Oxytocin receptor binding activity in cultured ovine endometrium

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Oxytocin receptor binding activity in explants of caruncular and intercaruncular endometrium collected from luteal phase ewes increased during culture. An initial rise in binding activity occurred during the first 24 h of culture in both tissues; binding activity in intercaruncular endometrium continued to increase until day 6, remained unchanged on day 8 and had decreased by day 10 of culture. The maximum concentration of receptors in caruncular endometrium was significantly lower than that in intercaruncular endometrium ($P < 0.001$) and did not change significantly between days 4 and 10 of culture. Apparent dissociation constants and maximal binding of oxytocin receptor in cultured caruncular and intercaruncular endometrium were 3.09 and 2.72 nmol l^{-1} and 249 and $459 \text{ fmol } [^3\text{H}]\text{oxytocin bound mg}^{-1} \text{ protein}$, respectively. Concentrations of oxytocin receptor remained constant in myometrium during 96 h of culture. The rise in endometrial oxytocin receptor concentration did not result from exposure to fetal calf serum, phenol red or insulin in the culture medium. Substituting fetal calf serum with sheep serum or BSA did not block the rise in receptor binding activity. Actinomycin D and cycloheximide inhibited the rise in receptor concentration in both tissues. Co-culture of lung or kidney with endometrium had no effect on binding activity, whereas co-culture with luteal tissue effectively reduced the rise in oxytocin receptor concentration. To establish whether synthesis of functional oxytocin receptors occurred during culture, the effect of oxytocin on prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) production was assessed in fresh tissue and after 48 h in culture. No $\text{PGF}_{2\alpha}$ was secreted in response to oxytocin on the day of tissue collection, but after 48 h oxytocin-induced $\text{PGF}_{2\alpha}$ production occurred in intercaruncular endometrium and to a lesser extent in caruncular endometrium. Indomethacin reduced basal and oxytocin-induced $\text{PGF}_{2\alpha}$ production in both cultured tissues. These data show that *de novo* synthesis of functional oxytocin receptors occurs during culture and can be inhibited by a secretory product of the corpus luteum.

Introduction

Uterine oxytocin receptors play an important role in regulating secretion of prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) during the ovine oestrous cycle (see Flint *et al.*, 1990; Silvia *et al.*, 1991 for reviews). Oxytocin interacts with the uterine oxytocin receptor to stimulate episodes of $\text{PGF}_{2\alpha}$ secretion during luteolysis, and the ability of oxytocin to increase $\text{PGF}_{2\alpha}$ secretion has been used to indicate the presence of a functional uterine oxytocin receptor in experiments *in vivo* (Vallet *et al.*, 1990) and *in vitro* (Roberts *et al.*, 1976). Concentrations of uterine oxytocin receptor rise during luteolysis, are maximal at oestrus and then decrease rapidly to reach low values by day 4 after oestrus (Roberts *et al.*, 1976; Sheldrick and Flint, 1985), with a low level of receptor being maintained throughout the luteal phase (days 5–13) of the oestrous cycle (Sheldrick and Flint, 1985) when luteal secretion of progesterone and oxytocin is maximal (Flint and Sheldrick, 1983). Both of these hormones have been implicated in the control of oxytocin receptor synthesis *in vivo*. Oxytocin given by continuous intravenous infusion to ewes from day 12 or 13 after oestrus prevents episodic secretion of $\text{PGF}_{2\alpha}$ and the increase in

uterine oxytocin receptor concentration associated with luteolysis and consequently delays luteal regression (Flint and Sheldrick, 1985; Sheldrick and Flint, 1990). However, this apparent downregulation of the oxytocin receptor by oxytocin can be reversed by administration of cloprostenol, a luteolytic $\text{PGF}_{2\alpha}$ analogue. Cloprostenol-induced luteolysis in ewes infused with oxytocin is associated with decreasing concentrations of progesterone in plasma and raised concentrations of uterine oxytocin receptor, similar to those seen in untreated ewes during luteolysis (Flint and Sheldrick, 1985). When plasma progesterone concentrations are maintained, by administration of exogenous progesterone in oxytocin-treated ewes corpora lutea regress in response to cloprostenol but oxytocin receptor concentrations remain low (Sheldrick, 1992). Thus progesterone appears to be a requirement for low uterine oxytocin receptor concentrations and reduced secretion of $\text{PGF}_{2\alpha}$.

In experiments designed to investigate control of oxytocin receptor synthesis *in vitro*, receptor synthesis in explants of rat uterus have been shown to be influenced by progesterone and oestradiol (Soloff *et al.*, 1983). However, as oxytocin has not been detected in the rat ovary (Clements and Funder, 1986), the mechanism by which oxytocin receptor production is controlled in the rat may be different from that in the ewe where

oxytocin produced by the ovary appears to play an important role in controlling oxytocin receptor synthesis and thereby regulating the duration of the oestrous cycle. Preliminary experiments have shown that a spontaneous increase in oxytocin receptor binding activity occurs when ovine endometrium, collected during the luteal phase of the oestrous cycle, is maintained in organ culture (Sheldrick, 1990; Sheldrick, 1991). The experiments described here were designed to investigate whether *de novo* synthesis of functional oxytocin receptors occurs during culture and, if it does, whether it results from stimulation by a hormonal factor in the culture medium or is independent of hormonal stimulation possibly resulting from disinhibition: that is, whether removal of the reproductive tract from the ewe releases the uterus from the influence of a circulating inhibitor of receptor synthesis. Identification of such a substance would further our knowledge of the factors involved in regulating the length of the ovine oestrous cycle.

