Activin and inhibin receptor gene expression in the ewe pituitary throughout the oestrous cycle

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Abstract

In mammals, activin and inhibin are important regulators of FSH secretion. Previous studies have demonstrated that primary ovine pituitary cells express different activin receptor subtypes: activin receptor-like (ALK)2, ALK4, activin type II receptor A (ActRIIA), ActRIIB and Smad proteins in vitro. Here, we have carried out physiological studies to investigate the pattern of mRNA expression of the activin receptor subunits in the ewe pituitary throughout the oestrous cycle. The oestrous cycles of ewes were synchronized with progesterone sponges. The animals were killed 36 h (before the preovulatory surge, n=4), 48 h (during the preovulatory surge, n=4), 72 h (during the second surge of FSH, n=6) and 192 h (during the luteal phase, n=4) after sponge removal. Using Northern blots, we have shown that the levels of ALK2, ALK4 and ActRIIB mRNA were significantly higher before the preovulatory surge and during the secondary surge of FSH as compared with both during the preovulatory surge and the luteal phase, whereas the level of the ActRIIA mRNA was similar throughout the oestrous cycle. Using Western blots we have also demonstrated that the level of phospho-Smad2 did not vary during the reproductive cycle. Inhibin binding protein (InhBP/p120) and the transforming growth factor-β type III receptor, betaglycan, have been identified as putative inhibin co-receptors. In this study, we cloned a fragment of both InhBP/p120 and betaglycan cDNAs in the ewe and showed by Northern blot that pituitary betaglycan and InhBP/p120 mRNA levels did not fluctuate across the oestrous cycle nor did they correlate with serum FSH levels.


Introduction

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) synthesis and secretion in the pituitary are regulated by a complex interplay of hypothalamic, gonadal and pituitary factors. Hypothalamic production of gonadotrophin-releasing hormone (GnRH) appears to be essential to maintain serum gonadotropins and, ultimately, fertility in mammals. Activin and inhibin originally identified as gonadal peptides but also expressed in the pituitary stimulate and inhibit, respectively, FSH secretion and synthesis from cultured anterior pituitary cells in several species (Ling et al. 1986, Carroll et al. 1989, Muttukrishna & Knight 1991). The generation of mice lacking either inhibin or activin receptor type IIA (ActRIIA) further supports the possibility that inhibin and activin are important regulators of pituitary FSH levels in vivo (Matzuk et al. 1992, 1995). Mice lacking inhibin have elevated FSH levels (Matzuk et al. 1992) whereas mice lacking ActRIIA (Matzuk et al. 1995) have suppressed FSH levels. In the rat (Arai et al. 1996, Bernard & Woodruff 2001) but also in the ewe (Pant et al. 1977), during natural and synchronized reproductive cycles, only one peak of LH occurs whereas two distinct phases of increased FSH levels are observed. Thus, some changes in the expression of activin, inhibin and/or their receptors could explain the variations of FSH expression and secretion observed during the female reproductive cycle (Ackland et al. 1990).

Activin is a dimeric protein comprised of two β subunits, βA or βB, which produce three isoforms of mature activin: activin A (βAβA), activin B (βBβB) and activin AB (βAβB). The activins signal through interaction with serine/threonine kinase receptors (Ethier & Findlay 2001, Welt et al. 2002). There are two known subtypes of activin receptors: type II (A and B) and type I (A and B also called ALK2 and ALK4 respectively). Ligand binding occurs with the type II receptor, which then recruits and activates a type I receptor. Consequently, activated type I receptors phosphorylate intracellular mediators Smad2 or Smad3, which then interact with Smad4, and this complex translocates to the nucleus.
where it promotes gene expression (Wrana & Attisano 2000). Inhibin is a dimeric protein comprised of a unique α subunit and one of the two activin β subunits, to produce inhibin A (αβA) or inhibin B (αβB) (Robertson et al. 1985, Esch et al. 1987). In the rat, inhibin production is tightly regulated throughout the oestrous cycle (Meunier et al. 1988, Woodruff et al. 1988, 1989). In the ewe, after the preovulatory LH/FSH surge, inhibin A falls to a nadir coincident with the peak of the postovulatory FSH rise (Knight et al. 1998). In the ewe pituitary, the α and βB subunit mRNAs were detected by RT-PCR or immunohistochemistry and the pituitary expression of βB subunit varied during the oestrous cycle (C Taragnat, J Fontaine & F Mathoùx, personal communication).

