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Bone morphogenetic protein-4 interacts with activin and GnRH to modulate gonadotrophin secretion in LβT2 gonadotrophs

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Abstract

We have shown previously that, in sheep primary pituitary cells, bone morphogenetic proteins (BMP)-4 inhibits FSHβ mRNA expression and FSH release. In contrast, in mouse LβT2 gonadotrophs, others have shown a stimulatory effect of BMPs on basal or activin-stimulated FSHβ promoter-driven transcription. As a species comparison with our previous results, we used LβT2 cells to investigate the effects of BMP-4 on gonadotrophin mRNA and secretion modulated by activin and GnRH. BMP-4 alone had no effect on FSH production, but enhanced the activin + GnRH-induced stimulation of FSHβ mRNA and FSH secretion, without any effect on follistatin mRNA. BMP-4 reduced LHβ mRNA up-regulation in response to GnRH (±activin) and decreased GnRH receptor expression, which would favour FSH, rather than LH, synthesis and secretion. In contrast to sheep pituitary gonadotrophs, which express only BMP receptor types IA (BMPRIA) and II (BMPRII), LβT2 cells also express BMPRIB. Smad1/5 phosphorylation induced by BMP-4, indicating activation of BMP signalling, was the same whether BMP-4 was used alone or combined with activin ± GnRH. We hypothesized that activin and/or GnRH pathways may be modulated by BMP-4, but neither the activin-stimulated phosphorylation of Smad2/3 nor the GnRH-induced ERK1/2 or cAMP response element–binding phosphorylation were modified. However, the GnRH-induced activation of p38 MAPK was decreased by BMP-4. This was associated with increased FSHβ mRNA levels and FSH secretion, but decreased LHβ mRNA levels. These results confirm 1. BMPs as important modulators of activin and/or GnRH-stimulated gonadotrophin synthesis and release and 2. important species differences in these effects, which could relate to differences in BMP receptor expression in gonadotrophs.

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Introduction

The gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are produced by the gonadotroph cells of the anterior pituitary and play important roles in reproductive function. Tight control over the production of these hormones is crucial, particularly in females, to coordinate follicle selection and terminal follicular growth as well as the timing and number of ovulations. Synthesis and release of LH and FSH are regulated by hypothalamic gonadotrophin-releasing hormone (GnRH) and gonadal steroids. Despite these common regulators, divergences in the circulating patterns of LH and FSH are often encountered, suggesting that these regulators act differentially on the production of LH and FSH and/or that other factors are specifically involved in the control of FSH synthesis.

While the pulsatile pattern of GnRH release is essential for the activation and secretion of both gonadotrophins, changes in pulse frequency observed during the oestrous cycle differentially affect the LH and FSH production. High frequencies of GnRH pulses are associated with greater LH secretion whereas lower frequencies favour FSH secretion (Wildt *et al.* 1981, Dalkin *et al.* 1989, Kaiser *et al.* 1997, Molter-Gerard *et al.* 1999). In addition, the FSH secretion is controlled, independently of LH, by transforming growth factor-β (TGFβ) superfamily members such as activins and inhibins. Activin, a dimer of two highly related β-subunits (βA and/or βB), stimulates FSHβ gene expression and FSH release, whereas inhibin, a heterodimer composed of one α-subunit and one β-subunit (βA or βB), decreases FSH synthesis and release (Carroll *et al.* 1989). Activin is secreted by the gonads and within the pituitary, where it acts as an autocrine and/or paracrine regulator of FSH synthesis. Recently, other members of the TGFβ superfamily, the bone morphogenetic proteins (BMPs), were shown to modulate preferentially FSH secretion. In ovine pituitary cells, BMP-4 (50 ng/ml) and BMP-6 (100 ng/ml) inhibited FSHβ mRNA expression and FSH release (Faure *et al.* 2005).

By contrast, in the murine LβT2 gonadotroph cell line and rat pituitary cells, BMP-6 and BMP-7, at higher concentrations (1 μg/ml), BMP-2 and BMP-15 (100 ng/ml) were able to stimulate basal FSH secretion and FSHβ promoter activity (Huang *et al.* 2001, Otsuka & Shimasaki 2002, Lee *et al.* 2007). This difference of BMP effect between the mouse and the sheep could reflect a dissimilar pattern of BMP receptors on gonadotroph cells driving different signalling pathways. Alternatively, different BMPs could differentially affect FSH synthesis.

