular epithelial

cells, and PGE₂ output by 15% from stromal cells after 8 h of culture. Oestradiol also significantly (*P* < 0.05) decreased the output of PGF_{2α} by 37% from glandular epithelial cells after 24 h of culture (Table 2). Melittin significantly (*P* < 0.05) increased the outputs of PGF_{2α}, PGE₂ and 6-keto-PGF_{1α} up to 1.6-fold after 2 h of culture, and PGF_{2α} output 1.4-fold after 24 h of culture from glandular epithelial cells. Melittin had no effect on the outputs of PGF_{2α}, PGE₂ and 6-keto-PGF_{1α} from stromal cells (Table 2).

Aristolochic acid significantly (*P* < 0.05) inhibited PGF_{2α} output by 52 to 56% from glandular epithelial cells after 8 and 24 h of culture. It had no effect on the outputs of PGE₂ and 6-keto-PGF_{1α} from glandular epithelial cells. Aristolochic acid significantly (*P* < 0.05) increased PGE₂ output from stromal cells by 1.2-fold after 2 h of culture, but had no effect on the outputs of PGF_{2α} and 6-keto-PGF_{1α} from stromal cells (Table 2).

Experiment 3: effects of protein synthesis inhibitors on PG output

Cycloheximide and puromycin (both at 10 and 50 µg ml⁻¹) significantly (*P* < 0.05) inhibited the output of PGF_{2α} from glandular epithelial cells by 26 to 45% after 2 h, by 62 to 84% after 8 h, and by 84 to 90% after 24 h. Actinomycin D (10 and 50 µg ml⁻¹) significantly (*P* < 0.05) inhibited PGF_{2α} output from glandular epithelial cells by 68% after 8 h and by 81% after 24 h (Table 3). PGE output from glandular epithelial cells was significantly (*P* < 0.05) inhibited between 18 and 28% by cycloheximide (10 µg ml⁻¹) after 2 h, by actinomycin D (10 and 50 µg ml⁻¹) and puromycin (50 µg ml⁻¹) after 8 h, and by puromycin (10 µg ml⁻¹) after 24 h (Table 3). The output of 6-keto-PGF_{1α} from glandular epithelial cells was significantly (*P* < 0.05) inhibited by actinomycin D (10 µg ml⁻¹),

Table 2. Effects of oestradiol ($3.7 \mu\text{mol l}^{-1}$), progesterone ($3.2 \mu\text{mol l}^{-1}$), melittin ($2 \mu\text{g ml}^{-1}$) and aristolochic acid (Arist. acid; $50 \mu\text{mol l}^{-1}$) on mean $\pm$ SEM ($n = 4$) outputs of prostaglandin (PG) F_{2u} , PGE_2 and 6-keto-PGF $_{1u}$ from glandular epithelial cells and stromal cells of guinea-pig endometrium during three consecutive periods of cell culture

