Endocrine (1995) 3, 151-158

Role of protein kinase C in the inhibitory action of trophoblast interferons on expression of the oxytocin receptor in sheep endometrium

D.R.E. Abayasekara[1,3], E.L. Sheldrick[2,4], H.C. Flick-Smith[2] & A.P.F. Flint[1,4]

[1]*Institute of Zoology, Zoological Society of London, Regent's Park, London NW1 4RY;* [2]*The Babraham Institute, Babraham, Cambridge CB2 4AT, UK*

Phospholipid/Ca^{2+}-dependent protein kinase C (PKC) and oxytocin receptor were measured in sheep endometrial explants after culture for up to 96 h. Oxytocin receptor binding and PKC activity were reduced by up to 90% in explants exposed to recombinant ovine trophoblast interferon (roIFN-τ), recombinant bovine IFN-α_1 or ovine conceptus secretory proteins (a source of IFN-τ). Inhibition occurred in both caruncular and intercaruncular endometrium taken between days 7 and 10 of the oestrous cycle and in intercaruncular (but not caruncular) endometrium on day 6. Down-regulation of PKC by continued exposure of explants to 4β-phorbol myristate acetate, or treatment with PKC inhibitors reduced both oxytocin receptor binding and PKC activity by up to 70%. Tyrosine kinase inhibitors were ineffective. Addition of oxytocin or progesterone, which reduce oxytocin receptor binding *in vivo*, also lowered oxytocin receptor binding *in vitro* in the absence of any effect on PKC. The data indicate that IFN-τ inhibits oxytocin receptor synthesis by a mechanism involving PKC inhibition, but that a non-PKC pathway also operates to control oxytocin receptor binding in non-pregnant animals. These conclusions were supported by measuring PKC activity and oxytocin receptor binding in endometrium without culture. Prolonged exposure of the endometrium to IFN-τ *in vivo* may lead to PKC down regulation by a mechanism analogous to that involved in the action of continuous activation by agonist, and this may represent one function of the prolonged secretion of IFN-τ over a 10-day period in early pregnancy.

Keywords: protein kinase C; oxytocin receptor; endometrium; interferons; sheep

Introduction

In domestic ruminants, the trophoblast of the developing blastocyst expresses genes coding for a number of characteristic Type I interferons, termed trophoblast interferons (IFN-τ; see Roberts *et al.*, 1992). These IFNs, which are structurally related to IFN-α_{II} (or IFN-ω), are secreted into the uterine lumen before the blastocyst attaches to the endometrium. Through interactions with specific receptors, they inhibit pulsatile uterine secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Fincher *et al.*, 1986; Flint *et al.*, 1991; Parkinson *et al.*, 1992). This in turn results in the blockade of corpus luteum regression (luteolysis) which would occur if the animal was not pregnant, thereby ensuring the continued luteal secretion of the progesterone required for the maintenance of pregnancy (Flint *et al.*, 1994 and references therein). This process, which has been termed the maternal recognition of pregnancy (Short, 1969) is necessary for the successful establishment of pregnancy, and its failure results in early embryonic loss.

Trophoblast IFNs exert a number of effects on their target cells, including modulation of immune functions (for instance, inhibition of lymphocyte activation and stimulation of endometrial synthesis of β_2-microglobulin; (Niwano *et al.*, 1989; Fillion *et al.*, 1991; Vallet *et al.*, 1991), changing patterns of protein synthesis (Godkin *et al.*, 1984; Stewart *et al.*, 1992), and presumably, reducing susceptibility to viral infection (Pontzer *et al.*, 1988). Generation of luteolytic episodes of $PGF_{2\alpha}$ secretion requires luteal secretion of oxytocin and the interaction of circulating oxytocin with its receptor, principally on endometrial epithelial cells (Flint *et al.*, 1990; Silvia *et al.*, 1991); a key response in terms of the antiluteolytic action of IFN-τ involves the inhibition of endometrial expression of the oxytocin receptor (Flint *et al.*, 1992; Mirando *et al.*, 1993). Northern blotting shows this effect to be mediated at the level of gene transcription (Stewart *et al.*, 1993). Trophoblast IFNs may have other, post-receptor effects which contribute to the inhibition of endometrial prostaglandin synthesis and secretion (Thatcher *et al.*, 1989; Salamonsen *et al.*, 1991), but because of the early nature of the oxytocin-oxytocin receptor interaction in the pathway leading to increased prostaglandin secretion in non-pregnant animals, the effect on receptor expression is particularly prominent.

Some effects of IFNs on certain target tissues involve activation of phospholipid/Ca^{2+}-dependent protein kinase C (PKC; Constantinescu *et al.*, 1990; Reich & Pfeffer, 1990); preliminary data obtained *in vitro* (Abayasekara *et al.*, 1992) and *in vivo* (in unilateral pregnancies, see below) suggest long-term exposure to IFN-τ reduces PKC activity in sheep endometrium. To test the hypothesis that PKC is involved in the action of IFN-τ on the endometrial oxytocin receptor, we have used endometrial explant cultures from non-pregnant (cyclic) sheep, which respond to hormones added to the culture medium with changes in oxytocin receptor expression (Sheldrick, 1990). Since in these

Correspondence: D.R.E. Abayasekara
Present address: [3]Department of Physiology, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF; [4]Department of Physiology and Environmental Science, University of Nottingham, Sutton Bonington, Loughborough, Leics LE12 5RD.
Received 16 August 1994; accepted 2 November 1994

explants oxytocin receptor binding rises spontaneously from the onset of culture, the effects of agents postulated to inhibit oxytocin receptor expression (such as IFN-τ) can be tested by addition to the culture medium. Effects of agents modulating PKC activity (such as calphostin C or the phorbol ester, 4β-phorbol myristate acetate, 4β-PMA) and those which affect endometrial oxytocin receptor binding during the oestrous cycle and in explant culture such as oxytocin and progesterone (Flint & Sheldrick, 1985; Vallet *et al.*, 1990) can also be investigated.

Results

Effects of IFN on oxytocin receptor binding and PKC activity

As reported previously (Sheldrick *et al.*, 1993), oxytocin receptor binding in explants of both caruncular and intercaruncular endometrium increased during culture (Tables 1–4). Addition of Type I IFN to the culture medium at concentrations in the range 7.5×10^4–10^9 i.u./ml (Table 1) either partially or (expt.