The TGFβ superfamily members act through two types of serine/threonine kinase receptors, type I and type II (for review: Miyazawa *et al.* 2002, Shimasaki *et al.* 2004). Activin signals through activin receptor types IIA (ActRIIA) or IIB (ActRIIB) and activin receptor type IB (ActRIB, also known as activin receptor-like kinase 4 or ALK-4). Specific BMP signalling requires BMP receptor type II (BMPRII) and BMP receptor types IA (BMPRIA or ALK-3) or IB (BMPRIB or ALK-6). The ligand-type II receptor complex induces phosphorylation and activation of type I receptors that in turn trans-phosphorylate receptor-activated Smad proteins within the cytoplasm. These then interact with common Smad4, and the resulting complex translocates to the nucleus to affect gene transcription. Activin leads to the specific activation of Smads 2 and 3, whereas BMPs activate Smads 1, 5 and 8. The combinations of activin and BMP ligands and receptors are not unique. For example, ActRIIA and ActRIIB can bind to BMP-6 (Ebisawa *et al.* 1999), whereas BMPRII binds specifically to BMP ligands (Liu *et al.* 1995, Nohno *et al.* 1995). Regarding type I receptors, ActRIA (also known as ALK-2) has been identified as a type-I receptor for BMPs (Macias-Silva *et al.* 1998).

To further explore BMP action at the gonadotroph cell level, we have examined the effects of BMP-4 on mouse LβT2 cell LH and FSH β-subunit mRNA levels and gonadotrophin release. Considering that activin and GnRH are important modulators of FSH synthesis, it is of interest to determine if BMP-4 interacts with these two factors and to explore the signalling pathways through which BMP-4 exerts its effect.

Materials and Methods

Cell culture

Mouse LβT2 cells (provided by Dr P Mellon, University of California, San Diego, CA, USA) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum (Hyclone, Logan, UT, USA) on flasks coated with Matrigel (1:29; BD Biosciences Clontech, Cowley, Oxford, UK). For experimental purposes LβT2 cells were set up as described previously (Nicol *et al.* 2004) at a density of $2·75 \times 10^6$/well. After overnight incubation, cells were treated with 0 or 50 ng/ml recombinant human activin A (R&D Systems, Abingdon, Oxon, UK) ±a daily 1-h pulse of

10 nM GnRH (Bachem, St Helens, Merseyside, UK), in the presence or absence of 50 ng/ml recombinant human BMP-4 (R&D Systems) for 3 days. Media were collected daily for measurement of secreted FSH and LH by RIA. On day 4, total RNA was extracted from cells for the measurement of FSHβ, LHβ, GnRH receptor (GnRH-R) and follistatin mRNA levels by quantitative RT-PCR. In addition, on day 4, after a final pulse of GnRH, protein extracts were prepared for western blot analysis.

Mouse FSH and LH RIAs

RIAs for mouse FSH and LH were performed as previously described (McNeilly *et al.* 1996), using reagents supplied by Dr A Parlow (NHPP, Harbor-UCLA, Torrance, CA, USA). The minimum detectable concentrations were 1 ng/ml and 200 pg/ml for FSH and LH respectively. The intra- and inter-assay coefficients of variation were <10% for both assays.

Qualitative RT-PCR

The expression of BMP receptor mRNA in LβT2 cells was assessed by RT-PCR, using adult mouse pituitary as a positive control. Total RNA was extracted using TRI Reagent (Sigma–Aldrich Ltd). Aliquots were treated with DNase I using DNA-free (Ambion (Europe) Ltd, Huntingdon, Cambs, UK) and reverse transcribed, using random hexamers, with Taqman Reverse Transcription reagents (PE Biosystems, Warrington, Cheshire, UK) according to manufacturers protocol. PCR was performed for 35 cycles using ThermoStart Taq DNA polymerase (Abgene, Epsom, Surrey, UK). Primer sequences were as follows: BMPRIA: fwd: CCTGTTGTTATAGGTCCGTT; rev: TCTCCT-ACTGGAATAAATGC; BMPRIB: fwd: AGATTGGAAA-AGGCCGCTATG; rev: GATGTCAACCTCATTTGTG-TC; BMPRII: fwd: GAGGACTGGCTTATCTTCAC; rev: AGCTCCTTCTAGCACTTCTG. Predicted product sizes were 194, 473 and 245 bp respectively.

Immunostaining

LβT2 cells, cultured on Labtek II chamber slides (Fisher Scientific UK, Manchester, UK) were washed with PBS, fixed in Bouin's solution for 5 min, washed and then permeabilized using 10% normal goat serum, 1% BSA in PBS (blocking solution) containing 0·2% Igepal CA-630 for 20 min at room temperature. After washing, cells were blocked for 1 h, then incubated at 4 °C overnight with rabbit polyclonal antibodies directed against BMPRIA, BMPRIB (ten Dijke *et al.* 1994) or BMPRII (Rosenzweig *et al.* 1995), diluted at 1:50. Antibodies were kindly donated by Dr C-H Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). Negative controls were performed with 1:100 goat serum in place of the primary antibody. Slides were washed and then incubated at room temperature for 1 h with goat anti-rabbit Alexa 488 (Molecular Probes, Eugene, OR, USA)

diluted 1:200. After washing, the slides were mounted using Permafluor fluorescent mounting medium (Beckman Coulter, High Wycombe, UK) and examined using a LSM 510 confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, Herts, UK).