Treatment	PG output ($\text{pg (1000 cell)}^{-1} \text{ h}^{-1}$)					
	0–2 h		Culture period 2–8 h		8–24 h	
	Glandular	Stromal	Glandular	Stromal	Glandular	Stromal
PGF$_{2u}$						
Control	176.8 $\pm$ 24.9	0.40 $\pm$ 0.06	101.2 $\pm$ 9.4	0.13 $\pm$ 0.02	59.3 $\pm$ 5.9	0.05 $\pm$ 0.01
Oestradiol	154.5 $\pm$ 12.2	0.34 $\pm$ 0.06	59.3 $\pm$ 8.2*	0.11 $\pm$ 0.01	36.8 $\pm$ 0.8*	0.05 $\pm$ 0.01
Progesterone	196.2 $\pm$ 25.8	0.37 $\pm$ 0.04	73.4 $\pm$ 11.6*	0.12 $\pm$ 0.01	55.2 $\pm$ 2.7	0.05 $\pm$ 0.01
Melittin	272.1 $\pm$ 57.1 [†]	0.39 $\pm$ 0.05	130.2 $\pm$ 46.1	0.12 $\pm$ 0.02	83.2 $\pm$ 1.2 [†]	0.06 $\pm$ 0.01
Arist. acid	154.3 $\pm$ 28.6	0.30 $\pm$ 0.04	44.4 $\pm$ 9.8*	0.10 $\pm$ 0.01	28.4 $\pm$ 7.9*	0.04 $\pm$ 0.01
PGE$_2$						
Control	163.1 $\pm$ 14.2	1.41 $\pm$ 0.02	72.1 $\pm$ 14.2	0.56 $\pm$ 0.02	27.8 $\pm$ 2.7	0.23 $\pm$ 0.01
Oestradiol	149.4 $\pm$ 3.8	1.34 $\pm$ 0.02	60.3 $\pm$ 6.9	0.48 $\pm$ 0.02*	26.5 $\pm$ 0.9	0.21 $\pm$ 0.01
Progesterone	170.2 $\pm$ 9.2	1.37 $\pm$ 0.03	59.2 $\pm$ 3.8	0.49 $\pm$ 0.02*	29.2 $\pm$ 1.8	0.24 $\pm$ 0.02
Melittin	196.8 $\pm$ 20.8 [†]	1.43 $\pm$ 0.02	65.2 $\pm$ 13.8	0.52 $\pm$ 0.02	33.4 $\pm$ 4.7	0.22 $\pm$ 0.01
Arist. acid	184.2 $\pm$ 13.9	1.70 $\pm$ 0.04 [†]	68.4 $\pm$ 10.9	0.59 $\pm$ 0.05	30.2 $\pm$ 1.6	0.26 $\pm$ 0.02
6-keto-PGF$_{1u}$						
Control	117.3 $\pm$ 10.1	0.83 $\pm$ 0.07	50.8 $\pm$ 7.8	0.29 $\pm$ 0.02	21.1 $\pm$ 3.8	0.12 $\pm$ 0.03
Oestradiol	116.5 $\pm$ 9.8	0.79 $\pm$ 0.17	44.2 $\pm$ 8.7	0.27 $\pm$ 0.08	18.1 $\pm$ 1.7	0.10 $\pm$ 0.01
Progesterone	122.1 $\pm$ 16.2	0.82 $\pm$ 0.14	42.9 $\pm$ 7.9	0.26 $\pm$ 0.05	17.8 $\pm$ 1.8	0.09 $\pm$ 0.01
Melittin	150.4 $\pm$ 32.6 [†]	0.90 $\pm$ 0.15	47.2 $\pm$ 9.6	0.30 $\pm$ 0.07	25.2 $\pm$ 7.2	0.11 $\pm$ 0.02
Arist. acid	114.3 $\pm$ 22.2	0.82 $\pm$ 0.09	48.1 $\pm$ 9.9	0.25 $\pm$ 0.02	19.1 $\pm$ 2.8	0.10 $\pm$ 0.01

*Significantly ($P < 0.05$) lower than corresponding control value.

[†]Significantly ($P < 0.05$) higher than corresponding control value.

cycloheximide ($50 \mu\text{g ml}^{-1}$) and puromycin ($50 \mu\text{g ml}^{-1}$) after 2 h and by cycloheximide ($10 \mu\text{g ml}^{-1}$), actinomycin D ($10 \mu\text{g ml}^{-1}$) and puromycin ($50 \mu\text{g ml}^{-1}$) after 8 h. By 24 h, both concentrations of actinomycin D, cycloheximide and puromycin significantly ($P < 0.05$) inhibited the output of 6-keto-PGF $_{1u}$ from glandular epithelial cells. The degree of inhibition ranged from 34 to 67% (Table 3).

On stromal cells actinomycin D, cycloheximide and puromycin (10 and $50 \mu\text{g ml}^{-1}$) significantly ($P < 0.05$) inhibited the outputs of PGF $_{2u}$, PGE $_2$ and 6-keto-PGF $_{1u}$, except for cycloheximide ($50 \mu\text{g ml}^{-1}$) which had no effect on PGE $_2$ output during all three culture periods. In addition, puromycin ($10 \mu\text{g ml}^{-1}$) and cycloheximide ($10 \mu\text{g ml}^{-1}$) had no effect on PGE $_2$ output during the first and the last periods of culture, respectively. The degree of inhibition ranged from 10 to 89% (Table 3).

Experiment 4: effect of PLA $_2$ on the intracellular free calcium concentration

In the presence of extracellular calcium, PLA $_2$ significantly ($P < 0.05$) increased the intracellular free calcium concentration of day 7 and day 15 epithelial and stromal cells 16.8- to 54.4-fold (Fig. 1). In calcium-depleted medium PLA $_2$ significantly ($P < 0.05$) increased the intracellular free calcium concentration in day 7 and day 15 epithelial cells and stromal cells 2.8- to 10.3-fold although, owing to the large scatter of results,

some of these increases were not significant on day 7 (Fig. 1). In the presence or absence of extracellular calcium, PLA $_2$ (4 to 10 U ml^{-1}) significantly ($P < 0.05$) increased the intracellular free calcium concentration to a greater extent in epithelial cells than in stromal cells obtained from day 15, but not day 7, endometrium (Table 1).

