Effect of FSH and cell localization on dimeric inhibin-A secretion from bovine granulosa cells in culture

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Abstract

We tested the hypotheses that the secretion of dimeric inhibin-A from cultured bovine granulosa cells is stimulated by FSH, and that antral cells secrete more inhibin-A than do mural cells. Cells from the antral or mural compartment of follicles were cultured in defined medium in two culture systems, and dimeric inhibin-A was measured by two-site ELISA or by Western immunoblotting. In the first culture system, dimeric inhibin-A secretion declined with time in culture, but was significantly (P<0.05) higher from antral than from mural cells (as was total inhibin-α measured by RIA). The secretion of dimeric inhibin-A and inhibin-α from antral but not mural cells was responsive to FSH. In the second culture system, dimeric inhibin-A secretion increased with time in culture, and was significantly stimulated by FSH, but FSH responsiveness was dependent on the concentrations of insulin in the culture medium. The major forms of inhibin-A secreted had molecular masses of approximately 58, 62, 103–116 and >116 kDa; the 32 kDa form was barely detectable. These different forms were all stimulated by FSH, but the >116 and 62 kDa forms were most responsive to FSH. We conclude that (i) FSH stimulates dimeric inhibin-A secretion from bovine granulosa cells, (ii) the 62 kDa form of inhibin-A may be more responsive to FSH than the 58 kDa form, and (iii) the spatial differentiation of granulosa cell function within the follicle previously observed for oestradiol secretion was also observed for inhibin-α and dimeric inhibin-A secretion.


Introduction

Inhibins are glycoprotein hormones of gonadal origin that suppress follicle-stimulating hormone (FSH) secretion from the pituitary gland without affecting the release of luteinizing hormone (LH) (reviewed by Ying 1988, Knight 1996). Biologically active inhibin consists of two subunits, and was initially isolated as a 58 kDa protein consisting of a 43 kDa α-subunit and a 15 kDa β-subunit. The ‘mature’ 32 kDa form consists of two subunits of 20 kDa and 15 kDa, suggesting that the 32 kDa form results from the cleavage of the 43 kDa α-subunit (Robertson et al. 1986). It is now generally recognized that the α-subunits are secreted in excess of the dimer (Knight et al. 1989, 1991), such that there are large amounts of monomeric α-subunit in biological fluids. The importance of this is that many assays utilize antibodies against the α-subunit, and thus recognize many forms of monomeric α-inhibin as well as the dimeric forms. In addition, there are two distinct β-subunits, termed βA and βB, which give rise to two dimeric inhibin populations, inhibin-A and inhibin-B (Ying 1988). In ruminants, however, inhibin-B has never been isolated from follicular fluid and assays for inhibin-B do not detect this inhibin form in sheep follicular fluid (Knight et al. 1998).

There appear to be major differences in dimeric inhibin secretion between rats and ruminants. In rats, 32 kDa inhibin is the predominant form secreted from cultured granulosa cells (Bicsak et al. 1988, Imai et al. 1996) and detected in blood plasma (Faby et al. 1995). In contrast, up to nine inhibin dimer/α-subunit forms have been described in bovine follicular fluid, the main forms having molecular masses of approximately 29, 34, 49, 53, 58, 77, 110 and >160 kDa (Hopko Ireland et al. 1994, Good et al. 1995, Sunderland et al. 1996). Of these forms, the 34, 58, 77 and >100 kDa forms are dimeric and biologically
active. Total dimeric inhibin concentrations are higher in atretic than in non-atretic bovine follicles (Guilbault et al. 1993, Hopko Ireland et al. 1994, Price et al. 1995a), and the relative amounts of some inhibin forms change differently during follicle growth; for example, abundance of the 32 kDa form increased with follicle atresia whereas that of the 58 kDa form decreased (Sunderland et al. 1996). These data suggest that the processing of the inhibin forms may be under endocrine control, probably by FSH (Mihm et al. 1997).

