Effects of active immunization of sheep against an amino terminal peptide of the inhibin α_C subunit on intrafollicular levels of activin A, inhibin A and follistatin

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Abstract

Active immunization of ewes against inhibin (IMM) consistently increases ovulation rate but this response is not always accompanied by the expected rise in plasma FSH. Inhibin-related molecules also have local auto/paracrine effects within the ovary and the ovulatory response to IMM could be due to neutralization of one of these effects, independent of changing FSH levels. To investigate this, ovaries were collected from long-term IMM ($n=6$) and control (CON; $n=8$) ewes killed 48 h after progestagen withdrawal (late follicular phase) and all follicles ≥ 3 mm were recovered to determine intrafollicular levels of inhibin A, activin A and follistatin by specific two-site immunoassay and oestradiol and testosterone by radio-immunoassay. Blood samples were collected to assess plasma FSH, oestradiol and inhibin antibody titres. Although plasma FSH levels were similar in IMM and CON ewes, IMM ewes had ~ 3 -fold more follicles ≥ 3 mm ($P<0.0001$) and ~ 3 -fold more oestrogenic follicles

($P<0.001$) than CON ewes. Compared with CON ewes, follicles from IMM ewes had much higher concentrations of activin A (~ 6 -fold; $P<0.001$) and inhibin A (~ 3 -fold; $P<0.001$) but only slightly more follistatin (~ 1.4 -fold; not significant). The activin A:follistatin ratio in follicles from IMM ewes ($\sim 1:1$) was significantly higher ($P<0.001$) than in follicles from CON ewes ($\sim 0.3:1$). Levels of inhibin antibody measured in follicular fluid (FF) from IMM ewes were similar to plasma levels. Given that activin A has been shown previously to up-regulate FSH receptors and aromatase activity in rat granulosa cells, the increase in intrafollicular activin A, unaccompanied by a rise in the concentration of its binding protein (follistatin), could explain how long-term IMM enhances follicle development and ovulation rate without necessarily promoting a sustained increase in FSH secretion.

Journal of Endocrinology (1998) **157**, 157–168

Introduction

Immunoneutralization experiments involving either active or passive immunization have provided good evidence that ovarian inhibin contributes to the negative feedback regulation of follicle-stimulating hormone (FSH) secretion in the ewe (for reviews see Findlay *et al.* 1993, Terqui *et al.* 1995). Various immunogens have been used to generate antibodies against inhibin, including partially purified follicular fluid (FF) preparations, recombinant inhibin subunits and chemically synthesized inhibin fragments. There is good agreement that active immunization of sheep using any of these different immunogens can increase the ovulation rate. However, the expected rise in circulating FSH levels has not been a consistent finding raising doubts about the mechanism through which active inhibin immunization enhances follicle development and ovulation rate (Findlay *et al.* 1993, Terqui *et al.* 1995).

In contrast to the equivocal FSH response to active immunization, during which inhibin antibodies are present for prolonged time periods, short-term passive immunization experiments have shown that plasma FSH levels increase abruptly in response to administration of inhibin antibodies (Mann *et al.* 1989, 1993, Wrathall *et al.* 1990, Campbell & Scaramuzzi 1995, Campbell *et al.* 1995). However, it has been noted that FSH levels soon revert to pretreatment levels despite the presence of high antibody titres (Mann *et al.* 1989, 1993, Campbell & Scaramuzzi 1995, Campbell *et al.* 1995), indicating that ovarian compensatory mechanisms, involving increased oestradiol and/or inhibin secretion, are operational. The existence of such ovarian compensatory mechanisms is also indicated by two studies carried out in this laboratory (Knight *et al.* 1991b, Fray *et al.* 1994) in which we failed to detect raised FSH levels in groups of long-term (>6 -month) actively immunized ewes. Previously, the same

ewes had shown raised FSH levels during their initial active immunization regimen (see Wrathall *et al.* 1990, 1992). Such inconsistencies in the FSH response to long-term inhibin immunoneutralization indicate that inhibin antibodies may have additional direct stimulatory effects on follicle development that are independent of changes in FSH secretion. Recent *in vitro* studies have indicated several putative actions of inhibin and related peptides within the follicle (for reviews see Findlay 1993, Woodruff & Mather 1995, Findlay *et al.* 1996, Knight 1996). Immunoneutralization of one or more of these local actions of inhibin or its free α subunit could provide an alternative mechanism for the enhancement of ovulation rate.