Discussion

Prostaglandin output is approximately 100- to 500-fold higher from glandular epithelial cells than from stromal cells. This difference occurred during the 6 days of culture before using the cells (data not shown), and on the seventh day when the cells were treated. On this experimental day, PG output per hour from both cell types decreased during the 24 h period. This may be a consequence of the increasing time intervals between changes of culture medium with the resultant slowing down of PG synthesis as nutrients are used up. It is clear that the glandular epithelial cells produce considerably more PGF $_{2u}$ than do the stromal cells and are therefore the source of the uterine luteolytic hormone in guinea-pigs. The epithelial cells have also been found to be the major endothelial cell type that synthesize PGF $_{2u}$ in rats, pigs, horses and cows (Peplow and Hurst, 1989; Zhang and Davis, 1991; Watson *et al.*, 1992; Danet-Desnoyers *et al.*, 1994). PGE $_2$ was the major PG synthesized by stromal cells in the guinea-pig.

Table 3. Effects of actinomycin D (Actin. D; 10 and 50 $\mu\text{g ml}^{-1}$); cycloheximide (Cyclo.; 10 and 50 $\mu\text{g ml}^{-1}$) and puromycin (Puromy.; 10 and 50 $\mu\text{g ml}^{-1}$) on mean $\pm$ SEM ($n = 4$) outputs of prostaglandin (PG) F_{2a} , PGE_2 and 6-keto- PGF_{1a} from glandular epithelial cells and stromal cells of guinea-pig endometrium during three consecutive periods of cell culture

