Expression of a cytosolic phospholipase A₂ by ovine endometrium on days 11–14 of a simulated oestrous cycle

G. A. Graf¹, P. D. Burns² and W. J. Silvia^{2*}

¹Department of Physiology, University of Kentucky, Chandler Medical Center, Lexington, KY, 40536-0084, USA; and ²Department of Animal Sciences, University of Kentucky, Lexington, KY 40546-0215, USA

Oxytocin stimulates the synthesis and secretion of PGF_{2α} from uterine tissues *in vivo* and *in vitro* late in the ovine oestrous cycle. The synthesis of eicosanoids is dependent upon the availability of free arachidonic acid which is released through the activity of arachidonate releasing phospholipases. In the present study, the following hypothesis was tested: the ovine endometrium expresses a cytosolic phospholipase A₂ (cPLA₂) and expression or activity of cPLA₂ increases as uterine secretory responsiveness to oxytocin develops late in the oestrous cycle. Endometrial tissue was collected from cyclic ewes on day 15 of the oestrous cycle for the preparation of tissue homogenates and isolation of mRNA to determine whether ovine endometrium expressed a cPLA₂. A 110 kDa band was detected by western blotting, indicating the presence of a putative ovine cPLA₂. A 834 bp fragment of the ovine cPLA₂ shared 87% homology with human and mouse cDNA, and northern blot hybridization analysis indicated a single 3.4 kb transcript. A total of 20 ewes were ovariectomized and treated with progesterone and oestrogen to simulate the oestrous cycle to determine whether the expression or activity of ovine cPLA₂ changed during the onset of uterine secretory responsiveness to oxytocin *in vivo*. On days 11–14 (*n* = 5 per day) of a simulated oestrous cycle, caruncular endometrium was evaluated for expression of ovine cPLA₂ mRNA and protein and the synthesis of PGF_{2α} in response to melittin (a potent stimulator of PLA₂ activity). Immunoreactive cPLA₂ and cPLA₂ mRNA were observed on all days and did not increase during the development of uterine responsiveness to oxytocin *in vivo*. Similarly, melittin increased the synthesis of PGF_{2α} irrespective of day, indicating the presence of a functional cPLA₂ on all days examined. These data indicate that the ovine endometrium expresses a functional cPLA₂, and that ample concentrations of cPLA₂ are present by day 11 of a simulated oestrous cycle.

Introduction

Prostaglandin F_{2α} (PGF_{2α}) is the endogenous luteolysin in sheep, and is secreted in response to oxytocin late in the ovine oestrous cycle (McCracken *et al.*, 1972). The synthesis of PGF_{2α} and other eicosanoids is dependent upon the availability of free arachidonic acid which is released from phospholipids by the activity of arachidonate releasing phospholipases (Irvine, 1982; Smith, 1989). Cytosolic phospholipase A₂ (cPLA₂) is an 85 kDa protein that preferentially hydrolyses arachidonic acid from the sn-2 position of phosphatidylcholine (Clark *et al.*, 1990; Kramer *et al.*, 1991). cPLA₂ has been implicated in a number of lipid-mediated signalling processes, including hormonally induced generation of prostanoids (Felder *et al.*, 1990; Lin *et al.*, 1992a,b).

Oxytocin-induced secretion of PGF_{2α} from ovine uterine endometrium includes the activation of cPLA₂. Aristilochic

acid, an inhibitor of PLA₂ activity, blocks oxytocin-induced PGF_{2α} synthesis *in vitro*, indicating that PLA₂ activity is required for this process (Lee and Silvia, 1994). Cyclic changes in uterine PLA₂ activity have been reported in the guinea-pig, in which activity was greater on day 15 versus day 7 of the oestrous cycle (Downing and Poyser, 1983). Ovarian steroids may control the synthesis of cPLA₂ during the oestrous cycle. In rat uterine tissues, oestradiol increases PLA₂ activity 12 and 24 h after injection, and this effect is blocked by cyclohexamide (Dey *et al.*, 1982). In sheep, uterine secretion of PGF_{2α} also increases during parturition, which is due in part to an increase in uterine responsiveness to oxytocin (Wu *et al.*, 1996). Increased PGF_{2α} secretion is associated with an increase in the expression of cPLA₂ mRNA and protein (Zhang *et al.*, 1996). On the basis of these observations, it is proposed that an increase in endometrial cPLA₂ also contributes to the increase in uterine PGF_{2α} secretion that occurs during luteolysis.