Materials and Methods

Animals

Reproductive tracts from 17 parous Clun Forest ewes were collected aseptically on eight separate days of the luteal phase of the oestrous cycle (day 5, $n = 2$; day 6, $n = 3$; day 7, $n = 2$; day 9, $n = 3$; day 10, $n = 3$; day 11, $n = 2$; day 12, $n = 1$; day 13, $n = 1$; day of oestrus = day 0), following administration of pentobarbitone sodium (Lethobarb: Duphar Veterinary Ltd, Southampton). Tissue from each ewe was cultured as a separate experiment. In co-culture experiments lung, kidney and corpora lutea were collected aseptically for co-culture with endometrium obtained from the same ewe. None of the ewes used in these experiments was given exogenous hormone treatment.

Tissue culture

The medium used in these experiments was Trowell T8 (Trowell, 1959) prepared in this laboratory with 2 mmol L-glutamine l^{-1} , 100 iu penicillin ml^{-1} , 100 μg streptomycin sulfate ml^{-1} and fetal calf serum (5% v/v; all purchased from Gibco, Paisley). This medium is referred to as 'control medium' in experiments where alterations to this formula are made. Trowell T8 contains 10 mg phenol red l^{-1} and 10 μmol bovine insulin l^{-1} (both Sigma Chemical Company, Poole, Dorset).

Aseptic procedures were used to dissect myometrium and endometrium from the uterus and caruncular and intercaruncular endometrium were separated into Petri dishes containing culture medium. The tissues were chopped into 1 mm cubes using a McIlwain tissue chopper (Mickle Laboratory Engineering Ltd, Guildford, Surrey). The explants were washed with medium before being placed onto sterile lens cleaning tissue (105: Whatman International Ltd, Maidstone) supported by expanded stainless steel grids (20 mm $\times$ 15 mm $\times$ 3 mm high). Three grids, representing 150–250 mg tissue, were placed into each Petri dish (6 cm diameter 1007: Falcon, Becton Dickinson, Plymouth). The volume of medium (approximately 9 ml per dish) was sufficient to bathe the lens tissue, but not submerge the explants (Trowell, 1959). Explants were cultured

for up to 10 days at 37°C in a humidified atmosphere of 95% air:5% CO_2 . Medium was changed daily and viability of explants assessed histologically after fixation in Bouin's solution and staining with haematoxylin–eosin. Histological examination of cultured explants confirmed that tissue viability was maintained throughout the experiments in all tissues used. All experiments were performed using three replicate dishes for each treatment for each tissue.

At the end of culture, explants were removed from the lens tissue and washed in 100 volumes of ice-cold 154 mmol NaCl l^{-1} containing 1 mmol EDTA l^{-1} . The explants were homogenized in ice-cold Tris–HCl (25 mmol l^{-1} ; pH 7.6) containing sucrose (0.25 mol l^{-1}) and EDTA (1 mmol l^{-1}), using an Ultra Turrax homogenizer (3 $\times$ 10 s). The tissue was then further homogenized using a Teflon–glass Dounce homogenizer. The homogenates were centrifuged at 2000 g for 10 min and the resulting supernatants centrifuged at 100 000 g for 1 h. Pellets were washed with Tris–HCl (25 mmol l^{-1} , pH 7.6) and then resuspended, by homogenization (Teflon–glass Dounce homogenizer), in this buffer and stored at -80°C before assay of oxytocin binding and protein concentration.

Oxytocin radioreceptor assay

Oxytocin receptor binding activity was measured by the method of Sheldrick and Flint (1985). Briefly, receptor fractions (50 μg protein) were incubated at 25°C for 15 min in 0.1 ml Tris–HCl (25 mmol l^{-1} , pH 7.6) containing 0.1% (w/v) BSA, MnCl_2 (1 mmol l^{-1}) and [$3,5\text{-}^3\text{H-Tyr}$]oxytocin (5 nmol l^{-1} , 1224 GBq mmol; NEN Research Products, Stevenage, Herts). Nonspecific binding was measured in the presence of 10 μmol unlabelled oxytocin l^{-1} (Bachem UK Ltd, Saffron Walden, Essex). After incubation, tubes were transferred to an ice-bath and 2 ml ice-cold Tris–HCl (25 mmol l^{-1} , pH 7.6) containing BSA (0.1% w/v) was added to each tube. Separation of free oxytocin from receptor-bound oxytocin was by filtration under vacuum using glass microfibre filters (GF/F; Whatman International Ltd, Maidstone) in a sampling manifold (No. 1225; Millipore UK Ltd). Inter- and intra-assay coefficients of variation were 12.7% and 8.0%, respectively. The limit of sensitivity of the assay (calculated as 2 $\times$ SD above nonspecific binding) was 7.8 fmol [^3H]oxytocin bound mg^{-1} protein.

Protein assay

Protein was measured by the method of Lowry *et al.* (1951). Volumes of reagents were reduced so that the assay could be carried out in 96-well microtitre plates (No. 611 F96; Sterilin, Hounslow) which were read at 720 nm (Argus 400 Microplate Reader: Canberra Packard, Pangbourne).