Table 1 Effects of Type I interferons added to culture media on oxytocin receptor binding and protein kinase C in sheep endometrial explants

Experiment	Ewe no.	Day of Cycle tissue collected	Addition	Oxytocin receptor (fmol [^{3}H]oxytocin bound/mg protein) CE	Oxytocin receptor (fmol [^{3}H]oxytocin bound/mg protein) ICE	Protein kinase C (fmol/mg protein/min) CE	Protein kinase C (fmol/mg protein/min) ICE
1	2	6	Control	56 ± 15(3)	196 ± 47(4)	250 ± 7	289 ± 9
			oCSP (10^9 i.u. ml^{-1})	75 ± 27(3)	97 ± 41(4)	275 ± 8	205 ± 9
2	5	7	Control	116 ± 4(3)	202 ± 47(3)	329 ± 8	200 ± 6
			roIFN (2.5 ± 10^5 i.u. ml^{-1})	10 ± 1(3)	36 ± 20(3)	33 ± 3	50 ± 6
3	9	9	Control	117 ± 12(4)	338 ± 25(4)	144 ± 15	323 ± 19
			rbIFN (10^6 i.u. ml.$^{-1}$)	35 ± 7(4)	210 ± 32(3)	125 ± 9	194 ± 17
4	11	9	Control	73 ± 0.3(3)	264 ± 26(3)	128 ± 8	94 ± 4
			roIFN (7.5×10^4 i.u. ml^{-1})	39 ± 7(3)	137 ± 33(3)	101 ± 3	73 ± 3
5	19	10	Control	138 ± 11(3)	516 ± 35(3)	310 ± 38	110 ± 2
			oCSP (10^9 i.u. ml^{-1})	43 ± 6(3)	319 ± 41(3)	65 ± 10	66 ± 11

Tissues were cultured for 96 h. Oxytocin receptor binding ([^{3}H]oxytocin bound/mg protein) before culture was as follows: experiment 1: CE 12, ICE 4; experiment 2: CE 20, ICE 7; experiment 3: CE 16, ICE 10; experiment 4: CE 19, ICE 9; experiment 5 CE 15, ICE 13. Results are expressed as mean ± SEM. All protein kinase C determinations were in quintuplicate and oxytocin receptor binding was measured in either three or four replicates (specific values are given within brackets as appropriate). IFN decreased oxytocin receptor binding ($P = 0.011$) and protein kinase C activity ($P = 0.011$) using Friedman repeated measures ANOVA on ranks in both cases. oCSP, ovine conceptus secretory proteins; roIFN recombinant ovine interferon; rbIFN recombinant bovine interferon. CE, caruncular endometrium; ICE, intercaruncular endometrium

Table 2 Effects of mixed protein kinase C/tyrosine kinase inhibitors on oxytocin receptor binding and protein kinase C activity in sheep endometrial explants

Experiment	Ewe no.	Day of Cycle tissue collected	Culture time (h)	Addition	Oxytocin receptor (fmol [^{3}H]oxytocin bound/mg protein) CE	Oxytocin receptor (fmol [^{3}H]oxytocin bound/mg protein) ICE	Protein kinase C (fmol/mg protein/min) CE	Protein kinase C (fmol/mg protein/min) ICE
6	5	7	96	Control	98 ± 17	107 ± 24	155 ± 3	264 ± 7
				Staurosporine (10^{-6} M)	17 ± 13	69 ± 2	105 ± 7	112 ± 4
7	9	9	96	Control	121 ± 5	149 ± 28	105 ± 5	80 ± 7
				Staurosporine (10^{-6} M)	59 ± 6	47 ± 14	55 ± 3	54 ± 7
8	7	9	96	Control	294 ± 27	547 ± 76	N.D.	N.D.
				Genistein (5×10^{-5} M)	214 ± 29	312 ± 29	N.D.	N.D.

Tissues were cultured for 96 h. Oxytocin receptor binding ([^{3}H]oxytocin bound/mg protein) before culture was as follows: experiment 8: CE 17, ICE 27; experiments 6 and 7 (ewes 5 and 9), see Table 1. N.D., not determined and other abbreviations as for Table 1. Results are means ± SEM. Oxytocin receptor binding was measured in three replicates and protein kinase C in five replicates. Staurosporine inhibited oxytocin receptor binding ($P = 0.014$) and protein kinase C activity ($P = 0.015$) in cultured endometrial tissue and genistein decreased oxytocin receptor binding ($P = 0.009$). All results from CE and ICE pooled for analysis, paired *t*-test on ln transformed data

Table 3 Effects of tyrosine kinase inhibitors on oxytocin receptor binding in cultured endometrial explants

Experiment	Ewe no.	Day of cycle tissue collected	Addition	Oxytocin receptor (fmol [^{3}H]oxytocin bound/mg protein) CE	Oxytocin receptor (fmol [^{3}H]oxytocin bound/mg protein) ICE
9	15	9	Control	347 ± 28	650 ± 55
			Tyrphostin A47 (2×10^{-5} M)	307 ± 14	630 ± 19
10	31	9	Control	336 ± 63	728 ± 97
			Methyl 2,5-dihydroxycinnamate (2.5×10^{-5} M)	324 ± 29	543 ± 39
11	4	9	Control	335 ± 29	427 ± 18
			Lavendustin A (10^{-6} M)	360 ± 56	486 ± 53

Tissues were cultured for 96 h. Abbreviations as for Table 1. Oxytocin receptor binding ([^{3}H]oxytocin bound/mg protein) before culture was as follows: experiment 9: CE 15, ICE 18; experiment 10: CE14, ICE 12; experiment 11: CE 16, ICE 4. Results are means ± SEM. All values means of six determinations

2) completely inhibited receptor binding, regardless of the source of IFN, and this effect was accompanied by decreased PKC activity in cultured tissue samples. These effects were evident ($P < 0.05$ in both cases) but of lower magnitude (mean 49% reduction in receptor, 16% in PKC) after 24 or 48 h of culture. They occurred with tissue obtained on day 6 of the cycle in intercaruncular endometrium (mean 50% reduction in receptor, 29% reduction in PKC), but were absent at this stage in caruncular endometrium; tissue obtained earlier in the cycle was not tested. After day 6 both tissues responded, and subsequent experiments were performed with tissue taken between days 7 and 10.

Effects of PKC activators and inhibitors

Addition of the protein kinase C inhibitors calphostin C or RO31-8220 to caruncular endometrium decreased oxytocin receptor binding (Figure 1). Similar effects were observed with the mixed PKC/tyrosine kinase inhibitors genistein and staurosporine (Table 2) but the pure tyrosine kinase inhibitors methyl 2,5-dihydroxycinnamate (2.5×10^{-5} M), lavendustin A (10^{-6} M) and tyrphostin A47 (2×10^{-5} M) had no effects on oxytocin receptor binding (Table 3). Separate cultures confirmed these compounds had no effect on PKC (data not shown). The protein kinase activator 4β-PMA, which down-regulates PKC following prolonged exposure, reduced oxytocin receptor binding (Figure 2 and Table 4) and PKC activity (Table 4). These effects were unrelated to time in culture (mean inhibition in caruncular and intercaruncular endometrium at 24 and 48 h, 56% and 50% for oxytocin receptor and PKC respectively (Table 4).