The objectives of the present study were (i) to clone the ovine cPLA₂ mRNA; (ii) to determine whether expression of

*Correspondence.

Received 29 June 1998.

Table 1. Progesterone treatment regimen

Day of treatment	Progesterone (mg)	
	08:00 h	20:00 h
1	0.50	0.63
2	0.75	1.00
3	1.50	2.00
4	3.00	4.00
5	5.00	6.00
6–7	8.00	8.00
8–15	12.00	12.00

cPLA₂ mRNA and protein accompanies the onset of uterine secretory responsiveness to oxytocin; and (iii) to determine whether PLA₂ enzymic activity is a factor that limits PGF_{2α} synthesis before the development of uterine secretory responsiveness to oxytocin.

Materials and Methods

Animals and treatments

Crossbred ewes ($n = 20$) were ovariectomized under sodium pentobarbital anaesthesia (20 mg kg⁻¹ body weight, i.v.) by mid-ventral laparotomy. Ovariectomies were performed at least 30 days before the beginning of the experiment. Ewes were pretreated with progesterone and oestradiol as described by Homanics and Silvia (1988). Progesterone was administered in corn oil at doses designed to mimic luteal phase concentrations of peripheral progesterone (Table 1). Oestradiol was given in the form of a subcutaneous Silastic implant. Commencing on day 0 of the simulated oestrous cycle, 5 ml blood was collected once a day by jugular venepuncture. Serum was harvested and stored at -20°C until assayed for progesterone. Progesterone concentration was determined by a solid phase radioimmunoassay (coat-a-count; Diagnostic Products Corp., Los Angeles, CA) as described by Silvia *et al.* (1992). On days 11, 12, 13 and 14 of the simulated oestrous cycle, ewes ($n = 5$ per day) were killed by captive bolt concussion and exsanguination. Uteri were immediately removed and taken to the laboratory for dissection within 10 min of collection. Carunculae from each ewe were dissected and placed in Krebs–Hensleit–bicarbonate buffer (KHB; 118 × 10⁻³ mol NaCl l⁻¹, 4.7 × 10⁻³ mol KCl l⁻¹, 2.56 × 10⁻³ mol CaCl₂ l⁻¹, 1.13 × 10⁻³ mol MgCl₂ l⁻¹, 25 × 10⁻³ mol NaHCO₃ l⁻¹, 1.15 × 10⁻³ mol NaH₂PO₄ l⁻¹, 5.55 × 10⁻³ mol glucose l⁻¹, 20 × 10⁻³ mol Hepes l⁻¹, 1.3 × 10⁻⁵ mol phenol red l⁻¹, pH 7.4). Sixteen carunculae were randomly selected from the pool of carunculae, weighed (80–100 mg), transferred to 12 mm × 75 mm borosilicate glass culture tubes and incubated to measure PGF_{2α} secretion rate (Silvia and Homanics, 1988). The remaining uterine endometrial tissues were dissected, frozen on dry ice and stored at -80°C for isolation and analysis of mRNA. All procedures involving animals were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Partial cloning and sequence analysis