Treatment	PG output ($\text{pg (1000 cell)}^{-1} \text{ h}^{-1}$)					
	0–2 h		Culture period 2–8 h		8–24 h	
	Glandular	Stromal	Glandular	Stromal	Glandular	Stromal
PGF_{2a}						
Control	89.2 $\pm$ 4.1	0.42 $\pm$ 0.01	49.2 $\pm$ 2.8	0.15 $\pm$ 0.09	37.4 $\pm$ 1.4	0.072 $\pm$ 0.003
Actin. D 10	70.4 $\pm$ 5.2	0.30 $\pm$ 0.02 [†]	15.4 $\pm$ 2.2 [†]	0.08 $\pm$ 0.01 [†]	7.1 $\pm$ 0.8 [†]	0.027 $\pm$ 0.003 [†]
Actin. D 50	80.3 $\pm$ 5.8	0.29 $\pm$ 0.02 [†]	16.3 $\pm$ 2.4 [†]	0.07 $\pm$ 0.01 [†]	7.2 $\pm$ 0.7 [†]	0.027 $\pm$ 0.003 [†]
Cyclo. 10	52.3 $\pm$ 5.9 [†]	0.28 $\pm$ 0.02 [†]	12.2 $\pm$ 1.4 [†]	0.08 $\pm$ 0.01 [†]	4.1 $\pm$ 0.3 [†]	0.026 $\pm$ 0.003 [†]
Cyclo. 50	49.4 $\pm$ 5.2 [†]	0.29 $\pm$ 0.01 [†]	11.8 $\pm$ 1.3 [†]	0.09 $\pm$ 0.01 [†]	5.2 $\pm$ 0.4 [†]	0.024 $\pm$ 0.001 [†]
Puromy. 10	66.1 $\pm$ 4.2 [†]	0.35 $\pm$ 0.02 [†]	19.2 $\pm$ 2.6 [†]	0.09 $\pm$ 0.01 [†]	6.2 $\pm$ 0.4 [†]	0.029 $\pm$ 0.003 [†]
Puromy. 50	52.3 $\pm$ 6.8 [†]	0.26 $\pm$ 0.01 [†]	8.2 $\pm$ 0.4 [†]	0.07 $\pm$ 0.01 [†]	4.9 $\pm$ 0.6 [†]	0.024 $\pm$ 0.002 [†]
PGE_2						
Control	113.2 $\pm$ 12.1	1.16 $\pm$ 0.02	35.3 $\pm$ 1.8	0.43 $\pm$ 0.03	18.2 $\pm$ 1.2	0.16 $\pm$ 0.01
Actin. D 10	96.4 $\pm$ 7.8	1.06 $\pm$ 0.03	29.2 $\pm$ 1.8 [†]	0.33 $\pm$ 0.01 [†]	17.4 $\pm$ 1.9	0.14 $\pm$ 0.01 [†]
Actin. D 50	89.8 $\pm$ 5.9	1.00 $\pm$ 0.04 [†]	28.4 $\pm$ 1.7 [†]	0.28 $\pm$ 0.02 [†]	15.3 $\pm$ 1.8	0.13 $\pm$ 0.01 [†]
Cyclo. 10	88.2 $\pm$ 6.2 [†]	0.98 $\pm$ 0.02 [†]	30.1 $\pm$ 3.9	0.32 $\pm$ 0.01 [†]	15.2 $\pm$ 1.6	0.14 $\pm$ 0.02
Cyclo. 50	120.2 $\pm$ 12.1	1.20 $\pm$ 0.11	39.2 $\pm$ 3.1	0.40 $\pm$ 0.02	17.4 $\pm$ 2.1	0.16 $\pm$ 0.01
Puromy. 10	106.8 $\pm$ 4.8	1.02 $\pm$ 0.03	36.2 $\pm$ 5.8	0.34 $\pm$ 0.01 [†]	13.1 $\pm$ 1.8 [†]	0.13 $\pm$ 0.01 [†]
Puromy. 50	104.7 $\pm$ 13.8	0.95 $\pm$ 0.04 [†]	26.8 $\pm$ 3.7 [†]	0.31 $\pm$ 0.02 [†]	13.9 $\pm$ 1.9	0.13 $\pm$ 0.01 [†]
6-keto-PGF_{1a}						
Control	105.2 $\pm$ 21.1	0.99 $\pm$ 0.04	18.1 $\pm$ 2.1	0.49 $\pm$ 0.14	12.2 $\pm$ 0.3	0.18 $\pm$ 0.04
Actin. D. 10	48.9 $\pm$ 2.1 [†]	0.68 $\pm$ 0.04 [†]	9.7 $\pm$ 1.6 [†]	0.12 $\pm$ 0.02 [†]	7.2 $\pm$ 0.8 [†]	0.04 $\pm$ 0.01 [†]
Actin. D 50	86.5 $\pm$ 25.5	0.68 $\pm$ 0.01 [†]	26.5 $\pm$ 4.8	0.10 $\pm$ 0.02 [†]	7.2 $\pm$ 0.7 [†]	0.04 $\pm$ 0.01 [†]
Cyclo. 10	86.0 $\pm$ 29.6	0.56 $\pm$ 0.03 [†]	12.1 $\pm$ 1.4 [†]	0.10 $\pm$ 0.02 [†]	6.0 $\pm$ 0.6 [†]	0.08 $\pm$ 0.02 [†]
Cyclo. 50	35.1 $\pm$ 2.2 [†]	0.55 $\pm$ 0.06 [†]	21.2 $\pm$ 5.8	0.10 $\pm$ 0.01 [†]	4.1 $\pm$ 0.3 [†]	0.04 $\pm$ 0.01 [†]
Puromy. 10	93.2 $\pm$ 24.2	0.68 $\pm$ 0.01 [†]	14.8 $\pm$ 3.8	0.14 $\pm$ 0.01 [†]	3.8 $\pm$ 0.4 [†]	0.05 $\pm$ 0.01 [†]
Puromy. 50	43.1 $\pm$ 3.2 [†]	0.57 $\pm$ 0.03 [†]	6.1 $\pm$ 0.4 [†]	0.10 $\pm$ 0.01 [†]	4.7 $\pm$ 0.5 [†]	0.04 $\pm$ 0.01 [†]

[†]Significantly ($P < 0.05$) lower than corresponding control value.

It has been proposed that, in the guinea-pig, oestradiol acting on a progesterone-primed endometrium stimulates the synthesis of proteins which, by some mechanism, increase the intracellular free calcium concentration and thereby activate PLA_2 to release arachidonic acid from phospholipids for increased PGF_{2a} synthesis after day 11 of the cycle (see Poyser, 1995). Although oestradiol and progesterone when administered *in vivo* cause a selective increase in endometrial PGF_{2a} synthesis (Poyser, 1983a, b), treatment with oestradiol or progesterone *in vitro* inhibits the outputs of PGF_{2a} and PGE_2 (but not of 6-keto- PGF_{1a}) from guinea-pig endometrium in culture (Riley and Poyser, 1987b). In the present study, oestradiol and progesterone inhibited PGF_{2a} output from glandular epithelial cells and PGE_2 output from stromal cells after 8 h of culture. Thus the two steroid hormones inhibit the production of different PGs by the two cell types. This differential inhibition would account for the reduced output of both PGE_2 and PGF_{2a} from guinea-pig endometrium when cultured *in vitro* in the presence of either steroid hormone. Progesterone treatment *in vitro* reduces the outputs of PGF_{2a} and PGE_2 from both cell types derived from pig endometrium, while oestradiol treatment has no effect (Zhang and Davies, 1991). On bovine epithelial cells, *in vitro* progesterone treat-