Effects of progesterone and oxytocin

In contrast to the effects of pharmacological activators or inhibitors, oxytocin and progesterone reduced oxytocin receptor binding in the absence of PKC inhibition (Figure 3). Inhibitory effects of oxytocin

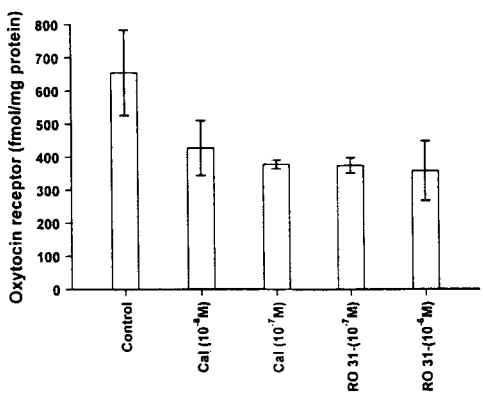

Figure 1 Effects of protein kinase C inhibitors, calphostin C (Cal) and RO 31-8220 (RO 31-) on oxytocin receptor binding in caruncular endometrium in organ culture. Inhibitors were present throughout the 96 h culture period. Cultures were initiated with tissue obtained from a sheep on day 9 after oestrus. Results are means ±SEM. Number of animals (N) = 1, Number of replicates (n) = 3

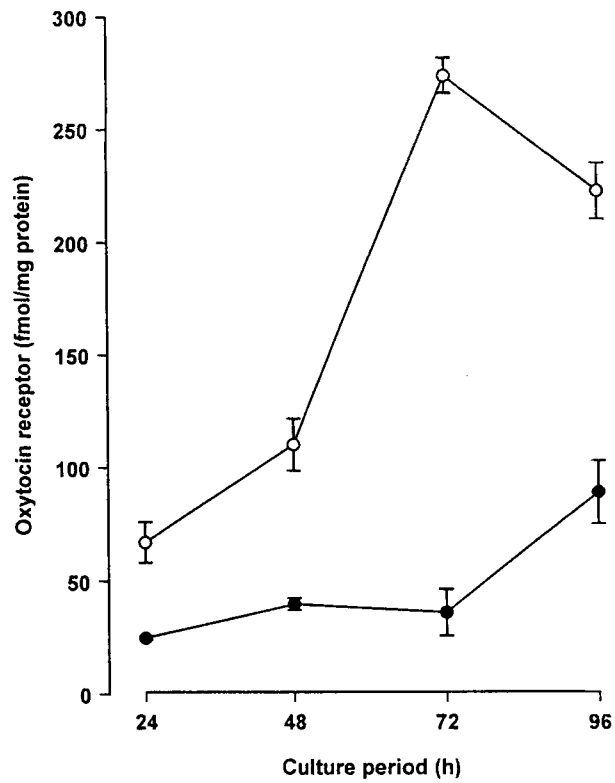

Figure 2 Effect of 4β-PMA (2×10^{-6} M) on oxytocin receptor binding in caruncular endometrium at various times in culture. Control (○); 4β-PMA (●). Cultures were initiated with tissue obtained on day 8 after oestrus. Results are means±SEM. N = 1, n = 3

Table 4 Effects of 4β-phorbol myristate acetate (PMA) on oxytocin receptor binding and protein kinase C in cultured endometrial explants

Experiment	*Ewe no.*	*Day of Cycle tissue collected*	*Culture time(h)*	*Addition*	*Oxytocin receptor (fmol [^{3}H]oxytocin bound/mg protein) CE*	*ICE*	*Protein kinase C (fmol/mg protein/min) CE*	*ICE*
12	3	8	24	Control	67 ± 9	32 ± 26	471 ± 86	399 ± 16
				4β-PMA (2×10^{-6} M)	25 ± 1	13 ± 2	221 ± 19	197 ± 9
13	3	8	48	Control	110 ± 11	42 ± 5	257 ± 40	127 ± 4
				4β-PMA (2×10^{-6} M)	40 ± 3	26 ± 6	181 ± 69	43 ± 5
14	9	9	96	Control	121 ± 5	149 ± 28	105 ± 5	80 ± 7
				4β-PMA (2×10^{-6} M)	69 ± 9	83 ± 15	43 ± 10	22 ± 2

Tissues were cultured for up to 96 h. Abbreviations as for Table 1. Oxytocin receptor binding ([^{3}H]oxytocin bound/mg protein) before culture was as follows: experiments 12 and 13: CE 58, ICE 2; experiment 14 (ewe 9) see Table 1. Results are expressed as means±SEM. Oxytocin receptor binding was measured in three replicates and protein kinase C in five replicates. 4β-PMA inhibited oxytocin receptor binding ($P = 0.001$) and protein kinase C activity ($P = 0.005$) at all time points studied (paired *t*-test on ln transformed data)