Total RNA was extracted from a day 15 cyclic ewe using Tri-Reagent® (Molecular Resources Inc., Cincinnati, OH). mRNA was isolated by poly-A selection (PolyAtract®; Promega Corp., Madison, WI) and reverse transcribed using random hexamers, deoxyribonucleoside triphosphates and MMLV reverse transcriptase (Gibco BRL Life Technologies, Grand Island, NY). The integrity of the cDNA was verified using a hot side reaction with [α -³²P]deoxyribocytidine 5'-triphosphate (specific activity: 3000 Ci mmol⁻¹; Amersham, Arlington Heights, IL) and alkaline agarose electrophoresis (Sambrook *et al.*, 1989). Partial cDNA clones of the ovine cPLA₂ mRNA were amplified using degenerate primers deduced from amino acid sequences of cPLA₂ in mouse, human, chicken and zebra fish (forward, 5'TGTGGAAYGARACCTTYGARTTYAT; reverse, 5'AAYTCTACCCARTCNG-CRAACAT), and the PCR. Primers were synthesized on a Perkin Elmer DNA synthesizer (Macromolecular Structure Analysis Facility, University of Kentucky, Lexington, KY). Primers, endometrial cDNA, dNTPs and Taq polymerase (Promega Corp., Madison, WI) were used in five degenerate cycles (94°C, 60 s; 37°C ramp, 1° (4 s)⁻¹; 72°C, 120 s) and 35 cycles of 94°C (60 s), 48°C (60 s) and 72°C (120 s) on a Thermolyne Temp Tronic® thermocycler (Barnstead, Dubuque, IA). The PCR product of the appropriate size was gel purified by band interception with NA45 dimethylaminoethyl (DEAE) nitrocellulose membrane (Schleicher and Schuell, Keene, NH). Products of five reactions were cloned into the pCR®II plasmid vector using the TA cloning kit (Invitrogen, San Diego, CA). Colonies from two of the reactions were cultured in Luria-Bertani media overnight and plasmid DNA was purified by alkaline lysis and anion exchange chromatography using the Plasmid Midi-Kit (QIAGEN Inc., Chatsworth, CA).

Partial cDNA clones were sequenced using the M13 forward and reverse universal primers and one internal primer (5'GGCTCTTGTTGACCAGGA) obtained from 5' sequences. Sequencing reactions were performed on a 373A DNA sequencer using the sequencing ready reaction with amplitaq DNA polymerase, FS (Perkin Elmer, Foster City, CA) at the Macromolecular Structure Analysis Facility, University of Kentucky (Lexington, KY). Complete sequences from four independent colonies were obtained in triplicate, and aligned using the Genalign program in the Intelligenetics software package. Sequences were compared with other nucleic acid sequences in the Genbank using the Gap and Fasta routines.

RNA isolation and analysis

Total RNA was extracted using Tri Reagent (Molecular Resources Inc., Cincinnati, OH). Briefly, 1 g endometrium was homogenized in Tri Reagent and centrifuged at 2500 g for 15 min at 4°C. The supernatant was removed, 0.2 volumes CHCl₃ were added, and the samples were then vortexed for 30 s and incubated for 10 min at 25°C. The samples were centrifuged at 10000 g for 15 min at 4°C. Isopropanol (0.5 vol) was added to the supernatant, and samples were centrifuged (10000 g, 15 min, 4°C). The

samples were decanted and pellets were washed twice with 5 ml 75% ethanol. Pellets were allowed to dry at room temperature for 10 min and dissolved in 0.5% (v/v) diethylpyrocarbonate (DEPC)-treated water. The integrity of RNA was examined by denaturing agarose gel electrophoresis and RNA was quantified spectrophotometrically (Ausebel, 1994).

The 834 bp fragment of the ovine cPLA₂ was cut from the pCRII vector using EcoR I restriction endonuclease, and gel purified as described earlier. Ovine cPLA₂ DNA probes were generated using cDNA, random hexamers, [α -³²P]deoxyribocytidine 5'-triphosphate, and the large Klenow fragment of DNA Pol I (Gibco BRL Life Technologies, Grand Island, NY). Probes were purified on a Sephadex microspin G-50 column (Pharmacia Biotech, Uppsala). The radioactivity associated with the DNA probes was determined by liquid scintillation counting.

The number and size of transcripts detected by random primed ovine cPLA₂ DNA probes were determined by northern blot hybridization analysis of total RNA as described by Ausebel (1994). Total cellular RNA (20 μ g) from two ewes on each day of the simulated oestrous cycle were subjected to electrophoresis in duplicate under denaturing conditions (sample buffer: 20 mmol Mops l⁻¹, 5 mmol NaOAc l⁻¹, 1 mmol EDTA l⁻¹, 50% (w/v) formamide, 2.2 mol formaldehyde l⁻¹, 65°C, 5 min; gel: 1.5% (w/v) agarose, 1.1 mol formaldehyde l⁻¹, 20 mmol Mops l⁻¹, 5 mmol NaOAc l⁻¹, 1 mmol EDTA l⁻¹). The first replicate was stained with ethidium bromide; the stain was removed and the sample was photographed to determine the integrity of RNA and the location of 18S and 28S ribosomal RNA subunit bands. The second replicate was transferred to a Nytran[®] nylon membrane (0.45 μ m; Schleicher and Schuell, Keene, NH) by capillary blotting, and RNA was fixed to the membrane by UV crosslinking.