Quantitative RT-PCR

Levels of mRNA for FSHβ, LHβ, GnRH-R and follistatin were measured using Taqman RT-PCR. Primer and probe sequences were designed using Primer Express software and synthesized by PE Biosystems or by Biosource Europe SA (Nivelles, Belgium). FSHβ, LHβ and GnRH-R primer/probe sequences have been previously reported (Nicol *et al.* 2004). The sequences for follistatin were as follows: fwd: GGGCTGGATGGGAAAACCTA; rev: CGGCTGCTC-TTTGCATCTG; probe (FAM labelled): CGCAAC-GAATGTGCACTCCTCAAGG. Ribosomal 18S primers and probe were from a Taqman Ribosomal RNA Control Reagents kit (VIC labelled probe). RNA was prepared, DNase I-treated and reverse transcribed as described above. RNA was added to RT reactions at 10 ng/μl. A PCR mix was prepared, consisting of Taqman Universal PCR Master Mix (1×), gene specific forward and reverse primers (300 nM each) and probe (200 nM), and ribosomal 18S forward and reverse primers (50 nM each) and probe (200 nM). This was aliquoted into tubes, then cDNA was added at 1 μl/25 μl reaction mix. Aliquots (25 μl) were transferred to wells in a 96-well PCR plate, with each sample run in duplicate. Plates were sealed with optical adhesive covers and the PCRs run on ABI Prism 7900HT PCR machine using standard conditions. Controls included cDNA prepared with omission of reverse transcriptase. Prior to analysis, a validation assay was performed to demonstrate that amplification of target genes and the reference (18S) were approximately equal. Quantification of specific mRNA levels was performed by the $\Delta\Delta C_t$ method (Bulletin no.2; PE Biosystems). Standard PCRs using each set of primers were run on an agarose gel to confirm amplification of a single product of the correct size. Unless otherwise stated all reagents, equipment and software were from PE Biosystems.

Western blotting

To investigate activation of Smads, ERK1/2, p38 MAPK and cAMP response element-binding protein (CREB), LβT2 cells were treated as described above except that on day 4, a final pulse of GnRH was given (duration of 1 h for Smad study and 10 or 20 min for ERK1/2, p38 and CREB studies). The cells were then washed with ice-cold PBS and lysed in buffer containing 10 mM Tris (pH 7·4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0·5% Igepal CA-630, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, protease inhibitor cocktail. The protein concentration was determined using a Bio-Rad DC protein assay (Bio-Rad Laboratories Ltd).

After 5 min at 95 °C in reducing buffer, proteins were separated on SDS-PAGE gels and electroblotted onto Immobilon membranes (Millipore Corporation, Bedford, MA, USA). For Smad and ERK analysis, membranes were blocked for 1 h at room temperature in Tris-buffered saline, 0·1% Tween (TBST) containing 5% fat-free dry milk and then incubated in primary antibody overnight at 4 °C. The P-Smad1/5 rabbit monoclonal, P-ERK1/2 and total ERK1/2 polyclonal antibodies were from Cell Signaling Technology (Danvers, MA, USA) and were all used at 1:1000 dilution. The Smad1/5 and Smad2/3 polyclonal antibodies were from Upstate Biotechnology (Lake Placid, NY, USA) and were used at 1:500 and 1:1000 dilutions respectively. The P-Smad2/3 antibody was provided by Dr C-H Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden) and used at 1:2000 dilutions. After washing in TBST, the membranes were incubated with Amersham ECL peroxidase-labelled anti-rabbit antibody (GE Healthcare UK Ltd, Little Chalfont, Bucks, UK) for 1 h at room temperature. Immunoreactive proteins were detected using Amersham ECL Plus western blotting detection reagents and exposure to Hyperfilm ECL (both from GE Healthcare UK Ltd). For quantification, the membranes were scanned on a Typhoon 9400 variable-mode imager (GE Healthcare UK Ltd). In each case, the membrane was probed first with the antibody against the phosphorylated protein, then stripped using buffer containing 2% SDS, 62·5 mM Tris (pH 6·8), 0·1 M 2-mercaptoethanol for 30 min at 75 °C. After washing, immunodetection was repeated using antibody against the total protein. For P-p38 and P-CREB analysis, membranes were blocked in Odyssey blocking buffer (Licor Biosciences UK Ltd, Cambridge, UK) and primary antibodies were diluted in Odyssey buffer diluted 1:1 with PBS, 0·1% Tween 20 (PBST). The primary antibody against the phosphorylated protein was combined with a monoclonal anti-α-tubulin to act as loading control for quantitation. The P-p38 and P-CREB antibodies (Cell Signaling Technology) were used at 1:1000 dilutions and α-tubulin monoclonal (Sigma) was used at 1:3000 dilutions. After washing in PBST the membranes were incubated with a mixture of goat anti-rabbit IR Dye 680 and goat anti-mouse IR Dye 800CW for 1 h at room temperature. Immunoreactive proteins were detected and quantified using an Odyssey Infrared Imaging System (Licor Biosciences UK Ltd).