ment increases and oestradiol treatment decreases the outputs of PGF_{2a} and PGE_2 . Neither hormone affects the output of PGF_{2a} and PGE_2 from stromal cells (Asselin *et al.*, 1996). On human endometrial epithelial cells, oestradiol treatment *in vitro* stimulates PGF_{2a} output, and this increase is largely prevented by progesterone (Schatz *et al.*, 1985). These observations, taken together with the findings in the present study, show that there are differences among species regarding the effects of the ovarian steroid hormones *in vitro* on PG production by the endometrial cell types cultured *in vitro*.

The administration of actinomycin D into the uterus of sheep and guinea-pigs prevents luteal regression and prolongs the oestrous cycle (French and Casida, 1973; Poyser, 1979). In guinea-pigs, this has been shown to be due to reduced PGF_{2a} output from the uterus (Poyser and Riley, 1987). Actinomycin D (an inhibitor of DNA-dependent RNA synthesis), cycloheximide (which prevents the movement of ribosomes along mRNA) and puromycin (an inhibitor of polypeptide chain elongation) inhibit protein synthesis by and the outputs of PGF_{2a} , PGE_2 and 6-keto- PGF_{1a} from day 7 guinea-pig endometrium in culture (Riley and Poyser, 1987b). However, the effect of cycloheximide at high concentrations (i.e. 50 $\mu\text{g ml}^{-1}$) is less marked since this compound also has a short-term

Fig. 1. Effects of cumulative increasing concentrations of phospholipase (PL) A_2 on the mean $\pm$ SEM ($n = 3$) intracellular free calcium concentration ($[Ca^{2+}]_i$), expressed as a percentage of the 'baseline' concentration in the absence of PLA_2 , in glandular epithelial cells ($\square$) and stromal cells ($\blacksquare$) of guinea-pig endometrium on (a, b) day 7 and (c, d) day 15 of the cycle in (a, c) calcium-containing and (b, d) calcium-depleted Krebs' solution. †Significantly ($P < 0.05$) higher than corresponding baseline value. *Significantly ($P < 0.05$) higher than corresponding value from stromal cells.

stimulatory effect on PG output, especially the output of PGE_2 (Matthews and Poyser, 1991). In the present study, the three inhibitors of protein synthesis reduced the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto- $PGF_{1\alpha}$ from both glandular epithelial cells and stromal cells. PGE_2 output was affected the least, particularly by cycloheximide ($50 \mu g \text{ ml}^{-1}$). This finding is in agreement with the observation that cycloheximide at higher concentrations has a stimulatory effect on PGE_2 output (Matthews and Poyser, 1991). Nevertheless, the results obtained with actinomycin D, cycloheximide and puromycin indicate that continued protein synthesis is necessary for the maintenance of PG synthesis in both the glandular epithelial cells and stromal cells of guinea-pig endometrium. Which proteins are involved needs further study, but it is known that PGH synthase is destroyed during PG biosynthesis so fresh enzyme has to be provided if the biosynthesis is to be maintained (Lands *et al.*, 1973).

The calcium ionophore A23187 stimulates PG output from the guinea-pig uterus superfused *in vitro* by a mechanism dependent upon the presence of extracellular calcium (Poyser and Brydon, 1983; Poyser, 1984). A23187 also stimulates $PGF_{2\alpha}$ output from guinea-pig endometrium cultured *in vitro* (Leaver and Seawright, 1982). In the present study, A23187

increased $PGF_{2\alpha}$ output from glandular epithelial cells and, to a lesser extent, from stromal cells after 2 h. This differential effect in favour of epithelial cells in the guinea-pig is similar to that seen in humans where A23187 stimulates increased arachidonic acid release and $PGF_{2\alpha}$ output from epithelial cells but not from stromal cells (Schatz *et al.*, 1987; Bonney *et al.*, 1991). A23187 did not increase the outputs of PGE_2 and 6-keto- $PGF_{1\alpha}$ from guinea-pig endometrial cells, so the stimulatory action of A23187 via calcium appears to be specific for $PGF_{2\alpha}$. The prolonged treatment with A23187 inhibited $PGF_{2\alpha}$ output from glandular epithelial cells and stromal cells, and 6-keto- $PGF_{1\alpha}$ output from stromal cells. The reason for this inhibitory effect is not known but may be due to a toxic action on the cells during the course of the culture period.