In rats, protein and mRNA for both α- and β-subunits, and dimeric inhibin secretion from granulosa cells are stimulated by FSH in vitro (Zhang et al. 1988, Turner et al. 1989, Michel et al. 1991, Aloï et al. 1995, Tate et al. 1996, Li et al. 1998). The effect of FSH on inhibin secretion in ruminants is less clear, partly owing to the different molecular weight forms that are not equally detected by all assay systems (Good et al. 1995), and partly because immunoreactive inhibin-α secretion decreases rapidly with time in culture of spontaneously luteinizing bovine granulosa cells (Henderson & Franchimont 1981, Luck et al. 1990). FSH stimulated bioactive inhibin secretion from granulosa cells of morphologically healthy but not atretic bovine follicles (Henderson et al. 1984). Immunoreactive inhibin-α secretion from non-luteinized granulosa cells cultured in serum-free medium was inhibited by FSH in one study (Wrathall & Knight 1993) and not affected by FSH in another (Campbell et al. 1996).

The objective of the present work was to study the secretion of dimeric inhibin-A from oestrogenic bovine granulosa cells in culture. We tested the hypothesis that FSH stimulates dimeric inhibin-A secretion from non-luteinized bovine granulosa cells, using two different culture systems in which oestriadiol secretion is responsive to FSH. A further objective was to determine if subpopulations of granulosa cells (antral vs mural) differ in their ability to secrete dimeric inhibin-A, as they differ in their ability to secrete oestriadiol (Rouillier et al. 1996, 1998).

Materials and Methods

Experiment 1

Granulosa cells were harvested from 3- to 4-month-old Holstein prepubertal female calves as described (Rouillier et al. 1996); in this culture system, aromatase activity decreases with time of culture but cells remain responsive to FSH. Animals were injected with 1500 IU equine chorionic gonadotrophin (eCG) (Equinex; Ayrest, Montreal, Canada) and ovaries were recovered by ovariectomy 96 h later. An antral granulosa cell suspension was prepared according to the method described by Saumande (1991) and modified by Rouillier et al. (1996); follicles ≥8 mm in diameter were punctured with an 18-gauge needle and rinsed ten times with 0·5 ml Menezo B2 medium supplemented with heparin (100 IU/ml; Organon Teknika, Toronto, Ontario, Canada). Following removal of the antral cell suspension, the collapsed follicles were dissected and the internal wall was scraped gently with a smooth inoculation loop to remove the adherent mural granulosa cells. Cell viability was determined by trypan blue exclusion (0·13% final concentration) using a haemocytometer, and varied between 65 and 86% for antral cells and between 42 and 67% for mural cells. Cell suspensions were adjusted with Menezo B2 medium to a final concentration of 6×10⁶ viable cells/ml, and cultured for 4 days in Ham’s F-12 medium, pH 7·4 containing NaHCO3 (1·176 mg/ml), Hepes (2·62 mg/ml), insulin (50 ng/ml), androstenedione (10⁻¹⁵ M), human transferrin (10 µg/ml), ascorbic acid (17·6 µg/ml), gentamycin (20 µg/ml) and nystatin (4 µg/ml), and supplemented with 0, 0·5, 2 or 10 ng/ml FSH (porcine FSH= 41× NIH-FSH-P1, LH activity<0·5%; gift from Dr Combarnous, INRA, Nouzilly, France). Culture medium was replaced daily, and spent medium stored at −20 °C until assayed for total inhibin-α and dimeric inhibin-A. Data were derived from three wells from each of three independent cultures.