Statistical analysis

Results are reported as mean ± s.e.m. from one representative experiment. Experiments were carried out at least three times. Analyses of interactions between BMP-4 and other treatment groups were carried out using two-way ANOVA and the Bonferroni method. Comparisons between treatments on FSH, LH concentrations, FSHβ and LHβ mRNA levels and phosphorylated protein intensities were analysed by one-way ANOVA followed by Tukey's multiple comparison test. The value of $P < 0.05$ was considered significant.

The statistical analyses were performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA).

Results

Expression of BMP receptors in LβT2 gonadotrophs and adult mouse pituitary

Qualitative RT-PCR analysis of total RNA prepared from mouse LβT2 gonadotroph cells and adult mouse pituitary was carried out using primers specific for BMPRIA (ALK-3), BMPRIB (ALK-6) and BMPRII. As shown in Fig. 1A, products of the expected size were observed for all three receptor types in both LβT2 cells and adult mouse pituitary. In negative controls, performed by omitting reverse transcriptase enzyme in RT reactions, no PCR products were detected (not shown). Particularly in LβT2 cells, the levels of BMPRIB mRNA appeared to be lower than the other two receptor types. Immunostaining using specific antibodies confirmed the presence of expressed protein for all three BMP receptor types in LβT2 cells when compared with a negative control (Fig. 1B).

Effects of BMP-4 on gonadotrophin secretion and FSHβ and LHβ mRNA expressions

To determine whether BMP-4 was capable of modifying gonadotrophin production and secretion in LβT2 gonadotrophs, cells given activin ± a daily 1 h pulse of GnRH for three days were also treated with BMP-4. Secretion profiles for FSH and LH, representing the total protein secreted during day 3 GnRH treatment and the subsequent overnight incubation, and the corresponding day 4 β-subunit mRNA levels are shown in Fig. 2. As expected, FSH secretion increased ($P < 0.001$) in response to activin and this effect was increased synergistically ($P < 0.001$) in the presence of GnRH, although GnRH alone had no effect (Fig. 2A). While BMP-4 alone had no effect on FSH secretion, it increased the release of FSH in response to activin and activin + GnRH ($P < 0.01$ and $P < 0.001$ respectively). Similarly, the FSHβ mRNA expression was up-regulated by activin ($P < 0.05$) and this effect was also increased synergistically by GnRH ($P < 0.001$; Fig. 2B). BMP-4 alone, or in combination with activin or GnRH, had no effect on FSHβ mRNA, but it further increased activin + GnRH-stimulated mRNA levels ($P < 0.01$).

LH release from LβT2 cells was stimulated by GnRH ($P < 0.001$) and these levels almost doubled when GnRH was combined with activin ($P < 0.001$, Fig. 2C). BMP-4 alone or in combination with activin and/or GnRH did not affect LH release. By contrast, while LHβ mRNA expression also increased in response to GnRH and activin + GnRH ($P < 0.001$), these levels were reduced ($P < 0.05$) by BMP-4 (Fig. 2D).

Effects of BMP-4 on GnRH-R and follistatin mRNA levels

To investigate the possible underlying mechanisms for the effects of BMP-4 on gonadotrophin production and release in LβT2 gonadotrophs, GnRH-R and follistatin mRNA levels on day 4 from cells treated as described were measured. As shown in Fig. 3A, activin and GnRH alone produced small increases in GnRH-R mRNA levels, but combined activin + GnRH was required to achieve a significant ($P < 0.001$) effect. BMP-4 reduced GnRH-R mRNA expression in both the GnRH- and activin + GnRH-treated cells ($P < 0.01$ and $P < 0.001$ respectively). Follistatin mRNA levels, on the other hand, increased in response to activin and activin + GnRH ($P < 0.01$ and $P < 0.001$ respectively, Fig. 3B). BMP-4 treatment significantly reduced the activin-stimulated increase in follistatin mRNA ($P < 0.05$), but had no effect on the increase observed in response to activin + GnRH.

Activation of Smad signalling pathways by BMP-4 and activin

To determine whether or not the BMP and activin signalling pathways could interact at the Smad activation level, LβT2 cells cultured in the presence or absence of activin ± a daily 1 h pulse of GnRH for 3 days were also exposed to BMP-4. On day 4, a final 1-h pulse of GnRH was given and proteins extracted for western blot analysis, using antibodies specific for P-Smad1/5 and P-Smad2/3, to look at the BMP and activin signalling pathways respectively. As shown in Fig. 4A, BMP-4, but not activin, induced the appearance of a band at ~60 kDa, corresponding to the phosphorylated form of Smad1/5. The presence of activin and/or GnRH with BMP-4 did not affect the intensity of this band when compared with BMP-4 treatment alone. By contrast, activin (± GnRH) induced activation of Smad2/3 as indicated by increased intensity of band(s) at ~55–58 kDa corresponding to phosphorylated Smad2/3 (Fig. 4B). When cells were treated with both BMP-4 and activin ± GnRH, band intensity did not change compared with activin ± GnRH alone. It should be noted that the results shown here were obtained using an antibody against P-Smad2/3 and that two bands were observed. However, it appeared that the upper band (P-Smad2) was mainly responsible for the increased signal in response to activin ± GnRH and indeed, identical results were obtained using an antibody specific for P-Smad2 (data not shown).