PLA_2 increases the outputs of $PGF_{2\alpha}$, PGE_2 and 6-keto- $PGF_{1\alpha}$ from the guinea-pig uterus superfused *in vitro* (Poyser, 1987). PLA_2 has now been found to increase the outputs of these three PGs from both glandular epithelial cells and stromal cells. Thus the action of PLA_2 (from *Naja naja* venom) is not cell specific or prostaglandin specific. PLA_2 also increased the intracellular free calcium in both cell types in the presence and absence of extracellular calcium. However, this effect was 10- to 20-fold greater in the presence of extracellular

calcium than in its absence, indicating that PLA₂ caused a large influx of calcium into the cells as well as causing the release of some intracellular calcium. PLA₂ was equally effective at raising the intracellular free calcium concentration in glandular epithelial cells and stromal cells obtained from day 7 endometrium, but was more effective on glandular epithelial cells than on stromal cells derived from day 15 endometrium. The mechanism by which PLA₂ increases the intracellular free calcium concentration in these cells is unknown, but it appears that the stage of the cycle has some influence on this mechanism. The question is raised as to whether PLA₂ releases arachidonic acid for PG synthesis by acting directly on phospholipids, or whether PLA₂ acts as a calcium ionophore (like A23187) and activates endogenous PLA₂ by increasing the intracellular free calcium concentration.

The action of PLA₂ is unlikely to be due solely to it increasing the free intracellular calcium concentration since (i) PLA₂ had a different PG-releasing profile from A23187 and (ii) unlike A23187, PLA₂ has been found to increase the release of PGs from the superfused guinea-pig uterus in the absence of extracellular calcium (R. S. Scotland, M. G. H. Scott and N. L. Poyser, unpublished observation). These findings suggest that PLA₂ acts directly on phospholipids to release arachidonic acid. However, the PG-forming effect of PLA₂, like that of A23187, is inhibited by the intracellular calcium antagonist, TMB-8 (Poyser, 1985; Johnson and Poyser, 1991). This finding indicates that the action of PLA₂ is dependent upon intracellular calcium (with the calcium acting as a co-factor), or that TMB-8 inhibits endometrial PG synthesis by a mechanism other than its ability to inhibit the action of intracellular calcium.

Melittin stimulates endogenous PLA₂ and has been found to increase the output of prostaglandins from the superfused guinea-pig uterus (Johnson and Poyser, 1991) and sheep endometrium *in vitro* (Lee and Silvia, 1994). Melittin stimulated the outputs of PGF_{2α}, PGE₂ and 6-keto-PGF_{1α} from glandular epithelial cells but had no effect on stromal cells. Thus the PG-releasing action of melittin is specific for the glandular epithelial cells.

Aristolochic acid is an inhibitor of PLA₂ and so prevents the release of arachidonic acid from phospholipids (Rosenthal *et al.*, 1989). Aristolochic acid reduced the output of PGF_{2α} from glandular epithelial cells, suggesting that endogenous PLA₂ is involved in the production of PGF_{2α} by this cell type. Aristolochic acid has been found to inhibit basal and oxytocin-induced PGF_{2α} release from sheep endometrium (Lee and Silvia, 1994), indicating that endogenous PLA₂ is involved in endometrial PGF_{2α} synthesis in sheep. Aristolochic acid did not inhibit PGF_{2α} output from stromal cells, or the outputs of PGE₂ and 6-keto-PGF_{1α} from glandular epithelial cells and stromal cells. Thus it is not clear what mechanisms are involved in the release of arachidonic acid for the synthesis of these PGs. In fact, aristolochic acid surprisingly increased PGE₂ output from stromal cells during the first 2 h of culture.

Overall, this study has shown that the glandular epithelial cells, as opposed to stromal cells, are the main cells synthesizing PG in the guinea-pig endometrium. Therefore, it is probable that these cells secrete the PGF_{2α} necessary for luteolysis. The present findings indicate that the synthesis of PGF_{2α} by these glandular cells depends upon fresh protein synthesis and endogenous PLA₂ activity.

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