Experiment 2

Antral cells from small (3–4·5 mm diameter) follicles were cultured in serum-free conditions as described by Gutiérrez et al. (1997). In this culture system, oestradiol secretion increases with time in culture and is responsive to FSH. Follicles were dissected from the ovaries, and follicles with an obvious atretic appearance (avascular theca layer and debris in the antrum) were discarded. Granulosa cells were isolated by hemisecting the follicles in Dulbecco’s PBS (without calcium or magnesium) at 37 °C and the follicle halves were flushed repeatedly up and down the barrel of a 1 ml syringe. The follicle halves were then allowed to settle and the resulting granulosa cell suspension was collected. Cells were resuspended in α-MEM (Minimum Essential Medium) with t-glutamine, containing sodium bicarbonate (10 mM), Hepes (20 mM), protease-free BSA (0·1%), selenium (4 ng/ml), transferrin (2·5 µg/ml), androstenedione (10⁻¹⁵ M), non-essential amino acid mix (1·1 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml). Cell viability (40–50%) was estimated using trypan blue exclusion.

Cells were plated at a density of 10⁶ viable cells into 24-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) in culture medium (α-MEM described above) containing human recombinant insulin-like growth factor-I (IGF-I) (10 ng/ml; Gibco BRL, Burlington, Ontario, Canada), and various doses (see below) of insulin and ovine FSH (oFSH) (NIADDK-oFSH-17; biological potency 20 U/mg relative to oFSH-S1). Cells were cultured for 8 days and the medium was changed at intervals of 48 h. Disturbance of the cells was minimized by gently removing and replacing 700 µl medium at each change.
The spent medium was stored at −20 °C before assay for dimeric inhibin-A by immunoblot analysis. At the end of culture, granulosa cells were sonicated for 5 min and the lysate used for DNA measurement with calf thymus DNA as standard (Labarca & Paigen 1980).

Three experimental treatments were applied to test the effects of FSH and insulin on dimeric inhibin-A secretion. Cells were cultured as above and were stimulated with 0·1, 1, 10 or 100 ng/ml FSH in the presence of 10 ng/ml insulin, with 1, 10 and 100 ng/ml FSH in the presence of 100 ng/ml insulin, or with 0, 10 or 100 ng/ml insulin without FSH. Intracellular inhibin-A forms were also examined by immunoblotting soluble cell extracts after culture with FSH and 10 ng/ml insulin. All data are derived from three wells from each of three or four independent cultures.

Assays

Oestradiol and progesterone concentrations measured in conditioned medium from Experiment 1 have been reported for treatments with 0, 2 and 10 ng/ml FSH (Rouillier et al. 1996). Oestradiol (Price et al. 1995a) and progesterone (Lafrance & Goff 1985) were measured in medium samples in duplicate from day 6 of Experiment 2 without extraction as described. Inter- and intra-assay coefficients of variation (CV) were less than 12% for oestradiol and less than 10% for progesterone. The sensitivity of these assays was equivalent to 0·25 and 1 ng/ml medium for oestradiol and progesterone respectively.

Immunoreactive inhibin-α was measured using the RIA developed by Vaughan et al. (1989). Briefly, the assay utilizes an antibody (#120; The Clayton Foundation Laboratories for Peptide Biology, The Silk Institute, La Jolla, CA, USA) raised against porcine inhibin-α(1–26), which was used at a final dilution of 1:750 000. The tracer was [δ-Ser,Ne]-human inhibin-α(1–25)-Gly-Tyr-NH₂ that was iodinated with Iodogen (Pierce, Rockford, IL, USA) and purified by HPLC (Vaughan et al. 1989), and ovine inhibin-α(1–25)-Gly-Tyr was used as the standard. Results of this assay are expressed as pmoL, derived from the mass of the standard (1 pg standard is 0·22 fmol; J M Vaughan, personal communication). The sensitivity for the synthetic peptide standard was 0·05 pmoL/ml. Intra- and inter-assay CVs were 9 and 19% respectively.