As one approach towards investigating potential direct intraovarian effects of inhibin antibodies, in this study we recovered ovaries from control and long-term inhibin-immunized ewes (IMM) during the late follicular phase of the oestrous cycle for comparison of follicle numbers and intrafollicular levels of oestradiol, testosterone, inhibin A, activin A and follistatin. These ewes had been actively immunized against inhibin for approximately 1 year and, from previous observations (Knight *et al.* 1991b, Fray *et al.* 1994), were predicted to have plasma FSH levels similar to those in non-immunized control ewes.

Materials and Methods

Animals

The study involved fourteen Mule (Blue faced Leicester $\times$ Swaledale) ewes which were kept under normal husbandry conditions at the University of Reading Animal Production Research Unit. About 1 year earlier, six of the ewes had been actively immunized against a synthetic peptide corresponding to the amino terminal region (amino acids 1–29) of the mature α_C subunit of bovine inhibin conjugated to tuberculin purified protein derivative (PPD) as described elsewhere (Wrathall *et al.* 1990, 1992). At the same time four of the ewes were actively immunized against PPD alone to serve as carrier-immunized controls. The remaining four ewes served as non-immunized controls. Hereafter, inhibin-immunized ewes are referred to as IMM ewes while PPD-immunized and non-immunized ewes are collectively referred to as CON ewes. Oestrous cycles were synchronized using intravaginal progestagen sponges (Intervet UK Ltd, Cambridge, Cambs, UK) which were inserted for 14 days. On the day of progestagen sponge insertion IMM ewes were given a booster immunization with the inhibin peptide–PPD conjugate while the four carrier immunized controls were boosted with PPD alone. Blood samples were collected by jugular venepuncture into heparinized tubes on the day of sponge insertion, weekly until the day of sponge removal and thereafter at 12-h intervals for 48 h at which time the animals were humanely killed. Blood

was centrifuged at 3000 r.p.m. for 30 min and plasma collected and stored at -20°C . Within 15 min of death ovaries were placed in ice-cold phosphate-buffered saline (PBS) for transfer to the laboratory where all visible ovarian follicles ≥ 3 mm in diameter were dissected out, measured accurately using a dissecting microscope fitted with an eye-piece graticule and then transferred to chilled microfuge tubes containing 1 ml PBS. Follicles were chopped coarsely using dissecting scissors to release the FF and the tubes were then stored at -20°C until analysis.

Classification of follicles

Only follicles estimated to exceed 3 mm in diameter were dissected from the ovaries. These were initially classified as medium ($\geq 3 \leq 5$ mm) and large (>5 mm) size. Subsequently, follicles were classified on the basis of FF steroid measurements as either oestrogen-active (OA; oestradiol:testosterone ratio >1) or oestrogen-inactive (OI; oestradiol:testosterone ratio <1). Follicle diameters were used to determine FF volumes according to the relationship derived by England *et al.* (1981) and intrafollicular hormones expressed on a per μl FF basis.

Measurement of inhibin antibody titre

Plasma and FF samples (1:1000 dilution) were tested for their ability to bind ^{125}I -labelled bovine inhibin (M_r 32 000) as described previously (Wrathall *et al.* 1990). Bovine inhibin was isolated and radiolabelled as described by Knight *et al.* (1989).

Hormone determinations

Plasma FSH Plasma concentrations of FSH were determined using an homologous ovine FSH RIA kit provided by NIDDK (Baltimore, MD, USA) as reported previously (Wrathall *et al.* 1990). The detection limit of the assay, expressed as the amount of FSH standard (NIDDK-oFSH-RP1) required to inhibit the binding of the iodinated tracer (NIDDK-oFSH-I1) to the antibody (NIDDK-anti-oFSH-1; dilution 1:16 000) by 20% was 25 pg/tube. Intra- and interassay coefficients of variation were 3.7 and 6.8% respectively.