The effect of day on ovine cPLA₂ expression was examined by dot blot hybridization analysis (Ausebel, 1994) to accommodate large sample numbers and to reduce differences in RNA loading during capillary blotting. Total cellular RNA (20 μ g) from each ewe was denatured as described earlier. RNA was immobilized on nylon membranes using a BioDot microfiltration apparatus (BioRad, Ann Arbor, MI). RNA (5, 10 and 20 μ g) from one ewe on each day was also immobilized on nylon membrane to determine linearity of the hybridization signal.

Membranes were prehybridized for 3 h (42°C) in 25 ml hybridization solution (50% (w/v) formamide, 5 $\times$ SSC (SSC: 150 mmol NaCl l⁻¹, 15 mmol Na₃ citrate-H₂O, pH 7.0), 5 $\times$ Denhardt's (Denhardt's: 2% (w/v) BSA (fraction V), 2% (w/v) Ficoll (Type 400), 2% (w/v) polyvinylpyrrolidone), 1% (w/v) SDS, 100 μ g salmon sperm DNA ml⁻¹). Membranes were hybridized for 16 h (42°C) in 25 ml hybridization solution containing cPLA₂ DNA probes with a specific activity of 1.2×10^9 c.p.m. μ g⁻¹. Hybridized membranes were washed twice in 2 $\times$ SSC, 1% (w/v) SDS for 5 min at room temperature, and twice with 0.2 $\times$ SSC, 1% (w/v) SDS for 15 min at 42°C. Blots were exposed to Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY) at -80°C. Blots were exposed to film for 24, 48, 72 and 96 h at -80°C, and the relative signal intensity was determined by densitometry (Bio Image Products, Ann

Arbor, MI). The ovine cPLA₂ transcript size was estimated by its relative mobility compared with the 18S (approximately 1900 bp) and 28S (approximately 4700 bp) ribosomal RNA subunits.

Western blotting

Endometrial tissues (0.5 g) were homogenized in 1.5 ml RIPA buffer (PBS + 1% (v/v) NP40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 μ g phenylmethylsulphonyl fluoride ml⁻¹ and 3.0% (v/v) Aprotinin) with a Teflon coated dounce on ice. Homogenates were incubated on ice for 20 min and centrifuged at 12000 g for 20 min at 4°C. Supernatants were collected and protein concentrations were determined using the Bradford protein assay according to the manufacturer's instructions (Sigma, St Louis, MO). Homogenates (50 μ g) were solubilized in sample buffer (5 $\times$: 0.31 mol Tris l⁻¹, pH 6.8, 2.5% (w/v) SDS, 50% (v/v) glycerol and 0.125% (w/v) bromophenol blue) and were separated on a 10% (w/v) polyacrylamide gel at 50 mA of constant current and subsequently transferred to polyvinylidene difluoride membrane at 50 V for 2 h. Membranes were blotted for the presence of immunoreactive cPLA₂ with a mouse IgG raised against an N-terminal peptide of the human cPLA₂ (Santa Cruz Biotech, Santa Cruz, CA) according to the manufacturer's protocol. Membranes were washed and visualized using enhanced chemiluminescence.