Dimeric inhibin-A was measured with the assay developed for human samples (Groome et al. 1994) and modified for use with bovine samples (Price et al. 1995b). Briefly, the capture antibody was a mouse monoclonal raised against the βA-subunit of inhibin (E4; Groome & Lawrence 1991), and the detection antibody was a mouse monoclonal antibody raised against the inhibin-α subunit (R1; Serotec, Oxford UK; Groome et al. 1990) and coupled to alkaline phosphatase. Samples and standard (32 kDa human recombinant inhibin; Genentech, South San Francisco, CA, USA) were diluted in assay diluent (0·1 M Tris HCl pH 7·5, containing 0·15 M sodium chloride, 5% Triton X-100, 10% BSA and 5% mouse serum) to a volume of 150 µl, incubated with 15 µl 10% hydrogen peroxide for 30 min at room temperature, then diluted to 300 µl with assay diluent. The oxidation step has been shown to improve antibody (E4) recognition of the β-subunit (Knight & Muttukrishna 1994). Aliquots of 100 µl sample solutions were assayed in duplicate. Bound alkaline phosphatase was detected using an ELISA Amplification System (Gibco BRL) as described by the manufacturer. Intra- and inter-assay CVs were 7 and 20%, and the sensitivity was equivalent to 0·1 fmol/ml. Results are expressed in fmol based on the 32 kDa dimeric standard.

Immunoblot analysis

Dimeric inhibin-A forms in culture medium and cell extracts from Experiment 2 were assessed by immunoprecipitating with anti-α-subunit IgG followed by immunoblotting with anti-βA-subunit IgG. Samples of culture medium (0·1–2 ml) and cell protein (100 µg) were immunoprecipitated with 0·2 ml 1:5000 dilution of antibody directed against the α-subunit of ovine inhibin (#120, The Salk Institute). After an overnight incubation at 4 °C on a rocking platform, 0·2 ml 1:40 goat anti-rabbit serum was added, and the incubation continued for 2 h at 4 °C. The samples were then centrifuged at 1000 g at 4 °C for 30 min, and the immunoprecipitate was washed twice with 1 ml 25 mM Tris–saline, pH 7·4 (TBS). The pellet was resuspended in 50 µl sample buffer (0·1 M Tris–HCl, pH 6·8, containing 10% SDS, 20% glycerol and 0·01% bromophenol blue) and heated to 90–100 °C for 10 min. Proteins were then separated on 12% polyacrylamide gels at 4 °C under non-reducing conditions (Laemmli 1970) using a Bio-Rad Mini gel apparatus (Mini-Protean II, Vertical Electrophoresis Cell; Bio-Rad, Mississauga, Ontario, Canada). Molecular sizes were estimated by comparison with molecular mass markers (prestained markers from Amersham Canada Inc., Oakville, Ontario, Canada or from Bio-Rad).
To verify the immunoprecipitation procedure, some aliquots of culture medium were pooled and concentrated by centrifugation against a 10 kDa filter (Filtron Technology Corporation, Northborough, MA, USA) and were electrophoresed alongside immunoprecipitated medium samples. Similarly, immunoprecipitated and untreated follicular fluid were compared. The non-precipitated samples were blotted with the βA-subunit antibody (E4). Different volumes of pooled medium samples were also immunoprecipitated to verify that band signal was linear with sample volume.

Statistical analysis
Concentrations of hormones assayed in the culture medium were log transformed before analysis to obtain a normal distribution (Shapiro–Wilk test). Data derived from the last day of culture were expressed relative to total DNA to correct for cell number. Inhibin-A forms detected by immunoblot were estimated by scanning each immunospecific band using a computer imaging system and software (Collage; Fotodyne Inc., New Berlin, WI, USA). The amount of total inhibin-A was estimated for each sample by summing densitometry values for all bands correcting for total DNA. The data were then expressed relative to total activity on each blot, as all samples from each culture replicate were included in the same blot. To calculate the proportions of each molecular form present, densitometry values for each band were expressed relative to total inhibin-A activity for each sample, and these data were transformed to arcsines before analysis. ANOVA was used to evaluate effects of day of culture, cell type (Experiment 1) and dose of FSH on concentrations or relative abundance of inhibin-A forms. Since culture replicate was nested within an experiment, it was included as a random variable in the F-test for the effect of experiment. Means comparisons were determined with orthogonal contrasts (Experiment 1) or with the Tukey–Kramer test (Experiment 2). Data are presented as least squares means ± s.e.m.