Steroids in plasma and FF Concentrations of oestradiol-17 β in plasma were determined using a commercial human oestradiol-17 β RIA kit (Serono Diagnostics, Welwyn G.C, Herts., UK) which was modified and validated for ovine plasma as reported elsewhere (Fray *et al.* 1996). Intra- and interassay coefficients of variation were 12.2 and 21.0% respectively and the detection limit of the assay was 0.25 pg/tube (equivalent to 0.5 pg/ml ovine plasma). Concentrations of oestradiol and testosterone in FF samples were determined using 'direct' (non-extraction) RIA procedures as described previously (Howles *et al.* 1980,

Wrathall & Knight 1993). The amount of oestradiol required to inhibit the binding of [3 H]oestradiol to the oestradiol antisera by 20% was 124 pg/ml and the intra- and interassay coefficients of variation were 9 and 12% respectively. The amount of testosterone required to inhibit the binding of [3 H]testosterone to the testosterone antisera by 20% was 236 pg/ml and the intra- and interassay coefficients of variation were 8 and 12% respectively.

Inhibin A in FF and HPLC fractions Inhibin A concentrations were determined using the two-site IRMA described previously (Knight & Muttukrishna 1994). Purified bovine M_r 32 000 inhibin A (Knight *et al.* 1989) was used as the standard and the detection limit was 50 pg/tube. Intra- and interassay coefficients of variation were 4.1 and 5.1% respectively. Although this assay is not suitable for measuring the very low inhibin A levels in sheep plasma it can comfortably detect inhibin A levels in high dilutions (1:500–1:8000) of ovine FF. The unique ability of this IRMA to quantify 'total' inhibin A (i.e. antibody-bound+free) in FF from IMM ewes relies on the fact that the capture antibody used (B18 at 1:100 dilution) is an ovine antiserum raised against a very similar inhibin α C subunit peptide to that used for immunizing the present ewes. Thus, addition of excess donkey anti-sheep IgG reagent on day 2 of the assay will immunoprecipitate sheep inhibin A- 125 I-labelled β A antibody complexes irrespective of whether they are bound to B18 ovine anti- α C capture antibody or to endogenous sheep anti-inhibin α C antibody. Validation of the assay for use with FF from CON and IMM ewes is presented in Results.

Activin A in FF and HPLC fractions Activin A concentrations were determined using the two-site ELISA recently described by Knight *et al.* (1996). Human recombinant (rh) activin A (provided by Dr J P Mather, Genentech Inc, San Francisco, CA, USA) was used as the standard and the detection limit was $\sim$ 10 pg/well. Intra- and inter-plate coefficients of variation were 9.3 and 10.4% respectively. Validation of the assay for use with FF from CON and IMM ewes is presented in Results.

Follistatin in FF and FPLC fractions Follistatin concentrations were determined using a new two-site ELISA which utilizes an immobilized mouse monoclonal antibody (MAb, clone 8/1; L W Evans & N P Groome, unpublished observations) in combination with a liquid-phase chicken polyclonal anti-follistatin serum raised in this laboratory. The immunogen used in both cases was rh follistatin-288 (NIDDK). MAb-coated microplates (Nunc Maxisorb, Life Technologies, Glasgow, UK) were prepared by overnight incubation with 0.1 M NaHCO₃ containing 10 μ g MAb/ml (50 μ l/well) followed by a blocking incubation (overnight) with 250 μ l follistatin assay diluent (PBS with 5% BSA and 0.1% sodium azide). Plates were then sealed and stored at 4 °C until required.

Immediately before use plates were washed (five cycles) with ELISA wash buffer consisting of Tris-buffered saline (TBS; 0.15 mol/l sodium chloride, 0.01 mol/l Tris-HCl, pH 7.4.) containing 0.1% Tween-20 and 0.1% sodium azide. Chicken anti-follistatin serum (100 μ l/well of 1:4000 dilution) and duplicate 100 μ l aliquots of serially-diluted standard, samples and diluent blanks were then added and the plate was incubated overnight on a microplate shaker. The plate was washed (ten cycles) before adding 50 μ l anti-chicken IgG conjugated to alkaline phosphatase (Sigma, Poole, Dorset, UK; diluted to 1:10 000 in TBS). After 2 h incubation on the microplate shaker the plate was washed thoroughly (15 cycles) before adding 200 μ l enzyme substrate solution (1 mg *p*-nitrophenyl phosphate/ml 0.2 M Tris buffer, pH 9.9; Sigma N-2770). After 3–4 h, absorbance values at 410 nm were determined on a microplate reader (MR700; Dynatech Laboratories, Billingham, Sussex, UK) and RIACALC software (LKB-Pharmacia, Milton Keynes, Bucks, UK) was used to fit the standard curve and calculate concentrations of follistatin in unknowns. The limit of detection of the assay was $\sim$ 30 pg/well when expressed in terms of the rh follistatin standard and the normal working range extended to $\sim$ 1000 pg/well. In practice, pooled bovine (b)FF (assigned an in-house units of 1000 bFS units/ml) was used as a working standard since the dilution curve for rh follistatin was consistently steeper than dilution curves for crude ovine (o)FF or bFF samples (Fig. 1), even though the dilution curve for a highly enriched bFF follistatin preparation (see below) was parallel to the rh follistatin curve.