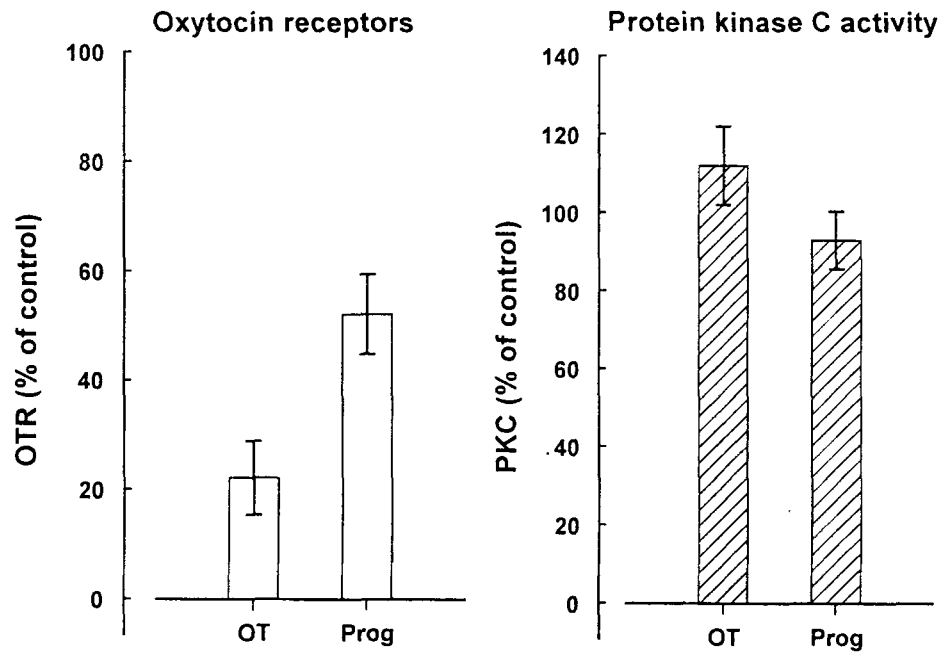

Figure 3 Effects of oxytocin (OT:10^{-8} M) and progesterone (Prog: 10^{-5} M) on oxytocin receptor binding (OTR) and protein kinase C (PKC) activity in caruncular and intercaruncular endometrium. Cultures were initiated with tissue obtained between days 7 and 10 after oestrus. Values are calculated as percent of controls for five separate experiments, where data from both caruncular and intercaruncular tissues were pooled for analysis. Results are expressed as mean ± SEM, $n = 3$, $P = 0.008$ (Friedman) for oxytocin and $P = 0.013$ (paired t test) for progesterone, on oxytocin receptor binding.

(maximum 65% reduction in receptor in caruncular endometrium after 96 h culture initiated on day 7, with similar effects in intercaruncular tissue) were observed in both endometrial tissues. Progesterone at 10^{-6} mol/l was ineffective; but at 10^{-5} mol/l progesterone inhibited oxytocin receptor binding in both tissues.

PKC activity in vivo

In non-pregnant ewes during the oestrous cycle mean PKC activities on days 8 and 10 after oestrus were 188 and 209 fmol/mg protein/min in caruncular and intercaruncular endometrium respectively. At oestrus these values were 132 and 58 fmol/mg protein/min. These data reveal a tendency for PKC activity to be reduced at oestrus; since the PKC activities in these tissues were comparable to those measured after 96 h culture, the data also confirm the viability of the tissue *in vitro*. Mean PKC activities in the pregnant and non-pregnant horns of unilaterally pregnant ewes are shown in Figure 4. PKC activity and oxytocin receptor binding were both reduced in the pregnant horns of these animals, where as expected concentrations of IFN were raised (Flint *et al.*, 1992).

Discussion

Prolonged exposure to IFN-τ decreased both PKC and oxytocin receptor binding activities in endometrial explants in organ culture. Rates of secretion of IFN-τ by the trophoblast (up to 10^7 i.u./day: Roberts *et al.*, 1992 and references therein) and concentrations of IFN-τ in the uterine lumen (normally 10^8 i.u. on day 16 of gestation, in a luminal fluid volume of less than 1 ml; A.P.F. Flint–unpublished observation) suggest that the effects observed here were obtained with physiological concentrations of IFN. PKC inhibitors (staurosporine, genistein, calphostin C and RO31-8220) also reduced oxytocin receptor binding, and these results therefore support the hypothesis that the inhibitory action of IFN on oxytocin receptor production involves inhibition of PKC. Parallel inhibitory effects on both PKC and oxytocin receptor also occurred following prolonged exposure to 4β-PMA, confirming the causative relationship.

The effects of oxytocin and progesterone on receptor binding in the absence of PKC inhibition point to the involvement of a second non-PKC pathway (or a pathway involving a PKC isoform not measured here) in regulating the oxytocin receptor. This was confirmed by the low PKC activity in ewes at oestrus, when receptor binding was raised 100–1000-fold. The existence of separate pathways is also suggested by *in vivo* data showing additive inhibitory effects of administered IFN and oxytocin on oxytocin-stimulated uterine $PGF_{2\alpha}$ secretion (Payne *et al.*, 1993). One potential non-PKC pathway involves tyrosine kinase, which phosphorylates transcription-activating proteins in HeLa cells exposed to IFN-α and thereby mediates the IFN anti-viral effect (David & Larner, 1992; Schindler *et al.*, 1992). However addition of tyrosine kinase inhibitors had no effect on oxytocin receptor binding.

The existence in mammalian tissues of ten isoforms of PKC (α, β1, βII, γ, δ, ε, η, θ, ζ, λ; Nishizuka, 1992) complicates the interpretation of the present data. These isoforms have been categorized as (i) classical or

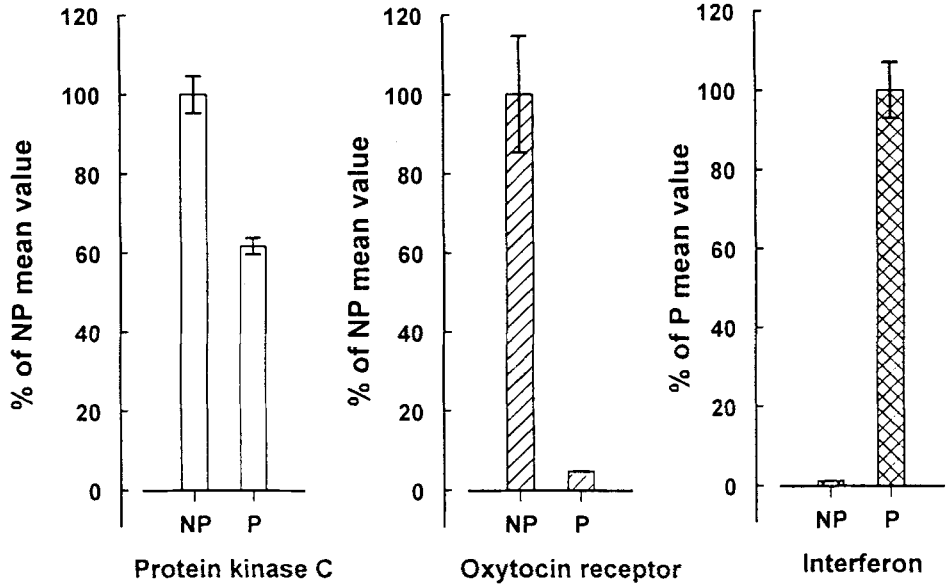

Figure 4 Protein kinase C activity and oxytocin receptor binding in endometrium, and interferon levels in uterine flushings, in the pregnant (P) and non-pregnant (NP) horns of unilaterally pregnant sheep. Data from caruncular and intercaruncular tissue were pooled for analysis. Values are expressed as percent levels in NP horns (PKC and receptor binding) or P horns (interferon levels). Results are expressed as mean ± SEM. $P < 0.001$ (Mann Whitney rank sum test) for oxytocin receptor binding ($n = 3$) and $P = 0.029$ (Mann Whitney rank sum test) for protein kinase C activity ($n = 5$), in the P horn compared to the NP horn