Effects of BMP-4 on the GnRH-induced activation of ERK1/2, p38 MAPK and CREB

As there is evidence that, as well as signalling through the Smad pathways, TGFβ family members may also utilize other downstream signalling pathways, we examined whether or not BMP-4 activated or influenced the activation of other pathways known to be active in gonadotrophs. The LβT2 cells were cultured in the presence or absence of activin ± a daily 1-h pulse of GnRH ± BMP-4 for 3 days.
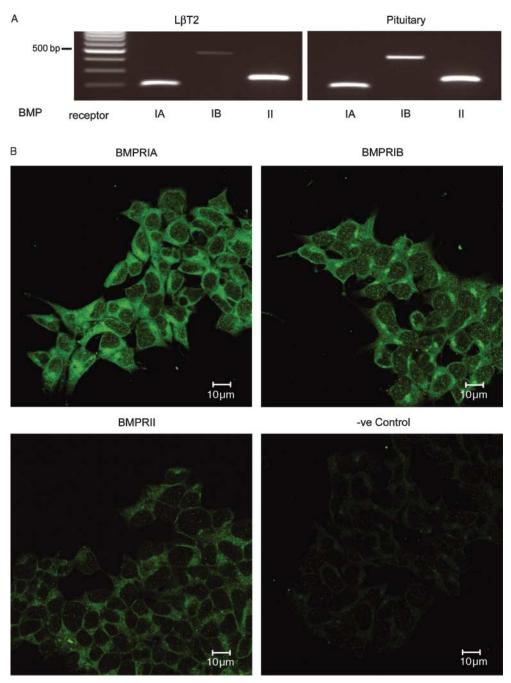
Figure 1 Expression of BMP receptors. (A) mRNAs for BMP receptors IA, IB and II were detected in untreated LβT2 cells and adult mouse pituitary by RT-PCR at 35 cycles. (B) BMP receptor proteins IA, IB and II were detected in untreated LβT2 cells by immunofluorescence. Bar represents 10 μm.

On day 4, a 10- or 20-min pulse of GnRH was given to cells previously exposed to GnRH and proteins extracted for western blot analysis, using antibodies specific for the phosphorylated forms of ERK1/2, p38 MAPK and CREB. As expected, GnRH induced ERK1/2 phosphorylation,

with the same effect observed in the presence and absence of activin ($P<0.001$; Fig. 5A). BMP-4 had no effect on ERK activation. The results shown here were from cells that are given GnRH for 20 min on day 4. After 10-min GnRH supply the same trend was observed, but the result did not
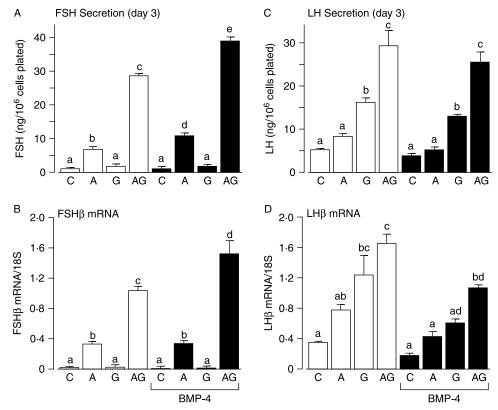
Figure 2 Effects of BMP-4 on gonadotrophin secretion and FSHβ and LHβ mRNA expression. (A) FSH and (C) LH secreted on day 3 from LβT2 cells cultured with 0 or 50 ng/ml BMP-4 in the presence and absence of 50 ng/ml activin A ± a daily 1 h pulse of 10 nM GnRH for 3 days were measured by RIA. Results represent total hormone secreted during the 1 h GnRH treatment and the subsequent overnight incubation; $n=5$. (B) FSHβ and (D) LHβ mRNA levels on day 4 in LβT2 cells, cultured as described, were measured by Taqman quantitative RT-PCR; $n=3$. Different letters indicate significant differences between treatment groups. Values represent means ± S.E.M. from one representative experiment. Five experiments were performed with similar results.

reach significance (data not shown). As shown in Fig. 5B, p38 MAPK was also activated by GnRH ($P<0.01$), but in this case activin increased the response observed with GnRH alone ($P<0.05$). BMP-4 treatment, in the presence of activin + GnRH, reduced phosphorylated p38 levels ($P<0.01$). In this case, the treatment 10-min GnRH supply gave the optimum result, as after 20 min GnRH levels of phosphorylated p38 were unmeasurable in our detection system. CREB was activated by GnRH alone ($P<0.001$; Fig. 5C) and neither activin nor BMP-4 had a significant effect. Again, 20-min GnRH produced the optimum result, although after 10 min a statistically similar effect was observed (data not shown).