The inhibin signalling mechanism has not been completely elucidated. However, a cell surface protein that binds inhibins with high affinity and provides a mechanism for inhibin A antagonism of activin action has been identified (Lewis et al. 2000). This protein, named betaglycan, increases inhibin affinity for the ActRII (Lewis et al. 2000). In addition, a cell adhesion molecule called inhibin binding protein/p120 (InhBP/p120), which does not bind inhibins (Chapman et al. 2002) associates in ligand-independent fashion with ALK4 (Chapman & Woodruff 2001). Moreover, in the presence of InhBP/p120, inhibin B antagonizes activin-dependent signalling (Chapman & Woodruff 2001). In the rat, the pituitary InhBP/p120 mRNA levels and the betaglycan immunoreactivity on the surface of the gonadotropes are regulated across the oestrous cycle and are negatively correlated with FSH secretion (Bernard & Woodruff 2001, Chapman & Woodruff 2003). In sheep pituitary, both betaglycan and InhBP/p120 expression patterns have not yet been identified. The present studies were designed to determine whether changes in the patterns of FSHβ gene expression and FSH secretion observed in vivo during the ewe oestrous cycle were associated with variations in expression of the different activin receptors, betaglycan and InhBP/p120, in the pituitary.

Materials and Methods

Materials

The radionucleotide [α<sup>32</sup>P]dCTP (6000 Ci/mmol) was obtained from Perkin Elmer Life Sciences (Boston, MA, USA). Taq DNA polymerase was provided by Promega (Madison, WI, USA). Avian myeloblastosis virus reverse transcriptase was purchased from Sigma Chemical Co. (St Louis, MO, USA). Antibodies against rabbit phospho-Smad2 (Ser 465/467) and rabbit Smad2 were purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Monoclonal anti-actin (clone AC) was obtained from Sigma Chemical Co. Polyclonal anti-ActRIIB was obtained from R&D Systems (Minneapolis, MN, USA).

All antibodies were used at 1/1000 dilution in Western blotting.

Animals

The oestrous cycles of 18 Ile de France ewes were synchronized by insertion of vaginal sponges impregnated with synthetic progestagen (40 mg fluorogestone acetate; Intervet, Beaucouze, France) during the breeding season (Ainsworth & Wollney 1982, Taragnat et al. 1998). Sponges remained in place for 12 days. Ewes were then killed and pituitaries collected at four time-points after removal of the sponges: 36 h, end of the follicular phase and before the gonadotrophin surge, group 1 (pre-LH, n=4); 48 h, during the preovulatory surge, group 2 (LH surge, n=4); 72 h, after the gonadotrophin surge and during the second surge of FSH, group 3 (2nd FSH surge, n=6); and 192 h, during the luteal phase, group 4 (luteal, n=4). Prior to killing, blood samples were collected from the jugular vein at 2-h intervals between 30 h and 36 h for group 1, 38 h and 48 h for group 2, 38 h and 72 h for group 3 and 38 h and 58 h for group 4. Plasma was recovered from these samples and assayed for LH, FSH and progesterone. Anterior pituitaries were cut into small pieces and stored at −80 °C until use. For cloning and gene expression studies, various Ile de France ewe tissues (including pituitaries and ovaries) and testes from Ile de France rams were collected (INRA, Nouzilly, France) after killing. Dissected organs were immediately frozen on powdered dry ice.

Gonadotrophin measurements

Concentrations of LH were measured in duplicate using the RIA method as described by Pelletier et al. (1968) and modified by Montgomery et al. (1985). The results are expressed as ng ovine LH (batch CY1051 equivalent to 2.5 NIH-LH-S1)/ml. The minimum detectable concentration of LH was 0.1 ng/ml. Mean intra- and interassay coefficients of variation (CV) were less than 8 and 10% respectively. The concentrations of plasma FSH were measured using the reagents supplied by NIADDK (Bethesda, MD, USA), and the results are expressed as ng ovine FSH (batch 19-SIAFP RP2)/ml. The minimum detectable concentration for FSH was 0.2 ng/ml. Mean intra- and interassay CV values were less than 9 and 11% respectively. The cross-reaction with ovine LH was 0.6%. The concentrations of plasma progesterone were measured by RIA as described previously (Thibier & Saumande 1985). The results are expressed as ng ovine progesterone (batch 19-SIAFP RP2)/ml. The minimum detectable concentration was 12.5 pg/ml. Mean intra- and interassay CV values were less than 9 and 10% respectively.