In order to calibrate the bFF working standard in terms of the pure rh follistatin standard, exactly 1 ml of the bFF pool was subjected to immunoaffinity extraction with $\sim$ 100 μ g anti-follistatin MAb (clone 1/1; L W Evans & N P Groome, unpublished observations) covalently linked to 0.2 ml Hi-Trap beads (Pharmacia) according to the suppliers instructions. After mixing by inversion for 48 h at 4 °C the MAb-coated beads were recovered carefully and washed repeatedly with PBS (ten cycles). The immuno-extraction procedure removed 100% of the follistatin immunoreactivity present in the 1 ml bFF pool. Follistatin immunoreactivity was then recovered quantitatively (confirmed by ELISA) from the beads by mixing for 15 min with 2 ml 6 M guanidine-HCl solution containing 0.1 M Tris-HCl buffer (pH 7.5). The guanidine-HCl fraction was applied to a reversed phase HPLC column (Vydac C4, Hichrom Ltd, Reading, Berks, UK) and the column was eluted at 1 ml/min with a 30-min linear gradient of 10% v/v acetonitrile:90% v/v water:0.1% v/v trifluoroacetic acid to 80% v/v acetonitrile:20% v/v water:0.1% v/v trifluoroacetic acid. Column eluant was monitored at 280 nm and 40 $\times$ 1 min fractions were collected into polypropylene tubes. A single peak of follistatin immunoreactivity eluted from the column (acetonitrile concentration 44%) accounting for 67% of the total

Figure 1 Comparison of response curves obtained in the follistatin ELISA for doubling dilutions of recombinant human (rh) follistatin, partially purified (pp) bovine follistatin, pooled bovine FF and pooled ovine FF. The pp bovine follistatin preparation was obtained from pooled bovine FF by immunoaffinity extraction and reverse-phase HPLC (see Materials and Methods for further details). Note that the dilution curves for pp bovine follistatin and rh follistatin were parallel but were consistently steeper than the dilution curves for ovine and bovine FF. For this reason a calibrated preparation of pooled bovine FF (assigned 1000 'in-house' units/ml and equivalent to 3.93 μ g rh follistatin/ml) was used as the working standard when assaying ovine FF samples.

immunoreactivity applied to the column. As mentioned above this highly enriched bovine follistatin preparation diluted in parallel to the rh follistatin standard in the ELISA. By comparing their relative potencies in the assay it was thus calculated that 1 ml standard bFF pool (1000 in-house units) is equivalent to 3.93 μ g rh follistatin. This estimate accords well with the value of 8–12 μ g follistatin/ml bFF reported by Klein *et al.* (1991), who calibrated their assay using a highly purified bovine follistatin preparation. In the interests of uniformity and to facilitate comparison with data from other laboratories, ovine FF follistatin concentrations in the present study are therefore expressed in terms of the rh follistatin standard, rather than 'in-house' units.

Mean recovery of follistatin standard (working standard equivalent to 95 or 190 pg rh follistatin/well) added to individual sheep FF samples before assay was quantitative ($104 \pm 9\%$; $n=18$). Within- and between-plate coefficients of variation determined for diluted bFF containing the equivalent of 190 pg rh follistatin/well were 4.7 and 6.9% respectively. Specificity tests showed that rh inhibin A (125 ng/well), bovine inhibin A (125 ng/well), rh activin A (125 ng/well), rh inhibin B (25 ng/well), rh activin B (25 ng/well), bovine pro- α C (500 ng/well) and human α 2 macroglobulin (100 μ g/well) did not cross react or interfere in the assay (values in parentheses indicate the

maximum levels tested). The observation that addition of a large (>25-fold) molar excess of activin A or B to the assay does not influence the dose-response curve indicates that the ELISA measures 'total' follistatin (i.e. free+activin-bound).