Discussion

Earlier studies have identified BMPs as modulators in the regulation of FSH synthesis and release. In rodent primary pituitary cells and the mouse LβT2 gonadotroph cell line, different BMPs (BMP-15, as well as BMP-6 and -7 at high

doses) exert a stimulatory effect on FSHβ transcription (Huang *et al.* 2001, Otsuka & Shimasaki 2002), whereas in ovine primary pituitary cells, BMP-4 and BMP-6 have inhibitory effects on FSHβ mRNA expression and FSH release (Faure *et al.* 2005).

In the present study we report that, in LβT2 mouse gonadotroph cells, BMP-4 alone had no effect on FSHβ mRNA and FSH release, as reported previously for BMP-2, -6 and -7 used at comparable doses (Huang *et al.* 2001, Lee *et al.* 2007). Remarkably, BMP-4 increased both FSHβ mRNA and FSH secretion when combined with activin A and GnRH. Recent studies observed synergistic effects on FSHβ transcription between BMP-2 and activin A in LβT2 cells (Lee *et al.* 2007). Moreover, our data show for the first time that LHβ mRNA and LH secretion remained unaffected by BMP-4 treatment but that, interestingly, the up-regulation of LHβ mRNA in response to GnRH was reduced.

Our results underline the opposite actions of BMP-4 on FSH expression in ewe primary pituitary cells and murine homogeneous gonadotroph cells. In ewe pituitary cells BMP-4 and BMP-6 decreased both basal and
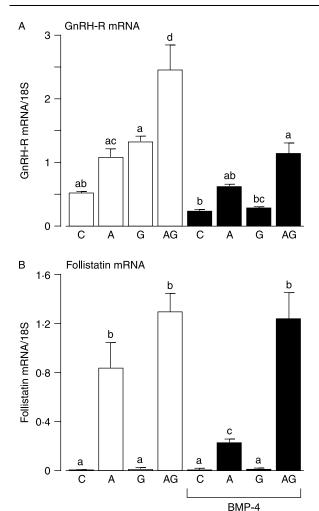
Figure 3 Effects of BMP-4 on GnRH-R and follistatin mRNA expression. LβT2 cells were cultured with 0 or 50 ng/ml BMP-4 in the presence and absence of 50 ng/ml activin A±a daily 1-h pulse of 10 nM GnRH for 3 days. On day 4, total RNA was prepared and (A) GnRH receptor and (B) follistatin mRNA levels were measured by Taqman quantitative RT-PCR. Different letters indicate significant differences between treatment groups. Values represent means± S.E.M. from one representative experiment; *n*=3. Five experiments were performed with similar results.

Figure 4 Activation of Smad signalling pathways. LβT2 cells were cultured with 0 or 50 ng/ml BMP-4 in the presence and absence of 50 ng/ml activin A±a daily 1 h pulse of 10 nM GnRH for 3 days. On day 4, the cells were given a further 1-h GnRH treatment and total protein extracts prepared. Levels of (A) phosphorylated Smad1/5 and (B) phosphorylated Smad2/3 were measured by western blotting and expressed relative to the respective total Smad proteins. Different letters indicate significant differences between treatment groups. Values represent means±S.E.M. from one representative experiment; *n*=2.

activin–stimulated FSHβ mRNA levels and FSH release (Faure *et al*. 2005). The reasons for this differential effect are not clear. They may reflect different specific patterns of BMP receptors between ewe gonadotroph cells and LβT2 cells leading to activation of different signalling pathways and/or different intra–cellular components. The present results demonstrate that LβT2 cells express mRNAs encoding the BMP receptors BMPRIA, BMPRIB and BMPRII, con–firming data from Lee *et al*. (2007), in contrast to another study in which only BMPRIA and BMPRII were found (Otsuka & Shimasaki 2002). However, in our study, BMPRIB mRNA does appear to be expressed at lower levels than BMPRIA and BMPRII. Furthermore,
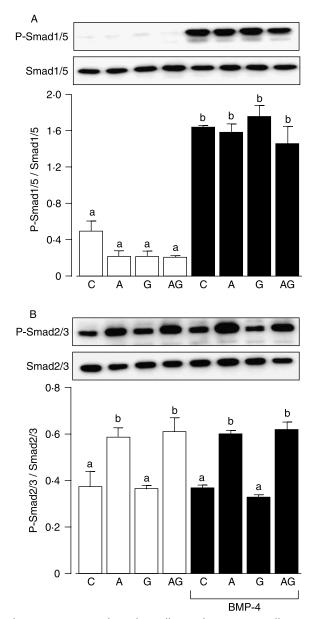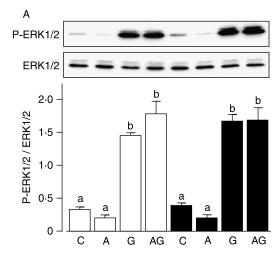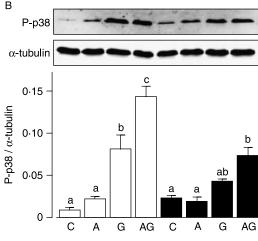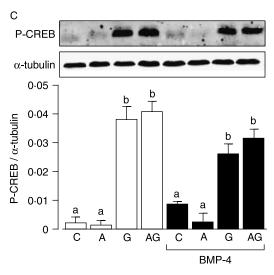
immunocytochemistry confirmed the presence of proteins corresponding to the three types of receptors. In contrast to LβT2 cells, BMPRIB was not detected in ewe gonadotroph cells (Faure *et al*. 2005). The bioavailability of different receptors could dictate the FSH response to BMPs. Even if