PGF_{2 α} synthesis

Explants ($n = 4$ per treatment) were subjected to an incubation protocol consisting of four consecutive periods of 90, 60, 5 and 30 min (Silvia and Homanics, 1988) to assess the synthesis and secretion of PGF_{2 α} . All incubations were carried out in a 37°C shaking waterbath. In periods 1–3, explants were incubated in KHB. During period 4, explants were incubated in KHB and one of four treatments: (i) control medium (KHB); (ii) oxytocin (10^{-7} mol l⁻¹); (iii) melittin (10^{-5} mol l⁻¹); or (iv) arachidonic acid (10^{-5} mol l⁻¹). Doses of melittin and arachidonic acid were based upon *in vitro* dose responses observed in experiments with steroid treated ewes (Graf *et al.*, 1998) and are the minimal doses that elicited an increase in PGF_{2 α} secretion. At the end of period 4, media were collected, diluted 1:10 in PBS and stored at -20°C until assayed for the content of PGF_{2 α} . The concentration of PGF_{2 α} in the media was determined by radioimmunoassay. Samples were run in four assays, and inter- and intra-assay coefficients of variation were 21% and 12%, respectively.

Statistical analysis

Least squares ANOVA was used to evaluate data with respect to variation due to day, treatment, and the interaction of treatment and day (treatment $\times$ day) using the General Linear Models procedure (SAS, 1985). Owing to the nesting of individual ewes within each day examined, the residual

Sheep	WNETFEFILD	PNQENVLEIT	LMDANYVMDE	TLGTTTFPIS	SMKVGEKKQV
Human	-----	-----	-----	---A---TV-	-----E-
Mouse	-----	-----	-----	---A---PV-	-----E-
Sheep	PFIFNQVTEM	ILEMSLEVCS	SPDLRFSMAL	CDQEKAFRQQ	RKENIKENMK
Human	-----	V-----	C-----	---T---	---H-R-S---
Mouse	-----	-----	C-----	---T---	-----
Sheep	KLLGPKNSEG	LHSTRDVPVV	AILGSGGGFR	AMVGFSGVMK	ALYDSGILDC
Human	-----	---A-----	-----	-----	---E-----
Mouse	-----K---	-Y-----	-----	-----	---E-----
Sheep	ATYIAGLSGS	TWYMSTLYSH	PDFPEKGPEE	INEELMKNVS	HNPLLLLTPO
Human	---V-----	-----	-----	-----	-----
Mouse	-----	-----	-----	-----	-----
Sheep	KIKRYVESLW	RKKSSGQPVT	FTDIFGMLIG	ETLIHNRMNT	TLSSLKEKVN
Human	-V-----	K-----	-----	-----	-----
Mouse	-V-----	K-----	-----	---Q---SM	-----
Sheep	TGQCPLPLFT	CLHVKPDVSE	LMFADWVE		
Human	-A-----	-----	-----		
Mouse	AAR-----	-----	-----		

Fig. 1. Inferred amino acid sequence from the 834 bp fragment of the ovine cytosolic phospholipase A₂ (cPLA₂) cDNA. The relative identities to both human and mouse cPLA₂ are 94%. Only divergent amino acids are shown for human and mouse sequences.

mean square for ewe nested within day was used to test the main effect of day on PGF_{2α} secretion and cPLA₂ expression. The main effect of treatment on PGF_{2α} secretion and the treatment × day interaction were tested using the residual mean square for treatment × ewe nested within day.

Results

Partial cloning and RNA analysis

Degenerate primers directed against cPLA₂ from several species were used to amplify putative ovine cPLA₂ to obtain a partial clone of the ovine cPLA₂ mRNA. Amplification of cDNAs by PCR generated four products (not shown). The major band was near the predicted length of approximately 834 bp. The nucleotide sequence shared 89.2 and 87.8% homology with human and mouse cPLA₂ mRNA, respectively (Clark *et al.*, 1991). The inferred amino acid sequence shared 94% identity with these two species (Fig. 1).

When the 834 bp fragment of ovine cPLA₂ cDNA was used as a probe, a single 3.4 ± 0.1 kb transcript was identified from endometrial total RNA on all days examined (Fig. 2b). Overexposure of membranes to film (14 days) did not reveal any additional transcripts. Ethidium bromide staining of the duplicate gel indicated that RNA was intact and differences in signal intensity were not due to loading (Fig. 2a,b). The signal intensity of cPLA₂ mRNA on day 14 appeared to be higher compared with days 11–13 (Fig. 2b). Dot blot hybridization analysis was used to quantify differences in

ovine cPLA₂ mRNA expression during the onset of uterine secretory responsiveness to oxytocin (Table 2). ANOVA indicated that day of simulated oestrous cycle had no effect on expression of ovine cPLA₂. Ovine cPLA₂ mRNA was detectable on all days examined, and steady state concentrations remained constant from day 11 to day 14 of the simulated oestrous cycle.