Radioimmunoassay for $\text{PGF}_{2\alpha}$

Triplicate samples of medium (25 μl) diluted 1:25 with assay buffer (0.1 mol Tricine l^{-1} , pH 8.0, containing 154 mmol sodium chloride l^{-1} , 0.1% sodium azide and 0.1% gelatine) were assayed with rabbit anti- $\text{PGF}_{2\alpha}$ –BSA (100 μl ; PG002: Steranti Research Ltd, London Road, St Albans, Herts) and [$5,6,8,9,11,12,14,15(\text{n})\text{-}^3\text{H}$] $\text{PGF}_{2\alpha}$ (25 μl containing 8000 d.p.m.;

7.51 TBq mmol; Amersham International plc, Aylesbury, Buckinghamshire). Assays were incubated for 20 h at 4°C. Separation of free from antibody-bound labelled $\text{PGF}_{2\alpha}$ was achieved by addition of 1 ml 1% activated charcoal (Sigma Chemical Company; previously sieved through 0.0053 mm mesh) in assay buffer containing 0.1% dextran (T-70; Pharmacia LKB, Uppsala), followed by centrifugation at 1000 g for 20 min. Supernatants were decanted into polyethylene vials (Canberra Packard) containing scintillation fluid (5 ml Lumac Aqua Luma Plus, Rhone Poulenc Laboratory Products, Eccles, Manchester) and counted on a liquid scintillation analyser (Tricarb 1900 CA; Canberra Packard) fitted with a radioimmunoassay software package (Securia 2200). Sensitivity of the assay was 16.8 fmol per tube and intra- and interassay coefficients of variation were 5.6% and 7.1%, respectively.

Effect of culture on endometrial and myometrial oxytocin receptor concentrations

Caruncular endometrium and intercaruncular endometrium from 14 ewes (range 5–13 days after oestrus) and myometrium from four ewes (day 5, $n = 2$; day 10, $n = 1$; day 13, $n = 1$) were cultured for up to 96 h. Concentrations of oxytocin receptor were measured at the time of tissue collection and after 24, 48, 72 and 96 h in culture. In a second experiment, caruncular and intercaruncular endometrium from one ewe on each of days 9 and 10 after oestrus were cultured for a maximum of 10 days. Receptor measurements were made at the time of tissue collection and after 2, 4, 6, 8 and 10 days in culture.

Receptor affinity and maximal binding were measured in pooled membrane fractions prepared from caruncular endometrium ($n = 6$ dishes) and intercaruncular endometrium ($n = 6$ dishes), collected from a ewe on day 9 of the cycle and cultured for 96 h. Binding of [^3H]oxytocin (seven concentrations ranging from 0.312 to 10.0 nmol l^{-1}) was analysed using the method of Scatchard (1949).

Effect of different sources of protein in the culture medium on concentrations of receptor in endometrial explants

Caruncular and intercaruncular endometrium from two ewes were cultured in triplicate dishes with (a) control medium (containing 5% v/v fetal calf serum); (b) medium containing charcoal-treated fetal calf serum (5% v/v) prepared by incubating serum with activated charcoal (8% w/v), at room temperature for 2 h followed by 4 h at 4°C; the charcoal was then removed by centrifugation and filtration and the serum sterilized by filtration; (c) ovariectomized sheep serum (5% v/v); (d) ovariectomized sheep serum that had been charcoal-treated (5% v/v); and (e) BSA (0.1% w/v; Sigma Chemical Company). Receptor concentrations were measured at the time of tissue collection and after 96 h of culture.

Effect of fetal calf serum, phenol red and insulin on endometrial receptor concentrations

Explants of caruncular and intercaruncular endometrium collected from three ewes were cultured in control medium,

medium without fetal calf serum or medium without insulin. Because serum proteins and insulin are required to ensure long-term viability of tissue *in vitro* these cultures were terminated after 24 h. Receptor measurements were made at the time of tissue collection and after 24 h in culture. Explants were cultured in medium without phenol red for up to 96 h. Oxytocin receptors were measured at the time of tissue collection and after 24, 48, 72 and 96 h in culture.

Effect of actinomycin D and cycloheximide on receptor concentrations

Explants of caruncular and intercaruncular endometrium from three ewes were cultured in medium containing actinomycin D (80 nmol l^{-1} ; Boehringer Mannheim, Germany) an RNA synthesis inhibitor or cycloheximide (3.5 $\mu\text{mol l}^{-1}$; Sigma Chemical Company), a protein synthesis inhibitor. Receptor concentrations were measured at the time of tissue collection and after 96 h in culture.

Co-culture experiment

Explants of caruncular and intercaruncular endometrium collected from three ewes were cultured on two grids in each culture dish with either one empty grid or with explants of lung, kidney or corpus luteum on the third grid (about 50 mg per grid). Receptor concentrations were measured at the time of tissue collection and after 48 or 96 h in culture.