Immunoblotting

A piece of tissue was homogenized on ice in lysis buffer A (10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA,
1 mM EGTA, 0·5% Nonidet P40) containing various protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin) and phosphatase inhibitors (100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate). Tissue lysates were incubated on ice for 30 min and subsequently centrifuged at 12 000 g for 20 min at 4°C as previously described (Dupont et al. 2001). The protein concentration in the resulting supernatants was then determined using the BCA protein assay. Samples (50 µg proteins) were directly subjected to SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked with 5% non-fat milk in Tris-buffered saline plus 0·1% Tween-20 for 1 h at room temperature and probed with α-ActRIIB, α-phospho-Smad2, α-Smad2 or α-actin. After extensive washing, immune complexes were detected with horseradish peroxidase conjugated to specific secondary antisera (Amersham, Piscataway, NJ, USA), followed by an enhanced chemiluminescence (ECL) reaction. Densitometry was performed by scanning the radiographs and then analysing the bands with the software MacBas V2·52 (Fuji PhotoFilm, Stamford, CT, USA).

**RT-PCR**

Total RNA from tissue was extracted using TRIzol reagent according to the manufacturer’s instructions (Gibco-BRL Life Technologies, Gaithersburg, MD, USA). RT-PCR was performed to assay the expression of betaglycan, InhBP/p120 and β-actin as control described in Table 1. PCRs were carried out using 2 µl of the RT reaction mixture in a volume of 50 µl containing 10 mM Tris–HCl (pH 9·0), 50 mM KCl, 1·5 mM MgCl2, 200 mM of each deoxynucleotide triphosphate, 10 pmol of each primer and 1 U Taq polymerase. In the absence of the enzyme, no amplification product was detected after 35 cycles. All samples were amplified by PCR with specific sets of primer pairs designed to amplify parts of the betaglycan, InhBP/p120 and β-actin as control as described in Table 1. PCR products were visualized in an agarose gel (1·5%) stained with ethidium bromide. All the amplified ovine cDNA fragments were cloned using the PCR2·1 Topo cloning kit (Invitrogen, Carlsbad, CA, USA) and automatically sequenced using an ABI Prism 377 automated sequencer (Perkin-Elmer, Courtaboeuf, France). To evaluate the risk of amplification from genomic DNA potentially present in our total RNA preparations, all samples were amplified by PCR with each primer pair in the absence of reverse transcriptase. In the absence of the enzyme, no amplification product was detected after 35 cycles.

**Table 1 Oligonucleotide primer sequences used for partial cloning and sequencing of ovine InhBP/p120 and betaglycan mRNAs and for the production of probes. The product size and the GenBank accession numbers are given**

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<tr>
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**Northern blot**

Total RNAs from various tissues (20 µg) were separated by denaturing formaldehyde electrophoresis, then transferred to a nylon membrane by capillary action overnight and immobilized by exposure to u.v. light as previously described (Dupont et al. 2001, 2002). Blots were pre-hybridized for 2 h at 42°C in a buffer containing 50% formamide, 5 × Denhardt’s, 1% SDS, 5 × SSC and 100 µg/ml denatured salmon sperm. Blots were then hybridized overnight at 42°C with 2 × 10⁶ c.p.m./ml [α³²P] of a dCTP-labelled DNA probe in a buffer 106 c.p.m./ml.

106 c.p.m./ml.
containing 50% formamide, 2× Denhardt’s, 1% SDS, 5× SSC, 1% dextran sulfate and 100 μg/ml denatured salmon sperm. The probes were labelled using the Rediprime labelling kit (Amersham). The full length of ALK2, ActRIIA, ALK4 and ActRIIB cDNAs provided by J F Ethier (Ottawa, Canada) was used as probes. The probes for ovine LHβ and FSHβ, and the GnRH-R, InhBP/p120 and betaglycan were generated by RT–PCR using the primers described in Table 1. All the fragments were purified from a 1% agarose gel using a Qiaex purification kit (Qagen, Valencia, CA, USA). After high stringency washings, membrane-incorporated radioactivity was quantified using a STORM apparatus (Molecular Dynamics, Paris, France) and autoradiography was also carried at −70 °C for 48 h using Kodak X-OMAT film with intensifying screens. The mRNA expression for each transcript was normalized to the level of 18S RNA expression (Ambion, Austin, TX, USA) and the mean of the ratio of each transcript/18S RNA from each group was represented \((n=4\) for groups 1, 2 and 4, and \(n=6\) for group 3).