Western blotting analysis

The expression of immunoreactive cPLA₂ was examined with a monoclonal IgG raised against an N-terminal peptide of the human cPLA₂. Immunoreactive ovine cPLA₂ was detected as a 110 kDa band in endometrial tissues from representative ewes on all days examined (Fig. 3). The mouse macrophage cell line (J774) expresses a cPLA₂ (Wijkander and Sundler, 1991), and lysates from these cells were included as a positive control for the detection of cPLA₂. Overexposure of blots (1 h) indicated the presence of additional immunoreactive bands (> 200 kDa and 75 kDa). However, no additional bands were observed in J774 cell lysates, indicating that these bands were not specific for cPLA₂. Densitometric analysis indicated that steady state concentrations of ovine cPLA₂ protein remained constant across all days examined (not shown). As with the mRNA, immunoreactive ovine endometrial cPLA₂ was present on days 11–14 of a simulated oestrous cycle and steady state concentrations did not increase at the onset of uterine secretory responsiveness to oxytocin.

Fig. 2. Northern blot hybridization analysis of total cellular RNA isolated from ovine endometrium on days 11–14 of a simulated oestrous cycle. Twenty micrograms total RNA was separated by electrophoresis in duplicate. (a) Ethidium bromide staining of total RNA from two ewes on each day examined. (b) A single 3.4 ± 0.1 kb transcript was detected on all days examined. Numbers represent the location of ribosomal RNA subunits.

PGF_{2 α} synthesis

Endometrial tissues were collected and examined for the synthesis of PGF_{2 α} in response to oxytocin and melittin *in vitro* to determine whether cPLA₂ activity is a limiting factor in the production of PGF_{2 α} (Fig. 4). Arachidonic acid was included as a positive control for the presence of functional prostaglandin synthase. The overall analysis of variance indicated a main effect of treatment (F 3.48; $P < 0.0001$). However, the main effect of day (F 3.16; $P = 0.9$) and the treatment $\times$ day interaction (F 9.48; $P = 0.26$) were not significant. Therefore, data were pooled across all days and differences between explants treated with control medium and each of the remaining treatments were examined by orthogonal contrasts. As expected, arachidonic acid increased synthesis of PGF_{2 α} irrespective of day ($P < 0.0001$). Oxytocin stimulated PGF_{2 α} synthesis irrespective of the day of the

Table 2. Dot blot hybridization analysis of steady state concentrations of cytosolic phospholipase A₂ mRNA in ovine endometrium

Day	Mean	Standard deviation
11	2.24	0.37
12	2.00	0.32
13	2.08	0.31
14	3.07	0.39

simulated oestrous cycle ($P < 0.05$); synthesis was greatest on day 12 (not shown). Similarly, melittin increased PGF_{2 α} synthesis irrespective of day ($P < 0.0001$), indicating that PLA₂ concentrations were sufficient to generate ample arachidonate for the synthesis of PGF_{2 α} on all days examined.

Fig. 3. Immunoblot detection of ovine cytosolic phospholipase A₂ (cPLA₂) in ovine endometrium collected on days 11–14 of a simulated oestrous cycle. Lanes 1–8: total cell lysates (50 µg) were separated by SDS-PAGE and immunoblotted with a mouse IgG raised against human cPLA₂; lane 9: 10 µg total cell lysate from J774 (mouse macrophage) cell line.

Discussion

The 834 bp fragment of the ovine cPLA₂ mRNA is highly homologous to the human and mouse sequences. While the full length cDNAs for human and mouse cPLA₂ are < 3.0 kb, northern analysis demonstrated that the transcripts migrate at 3.4 kb (Clark *et al.*, 1991; Sharp *et al.*, 1991). The observed mobility of ovine cPLA₂ mRNA is in close agreement with that observed by Zhang *et al.* (1996) using human specific probes. However, the size of the full-length ovine cPLA₂ cDNA has yet to be determined.