SDS-PAGE/immunoblotting

To provide an independent semi-quantitative comparison of their inhibin A and activin A contents, pooled FF samples from CON and IMM ewes were subjected to SDS-PAGE/immunoblotting (reducing and non-reducing conditions) with MAb against the α and β A subunits. Electrophoresis and electrophoretic transfer were performed as described previously (Knight *et al.* 1989). After transfer, nitrocellulose membranes were blocked overnight at 4 °C in TBS with 1% (w/v) BSA before a further overnight incubation at 4 °C in TBS-Tween/1% BSA containing 10^5 c.p.m./ml 125 I-labelled MAb against either α (clone 173/29; N P Groome, unpublished observations) or β A (clone E4; Groome & Lawrence 1991) subunit. Antibodies were radio-iodinated to a specific activity of ~ 2.5 Ci/ μ mol as described by Knight *et al.* (1991a). Membranes were washed thoroughly in TBS/Tween, dried and covered in Saran wrap before exposure to X-ray film with an intensifying screen for 4 days at -70 °C.

Gel-permeation fast protein liquid chromatography (FPLC)

To determine whether IMM affected the chromatographic behaviour of FF inhibin A, activin A and follistatin, pooled FF samples from IMM and CON ewes were run on gel-permeation FPLC under non-dissociating conditions as described by Knight *et al.* (1996). A Superose 12 FPLC column (Pharmacia) was equilibrated with PBS containing 0.1% polypep (Sigma) and 0.05% sodium azide and the same buffer was used to elute the column at a flow rate of 0.5 ml/min. Fractions (1 min) were collected for measurement of inhibin A, activin A and follistatin. Marker proteins used to calibrate the column were α 2macroglobulin (750 kDa), apoferritin (450 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa) and rh activin A (25 kDa).

Statistical analysis

Repeated-measures analysis of variance with *post hoc* Bonferroni *t*-test was used to compare plasma hormone profiles in CON and IMM groups. Student's unpaired *t*-test was used to evaluate the effect of IMM on the numbers of follicles in specified categories and on FF hormone levels (expressed on a per μ l FF basis). Student's paired *t* test was used to make within-group comparisons of numbers of follicles in specified categories and of FF hormone levels. Simple regression analysis was

used to examine the relationship between different follicular parameters. Data were log transformed before statistical analysis but values are presented as arithmetic means $\pm$ S.E.M.

Results

Validation of inhibin, activin and follistatin assays for FF from IMM and CON ewes

In each assay pooled FF from both IMM and CON ewes gave response curves parallel to the respective standard curve (Fig. 2). Mean recoveries of inhibin A spike (125 pg/tube) added to FF samples from CON and IMM ewes were $99 \pm 8\%$ ($n=9$) and $85 \pm 4\%$ ($n=16$); mean recoveries of activin A spike (250 pg/well) were $87 \pm 12\%$ ($n=9$) and $99 \pm 8\%$ ($n=9$); mean recoveries of follistatin spike (0.05 units bFF standard, equivalent to 190 pg rh follistatin/well) were $118 \pm 9\%$ ($n=15$) and $94 \pm 5\%$ ($n=17$) respectively. In addition, mean recoveries of endogenous activin A, inhibin A and follistatin in control oFF samples assayed in the presence of pooled plasma (1:100 dilution) from IMM ewes were $101 \pm 4\%$, $102 \pm 3\%$ and $101 \pm 4\%$ ($n=8$) confirming that inhibin α antibodies do not interfere in any of these assays. As shown in Fig. 3, immunoblotting of sheep FF with ^{125}I -labelled βA antibody revealed two major immunostained bands under non-reducing conditions, corresponding to molecular masses of ~ 32 kDa (inhibin A) and ~ 26 kDa (activin A). Faint bands were also observed at ~ 100 and ~ 60 kDa. Under reducing conditions major bands corresponding to ~ 15 kDa (mature βA subunit) and ~ 60 kDa (full-length βA precursor) were detected. Immunodetection with ^{125}I -labelled α antibody revealed, under non-reducing conditions, bands of 100, 70, 46–60, 26 and 20 kDa. Under reducing conditions only two major bands were evident at ~ 46 –55 and ~ 22 kDa, corresponding to free α subunit forms. The intensity of each α and βA immunostained band was markedly stronger in FF samples from IMM compared with CON ewes. This observation concurs with the quantitative difference measured using the inhibin A IRMA and activin A ELISA and provides further assurance that antibodies to the α subunit present in FF of IMM ewes do not compromise these assays. It also indicates FF from IMM ewes contains more total α (i.e. dimeric inhibins and monomeric α forms) and total βA immunoreactivity than FF from CON ewes.