A

P-ERK1/2

ERK1/2

B

P-p38

α-tubulin

C

P-CREB

α-tubulin

BMP-4

BMP-4 is able to bind complexes of BMPRII and BMPRIA or BMPRIB (ten Dijke *et al.* 1994), the two receptors may not be equivalent in activating signalling. Alternatively, receptors other than BMPRIA or IB could be involved. A previous report has shown that BMP-2 acts through BMPRII and ALK-2 in LβT2 cells (Lee *et al.* 2007). Moreover, ewe primary pituitary cells are composed of different cell types and the target cell type(s) for BMP-4 are not known. Whether BMP-4 acts directly on gonadotroph cells in sheep primary pituitary cells has yet to be determined.

The nature of the mechanisms by which BMP-4 interacts with activin and GnRH is still unclear. The classical signalling pathway used by BMPs involves Smads 1, 5 and 8 activated by BMP receptors type II and type I. As demonstrated by the phosphorylation of Smad1/5, BMP-4 activated the BMP pathway in LβT2 cells. However, BMP-4 alone failed to modify the level of FSHβ or LHβ mRNA and gonadotrophin release. It is possible that Smads 1/5 mediate other responses to BMP in LβT2 cells such as cell proliferation (Otsuka & Shimasaki 2002, Takeda *et al.* 2007). BMP-4 action required the presence of activin and was amplified when GnRH was added in combination with activin. This action was not accompanied by changes in the level of P-Smad1/5, suggesting that other signalling proteins and/or pathways are involved. A recent study suggested that Smad8 might be the preferred signalling protein in the BMP pathway since overexpression of Smad8, but not Smad1 or 5, enhanced the FSHβ promoter activity in response to BMP-2 (Lee *et al.* 2007). It is possible that BMP-4 may be acting through the modulation of activin and/or GnRH pathway. Previous data have shown that activin transmits its signal through Smad2 and/or Smad3 phosphorylation (Dupont *et al.* 2003, Suszko *et al.* 2003, Bernard 2004). While we detected an increase in the phosphorylated forms of Smad2/3 in the presence of activin, the addition of BMP-4± GnRH in LβT2 cell medium did not change the level of P-Smad2/3. Thus, the interaction between BMP-4, activin and GnRH does not modify Smad1/5 or Smad2/3 phosphorylation.

It is unclear whether follistatin plays a role in this system, as the effects of BMP-4 on expression of follistatin mRNA were inconsistent. Activin-stimulated mRNA was reduced by BMP-4 treatment, in contrast to activin+GnRH-stimulated mRNA which remained unaffected. In addition, increased follistatin synthesis did not have any effect on Smad2/3

Figure 5 Activation of ERK, p38 MAPK and CREB signalling. The LβT2 cells were cultured with 0 or 50 ng/ml BMP-4 in the presence and absence of 50 ng/ml activin A±a daily 1-h pulse of 10 nM GnRH for 3 days. On day 4, the cells were given a final GnRH pulse for 10 or 20 min and levels of (A) phosphorylated ERK1/2 expressed relative to total ERK1/2, (B) phosphorylated p38 expressed relative to α-tubulin and (C) phosphorylated CREB expressed relative to α-tubulin were measured by western blotting. Different letters indicate significant differences between treatment groups. Data shown for ERK1/2 and CREB are from 20-min GnRH treatment groups and for p38 from the 10-min GnRH treatment. Values represent means±S.E.M. from one representative experiment; *n*=2.

phosphorylation. However, it should be emphasized that the activin and BMP-4 levels used in this study would most likely be sufficient to completely override any possible effects of endogenous follistatin, which may be more relevant *in vivo*, where levels of endogenous activin and BMPs would be lower. The ability of follistatin to bind BMPs, albeit with a lower affinity than activin (Balemans & Van Hul 2002), suggests it may well be involved in the BMP-mediated regulation of FSH production *in vivo*.