Effect of oxytocin on $\text{PGF}_{2\alpha}$ production by endometrial explants

Caruncular and intercaruncular endometrium from five ewes were used in experiments designed to investigate the ability of oxytocin to stimulate secretion of $\text{PGF}_{2\alpha}$ in freshly collected endometrium and cultured explants. Explants were cultured in 3.5 cm dishes (Falcon 1008) containing 4.5 ml medium with one stainless steel grid (23 mm $\times$ 20 mm $\times$ 4 mm) per dish. Endometrium was prepared as for the other experiments and then the explants were blotted on sterile filter paper and 100 mg tissue weighed onto each grid. Each measurement was made using triplicate dishes. Explants of endometrium from two ewes, both day 10 of the cycle, were challenged with oxytocin on the day of tissue collection: explants were incubated in control medium for 30 min at 37°C; aliquots of medium (2 $\times$ 0.3 ml) were removed from each culture dish for $\text{PGF}_{2\alpha}$ estimation at a time designated as 0 min and immediately replaced with either the same volume of control medium or medium containing 2.25 nmol oxytocin l^{-1} (Bachem UK Ltd, Saffron Walden, Essex), to give a final concentration of 0.5 $\mu\text{mol l}^{-1}$. Aliquots of medium were collected after incubation for a further 60 min ($t = 1$ h). Medium was immediately frozen on dry-ice and stored at -80°C until required for assay of $\text{PGF}_{2\alpha}$. Endometrium collected from ewes on days 6, 9 and 10 of the cycle was cultured for 48 h before exposure to oxytocin. Medium was changed after 24 h and again after a further 24 h when six dishes of each tissue from each of the three ewes received control medium and six dishes received medium containing the $\text{PGF}_{2\alpha}$ synthesis inhibitor indomethacin (11.2 $\mu\text{mol l}^{-1}$; Sigma Chemical Company). After a preincubation

Fig. 1. Concentrations of oxytocin receptor in ovine caruncular endometrium (◆), intercaruncular endometrium (●) and myometrium (■) at time of tissue collection and after 24, 48, 72 or 96 h of culture (mean $\pm$ SEM).

period of 30 min, aliquots of medium (2×0.3 ml) were removed for determination of $\text{PGF}_{2\alpha}$ concentration and replaced with either control medium, medium containing indomethacin, medium containing oxytocin or oxytocin and indomethacin so that the concentration of indomethacin was maintained during the experiment. Aliquots of medium were collected after a further 1 h and stored as described above.

The concentration of $\text{PGF}_{2\alpha}$ in a culture dish before addition of oxytocin or control medium at 0 min was subtracted from the concentration of $\text{PGF}_{2\alpha}$ present in the medium at 1 h. Results are expressed as ng $\text{PGF}_{2\alpha}$ secreted per 100 mg tissue h^{-1} .

Statistical analysis

All values are reported as means $\pm$ SEM where n is the number of animals from which tissue was obtained. Statistical differences were determined by Student's t test.

Results

Effect of culture on oxytocin receptor concentration in caruncular and intercaruncular endometrium and myometrium

The initial concentrations of oxytocin receptor were 10.4 ± 1.8 fmol [^3H]oxytocin bound mg^{-1} protein (range 1.1–21.9; $n = 14$ ewes) and 9.6 ± 2.9 fmol [^3H]oxytocin bound mg^{-1} protein (range 0.6–29.4; $n = 14$) in caruncular and intercaruncular endometrium, respectively (Fig. 1). The rate of increase in endometrial receptor concentration was similar at

each stage of the oestrous cycle examined. Concentrations of oxytocin receptor in myometrium were 55.5 ± 10.0 fmol [^3H]oxytocin bound mg^{-1} protein at the time of tissue collection (range 27.1–70.6; $n = 4$) and remained unchanged throughout the 96 h culture. Mean values for receptor binding after 0, 2, 4, 6, 8 and 10 days in culture were 8.5, 28.9, 58.4, 67.5, 72.3 and 76.7 fmol [^3H]oxytocin bound mg^{-1} protein for caruncular endometrium and 5.5, 69.7, 305.2, 411.9, 404.3 and 164.5 fmol mg^{-1} protein for intercaruncular endometrium. Scatchard plots of binding parameters were linear for caruncular and intercaruncular endometrium cultured for 96 h. Apparent dissociation constants and maximal binding were 3.09 and 2.72 nmol l^{-1} and 249 and 459 fmol mg^{-1} protein in caruncular and intercaruncular endometrium, respectively.

Effects of different sources of serum protein on receptor binding activity

In medium containing fetal calf serum, binding activity in caruncular and intercaruncular endometrium increased from mean concentrations of 16.6 and 18.8 fmol [^3H]oxytocin bound mg^{-1} protein at the time of tissue collection to 106.2 and 333.3 fmol mg^{-1} protein after 96 h in culture. When cultured in medium containing charcoal-treated fetal calf serum, ovariectomized sheep serum, charcoal-treated sheep serum and BSA, binding activities of explants from one ewe were not significantly different from the control at 96 h. In the second ewe binding activities were similar to the control except in explants of intercaruncular endometrium cultured with ovariectomized sheep serum (189.9 ± 37.9 fmol mg^{-1} protein) and in caruncular and intercaruncular endometrium cultured with BSA (45.6 ± 1.3 and 253.3 ± 10.2 fmol mg^{-1} protein, respectively) where binding activities were significantly reduced ($P < 0.05$).

Effects of fetal calf serum, phenol red and insulin on concentrations of endometrial receptor binding activity

No inhibition of receptor production was evident after 24 h of culture in the absence of fetal calf serum or insulin (Table 1) and no significant differences were detected between oxytocin receptor concentrations in explants cultured in control medium and those cultured for up to 96 h in medium that did not contain phenol red (Table 2).