Inhibin antibody levels in plasma and FF

All IMM ewes responded to the booster with an increase in plasma ^{125}I inhibin binding capacity from $5.2 \pm 2.1\%$ (tracer binding at 1:1000 dilution) to $21.5 \pm 5.7\%$ 1 week after boosting. Antibody levels remained at $\sim 20\%$ throughout the remainder of the experiment (Fig. 4a). No

^{125}I inhibin binding was detected in plasma from CON ewes. When tested at the same dilution as plasma samples, FF from IMM ewes bound $17.1 \pm 3.2\%$ tracer while that from CON ewes showed no detectable binding.

Plasma FSH and oestradiol

Mean plasma FSH concentrations did not differ between IMM and CON groups at any time prior to the final blood sample taken at slaughter (Fig. 4a). Between 36 and 48 h after progestagen withdrawal, plasma FSH increased more markedly in IMM than in CON ewes ($P<0.05$), apparently due to an advancement in the timing of the preovulatory gonadotrophin surge as a consequence of the greater number of OA follicles present (see Table 1). This was reflected in a ~ 2 -fold higher ($P<0.01$) plasma oestradiol concentration observed in IMM ewes compared with CON ewes 36 and 48 h after progestagen withdrawal (Fig. 4b).

Follicle distribution and intrafollicular hormone levels

Follicle distribution IMM promoted a 3-fold increase in the total number of follicles/ewe ≥ 3 mm ($P<0.0001$) compared with CON ewes. As shown in Table 1 the increase was evident in all four categories of follicle: large (3.8-fold increase; $P<0.001$), medium (2.3-fold increase; $P<0.05$), OI (2.7-fold increase; $P<0.01$) and OA follicles (3.6-fold increase; $P<0.01$). There was no difference in the mean diameter of the largest follicle present on the ovary at the time of slaughter (IMM 7.6 ± 0.2 mm, CON 7.2 ± 0.3 mm).

Oestradiol As expected, the mean FF oestradiol level in both CON and IMM groups was significantly higher in large follicles compared with medium follicles ($P<0.01$; Table 1a) and in OA follicles compared with OI follicles ($P<0.001$; Table 1b). IMM was associated with a significant increase in FF oestradiol levels in OI follicles ($P<0.001$ compared with CON) and in medium follicles ($P<0.01$ compared with CON) but not in OA or large follicle categories.

Testosterone In both medium and large follicle categories mean FF testosterone level tended to be higher in IMM compared with CON ewes (Table 1a) although the difference was only significant in medium follicles (94%; $P<0.01$). Testosterone levels in OI follicles were higher in IMM compared with CON ewes (98%; $P<0.01$; Table 1b). Within the IMM group testosterone levels were higher (70%; $P<0.01$) in OI than in OA follicles.

Inhibin A Table 1a,b shows that IMM significantly increased FF inhibin A concentration in all follicle categories compared with CON ewes (~ 2 - to 4-fold; $P<0.001$). Within the CON group large size follicles and

follicles classified as OA had significantly higher (both $P<0.01$) inhibin A than medium size and OI follicles respectively. No such differences were seen within the IMM group. In the CON group inhibin A concentration

was positively correlated with follicle diameter ($r=+0.57$, $P<0.01$) and oestradiol concentration ($r=+0.51$, $P<0.01$) but not with testosterone, activin A or follistatin. In the IMM group inhibin A was positively correlated with activin A ($r=+0.75$, $P<0.0001$) and follistatin ($r=+0.59$, $P<0.001$) but not with follicle diameter or with oestradiol or testosterone concentration.

Activin A IMM was associated with a marked increase in FF activin A concentration in all follicle categories compared with CON ewes (~ 6 -fold; $P<0.001$; Table 1). Follicles categorised as OI tended to have higher activin A concentrations than OA follicles (Table 1b) but the difference was statistically significant only in the IMM group ($P<0.02$). Likewise, medium-size follicles tended to have higher activin A concentrations than large follicles ($\sim 60\%$) in both IMM and CON groups but the differences were not significant.