**Statistics**

The levels of hormone, mRNA and protein during the oestrous cycle were compared using one-way ANOVAs followed by the Newman–Keuls test. The data from three repetitions using different RNA or protein samples from different pieces of pituitary from the same animal of each group were analyzed separately and the data presented are from one representative experiment. All results are expressed as means ± s.d. Values of \(P<0.05\) were considered statistically significant.

**Results**

**Pituitary mRNA expression and plasma gonadotrophin levels during the oestrous cycle**

Plasma hormone concentrations were determined to verify the timing of the pituitary collection. Data shown in Fig. 1 confirmed that ewes were killed before (group 1, pre-LH) or during the preovulatory LH surge (group 2, LH surge), during the second FSH surge (group 3, 2nd FSH surge) and during the luteal phase (group 4, luteal). For groups 3 and 4, plasma concentrations determined between 38 h and 58 h after sponge removal demonstrated that the ewes had produced a gonadotrophin surge (Fig. 1). Plasma concentrations of progesterone in blood samples recovered at the time of killing gave further evidence for the correct timing (Fig. 1, boxed inserts). Indeed, the plasma progesterone level was significantly increased in group 4 (luteal phase) as compared with the other groups. We also determined the mRNA levels of both LHβ and FSHβ subunits by Northern blot in the pituitaries from various groups (Fig. 2). Mean LHβ subunit mRNA levels remained constant throughout the oestrous cycle (Fig. 2A) whereas mean FSHβ subunit mRNA levels varied (Fig. 2B). Mean FSHβ subunit mRNA levels were significantly decreased \((P<0.001)\) and increased \((P<0.001)\) during the ovulation phase (group 2) and 24 h postovulation (group 3) respectively, as compared with either before the preovulatory LH surge or during the luteal phase (Fig. 2B). In the luteal phase (group 4), the mean FSHβ subunit mRNA levels were significantly higher \((P<0.05)\) as compared with before the preovulatory LH surge (group 1, Fig. 2B).

**mRNA expression of GnRH-R and activin receptor subunits during the oestrous cycle**

The mRNA levels of GnRH-R and activin receptor subunits were evaluated by Northern blot in the ewe pituitary gland during the oestrous cycle. As shown in Fig. 2C, Northern blot analysis of GnRH-R mRNA in the pituitary gland revealed three major and one minor transcripts of 5·4, 3·6, 2·3 and 1·3 kb respectively (data not shown for the minor transcript). The levels of the three major transcripts were similar before (pre-LH) and during (LH surge) the preovulatory LH surge and were significantly higher that the levels observed after the preovulatory LH surge (2nd FSH surge) and during the luteal phase (luteal) respectively (Fig. 2C). The level of the 5·4 kb transcript was significantly lower during the secondary FSH surge than during the luteal phase whereas no difference was observed during these two phases for both 3·6 and 1·3 kb transcripts (Fig. 2C). For ALK2, ActRIIA, ALK4 and ActRIIB, transcripts of 4 kb, 5 kb, 4 kb and 10 kb were detected by Northern blot in the ewe pituitary gland (Fig. 3) as previously shown in the bovine corpus luteum (Jones et al. 2002). As shown in Fig. 3A, C and D, the level of ALK2, ALK4 and ActRIIB mRNA varied across the oestrous cycle whereas the level of ActRIIA was unchanged (Fig. 3B). The levels of ALK2, ALK4 and ActRIIB mRNA were significantly higher \((P<0.05)\) before and after the preovulatory LH surge as compared with either during the preovulatory LH surge or the luteal phase.

**ActRIIB and Smad2 protein level during the oestrous cycle**

Next, we determined the amount of ActRIIB protein (56 kDa) across the oestrous cycle by using Western blots. We did not determine the protein level of the other activin receptor types since we failed to reveal them by using the appropriate antibodies in vivo in the ewe pituitary. Validation of the ActRIIB for use in sheep was checked by pre-absorbing the antibody with an immunogenic peptide (data not shown). As shown in Fig. 4A, the amount of ActRIIB protein was significantly increased before and after the preovulatory LH surge as compared with those observed during the preovulatory LH surge and the luteal
phase (Fig. 4A). Thus, the variations of ActRIIB protein expression across the oestrous cycle correlated well with those observed in the expression of ActRIIB mRNA (Fig. 3D). To determine the changes of phosphorylated pSmad2 throughout the oestrous cycle, pituitary lysates from different groups of ewes were subjected to Western blot and membranes were blotted with antibodies against phospho-Smad2 and then Smad2. As shown in Fig. 4B, we did not detect changes in the level of phospho-Smad2 (determined by the ratio of phospho-Smad–2 and total Smad2) in the ewe pituitary across the oestrous cycle (Fig. 4B).