Effects of actinomycin D and cycloheximide on receptor binding activity

Both actinomycin D and cycloheximide reduced receptor binding activity in caruncular and intercaruncular endometrium (Table 3). Viability of the explants cultured with cycloheximide or actinomycin was confirmed by the observation that basal secretion of $\text{PGF}_{2\alpha}$ by caruncular endometrium cultured with actinomycin or cycloheximide and intercaruncular endometrium cultured with actinomycin occurs at a rate similar to that in tissue cultured in control medium. Cycloheximide reduces $\text{PGF}_{2\alpha}$ secretion in intercaruncular endometrium in this culture system (data not shown).

Table 1. Effect of fetal calf serum (5% v/v) and insulin ($10 \mu\text{mol l}^{-1}$) on ovine oxytocin receptor binding activity (fmol [^3H]oxytocin bound mg^{-1} protein) after 24 h in culture

Endometrium	+ Fetal calf serum	– Fetal calf serum	+ Insulin	– Insulin
Caruncular	94.7 ± 4.5	103.3 ± 10.1	109.7 ± 15.7	92.7 ± 10.0
Intercaruncular	52.4 ± 3.1	83.1 ± 27.2	53.3 ± 6.1	56.8 ± 9.1

All values are means $\pm$ SEM ($n = 3$ ewes).

Mean oxytocin receptor concentrations at time of tissue collection were 10.6 ± 2.8 fmol [^3H]oxytocin bound mg^{-1} protein in caruncular endometrium and 2.5 ± 1.0 fmol mg^{-1} protein in intercaruncular endometrium.

Table 2. Effect of phenol red on ovine oxytocin receptor concentration (fmol [^3H]oxytocin bound mg^{-1} protein) in explants of caruncular and intercaruncular endometrium cultured for 24, 48, 72 or 96 h

Endometrium	Phenol red	Incubation time (h)				
		0	24	48	72	96
Caruncular	+	12.7 ± 2.0	135.4 ± 26.0	110.9 ± 21.5	217.4 ± 68.4	218.0 ± 18.8
	–		110.5 ± 27.9	71.2 ± 3.6	187.1 ± 16.6	217.7 ± 13.9
Intercaruncular	+	13.8 ± 2.4	63.7 ± 7.8	64.5 ± 12.9	192.5 ± 35.0	325.9 ± 51.0
	–		43.5 ± 2.0	94.4 ± 16.6	164.0 ± 18.1	354.1 ± 52.1

Values are means $\pm$ SEM ($n = 3$ ewes).

Table 3. Oxytocin receptor binding activity (fmol [^3H]oxytocin bound mg^{-1} protein) in ovine caruncular and intercaruncular endometrium cultured for 96 h with actinomycin or cycloheximide

Treatment	Caruncular endometrium	Intercaruncular endometrium
Control	170.4 ± 65.3	565.9 ± 172.6
Actinomycin D (80 nmol l^{-1})	15.5 ± 5.2	25.6 ± 4.9
Cycloheximide ($3.5 \mu\text{mol l}^{-1}$)	61.8 ± 19.7	29.6 ± 10.6

All values are means $\pm$ SEM ($n = 3$ ewes, triplicate cultures for each ewe).

Oxytocin receptor binding activities at time of tissue collection in caruncular and intercaruncular endometrium were 3.0 ± 1.5 and 4.0 ± 1.3 fmol [^3H]oxytocin bound mg^{-1} protein, respectively.

Co-culture experiment

Co-culture with kidney and lung did not affect the concentration of receptor binding activity in explants of endometrium, but luteal tissue reduced binding activity in both intercaruncular and caruncular endometrium (Fig. 2). This effect was greater at 48 h than at 96 h, possibly because the luteal tissue was becoming depleted of the inhibitor.

Effect of oxytocin on $\text{PGF}_{2\alpha}$ production by fresh and cultured endometrium

No detectable $\text{PGF}_{2\alpha}$ was produced in response to oxytocin by tissue challenged on the day of tissue collection (day 10 of

the cycle). Effects of oxytocin on $\text{PGF}_{2\alpha}$ production by caruncular endometrium previously cultured for 48 h were variable with a significant response occurring only in tissue collected on day 10 of the cycle (Table 4). However, oxytocin significantly increased $\text{PGF}_{2\alpha}$ production by all cultured intercaruncular endometria. Indomethacin reduced mean ($\pm$ SEM) basal $\text{PGF}_{2\alpha}$ production by caruncular endometrium from 44.3 ± 18.2 to $9.1 \pm 7.9 \text{ ng (100 mg)}^{-1} \text{ h}^{-1}$ and oxytocin-induced $\text{PGF}_{2\alpha}$ production from 79.5 ± 35.9 to $3.3 \pm 3.3 \text{ ng (100 mg)}^{-1} \text{ h}^{-1}$. Basal production of $\text{PGF}_{2\alpha}$ in intercaruncular endometrium was reduced from 134.5 ± 26.8 to $66.5 \pm 39.6 \text{ ng (100 mg)}^{-1} \text{ h}^{-1}$ by indomethacin and oxytocin-induced $\text{PGF}_{2\alpha}$ production was reduced from 401.7 ± 74.7 to $68.5 \pm 18.2 \text{ ng (100 mg)}^{-1} \text{ h}^{-1}$. These data indicate that low concentrations of presynthesized $\text{PGF}_{2\alpha}$ are present in the tissues but *de novo* synthesis is required for oxytocin stimulated $\text{PGF}_{2\alpha}$ secretion to occur.