The phospholipase encoded by these transcripts contains a regulatory calcium-dependent lipid binding domain and a calcium-independent catalytic domain (Nalefski *et al.*, 1994; Schievella *et al.*, 1995). The first ten inferred amino acids of the ovine cPLA₂ sequence are within the calcium-dependent translocation domain of the murine cPLA₂ (Clark *et al.*, 1991). Mouse, human and ovine sequences share 100% homology within this region, indicating that calcium-dependent membrane localization is also a property of ovine cPLA₂. Calcium-dependent membrane localization is of particular interest since treatment of uterine tissues with a calcium ionophore (A23187) alone increases synthesis and secretion of PGF_{2α} (Silvia and Homanics, 1988). Oxytocin may increase intracellular calcium concentrations in the ovine endometrium. Oxytocin receptors are coupled to the G_{αq} subtype of G-proteins (Phaneuf *et al.*, 1993; Ku *et al.*, 1995) and probably increase cytosolic calcium by activation of phospholipase C and generation of inositol trisphosphate (IP₃). Oxytocin increases IP₃ in ovine uterine tissues (Flint *et al.*, 1986; Silvia and Homanics, 1988), presumably leading to an increase in intracellular calcium.

In addition to an increase in calcium, maximal activation of cPLA₂ requires phosphorylation of serine 505, a substrate for mitogen activated protein kinase (MAPK; Lin *et al.*, 1993). Oxytocin may fully activate cPLA₂ by protein kinase C, which has been shown to activate MAPK in other systems (Posada and Cooper, 1992; Seger and Krebs 1995). This appears to be true in sheep by virtue of the enhanced ability of A23187 to stimulate PGF_{2α} synthesis in the presence of phorbol 12-myristate 13-acetate (Silvia and Homanics, 1988). Conversely, the role of protein kinase C in PGF_{2α} synthesis may be dependent upon the isoform(s) of the enzyme in uterine tissues. Protein kinase Cα modulates cPLA₂ activity

Fig. 4. Rate of release of PGF_{2α} by ovine caruncular endometrial tissue incubated *in vitro* in the presence of control medium, oxytocin (10⁻⁷ mol l⁻¹), melittin (10⁻⁵ mol l⁻¹), or arachidonic acid (10⁻⁵ mol l⁻¹). Tissue was collected from ovariectomized ewes on days 11, 12, 13 and 14 of a simulated oestrous cycle (*n* = 5 per day). Bars represent anti-log values of the least squares means of logarithmically transformed data. Lines above bars represent anti-log values of standard errors of the least squares means (**P* < 0.05; ***P* < 0.0001).

independently of MAPK (Xing *et al.*, 1997). The roles of protein kinase C and MAPK on cPLA₂ activation have not been examined in the ovine endometrium.

The uterus develops the capacity to synthesize PGF_{2α} in response to oxytocin between days 12 and 13 *in vivo*, and between days 11 and 12 *in vitro* in a simulated oestrous cycle (Graf *et al.*, 1998). In the present study, the response to melittin was consistent on all days examined, indicating that ample cPLA₂ is present in advance of a change in uterine responsiveness to oxytocin *in vivo* and *in vitro*. This is supported by the finding that cPLA₂ mRNA and protein expression did not change between days 11 and 14 of the simulated oestrous cycle. Therefore, regulation of cPLA₂ expression does not appear to be a limiting factor in the development of the luteolytic signal in sheep. It is possible that changes in cPLA₂ expression during the periluteolytic period occur only in the luminal epithelium, as was observed for the oxytocin receptor (Ayad *et al.*, 1991a,b). However, whether or not such changes do occur, they appear to be inconsequential since PLA₂ activity is consistent before the development of oxytocin responsiveness in the ovine uterine endometrium.

The authors wish to thank Susan Hays and Doug Yelton for their extensive efforts in this experiment. This research was supported in part by grants from the Kentucky Artificial Breeders Association (KABA/Select Sires) and the Kentucky Agricultural Experiment Station. It is published with the approval of the director of the Kentucky Agricultural Experiment Station (publication number 98-07-91).

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