Results

Experiment 1
There were significant effects of time in culture (P < 0.001), of FSH (P < 0.01) and of cell type (P < 0.001) on the secretion of inhibin-α immunoactivity (Fig. 1A). The release of inhibin-α into the culture medium from antral and from mural granulosa cells declined by over 90% (P < 0.001) between days 1 and 2 of culture, and did not decrease further between days 2 and 3 of culture (data for day 3 not shown). Inhibin-α was not detected on day 4 of culture, so concentrations could not be corrected for cell number (DNA content) at the end of culture; however, cell number did not differ significantly between mural or antral cells (data not shown). Antral cells secreted significantly more inhibin-α than did mural cells on day 1 (P < 0.001) and on day 2 of culture (P < 0.05), and there was no difference between cell types on day 3 of culture (P > 0.05). There was a significant effect of FSH on inhibin-α concentrations on days 1 and 2 of culture for
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Figure 2 Validation of dimeric inhibin-A immunoprecipitation. (A) The main inhibin-A forms in two samples of bovine follicular fluid (0.5 µl) as revealed by blotting with a βA-subunit antibody (E4); the band at approximately 21 kDa probably represents activin. (B) The effect of sample volume on inhibin-A forms immunoprecipitated from pooled culture medium from cells of small follicles (2–5 mm) cultured for 6 days. (C) The same forms of inhibin-A were observed in concentrated (‘C’) and immunoprecipitated (‘P’) culture medium from small and large follicles. For immunoprecipitation, samples were precipitated with α-subunit antibody (#120) and blotted with βA-subunit antibody (E4; see Material and Methods). Arrows indicate the position of molecular mass markers.

antral (P<0.05) but not mural cells (P>0.05). On day 3, there was a significant effect of FSH irrespective of cell type (0.09 ± 0.01 vs 0.22 ± 0.04 pmol/10⁵ cells, for 0 and 10 ng/ml FSH respectively; P<0.05).

There were significant effects of time in culture (P<0.001), of FSH and of cell type (P<0.05) on the secretion of dimeric inhibin-A (Fig. 1B). Dimeric inhibin-A secretion by mural and antral granulosa cells decreased by 70–80% (P<0.01) between days 1 and 2 of culture (Fig. 1B), and was not detected on days 3 or 4 of culture. On day 1 of culture, antral cells secreted significantly more dimeric inhibin-A than did mural cells (P<0.05), and there was no difference between cell types on day 2 of culture (P>0.05). There was a significant effect of FSH on inhibin-A secretion by antral (P<0.01) but not mural cells (P>0.05; Fig. 1), and there was a significant interaction between cell type and FSH on dimeric inhibin-A concentrations on day 2 of culture (P<0.05).

Experiment 2

The immunoprecipitation procedure detected proteins of approximately 32, 55–62, 70, 100, 116 and >116 kDa in bovine follicular fluid. Immunoblotting raw follicular fluid with the anti-βA-subunit IgG revealed similar bands with additional strong staining at approximately 21 kDa (Fig. 2A). The 55–60 kDa proteins may have been displaced by the large amount of non-specific protein observed at 45–70 kDa in non-precipitated follicular fluid. Immunoblotting with the anti-α-subunit IgG recognized 25, 32 and 48 kDa forms, as well as 77 and >100 kDa forms (not shown); again, the presence of follicular proteins (probably albumin) obscured bands present in the 50–80 kDa range.