Expression of InhBP/p120 and betaglycan in sheep organs
We also determined both InhBP/p120 and betaglycan mRNA expression in various sheep tissues by either
A. LH β

B. FSH β

C. GnRH receptor

RT-PCR (Fig. 5A) or Northern blot as described in Materials and Methods (Fig. 5B). PCR products targeted for InhBP/p120 and betaglycan were obtained at the expected sizes: 668 and 329 bp respectively (Fig. 5A). The specificity of the amplified products was assessed by sequencing. Each partial sequence has been submitted to GenBank (accession numbers in Table 1). Ovine InhBP/p120 and betaglycan exhibited a high homology (90–95%) with rat InhBP/p120 and betaglycan respectively. Using both RT-PCR and Northern blot techniques, we observed a high level of InhBP/p120 expression in pituitary and testis total RNA, while no expression was detected in the other tissues. Using Northern blot, we detected two major InhBP/p120 mRNA species (about 4·4 and 1·8 kb) in sheep pituitary using a probe directed against the 5’-end of sheep cDNA. In comparison with the rat, the 4·4 kb transcript likely corresponds to the full-length InhBP/p120, while the 1·8 kb transcript may encode a truncated form of the receptor. In the testsis, two major transcripts (3·7 and 1·8 kb) and larger transcripts (>6 kb) of InhBP/p120 were detected. In contrast to InhBP/p120, betaglycan was detected in the majority of tissues as determined by RT-PCR and Northern blot. The major transcript (about 7 kb) was observed mainly in the pituitary but also in the ovary and kidney (Fig. 5B).

Expression of pituitary InhBP/p120 and betaglycan across the oestrous cycle

We next used RNA blot analyses to examine pituitary mRNA levels of InhBP/p120 and betaglycan across the ewe oestrous cycle. As shown in Fig. 6A, InhBP/p120 (long and short transcript (Fig. 6A)) and betaglycan (Fig. 6B) did not vary significantly across the ewe oestrous cycle.

Discussion

We have previously shown that ovine pituitary cells express in vitro the different activin receptors (ActRIIB, ActRIIA, ALK2, ALK4) and Smad proteins (Dupont et al. 2003). Moreover, we have demonstrated that activin was able to activate different signalling pathways including Smad2. The first objective in this study was to investigate the changes in activin receptor mRNA expression in vivo in pituitary and the potential involvement of Smad2 signalling throughout the ovine oestrous cycle. Four phases of the oestrous cycle with variations of plasma LH and FSH were studied: before the gonadotrophin surge, during the gonadotrophin surge, during the second surge of FSH and during the luteal phase. As previously shown, the level of FSHβ and GnRH-R mRNA levels varied substantially throughout the oestrous cycle in the ewe. FSHβ mRNA is strongly decreased during the preovulatory surge as compared with the other phases of the cycle (Leung et al. 1988). Our investigation, like other previous studies (Crowder & Nett 1984, Brooks et al. 1993, Turzillo et al. 1994, Padmanabhan et al. 1995) showed increased GnRH-R mRNA levels in the ewe pituitary prior to the onset of the LH surge. A rise in GnRH-R mRNA also occurs on the afternoon of pro-oestrus in rats (Bauer-Dantoin et al. 1993). In contrast, LHB mRNA does not change throughout the oestrous cycle.

Interestingly, our results showed that the levels of ActRIIB, ALK4 and ALK2 mRNA in the ewe pituitary were higher before the preovulatory peak (late follicular phase) and during the second surge of FSH than during the preovulatory peak and the luteal phase. In contrast, the levels of ActRIIA mRNA did not change during the oestrous cycle. Similarly, Halvorson et al. (1994) showed that pituitary ActRIIA mRNA levels do not change during the rat oestrous cycle; however, ActRI and ActRIIB were not examined. It is not possible to relate these results to the activin plasma levels since until now any specific activin assay has not been available for sheep. Here, we have demonstrated that the increase in the level of ActRIIB mRNA in the whole pituitary of the ewe before the gonadotrophin surge and the second surge of FSH was well correlated with an increase in the production of ActRIIB protein in these conditions. Since activin is known to increase the synthesis and secretion of pituitary FSH in various species including the ewe, we can hypothesize that if such results are observed specifically on the ovine gonadotrope cells the higher level of ActRIIB observed before and after the gonadotrophin surge could explain the maintenance of constitutive FSH release and the generation of the secondary FSH surge respectively. However, in vivo hybridization or immunohistochemistry must be performed to confirm this hypothesis. In the rat, there is some evidence that activin contributes to the generation of the secondary FSH surge, which occurs.