conventional PKCs (cPKC: α, βI, βII, γ), (ii) new PKCs (nPKC: δ, ε, η, θ) and (iii) atypical PKCs (aPKC: ζ, λ). Western blotting with specific antisera (Marais & Parker, 1989; Dekker *et al.*, 1992; Ways *et al.*, 1992) confirmed the presence of α, β and δ isoforms in caruncular and intercaruncular sheep endometrium, and showed that the γ isoform, which is thought to be only present in neural tissue (Nishizuka, 1988), is absent (D.R.E. Abayasekara, E.L. Sheldrick and A.P.F. Flint, unpublished). PKC was assayed in the present study utilising histone IIIs as the substrate; histone IIIs is a poor substrate for PKC isoforms of the nPKC and aPKC categories (Schaap *et al.*, 1989; Dekker *et al.*, 1992; Hug & Sarre, 1993) and therefore these isoforms may not have been detected.

As reported in the introduction IFNs activate PKC in a variety of different cells (see Pfeffer & Tan, 1991, and references therein; James *et al.*, 1992). In some tissues activation of PKC leads to down-regulation of cell surface receptors (Shahabi & Sharp, 1993) and the inhibition of PKC activity by IFN observed here in prolonged cultures in consistent with short-term activation followed by agonist-induced down-regulation (Akita *et al.*, 1990; Kiley *et al.*, 1990; Cook *et al.*, 1992). The connection between lowered PKC activity and decreased oxytocin receptor binding observed here is revealed by the effects of the inhibitors tested: the PKC inhibitors reduced oxytocin receptor binding, whereas the tyrosine kinase inhibitors did not. Therefore it must be concluded it is by PKC inhibition, rather than by transient activation or prolonged activation of one subcellular fraction, that IFN exerts its effect on the oxytocin receptor. The concomitant decrease in PKC and oxytocin receptors in uterine explant cultures following prolonged exposure to IFN is consistent with the results of Hu *et al.* (1992) who showed that prolonged exposure of vascular smooth muscle to noradrenaline decreased both adrenoreceptors and PKC activity. Measurement of $PGF_{2\alpha}$ produced during culture showed that the tissue remained viable for 96 h and therefore confirmed the conclusion based on histological examination. The oxytocin receptor synthesized during culture has been shown to be functional in terms of increased $PGF_{2\alpha}$ production in response to added oxytocin (Sheldrick *et al.*, 1993). It should be noted that concentrations of $PGF_{2\alpha}$ reached in the cultures (up to 10^{-5} mol/l) were high relative to levels required to activate prostaglandin receptors; it is uncertain whether $PGF_{2\alpha}$ receptors are present in the ovine endometrium, but one consequence of their activation, if present, may be activation of PKC (Wiltbank *et al.*, 1991; Abayasekara *et al.*, 1993).

It is significant that on the basis of the data presented here, the continuous exposure of the endometrium to IFN-τ which occurs over a relatively long period *in vivo* would be expected to result in decreased PKC activity. The culture periods utilised here (up to 96 h) are, in fact, short compared to the 10-day period over which IFN-τ is characteristically produced by the ruminant blastocyst. It is possible therefore that down-regulation of PKC, hitherto considered an *in vitro* artifact of PKC activation by phorbol esters, represents a physiological response to prolonged IFN secretion in early pregnancy.

Materials and methods

Bovine serum albumin (BSA, fraction V), phenylmethylsulphonyl fluoride (PMSF), Nonidet P-40, diolein, phosphatidylserine, histone type IIIs, adenosine 5′-triphosphate (ATP), ethylenediamine tetra-acetic acid (EDTA) and ethylene glycol-bis(β-aminoethyl ether) N′,N′,N′,N′-tetra-acetic acid (EGTA) were purchased from Sigma Chemical Company (Poole, Dorset, UK). Diethyl aminoethyl cellulose

(DEAE-DE52) was from Whatman Biosystems Ltd (Kent, UK), and leupeptin was from Scientific Marketing Associates (Barnet, Herts, UK). Phorbol myristate acetate (PMA: 4α and 4β stereoisomers), calphostin C, lavendustin A and B, tyrphostin A1 and A47, methyl 2,5-dihydroxycinnamate, genistein, daidzein and staurosporine were from Calbiochem (Beeston, Notts, UK). Adenosine 5'-[γ-^{32}P]triphosphate (ATP) was obtained from Amersham International, Bucks, U.K. RO31-8220 was the kind gift of Roche Products (Welwyn Garden City, Herts). All other reagents were of analytical grade and were supplied by BDH (Poole, Dorset, UK). The culture medium used in these experiments was Trowell T8 (Trowell, 1959) prepared in this laboratory (Cambridge) with 2 mmol/l L-glutamine, 100 i.u./ml penicillin, 100 μg/ml streptomycin sulphate and fetal calf serum (5% v/v; all purchased from Gibco, Paisley, UK).

Animals

Reproductive tracts from parous Clun Forest ewes kept in an open-sided barn with a raddled vasectomized ram were collected aseptically on days 6, 7, 8, 9 or 10 of the luteal phase of the oestrous cycle and from two ewes in oestrus (mean cycle length, 16.7 ± 0.2 days, $n = 37$ cycles; day of oestrus = day 0), following administration of pentobarbitone sodium (Lethobarb, Duphar Veterinary Ltd., Southampton, UK). Uteri were also obtained from two ewes on day 16 after mating in which one uterine horn had been surgically transected to cause a unilateral pregnancy (a procedure similar to that of Bazer *et al.*, 1979; see also Flint *et al.*, 1992).

Organ culture

Endometrium was dissected from the uterus using aseptic procedures and caruncular and intercaruncular endometrium 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, UK). The explants were washed with medium before being placed onto sterile lens cleaning tissue (105, Whatman International Ltd., Maidstone, UK) supported by expanded stainless steel grids (23 × 20 × 4 mm high; 150–250 mg tissue per grid) and placed into a petri-dish (3.5 cm diameter, cat. no. 1008; Falcon, Becton Dickinson, Plymouth, UK). The volume of medium (4.5 ml per dish) was sufficient to bathe the lens tissue, but not submerge the explants (Trowell, 1959). Explants were cultured for up to 96 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air. Medium was changed daily and viability of explants assessed by measuring $PGF_{2\alpha}$ in culture medium and histologically after fixation in Bouins solution and staining with haemotoxylin-eosin. Both techniques confirmed that tissue viability was maintained throughout the experiments in all tissues described here. Tissue from each ewe constituted a separate experiment and all experiments were performed using three or four replicate dishes for each treatment for each tissue. For experiments with kinase (tyrosine kinase and C-kinase) inhibitors, inactive homologues or isomers were added to control cultures where appropriate; these were 4α-PMA, tyrphostin A1, daidzein and lavendustin B.