Immunoprecipitation of culture medium from granulosa cells detected four major bands, with molecular masses of approximately 58, 62, 116 and >116 kDa, and band intensity increased linearly between 50 and 300 µl sample volume (Fig. 2B). A similar band pattern was observed in concentrated culture medium (Fig. 2C), although bands at 58–62 kDa were obscured by the large amount of albumin present in the concentrated medium. Very weak staining was detected at approximately 32, 42 and 95 kDa when medium was pooled and a large volume immunoprecipitated (not shown). Substitution of either precipitating or detecting antibodies with a non-specific antibody, or immunoprecipitation of fresh (pre-culture) culture medium resulted in loss of signal (not shown).

There was a significant effect of FSH on the secretion of inhibin-A forms from cells incubated with insulin at a concentration of 10 ng/ml (Fig. 3A). In the absence of FSH, inhibin–A was barely detectable by immunoblotting, and was significantly (P<0.01) increased by the addition of 1 ng/ml FSH. The addition of greater amounts of FSH did not further stimulate inhibin-A secretion. The major forms of inhibin–A detected had molecular masses of approximately 58, 62, 116 and >116 kDa, and most forms were affected by FSH. There was a linear increase (P<0.01) in the>116 kDa form of inhibin–A, and the response of the 62 kDa form to FSH was quadratic (P<0.05; Fig. 3B). There was slight quadratic effect of FSH on the 116 kDa...
form \( (P=0.06) \), and no effect upon the 58 kDa form \((P>0.1)\).

Analysis of steroid production by these cells showed that the cells were oestrogenic, and that lower doses of FSH stimulated oestradiol secretion \((92 \pm 6, 296 \pm 28, 2345 \pm 419 \text{ pg/µg DNA for } 0, 0.1 \) and 1 ng/ml FSH respectively; \( P<0.01 \)). Higher doses did not further

stimulate oestradiol secretion \((2543 \pm 124 \text{ and } 2480 \pm 459 \text{ pg/µg DNA for } 10 \text{ and } 100 \text{ ng/ml FSH respectively}) \).

Progesterone secretion did not increase significantly with lower dose FSH treatment, but increased with 10 and 100 ng/ml FSH \((124 \pm 19 \text{ and } 154 \pm 20 \text{ ng progesterone/µg DNA respectively, compared with } 27 \pm 5 \text{ ng/µg DNA for control, } P<0.05)\).

There was a significant effect of insulin on dimeric inhibin-A secretion, although as cells incubated in the absence of insulin did not survive, only data from cells cultured with 10 or 100 ng/ml insulin were compared. Total inhibin-A immunoblot activity as well as the activity of each inhibin-A form was increased by culture with 100 ng/ml insulin \((P<0.05; \text{ Fig. 4})\). In the presence of 10 ng/ml insulin, the >116 and 62 kDa forms were significantly \((P<0.05)\) more abundant than the 116 and 58 kDa forms, whereas these forms were equally abundant after culture in the presence of 100 ng/ml insulin (Fig. 4). In the presence of 100 ng/ml insulin, FSH had no significant effect on dimeric inhibin-A secretion \((P>0.05; \text{ data not shown})\).

Oestradiol secretion from these cells was stimulated by insulin \((91.9 \pm 5.5 \text{ vs } 3376 \pm 620 \text{ pg/µg DNA for } 10 \text{ and } 100 \text{ ng/ml insulin respectively; } P<0.001)\) whereas...
progestosterone secretion was not affected. In the presence of 100 ng/ml insulin, FSH did not stimulate oestradiol or progestosterone secretion ($P > 0.05$).

Immunoblot analysis of cell proteins after 6 days of incubation (with insulin at 10 ng/ml) showed weak immunostaining at 116 and $>116$ kDa (not shown), and a weak band at approximately 32 kDa was occasionally observed. Intracellular inhibin-A forms could not be reliably quantified.