Discussion

Concentrations of oxytocin receptor binding activity have previously been shown to increase in explants of ovine endometrium during culture (Sheldrick, 1990; Sheldrick, 1991). The experiments described here were designed to investigate the mechanism by which the apparent increase in receptor concentration occurs. Furthermore, we wished to establish whether the receptors synthesized are functional and, if so, what this tells us about control of oxytocin receptor synthesis in the cyclic ewe.

Oxytocin receptor binding increased in both caruncular and intercaruncular endometrium during culture with maximal binding being greater in intercaruncular than in caruncular

Fig. 2. Concentrations of oxytocin receptor in (a) ovine caruncular endometrium and (b) intercaruncular endometrium after culture for 48 or 96 h in control medium (C) or with explants of kidney (K), lung (L) or corpus luteum (CL). Results are expressed as a percentage of the level of receptor in explants cultured in control medium at 48 h or at 96 h. Concentrations of receptor in caruncular endometrium and intercaruncular endometrium at time of tissue collection (0) were 3.4 ± 1.5 and 1.2 ± 0.2 fmol [3 H]oxytocin bound mg^{-1} protein, respectively. All values are means $\pm$ SEM.

Table 4. Effect of oxytocin ($0.5 \mu\text{mol l}^{-1}$) on $\text{PGF}_{2\alpha}$ secretion ($\text{ng (100 mg)}^{-1} \text{h}^{-1}$) by ovine endometrium previously cultured for 48 h. Values represent the total amount of $\text{PGF}_{2\alpha}$ secreted during 1 h following addition of either control medium or medium containing oxytocin

Number of ewes	Day of tissue collection	Control	Oxytocin
Caruncular endometrium			
3	6	30.4 ± 2.7	33.9 ± 5.3
15	9	80.5 ± 26.2	146.9 ± 29.2
14	10	22.1 ± 13.0	$163.1 \pm 31.9^{**}$
Intercaruncular endometrium			
3	6	91.6 ± 57.4	$286.0 \pm 25.1^*$
15	9	128.1 ± 39.7	$541.5 \pm 5.0^{**}$
14	10	183.7 ± 15.8	$377.7 \pm 44.4^{**}$

All values are means $\pm$ SEM for three replicate dishes.

* $P < 0.05$; ** $P < 0.005$.

endometrium. Apparent dissociation constants in cultured caruncular and intercaruncular endometrium were similar to those previously reported in cyclic ewes (1.96 nmol l^{-1} and 1.87 nmol l^{-1} , respectively; Sheldrick and Flint, 1985). The rate of increase in receptor binding activity was similar irrespective of the day of the cycle on which culture was started, indicating that the rise in binding did not result from a predetermined event programmed to occur at the onset of luteolysis. Levels of binding in myometrium were higher than those in endometrium at the time of tissue collection and remained unchanged throughout 96 h of culture. The reason for the different responses by the three tissues studied here is not known but may indicate either that they respond differently to the culture conditions or that there are different populations of oxytocin receptor in the ovine uterus.

The increase in receptor binding did not appear to be the result of stimulation by hormonal factors in the medium. Oestrogen has been cited as a stimulator of oxytocin receptor synthesis *in vivo* and *in vitro* (Roberts *et al.*, 1976; Soloff *et al.*, 1983; McCracken *et al.*, 1984; Matthews *et al.*, 1991), but oestradiol has previously been shown to have no stimulatory effect on oxytocin receptor synthesis by ovine endometrium *in vitro*, and at a high concentration inhibited receptor synthesis (Sheldrick, 1990; Sheldrick and Flick-Smith, 1993). This hypothesis is supported by the experiments reported here where factors with possible oestrogenic activity were omitted from the medium without effect on oxytocin receptor binding activity. For example, phenol red has been shown to have oestrogenic activity in rat anterior pituitary cells (Hubert *et al.*, 1986), MCF-7 human breast carcinoma cells (Berthois *et al.*, 1986) and, to some extent, immature rat uterine cells (Welshons *et al.*, 1988), but in the experiments reported here there was no effect of phenol red on oxytocin receptor binding activity. Fetal calf serum is also a possible source of oestrogen. However, explants cultured for 96 h with calf serum that had been treated with charcoal to remove steroid hormones (Jones *et al.*, 1986) had receptor binding activity similar to that of explants cultured with untreated calf serum.

Receptor binding activity was generally unrelated to the nature of protein added to the culture medium. Substituting fetal calf serum with serum from ovariectomized sheep or ovariectomized sheep serum treated with charcoal did not inhibit the rise in receptor binding although the binding activity achieved in intercaruncular endometrium of one ewe cultured for 96 h with whole sheep serum was reduced, compared with the activity in tissue from the same ewe cultured with calf serum. Substituting BSA for fetal calf serum resulted in a rise in receptor binding activity, but binding at 96 h in endometrium from one ewe was significantly lower than in tissue cultured in the presence of calf serum. This result may reflect the requirement of both serum albumin and globulins for maintenance of mammalian cells in culture (Tozer and Pirt 1964). However, removing the source of serum protein for a shorter incubation (24 h) did not inhibit the rate of increase of receptor binding activity and therefore serum proteins in the medium must be excluded as a stimulus of receptor binding activity. The concentration of insulin in Trowell T8 ($10 \mu\text{mol l}^{-1}$) is considerably higher than in most other culture media but omitting insulin from the medium did not affect the increase in oxytocin receptor binding activity in both cultured tissues. We conclude from these data that the rise in receptor

binding activity during culture does not result from stimulation by constituents in the medium.