Figure 2  (A) LHβ, (B) FSH β and (C) GnRH-R mRNA levels across the ewe oestrous cycle. Pituitaries were collected from ewes killed at four time-points during the oestrous cycle (groups1, 2 and 4, n=4 and group 3, n=6) as described in Materials and Methods. Total RNA was then extracted from the pituitaries of each animal, and samples containing 20 μg total RNA were analyzed by Northern blotting. LHβ, FSHβ and GnRH-R probes were generated from cDNA fragments obtained by RT-PCR as described in Materials and Methods. In each case, the membrane was stripped and reprobed with 18S RNA to confirm equal loading and to quantify signal intensity. Data are normalized to the 18S level and expressed as the means ± S.D. of the ratio of LHβ, FSHβ or GnRH-R (transcripts 5·4, 3·6 and 2·3 kb) mRNA level/18S level. All Northern blotting procedures were repeated three times using different RNA samples from different pieces of pituitary from the same animal. The data presented are from one representative experiment. Bars with different letters indicate a significant difference (P<0.05) between the groups.
Figure 3 Activin receptor mRNA expression during the oestrous cycle in the ewe pituitary. Pituitaries were collected from ewes killed at four time-points during the oestrous cycle (groups 1, 2 and 4, n=4 and group 3, n=6) as described in Materials and Methods. Total RNA was then extracted from the pituitaries of each animal, and samples containing 20 μg total RNA were analyzed by Northern blotting. The full length of different activin receptor subtype cDNAs ((A) ALK2 (or ActRIIB), (B) ActRIIA, (C) ALK4 (or ActRIIB) and (D) ActRIIB) were used as probes. In each case, the membrane was stripped and reprobed with 18S RNA to confirm equal loading and to quantify signal intensity. Data are normalized to the 18S level and expressed as the means ± S.D. of the ratio of ALK2, ALK4, ActRIIA and ActRIIB receptor mRNA level/18S level. All Northern blotting procedures were repeated three times using different RNA samples from different pieces of pituitary from the same animal. The data presented are from one representative experiment. Bars with different letters indicate a significant difference (P<0.05) between the groups.
Figure 4 (A) ActRIIB protein expression and (B) Smad2 phosphorylation during the oestrous cycle in the ewe pituitary. Pituitary proteins from ewes killed at four time-points during the oestrous cycle (groups 1, 2 and 4, n = 4 and group 3, n = 6) were isolated and resolved on 10% SDS-PAGE (50 μg protein) and transferred to nitrocellulose membrane by electroblotting as described in Materials and Methods. Blots were probed with an (A) anti-actRIIB or (B) phospho-Smad2 antibody (pSmad2), and bands were revealed by ECL. As control for protein loading, the blots (probed with the anti-actRIIB) were stripped and reprobed with an actin monoclonal antibody. To determine the level of Smad2 phosphorylation, the blot probed with phospho-Smad2 was stripped and reprobed with a polyclonal Smad2 antibody (αSmad2). Band densities were quantified by scanning densitometry and expressed as the means ± s.e. of the ratio of (A) ActRIIB/actin and (B) phospho-Smad2 (pSmad2)/Smad2 protein. These experiments were repeated three times using different protein samples from different pieces of pituitary from the same animal. The data presented are from one representative experiment. Bars with different letters indicate a significant difference (P < 0.05) between the groups.
independently of LH release and initiates follicular recruitment (DePaolo et al. 1992). In good agreement with this hypothesis, in the sheep and the rat, the FSHβ subunit gene is a direct transcriptional target of activin signals (Dupont et al. 2003, Suszko et al. 2003). Moreover, in the mouse gonadotrope LβT2 cell line, using the Northern blot technique and the transient transfection of a Smad2 dominant negative construct, our group has previously shown that the Smad2 signalling pathway is partly involved in the activin-induced FSH synthesis and release (Dupont et al. 2003). Our results have very recently been confirmed by Bernard (2004), using the RNA inhibitor approach.