**Discussion**

This report is, to our knowledge, the first to document the secretion of dimeric inhibin-A by ruminant granulosa cells in vitro. The novel finding of these studies is the significant effect of FSH on dimeric inhibin-A secretion. This is in contrast to previous reports for ruminants, which have suggested that the secretion of immunoreactive inhibin (dimer plus free α-forms) was inhibited or not affected by FSH in vitro (Henderson & Franchimont 1981, Wrathall & Knight 1993, Campbell et al. 1996). These previous reports, coupled with data derived from experiments in vivo (Campbell et al. 1991), led to the hypothesis that increased inhibin production in ruminants, in contrast to rats and humans, is a result of follicle growth and not direct stimulation by FSH per se (Campbell et al. 1996). The present data refute this hypothesis, and as such present an important clarification of our understanding of inhibin physiology in ruminants. Previous studies may not have detected effects of FSH because of inappropriate assays (those cross-reacting with free α-subunit, e.g. Wrathall & Knight 1993), culture conditions (luteinized cells or cells that did not respond to FSH, e.g. Luck et al. 1990), or the use of supraphysiological concentrations of FSH (e.g. Henderson & Franchimont 1981). This conclusion is consistent with data from rats (see Introduction), and is in accord with the negative-feedback relationship between FSH and dimeric inhibin-A (Knight et al. 1998).

The secretion of dimeric inhibin-A from antral granulosa cells was higher than that from mural granulosa cells, and responded to FSH in a dose-dependent manner. This is consistent with the response of these cells with respect to oestradiol secretion (Rouillier et al. 1996). These data add to the literature by showing that protein as well as steroid hormones are affected by location of cells within the follicle (Roberts & Echternkamp 1994, Rouillier et al. 1996, 1998). One explanation for this difference is that a greater disruption of cell-to-cell contact occurs when harvesting mural cells, which reduces subsequent endocrine function of these cells in vitro (Rouillier et al. 1996).

Although the culture system described in Experiment 1 is useful for demonstrating differences between antral and mural cells, the secretion of both these hormones declines significantly with time in culture, suggesting that these cells may be luteinizing in vitro. To verify that FSH also stimulates inhibin-A secretion from non-luteinizing cells, we also employed a culture system in which oestradiol secretion increases with time in culture (Campbell et al. 1996, Gutiérrez et al. 1997). In good agreement with the data from Experiment 1, FSH stimulated dimeric inhibin-A secretion from cells cultured in the presence of 10 ng/ml insulin. Owing to difficulties in measuring dimeric inhibins in blood plasma of ruminants, relatively little is known about the control of dimeric inhibin-A secretion in vivo. FSH and/or eCG increased plasma dimeric inhibin-A concentrations in cattle (Price et al. 1995b) and sheep (Knight et al. 1998). Together, these data support the concept that FSH stimulates inhibin-A secretion in vivo in ruminants.

Insulin stimulated dimeric inhibin-A secretion from bovine granulosa cells in the present study, which is in agreement with previous studies showing a stimulatory effect of insulin on total inhibin–α secretion from cultured sheep granulosa cells (Campbell et al. 1996) and of IGF-I on subunit-α secretion from cultured rat granulosa cells (Aloi et al. 1995, Li et al. 1998). Interestingly, FSH did not stimulate dimeric inhibin-A secretion from cells cultured in the presence of 100 ng/ml insulin. This dose of insulin is supraphysiological and may act through both insulin and IGF-I receptors (Poresky et al. 1999). A similar observation was made for inhibin-α secretion from cultured sheep granulosa cells cultured with 10 ng/ml insulin and 10 ng/ml of a potent IGF-I analogue (Campbell et al. 1996). Thus it is likely that activation of insulin and IGF-I signalling pathways may maximally stimulate inhibin-A secretion, or there may be cross-talk between the insulin/IGF-I and FSH pathways.