Addition of actinomycin to the medium reduced oxytocin receptor binding, indicating that the increase in binding activity during culture requires RNA synthesis and therefore results from *de novo* synthesis of either the receptor itself or of a protein required for processing or activating the receptor. Inhibition of the rise in binding by cycloheximide confirms that binding increases as a result of protein synthesis. Cycloheximide was more effective in reducing oxytocin binding activity in intercaruncular than in caruncular endometrium, which implies that the increase in receptor binding seen in these two tissues may occur in different ways, with *de novo* synthesis accounting for all increased binding in intercaruncular endometrium and a combination of *de novo* synthesis and receptor activation occurring in caruncular endometrium. Receptor activation could result from dissociation of ligand from receptor during culture giving an apparent rise in binding; however, this is unlikely as the method used to prepare tissues for receptor assay causes dissociation of oxytocin-receptor complexes and as a result total receptor is measured in the assay (Sheldrick and Flint, 1985). The action of actinomycin D in inhibiting oxytocin receptor synthesis is consistent with the data of French and Casida (1973) who found that intra-uterine administration of actinomycin D blocked both naturally occurring and oestradiol-induced luteal regression in cyclic ewes.

To investigate whether the increase in receptor binding activity represented synthesis of functional oxytocin receptors, explants were challenged with oxytocin on the day of tissue collection and after 48 h in culture. The effect of oxytocin on PGF_{2α} secretion during the luteal phase of the ovine oestrous cycle is attenuated *in vivo* (McCracken, 1980) and *in vitro* (Roberts *et al.*, 1976) and in the experiments described here no detectable PGF_{2α} was produced in response to oxytocin by endometrium collected in mid-cycle, when receptor concentrations were at basal values. However, after 48 h in culture, oxytocin stimulated PGF_{2α} production in all three samples of intercaruncular endometrium and in two of three samples of caruncular endometrium. The response of caruncular endometrium to oxytocin may be related to the day of the cycle on which the tissue was collected. In general, the degree of stimulation of PGF_{2α} production by oxytocin was greater in intercaruncular than in caruncular endometrium and this is consistent with the higher production of receptor in this tissue during culture. The fact that cultured endometrium responds to oxytocin by producing PGF_{2α} confirms that increased receptor binding results from synthesis of functional oxytocin receptors. Inhibition of oxytocin-induced PGF_{2α} production by indomethacin has been reported to occur in endometrium from ovariectomized ewes in which oxytocin receptors had been coupled to PGF_{2α} secretory pathway by steroid hormones administered *in vivo* (Raw and Silva, 1991).

The results presented here have implications for the control of oxytocin receptor synthesis during the oestrous cycle. During the luteal phase of the oestrous cycle when the corpus luteum secretes oxytocin and progesterone at maximal rates, endometrial oxytocin receptor concentrations are low and both hormones have been shown to affect concentrations of uterine oxytocin receptor (Flint and Sheldrick, 1985; Leavitt *et al.*, 1985; Vallet and Lamming, 1991; Sheldrick, 1992; Zhang *et al.*, 1992).

It can be assumed that progesterone and oxytocin were affecting oxytocin receptor synthesis at the time that the tissues were collected for use in the experiments described here. Co-culture of endometrium with explants of corpora lutea inhibited receptor synthesis *in vitro* and this result indicates that secretory products of the corpus luteum act directly on the endometrium to control receptor production. This finding is supported by the evidence that uterine explants obtained from ovariectomized ewes, and, therefore, not exposed to inhibitors of ovarian origin *in vivo*, had higher basal receptor binding activity than that seen in ewes in the mid-luteal phase of the cycle and this high level of activity remained unchanged during 96 h in culture (Sheldrick and Flick-Smith, 1993). Although progesterone or oxytocin are obvious candidates for this inhibitory role, other luteal products cannot be discounted, as recent studies on the effect of α -interferon on endometrial oxytocin receptor binding in culture suggest that any agent affecting protein kinase C activity may influence concentrations of oxytocin receptor (Abayasekara *et al.*, 1992).