At the end of culture, explants were removed from the lens tissue and washed in 100 volumes of ice-cold NaCl (0.9% containing EDTA, 1 mmol/l) and homogenized in Tris-HCl (25 mmol/l; pH 7.6) containing sucrose (0.25 mol/l) and EDTA (1 mmol/l), using an Ultra Turrax homogenizer (3 × 10 s). The tissue was then further homogenized using a Teflon/glass Dounce homogenizer. The homogenates were centrifuged at 2000 *g* for 10 min at 4°C and the resulting supernatants centrifuged at 100 000 *g* for 60 min at 4°C. Pellets were washed with Tris-HCl (25 mmol/l; pH 7.6) and then resuspended, by homogenization (Teflon/glass Dounce homogenizer), in this buffer and stored at − 80°C prior to assay of oxytocin receptor binding and protein concentrations.

Preparation and assay of interferons

Three preparations of IFN were used. As a source of natural trophoblast IFN (IFN-τ), ovine conceptus secretory proteins (oCSP) were obtained by culturing sheep blastocysts, after flushing from the uterine lumen on day 16 of pregnancy, in Minimal Essential Medium with Earle's salts (MEM) (Gibco Ltd., Paisley, Scotland) for 30 h (Godkin *et al.*, 1982). After removing tissue, the culture media were dialysed and concentrated (Amicon, 500 kD cut-off) up to 10-fold and sterilized by filtration. Preparations contained 10^8–10^{10} i.u./ml IFN antiviral activity and generated dose response curves (effects on oxytocin receptor and PKC) *in vitro* in the range 10^5–10^9 i.u./ml. This material represents a mixture of IFN-τ isoforms in unequal quantities; how many genes are expressed in each conceptus is unknown (Roberts *et al.*, 1992). Control medium was prepared by the same method but in the absence of blastocyst tissue.

Recombinant ovine IFN-τ (rolFN-τ) was obtained by culturing *Spodoptera frugiperda* cells infected with recombinant baculovirus, *Autographa californica* nuclear polyhedrosis virus (Matsuura *et al.*, 1987). For preparation of recombinant baculovirus a cloned ovine IFN-τ cDNA (Stewart *et al.*, 1989) was ligated into the BamHI site in the transfer plasmid pAcYM1 and recombinant virus isolated by picking colourless plaques following co-transfection of recombinant transfer plasmid and non-recombinant virus; under these conditions recombinant virus is produced by homologous recombination. The resulting virus was plaque purified three times before use. Peak IFN production occurred 96 h after transfection in cells kept at 28°C; culture media (TC 100 containing 10% fetal calf serum) contained up to 10^7 i.u./ml IFN and was used following appropriate dilution. Recombinant bovine IFN-α_1 was the kind gift of Ciba-Ceigy; control cultures contained vehicle provided by the Company.

Antiviral activity of IFN preparations was determined by cytopathic effect inhibition assay (Meager, 1987) using Madin Darby bovine kidney (MDBK) cells and Semliki Forest virus (SFV). On day 1, 2.5×10^4 MDBK cells were plated into culture grade 96-well microtitre plates (Nunc, Kamstrup, Denmark) in 150 μl culture medium containing serial dilutions of bovine recombinant IFN-α_1 (Ciba-Geigy) in the range 0–13.34 ng/ml (laboratory standard) or unknowns. On day 2, SFV was added to each well in 25 μl culture medium, and on day 3 the medium was removed from the wells by tipping the contents into 10% sodium hypochlorite. Cells adhering to the plate were rinsed with methanol before being fixed and stained with 100 μl 0.1% crystal violet in formal saline for 20 min. The plates were rinsed with tap water and air dried before the optical densities were read with an automated microtitre plate reader at 570 nm. Each concentration of unknown was assayed in duplicate. All additions were made in culture medium containing 10% fetal calf serum, 50 i.u./ml penicillin, 50 μg/ml streptomycin and 2 mmol/l L-glutamine in MEM, and cultures were at 37°C in 5% CO_2 in air. Stocks of SFV (kindly donated by Dr F.R. Balkwill, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London) were kept at − 70°C; the lowest concentration of SFV required to cause 100% cell death was determined by serial dilution and culture under assay conditions, and this concentration was subsequently used throughout. MDBK cells (the kind gift of Miss L. Griffiths, Royal Veterinary College, Camden, London) were kept in liquid N_2 when not in culture. To provide MDBK cells for transfer to multi-well plates, stocks grown to confluence in 250 ml bottles, in the culture medium described above, were rinsed in phosphate-buffered saline and removed with 0.25% trypsin (Gibco) for 2 min at 37°C in the absence of fetal calf serum.

Stock cultures were trypsinized every 7 days, and contained approximately 10^7 cells.

Concentrations of bovine recombinant IFN (laboratory standard) were standardized against the 1st International Standard (1987), containing human recombinant IFN-α_{II}, obtained from the National Institute of Biological Standards and Control, Potters Bar, Herts. Potency of the laboratory standard was 1.57 i.u./ng. In addition to standards and unknowns, multiple wells containing cells alone (no virus controls) and cells plus virus (no IFN controls) were included on each plate, and the mid-point between these values was used to calculate concentrations of IFN, from dilutions of unknowns resulting in 50% inhibition of cytopathic effect. These end points were then expressed in terms of the International Standard. When assaying sheep uterine flushings or oCSP, it was necessary to dilute unknowns between 10^3- and 10^7-fold to obtain an ED_{50}.

Extraction and partial purification of protein kinase C

Extraction and purification of protein kinase C (PKC) was carried out using a modification of the method previously described for human granulosa cells (Abayasekara *et al.*, 1993). Either freshly isolated or cultured endometrial explants were homogenized and sonicated (MSE Soniprobe, 4 × 15 s, 10 μm) in 1 ml of ice cold buffer A (20 mmol/l Tris-HCl, 2 mmol/l EDTA, 0.5 mmol/l EGTA, 50 μg/ml leupeptin, 1 mmol/l PMSF, 0.002% (v/v) Triton X-100 and 0.1% (v/v) mercaptoethanol, pH 7.5). Homogenates were centrifuged at 1000 g for 10 min at 4°C. Enzyme activity was solubilised in the resulting supernatant (600 μl) by incubation (30 min, 4°C) with 1% (v/v) Nonidet-P40. An aliquot was removed and stored at − 20°C prior to protein assay (Bradford, 1976). The solubilized supernatants were applied to DEAE-cellulose DE52 columns (600 μl, pre-equilibrated with two column volumes of 20 mmol/l Tris-HCl buffer and 2 ml of buffer A) and washed with 6 ml of buffer A. The enzyme was eluted from the columns with 600 μl buffer A supplemented with 120 mmol/l NaCl.