As has been amply demonstrated (Hopko Ireland et al. 1994, Good et al. 1995, Sunderland et al. 1996, Mihm et al. 1997), no analysis of inhibin is complete without an assessment of the different molecular forms present in the biological fluid studied. In the present study, the predominant forms of inhibin–A immunoprecipitated from culture medium had molecular masses of approximately 58, 62, 116 and $>116$ kDa, which correspond well to the 58, 65–68, 105–122 and $>120$ kDa forms reported by various laboratories (Sugino et al. 1992, Hopko Ireland et al. 1994, Good et al. 1995, Sunderland et al. 1996, Mihm et al. 1997). These forms are presumed to be dimeric proteins (Sugino et al. 1992, Good et al. 1995), and have similar bioactivities (Good et al. 1995). The immunoprecipitation procedure did not appear to selectively alter the pattern of inhibin-A forms detected, as immunoprecipitated and concentrated medium samples showed very similar inhibin-A forms, although the high concentration of non-specific proteins of 58–70 kDa (presumably albumin) in concentrated samples obscured and displaced the expected 58 kDa inhibin-A band. In neither case was a 32 kDa form readily detected. In contrast, immunoprecipitation of follicular fluid revealed the major forms of 32, 58, 62, 77, 95 and $>103$ kDa expected (Hopko Ireland et al. 1994, Good et al. 1995).

In the present study, FSH stimulated the major forms of dimeric inhibin-A, although this was more apparent for some forms than for others. This was most evident for the >116 and 62 kDa forms, which were barely detectable in the absence of FSH or with 0.1 ng/ml FSH. These data should be interpreted with caution, as quantification of very weak densitometry signals is error prone. In vivo, FSH administration increased the relative amounts of the larger (>32 kDa) forms of inhibin in follicular fluid of cattle (Mihm et al. 1997). The present data suggest that the increase in larger forms observed by Mihm et al. (1997) was a result of an increase of all (or most) secreted (58, 62, 116 and >116 kDa) forms, with relatively little degradation to the 32 kDa form.

The intracellular forms of dimeric inhibin-A detected in the present study were generally >116 kDa forms, with no evidence of the 58, 62 or 103–116 kDa forms found in culture medium. The relative abundance of intracellular dimeric inhibin-A was much lower than that of secreted inhibin-A, as the amount of protein immunoprecipitated represents the cell content of approximately six culture wells, whereas the volume of culture medium immunoprecipitated is equivalent to less than that of one well. This suggests that dimer formation occurs immediately before or during secretion, which is consistent with studies of rat granulosa cells (Bicsak et al. 1988). Indeed, Wrathall & Knight (1993), immunoblotting bovine granulosa cell extracts with an inhibin-α antibody, suggested that granulosa cells do not store inhibin subunits.

While the studies reported herein did not address the negative feedback role of inhibin in vivo, recent studies have shown significant correlations between circulating FSH, oestradiol and dimeric inhibin-A concentrations in sheep (Knight et al. 1998, Souza et al. 1998). In rats and in humans, it has become clear that both inhibin-α and inhibin-B are important for the regulation of FSH secretion (Woodruff et al. 1996, Groome et al. 1996, Reame et al. 1998, Smitz & Cortvriendt 1998); however, inhibin-B has never been isolated from ruminant follicular fluid and assays for inhibin-B do not detect this inhibin form in sheep follicular fluid (Knight et al. 1998). It is at present likely that inhibin-B is not a major secretory product of the ruminant follicle.

In summary, these data show for the first time that (i) FSH stimulates dimeric inhibin-A secretion from bovine granulosa cells, (ii) that the 62 kDa form of inhibin-A may be more responsive to FSH than the 58 kDa form, and (iii) that the spatial differentiation of granulosa cell function within the follicle previously observed for oestradiol secretion was also observed for α- and dimeric inhibin-A. These data also support the concept that in ruminants, in contrast to rodents, the ‘mature’ 32 kDa inhibin-A is a minor by-product of extracellular cleavage of larger forms.

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