References

- Abayasekara DRE, Sheldrick EL, Flick-Smith HC, Commander H and Flint APF (1992) Protein kinase C in the sheep endometrium: mechanism by which interferon inhibits oxytocin receptor expression *Journal of Reproduction and Fertility Abstract Series* **9** Abstract 65
- Berthois Y, Katzenellenbogen JA and Katzenellenbogen BS (1986) Phenol red in tissue culture media is a weak estrogen: Implications concerning the study of estrogen-responsive cells in culture *Proceedings of the National Academy of Sciences USA* **83** 2496–2500
- Clements JA and Funder JW (1986) Arginine vasopressin (AVP) and AVP-like immunoreactivity in peripheral tissues *Endocrine Reviews* **7** 449–460
- Flint APF and Sheldrick EL (1983) Evidence for a systemic role for ovarian oxytocin in luteal regression in sheep *Journal of Reproduction and Fertility* **67** 215–225
- Flint APF and Sheldrick EL (1985) Continuous infusion of oxytocin prevents induction of uterine oxytocin receptor and blocks luteal regression in cyclic ewes *Journal of Reproduction and Fertility* **75** 623–631
- Flint APF, Sheldrick EL, McCann TJ and Jones DSC (1990) Luteal oxytocin: characteristics and control of synchronous episodes of oxytocin and PGF_{2α} secretion at luteolysis in ruminants *Domestic Animal Endocrinology* **7** 111–124
- French LR and Casida LE (1973) Effect of actinomycin D on corpus luteum regression in ewes *Journal of Animal Science* **37** 1218–1221
- Hubert J-F, Vincent A and Labrie F (1986) Estrogenic activity of phenol red in rat anterior pituitary cells in culture *Biochemical and Biophysical Research Communications* **141** 885–891
- Jones A, Ratcliffe JG and Swift AD (1986) A comparison of physical methods of obtaining analyte-free serum suitable for use in radioimmunoassay of thyroxine and triiodothyronine *Communications in Laboratory Medicine* **2** 128–130
- Leavitt WW, Okulicz WC, McCracken JA, Schramm W and Robidoux WF (1985) Rapid recovery of nuclear estrogen receptor and oxytocin receptor in the ovine uterus following progesterone withdrawal *Journal of Steroid Biochemistry* **22** 687–691
- Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent *Journal of Biological Chemistry* **193** 265–275
- McCracken JA (1980) Hormone receptor control of prostaglandin F_{2α} secretion by the ovine uterus *Advances in Prostaglandin and Thromboxane Research* **8** 1329–1344
- McCracken JA, Schramm W and Okulicz WC (1984) Hormone receptor control of pulsatile secretion of PGF_{2α} from the ovine uterus during luteolysis and its abrogation in early pregnancy *Animal Reproduction Science* **7** 31–55
- Matthews EL, Ayad VJ and Wathes DC (1991) The effect of oestradiol-17 β on oxytocin receptor concentrations in the reproductive tract of the anoestrous ewe *Journal of Reproduction and Fertility Abstract Series* **8** Abstract 81

- Raw RE and Silvia WJ** (1991) Activity of phospholipase C and release of prostaglandin $F_{2\alpha}$ by endometrial tissue from ovariectomized ewes receiving progesterone and estradiol *Biology of Reproduction* **44** 404–412
- Roberts JS, McCracken JA, Gavagan JE and Soloff MS** (1976) Oxytocin-stimulated release of prostaglandin $F_{2\alpha}$ from ovine endometrium *in vitro*: correlation with estrous cycle and oxytocin-receptor binding *Endocrinology* **99** 1107–1114
- Scatchard G** (1949) The attraction of proteins for small molecules and ions *Annals of the New York Academy of Sciences* **51** 660–672
- Sheldrick EL** (1990) Effect of oestradiol-17 β on uterine oxytocin receptor synthesis *in vivo* and *in vitro* *Journal of Reproduction and Fertility Abstract Series* **5** Abstract 15
- Sheldrick EL** (1991) Oxytocin and luteal maintenance in the ewe *Journal of Reproduction and Fertility Supplement* **43** 105–107
- Sheldrick EL** (1992) Effect of continuous infusion of oxytocin on ovarian function and uterine oxytocin receptor concentrations in the cyclic ewe *Reproduction Fertility and Development* **4** 505–513
- Sheldrick EL and Flick-Smith HC** (1993) Effect of ovarian hormones on oxytocin receptor concentrations in explants of uterus from ovariectomized ewes *Journal of Reproduction and Fertility* **97** 241–245
- Sheldrick EL and Flint APF** (1985) Endocrine control of uterine oxytocin receptors in the ewe *Journal of Endocrinology* **106** 249–258
- Sheldrick EL and Flint APF** (1990) Effect of continuous infusion of oxytocin on prostaglandin $F_{2\alpha}$ secretion and luteolysis in the cyclic ewe *Reproduction Fertility and Development* **2** 89–99
- Silvia WJ, Lewis GS, McCracken JA, Thatcher WW and Wilson, Jr** (1991) Hormonal regulation of uterine secretion of prostaglandin $F_{2\alpha}$ during luteolysis in ruminants *Biology of Reproduction* **45** 655–663
- Soloff MS, Fernstrom MA, Periyasamy S, Soloff S, Baldwin S and Weider M** (1983) Regulation of oxytocin receptor concentration in rat uterine explants by estrogen and progesterone *Canadian Journal of Biochemistry and Cell Biology* **61** 625–630
- Tozer BT and Pirt SJ** (1964) Suspension culture of mammalian cells and macromolecular growth-promoting fractions of calf serum *Nature* **201** 375–378
- Trowell OA** (1959) The culture of mature organs in a synthetic medium *Experimental Cell Research* **16** 118–147
- Vallet JL and Lamming GE** (1991) Ovine conceptus secretory proteins and bovine recombinant interferon α_1 -I decrease endometrial oxytocin receptor concentrations in cyclic and progesterone-treated ovariectomized ewes *Journal of Endocrinology* **131** 475–482
- 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
- Welshons WV, Wolf MF, Murphy CS and Jordan VC** (1988) Estrogenic activity of phenol red *Molecular and Cellular Endocrinology* **57** 169–178
- Zhang J, Weston PG and Hixon JE** (1992) Role of progesterone and oestradiol in the regulation of uterine oxytocin receptors in ewes *Journal of Reproduction and Fertility* **94** 395–404