Protein kinase C assay

PKC activity in eluate samples was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into histone type III (Persaud *et al.*, 1989). Aliquots of the column eluates were incubated at 30°C in the presence of 11.1 mmol/l magnesium acetate, 1.3 mmol/l $CaCl_2$, 96 μg/ml phosphatidlyserine, 6.4 μg/ml diolein, 1.11 mg/ml histone and 111 μmol/l ATP, in a final volume of 45 μl. Reactions were started by the addition of [γ-^{32}P]ATP (final spec. act. 0.3 Ci/mmol), and terminated by the addition of 1 ml of ice cold 10% (v/v) trichloroacetic acid containing 10 mmol/l sodium pyrophosphate and 1 mmol/l ATP. Bovine serum albumin (50 μl; 10 mg/ml) was added as a carrier and the precipitated protein was collected onto glass microfibre filters (GF/C; Whatman International Ltd, Maidstone, UK) using a Millipore 1225 filtration manifold, then washed twice with 10 ml of 5% (v/v) trichloroacetic acid. [^{32}P] incorporation was estimated by liquid scintillation counting of the filters in Tri-carb 1900 CA, Canberra Packard, Pangbourne, UK. All samples were corrected for [^{32}P] binding to the filter in the absence of added enzyme (<0.25% of total radiolabel). Each eluate sample was assayed in quintuplicate in the absence and presence of phosphatidylserine and diolein, and the difference in [^{32}P] incorporation between these conditions was taken to reflect PKC activity in the sample.

Oxytocin receptor binding

Oxytocin receptor binding was measured by the method of Sheldrick & Flint (1985). Briefly, receptor fractions (50 μg protein) were incubated at 25°C for 15 min in 0.1 ml Tris-HCl (25 nmol/l; pH 7.6) containing 0.1% (w/v) bovine serum albumin, $MnCl_2$ (1 mmol/l) and [3,5-^{3}H-Tyr]oxytocin (5 mmol/l; 30 Ci/mmol; NEN Research Products, Stevenage, Herts, UK). Non-specific binding was measured in the presence of 10 μmol/l unlabelled oxytocin (Bachem UK Ltd, Saffron Walden, Essex, UK). After incubation, tubes were transferred to an ice-bath and 2 ml ice-cold Tris-HCl (25 mmol/l; pH 7.6) containing bovine serum albumin (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) in a filtration manifold. Membrane-bound [^{3}H]oxytocin was estimated by liquid scintillation counting (Ultima Gold, Tri-Carb 2500 TR; Canberra Packard). Inter- and intra-assay coefficients of variation were 9.8% and 10.2% respectively. The mean limit of sensitivity of the assay (calculated as 2 × SD above non-specific binding) was 10.5 fmol [^{3}H]oxytocin bound/mg protein.

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 (Sero-well No. 611 F96, Sterilin, Bibby, Stone, Staffordshire, UK) which were read at 720 nm (Argus 400 Microplate Reader, Canberra Packard). Intra- and inter-assay co-efficients of variation were 4.92% and 4.75% respectively.

Statistics

For oxytocin receptor measurements replicates represent separate culture dishes; for PKC estimation replicates were repeated assays carried out on tissue pooled from several culture dishes. The need to purify PKC before assay prevented cultures being treated separately (this approach is used routinely in these circumstances). Values for control cultures were not affected by the day of the cycle on which culture was started ($P > 0.1$, Pearson product moment correlation); therefore data were pooled with respect to day of cycle for analysis. Means are given ± SEM; N = number of animals, n = number of replicate assays or cultures.

Acknowledgements

We thank F.R. Balkwill, ICRF Laboratories, London and L. Griffiths, Royal Veterinary College, London, for assistance with the interferon assay; R.D. Possee, NERC Institute of Virology, Oxford, for help with the baculovirus system; J. Payne and G.E. Lamming for providing ovine conceptus secretory protein and tissue from unilaterally pregnant ewes; H. Commander and M.J. Searle for assistance with interferon production and assay; and Ciba-Geigy, Basle, Switzerland for gifts of bovine recombinant IFN-α_I; G.F. Lawton, Roche Products, Welwyn Garden City, Herts, for the gift of Ro 31-8220.

References

Abayasekara D.R.E., Sheldrick, E.L.R., Flick-Smith, H.C., Commander, H. & Flint, A.P.F. (1992). *J. Reprod. Fert.*, Abstr. Ser. No. 9, Abstr. 65.

Abayasekara, D.R.E., Jones, P.M., Persaud, S.J., Michael, A.E. & Flint, A.P.F. (1993). *Mol. Cell. Endocr.*, **91**, 51–57.

Akita, Y., Ohno, S., Yajima, Y. & Suzuki, K. (1990). *Biochem. Biophys. Res. Commun.*, **172**, 184–189.

Bazer, F.W., Roberts, R.M., Basha, S.M.M., Zavy, M.T., Caton, D. & Barron, D.H. (1979). *J. Anim. Sci.*, **49**, 1522–1527.

Bradford, M.M. (1976). *Anal. Biochem.*, **72**, 248–254.

Constantinescu, S.N., Cernescu, C., Balta, F., Maniu, H. & Popescu, L.M. (1990). *J. Interferon. Res.*, **10**, 589–597.