**A. RT-PCR**

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<td>Actin</td>
<td>329</td>
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**B. Northern-blot**

- **InhBP/p120**
  - Pituitary: 9 kb
  - Liver: 7 kb
  - Ovary: 4 kb
  - Kidney: 2 kb
  - Heart: 2 kb
  - Muscle: 2 kb
  - Intestine: 2 kb

- **Betaglycan**
  - Pituitary: 7 kb
  - Liver: 2 kb
  - Ovary: 2 kb
  - Kidney: 2 kb
  - Heart: 2 kb
  - Muscle: 2 kb
  - Intestine: 2 kb

- **18S**
  - Pituitary: 2 kb
  - Liver: 2 kb
  - Ovary: 2 kb
  - Kidney: 2 kb
  - Heart: 2 kb
  - Muscle: 2 kb
  - Intestine: 2 kb

*Figure 5* InhBP/p120 and betaglycan mRNA expression in sheep tissues. Total RNAs extracted from various ewe tissues and testes from rams were subjected to either (A) RT-PCR using primer pairs designed to amplify fragments from betaglycan and InhBP/p120 transcripts or (B) Northern blot as described in Materials and Methods. Expression of the β-actin for the RT-PCR experiment and 18S for the Northern blot were used as positive control. The expected sizes of the different amplified products by RT-PCR are indicated on the right. RT− corresponds to an RT-PCR carried out without reverse transcriptase. These experiments were repeated twice using different animals.
whereas Suszko et al. (2003), using transient transfection, have shown that Smad3 and Smad4 mediate activin-stimulated activity of the rat FSHβ promoter. In our study, we have shown that the level of phosphorylated Smad2 is unchanged in the whole pituitary across the oestrous cycle in the ewe. However, immunohistochemical studies are

Figure 6 InhBP/p120 and betaglycan mRNA expression during the oestrous cycle in the ewe pituitary. Total RNA (20 μg) was analyzed by Northern blotting. (A) InhBP/p120 and (B) betaglycan probes were generated from DNA fragments obtained by RT-PCR as described in Materials and Methods. In each case, the membrane was stripped and reprobed with 18S RNA to confirm equal loading and to quantify signal intensity. Data are normalized to the 18S level and expressed as the means ± S.D. of the ratio of InhBP/p120-L (InhBP/p120-long) or InhBP/p120-S (InhBP/p120-short) and betaglycan mRNA level/18S level. All Northern blotting procedures were repeated three times using different RNA samples from different pieces of pituitary from the same animal. The data presented are from one representative experiment.
necessary to measure the level of Smad2 phosphorylation specifically in ovine gonadotrope cells to determine whether the Smad2 signalling pathway participates in vivo in the regulation of FSH secretion and expression during the oestrous cycle in the ewe.

Surprisingly, we have shown here that ActRIIA was differently regulated from the other types of activin receptors. However, various studies have already described a differential regulation in the expression of types of activin receptors in the pituitary and different tissues. For example, in rat primary pituitary cells, activin increases ActRI and ActRIIB but does not alter ActRIIA mRNA levels (Dalkin et al. 1996). Also, in the infantile female rat pituitary, ActRIIA was not expressed in FSH-immunoreactive cells, while ActRIIB was expressed in FSH-immunoreactive cells, suggesting that ActRIIB is more likely associated with activin signalling for regulating FSH expression (Wilson & Handa 1998). In whole rat pituitary cultures, the amount of ActRIIA and ActRIIB are different. The ActRIIA expression is higher than that of ActRIIB (Dalkin et al. 1996) and, in the adult female rat, ActRIIA expression is confined to the intermediate and anterior lobes of the pituitary, while ActRIIB appears to be more diffusely expressed across all three lobes (Cameron et al. 1994). In our work, we did not study the distribution of activin receptor mRNA in the ewe pituitary by in situ hybridization. Thus, it remains possible that the distribution of activin receptors changes throughout the oestrous cycle in the ewe. ALK-2 has been initially shown to bind activin in concert with ActRIIA and IIB, but some evidence now indicates that it functions as a bone morphogenetic protein (BMP) type I receptor (Ten Dijke et al. 1994, Macias-Silva et al. 1998). Thus, our results suggest that the BMP receptors could vary in the ewe pituitary across the oestrous cycle. However, this hypothesis remains to be confirmed.