Follistatin In contrast to its marked effect on intra-follicular activin A and inhibin A, IMM had only a slight stimulatory effect on mean FF follistatin levels (10–40% increase; not significant). As observed for activin A, medium-size follicles tended to have higher follistatin concentrations than large follicles in both IMM and CON groups ($P<0.05$; Table 1a) and OI follicles tended to have higher follistatin concentrations than OA follicles (Table 1b) the latter being significant only in the IMM group ($P<0.02$). There was a positive correlation between follistatin and activin A in IMM ewes ($r=+0.58$, $P<0.0001$) but not in CON ewes ($r=0.18$, $P=0.36$). A negative correlation between follistatin and follicle diameter was observed in both IMM ($r=-0.26$, $P<0.05$) and CON ($r=-0.53$, $P<0.005$) ewes.

Activin A:follistatin ratio As shown in Table 1a,b, the activin A:follistatin ratio in FF from IMM ewes ($\sim 1:1$) was significantly ($P<0.001$) greater than the $\sim 0.3:1$ ratio observed in CON ewes, irrespective of follicle category. There was a weak positive correlation between activin A:follistatin ratio and follicle diameter in both IMM ($r=+0.28$, $P<0.05$) and CON ($r=+0.37$, $P<0.05$) groups.

Relationships between intrafollicular inhibin antibody titres and hormone levels Inhibin antibody levels measured in individual follicles from IMM ewes were

Figure 2 Dose-response curves in the (a) inhibin A IRMA, (b) activin A ELISA and (c) follistatin ELISA for pooled FF samples collected from inhibin-immunized (IMM; ■) and control (CON; □) ewes. In each panel, the dilution curve for the assay standard is represented by ●. Points represent the mean of duplicate determinations. Parallelism between dose-response curves for test samples and standards was confirmed by regression analysis of log-log transformed data which showed that their slopes had overlapping 95% confidence intervals.

Figure 3 SDS-PAGE/immunoblotting analysis of pooled follicular fluid samples from inhibin-immunized (IMM; odd-numbered lanes) and control (CON, even-numbered lanes) ewes. Samples were electrophoresed under reducing (R) and non-reducing (NR) conditions and transfer membrane immunostained using either [125 I]-labelled β A subunit specific monoclonal antibody (E4; lanes 1–4) or [125 I]-labelled α subunit specific monoclonal antibody (lanes 5–8).

weakly correlated with intrafollicular levels of inhibin A ($r=+0.32$, $P<0.05$), activin A ($r=+0.31$, $P<0.05$), follistatin ($r=+0.49$, $P<0.01$) and oestradiol ($r=+0.33$, $P<0.05$) but not with testosterone ($r=+0.21$) or follicle diameter ($r=+0.10$).

Gel-permeation chromatography of FF from CON and IMM ewes Figure 5 shows the FPLC elution profiles for inhibin A, activin A and follistatin immunoreactivity in pooled FF from IMM and CON ewes. As expected, most of the inhibin A in FF from IMM ewes eluted earlier (fractions corresponding to 750–450 kDa) than in FF from CON ewes (~ 66 –150 kDa) due to the presence of inhibin α antibodies which would have formed immune complexes with inhibin in the former (Fig. 5a). No inhibin A eluted in fractions corresponding to 32 kDa in either FF pool. Most of the activin A (Fig. 5b) and follistatin (Fig. 5c) in both FF pools coeluted in the column void volume fractions (>700 kDa) and no immunoreactivity was detected in fractions corresponding to the elution positions of free activin (25 kDa) or follistatin (30–40 kDa). Comparison of 'areas under the curve' confirmed that FF pooled from IMM ewes contained much more inhibin A (~ 4 -fold) and activin A (~ 8 -fold) but only slightly more follistatin (~ 1.5 -fold) than FF from CON ewes.