Since there is evidence that BMPs can operate through Smad-independent pathways such as MAPK signalling molecules (Lou *et al.* 2000, Xiao *et al.* 2002, Derynck & Zhang 2003), changes in levels of phosphorylated ERK1/2 and p38 were measured. As expected, GnRH activated the phosphorylation of ERK1/2 (Liu *et al.* 2002), but this response was not modified by activin, corroborating previous data (Gregory *et al.* 2005). The presence of BMP-4 did not affect GnRH-mediated activation of ERK1/2. Previous studies have shown that BMPs can induce the p38 MAPK-dependent pathway (Iwasaki *et al.* 1999, Nakamura *et al.* 1999). In the current study we observed that the level of GnRH-enhanced p38 activation was increased by activin, confirming a previous report (Coss *et al.* 2007), and this correlated with increased FSHβ mRNA and FSH secretion. This p38 activation in response to GnRH+activin was reduced by BMP-4, contrary to the increase in FSHβ mRNA and FSH secretion. As GnRH alone induced the p38 activation, but had no effect on FSHβ mRNA and FSH secretion, which remained equivalent to those in untreated cells, the exact role of p38 in regulating FSH production is unclear. The BMP-induced reduction in p38 MAPK does, however, correlate with a decrease in LHβ mRNA expression. GnRH is also known to activate CREB protein in gonadotroph cell lines (Duan *et al.* 1999, Shafiee-Kermani *et al.* 2007). Our results confirm that GnRH activates CREB, but CREB phosphorylation was not significantly affected by activin and/or BMP-4. Our current data, therefore, indicate that the classical upstream activin (Smad2/3) or GnRH (ERK, CREB) pathways are not modified in the presence of BMP-4. The p38 MAPK signalling appears to be modulated by BMP-4, but whether this has direct effects on FSH production/secretion remains to be determined. Considering the synergistic effects of BMP-2 and activin on FSHβ transcription in LβT2 cells, it has recently been suggested that each ligand employs different, non-overlapping mechanisms to generate their effects (Lee *et al.* 2007). A summation of the independent signalling pathways activated by BMP-4, activin and GnRH could mediate their combined response with an amplified action occurring specifically at the level of the FSHβ promoter. So far, it is not known if BMP-4 exerts its effect directly on the FSHβ promoter and detailed analyses will be necessary to localize the site of BMP action.

In addition to its effect on FSHβ mRNA and FSH release, BMP-4 also regulated LHβ expression. In the present study, we obtained clear evidence that BMP-4 decreased levels of GnRH and activin-induced LHβ mRNA. This effect appeared to be mediated, at least partly, by the down-regulation of GnRH-R mRNA. The inhibitory effect of BMP-4 on LHβ and GnRH-R mRNA levels is contradictory to the stimulatory action of activin (Pernasetti *et al.* 2001, Nicol *et al.* 2004, Yamada *et al.* 2004). Several studies indicate a correlation between GnRH-R density and a differential gonadotrophin response to GnRH (Kaiser *et al.* 1995, Bedecarrats & Kaiser 2003). High levels of GnRH-R are associated with preferential LHβ gene expression whereas lower levels favour the stimulation of FSHβ gene expression, suggesting a role for GnRH-R levels in mediating these differential responses. Thus, we propose that BMP-4, partly by inhibiting GnRH-R expression, could induce an inhibition of the LHβ mRNA level, potentially through p38 MAPK activation, and promote an increase in the level of FSHβ mRNA and FSH release. The absence of any effect of BMP-4 on LH release compared with its effect on LHβ mRNA levels may simply reflect the release of stored LH via the regulated secretory pathway (Nicol *et al.* 2002, 2004).

In conclusion, our results show that BMP-4, activin and GnRH act in a synergistic manner to up-regulate FSHβ mRNA and FSH release. By contrast, BMP-4 reduces the GnRH and/or activin-induced up-regulation of LHβ and GnRH receptor mRNA. We demonstrated that the classical upstream BMP signalling molecule(s) are activated in the LβT2 cells by BMP-4, but other signalling molecules/pathways must be involved in order to obtain the effects on FSH production observed in response to the interaction of BMP-4 with activin/GnRH. Naturally, these results need to be checked in primary cell cultures to determine if the difference in the effects of BMP-4 in the present mouse LβT2 gonadotroph cell line and our previous studies in sheep primary cells is due to different paracrine or autocrine mechanisms involving an interaction with other pituitary cell types. In contrast to mouse pituitary, BMP-4 mRNA was not detected in LβT2 cells (Lee *et al.* 2007, our unpublished data) arguing for a paracrine role. Collectively, the present data reinforce the concept of a role for BMP-4 in the control of FSH and reveal a novel involvement in LH control.

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