Inhibin is a dimeric protein originally isolated from porcine and bovine follicular fluid as an FSH-suppressing protein (Ling et al. 1985, Miyamoto et al. 1985, Rivier et al. 1985, Robertson et al. 1985). Thereafter, inhibin proved to be a major FSH-suppressing factor during the rat oestrous cycle (Rivier et al. 1986, Arai et al. 1996). It is generally accepted that a decline of plasma inhibin during the periovulatory period permits the secondary surge of FSH. This hypothesis is supported by an inverse relationship between plasma concentrations of inhibin and FSH during the periovulatory period (Kimura et al. 1983, Hasegawa et al. 1989, Watanabe et al. 1990, Woodruff et al. 1996). In addition, administration of an inhibin preparation or recombinant human inhibin A in the rat abolishes the rise of FSH during oestrus (Schwartz & Clamann 1977, Rivier & Vale 1991). In contrast, the blockade of GnRH does not eradicate the secondary surge of FSH in the rat. In the ewe, passive immunization against an inhibin peptide before the preovulatory period increases FSH secretion and the ovulation rate (Kusina et al. 1995). Inhibin-binding sites with high affinity and specificity have been identified on ovine pituitary cells, consistent with both inhibin action on the pituitary and the presence of a putative inhibin receptor (Hertan et al. 1999).

In the rat, the transforming growth factor-β type III receptor, also called betaglycan, binds both inhibin A and B with very high affinity and increases the affinity of inhibin for the activin type II receptors and thereby can antagonize activin signalling in brain, pituitary and gonads (Lewis et al. 2000, Esparza-Lopez et al. 2001, Chapman et al. 2002, MacConell et al. 2002). In the rat pituitary, betaglycan protein and its subcellular localization is regulated across the rat oestrous cycle. Betaglycan immunoreactivity on the surface of pituitary gonadotropes is highly correlated with high serum and pituitary inhibin levels and low FSHβ and FSH during the early part of the oestrous cycle and just after the secondary FSH surge (Chapman & Woodruff 2003). Here we have shown that betaglycan is also expressed in the ewe pituitary. Moreover, the level of betaglycan mRNA did not change throughout the oestrous cycle. Again, it remains possible that the distribution of betaglycan varies across the oestrous cycle.

A second, recently identified putative inhibin receptor, InhBP/p120, provides an alternative mechanism for the antagonism of activin by inhibin (Chong et al. 2000, Chapman & Woodruff 2001, Chapman et al. 2002). Unlike the case for betaglycan, InhBP/p120 forms a complex with the activin type I receptor. In the presence of InhBP/p120, inhibin B but not inhibin A blocks activin-stimulated gene transcription (Chapman & Woodruff 2001). In the ewe pituitary, we have cloned and sequenced a fragment of InhBP/p120 cDNA. The two isoforms of InhBP/p120 are expressed only in the pituitary and testis of sheep. Moreover, we did not detect any change in the InhBP/p120 mRNA levels over the oestrous cycle. These results are opposite to those observed in the rat pituitary. Indeed, in the rat pituitary, both isoform of InhBP/p120 are dynamically regulated across the oestrous cycle. These results are opposite to those observed in the rat pituitary. Moreover, the same authors have recently shown that mice deficient for both isoforms of InhBP/p120 are fertile and exhibit no obvious alterations in pituitary FSH synthesis or release and normal gonadal physiology and histology (Bernard et al. 2003). Thus, these results support our data obtained in the ewe indicating no change in the expression of InhBP/p120 in the pituitary throughout the oestrous cycle, suggesting that InhBP/p120 may not be important for the synthesis and release of FSH. However, here we have determined the mRNA level of InhBP/p120 in the whole pituitary gland and consequently we do not know the variation of
InhBP/p120 mRNA level specifically in the ovine gonadotrope cells. Hence it is difficult to relate our results with the synthesis and release of FSH until we perform in situ hybridization or immunohistochemistry.

In conclusion, the present study indicates that the levels of ALK2, ALK4 and ActRIIB mRNA in vivo in the ewe pituitary are increased before and after the gonadotrophin surge as compared with those observed during the gonadotrophin surge and the luteal phase. However, no change in the miRNA level of either ActRIIA or two inhibin co-receptors, betaglycan and InhBP/p120, was detected. Identification of the cells expressing the different types of activin receptors, betaglycan and InhBP/p120, as well as the cellular distribution of these components in the ewe pituitary gland across the oestrus cycle remain to be determined.

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