Cook, P.P., Chen, J. & Ways, D.K. (1992). *Biochem. Biophys. Res. Commun.*, **185,** 670–675.
David, M. & Larner, A.C. (1992). *Science*, **257,** 813–815.
Dekker, L.W., Parker, P.J. & McIntyre, P. (1992). *FEBS Lett.*, **312,** 195–199.
Fillion, C., Chaouat, G., Reinaud, P., Charpigny, J.C. & Martal, J. (1991). *J. Reprod. Immunol.*, **19,** 237–249.
Fincher, K.B., Bazer, F.W., Hansen, P.J., Thatcher, W.W. & Roberts, R.M. (1986). J. Reprod. Fertil., **76,** 425–433.
Flint, A.P.F. & Sheldrick, E.L. (1985). *J. Reprod. Fertil.*, **75,** 623–631.
Flint, A.P.F., Sheldrick, E.L., McCann, T.J. & Jones, D.S.C. (1990). *Dom. Ani. Endocrinol.*, **7,** 111–124.
Flint, A.P.F., Parkinson, T.J., Stewart, H.J., Vallet, J.L. & Lamming, G.E. (1991). *J. Reprod. Fert. Suppl.*, **43,** 13–25.
Flint, A.P.F., Stewart, H.J., Lamming, G.E. & Payne, J.H. (1992). *J. Reprod. Fertil. Suppl.*, **45,** 53–58.
Flint, A.P.F., Lamming, G.E., Stewart, H.J. & Abayasekara, D.R.E. (1994). *Phil. Trans. R. Soc. Lond. B.*, **344,** 291–304.
Godkin, J.D., Bazer, F.W., Moffatt, J., Sessions, F. & Roberts, R.M. (1982). *J. Reprod. Fertil.*, **65,** 141–150.
Godkin, J.D., Bazer, F.W. & Roberts, R.M. (1984). *Endocrinology*, **114,** 120–130.
Hu, Z., Azhar, S. & Hoffman, B.B. (1992). *J. Cardiovasc. Pharm.*, **20,** 982–989.
Hug, H. & Sarre, T.F. (1993). *Biochem. J.*, **291,** 329–343.
James, R.I., Menaya, J., Hudson, K., Devalia, V., Ryves, J., Evans, F.J., Thomas, S. & Clemens, M.J. (1992). *Eur. J. Biochem.*, **209,** 813–822.
Kiley, S., Schaap, D., Parker, P., Hsieh, L.L. & Jaken, S. (1990). *J. Biol. Chem.*, **265,** 15704–15712.
Lowry, O.H., Roseborough, N.J., Farr, A.L. & Randall, R.J. (1951). *J. Biol. Chem.*, **193,** 265–275.
Marais, R.M. & Parker, P.J. (1989). *Eur. J. Biochem.*, **182,** 129–137.
Matsuura, Y., Possee, R.D., Overton, H.A. & Bishop, D.H.L. (1987). *J. Gen. Virol.*, **68,** 1233–1250.
Meager, A. (1987). *Lymphokines and Interferons: a Practical Approach.* Clemens, M.J., Morris, A.G. & Gearing, A.J.H. (eds.), IRL Press: Oxford, pp. 129–147.
Mirando, M.A., Harney, J.P., Zhou, Y., Ogle, T.F., Ott, T.L., Moffat, R.J. & Bazer, F.W. (1993). *J. Mol. Endocrinol.*, **10,** 185–192.
Nishizuka, Y. (1992). *Science*, **258,** 607–614.
Nishizuka, Y. (1988). *Nature*, **334,** 661–665.
Niwano, Y., Hansen, T.R., Kazemi, M., Malathy, P.V., Johnson, H.D., Roberts, R.M. & Imakawa, K. (1989). *Am. J. Reprod. Immunol.*, **20,** 21–26.
Parkinson, T.J., Lamming, G.E., Flint, A.P.F. & Jenner, L.J. (1992). *J. Reprod. Fert.*, **94,** 489–500.
Payne, J.H., Mann, G.E. & Lamming, G.E. (1993). *J. Reprod. Fertil.*, Abst. Ser 11, Abst 57.
Persaud, S.J., Jones, P.M., Sugden, D. & Howell, S.L. (1989). *FEBS Lett.*, **245,** 80–84.
Pfeffer, L.M. & Tan, Y.H. (1991). *Trends Biochem. Sci.*, **16,** 321–323.
Pontzer, C.H., Torres, B.A., Vallet, J.L., Bazer, F.W. & Johnson, H.M. (1988). *Biochem. Biophys. Res. Commun.*, **152,** 801–807.
Reich, N.C. & Pfeffer, L.M. (1990). *Proc. Natl. Acad. Sci. USA*, **87,** 8761–8765.
Roberts, R.M., Cross, J.C. & Leaman, D.W. (1992). *Endocr. Rev.*, **13,** 432–452.
Salamonsen, L.A., Cherny, R.A. & Findlay, J.K. (1991). *J. Reprod. Fertil.*, Suppl. **43,** 27–38.
Schaap, D., Parker, P.J., Bristol, A., Kriz, R. & Knopf, J. (1989). *FEBS Lett.*, **243,** 351–357.
Schindler, C., Shuai, K., Prezioso, V.R. & Darnell, J.E. (1992). *Science*, **257,** 809–813.
Shahabi, N.A. & Sharp, B.M. (1993). *J. Pharm. Exp. Ther.*, **264,** 276–281.
Sheldrick, E.L. & Flint, A.P.F. (1985). *J. Endocrinol.*, **106,** 249–258.
Sheldrick, E.L. (1990). *J. Reprod. Fertil.*, Suppl. **43,** 105–107.
Sheldrick, E.L., Flick-Smith, H.C. & Dos Santos Cruz, G.J. (1993). *J. Reprod. Fertil.*, **98,** 521–528.
Short, R.V. (1969). *Foetal Autonomy.* Wolstenholme, G. & O'Connor, M. (eds.). Ciba Foundation, Churchill, London. pp. 2–26.
Silvia, W.J., Lewis, G.S., McCracken, J.A., Thatcher, W.W. & Wilson, L. (1991). *Biol. Reprod.*, **45,** 655–663.
Stewart, H.J., McCann, S.H.E., Northrop, A.J., Lamming, G.E. & Flint, A.P.F. (1989). *J. Mol. Endocrinol.*, **2,** 65–70.
Stewart, H.J., Guesdon, F.M.J., Payne, J.H., Charleston, B., Vallet, J.L. & Flint, A.P.F. (1992). *J. Reprod. Fertil.*, Suppl. **45,** 59–68.
Stewart, H.J., Stevenson, K.R. & Flint, A.P.F. (1993). *J. Mol. Endocrinol.*, **10,** 359–361.
Thatcher, W.W., Hansen, P.J., Gross, T.S., Helmes, S.D., Plante, C. & Bazer, F.W. (1989). *J. Reprod. Fert.*, Suppl. **37,** 91–99.
Trowell, O.A. (1959). *Exp. Cell Res.*, **16,** 118–147.
Vallet, J.L., Barker, P.J., Lamming, G.E., Skinner, N. & Huskisson, N.S. (1991). *J. Endocrinol.*, **130,** R1–R4.
Vallet, J.L., Lamming, G.E. & Batten, M. (1990). *J. Reprod. Fertil.*, **90,** 625–634.
Ways, D.K., Cook, P.P., Webster, C. & Parker, P.J. (1992). *J. Biol. Chem.*, **267,** 4799–4805.
Wiltbank, M.C., Diskin, M.G. & Niswender, G.D. (1991). *J. Reprod. Fertil.*, Suppl. **43,** 65–75.