Discussion

It should be emphasized that the present study concerns the long-term (>1 year) consequences of active immunization against inhibin, and that these are likely to be quite

different from the acute effects on the pituitary–ovarian axis resulting from passive immunization against inhibin (Mann *et al.* 1989, 1993, McNeilly *et al.* 1991, Campbell *et al.* 1995). The results confirm and extend previous evidence (Knight *et al.* 1991b, Fray *et al.* 1994, Bleach *et al.* 1996) suggesting that the long-term stimulatory effect of IMM on follicle development may not be due exclusively to neutralization of the negative feedback action of inhibin on pituitary FSH. This contrasts with the acute increase in FSH secretion observed after passive immunization against inhibin (Mann *et al.* 1989, 1993, Campbell & Scaramuzzi 1995, Campbell *et al.* 1995) where the associated increase in follicular development can be directly attributed to the FSH rise. The most surprising and novel finding was that IMM ewes had greatly increased intrafollicular concentrations of activin A ($\beta\alpha\beta$ dimer), a molecule that has no sequence homology with the α subunit hapten used to generate inhibin antibodies in these animals. This change occurred despite the presence of similar plasma FSH levels in IMM and CON groups. As such, the study provides evidence that long-term exposure to inhibin α antibodies may have direct effects on granulosa cell function that are not mediated by changes in pituitary FSH secretion. This evidence contrasts with a previous study in which acute exposure to exogenous inhibin (oFF) or inhibin antiserum had no effect on FSH-induced follicular growth in gonadotrophin-releasing hormone (GnRH) agonist-suppressed sheep (McNeilly *et al.* 1991). However, other studies (Cahill *et al.* 1985a,b) have shown that exogenous inhibin (oFF) can reduce gonadotrophin-induced follicle development in both intact and hypophysectomized ewes, findings which are more supportive of a direct ovarian action of inhibin.

Although the present experimental design did not include a detailed analysis of plasma FSH profiles, the limited data available showed no difference in plasma FSH between IMM and CON groups at any time until the final blood sample at the time of killing. This agrees with previous evidence from this laboratory (Knight *et al.* 1991b, Fray *et al.* 1994) indicating that plasma FSH levels revert to normal in long-term IMM ewes, presumably due to a compensatory increase in the negative feedback actions of ovarian oestradiol secreted by the additional follicles. It is possible that IMM might affect the relative abundance of different FSH isoforms secreted by the pituitary perhaps leading to increased bioactivity in the absence of detectable change in immunoactivity. However, there is no evidence to support this possibility (Ulloa-Aguirre *et al.* 1995). Although we cannot exclude the possibility that ovarian exposure to FSH may be raised in long-term IMM ewes, the question arises of how multiple follicular development is sustained in ewes without an increase in FSH. Taken together with recent *in vitro* evidence that activin has autocrine actions on granulosa cells (Findlay 1993, Findlay *et al.* 1996), the present findings lead us to propose two alternate (but not mutually

Figure 4 Mean ($\pm$ S.E.M.) plasma levels of (a) FSH (circles) and inhibin antibody (squares) and (b) oestradiol in six inhibin-immunized ewes (IMM, closed symbols) and eight control ewes (CON, open symbols). Inhibin antibody titres are expressed as the percentage of [125 I]-labelled bovine 32 kDa inhibin bound at a plasma dilution of 1:1000. Data are plotted relative to the day of progestagen sponge removal. * $P < 0.05$, ** $P < 0.025$, *** $P < 0.005$ compared with CON. Note that plasma FSH levels did not differ between immunized and control groups until the final blood sample taken at the time of slaughter.

exclusive) hypotheses, both of which are compatible with the finding that inhibin antibodies are present in FF of IMM ewes. According to the first hypothesis, inhibin α antibodies promote increased granulosa cell expression of activin A which, in turn, enhances their expression of FSH receptors and increases follicular responsiveness to the prevailing level of FSH. By lowering their individual FSH

'thresholds' this could permit those follicles which would normally undergo atresia to survive, maintain aromatase activity and ultimately reach ovulatory potential through FSH-induced acquisition of granulosa cell luteinizing hormone (LH) receptors at the late antral stage (Fortune 1994). In support of this hypothesis there is recent *in vitro* evidence in rats that activin can promote preantral follicle

Table 1 Mean ($\pm$ S.E.M.) numbers of follicles/ewe, FF hormone concentrations, oestradiol:testosterone ratios (O:T ratios) and activin A:follistatin ratios (Act:FS ratios) in inhibin-immunized (IMM) and control (CON) ewes. In (a) follicles are classified according to size as either medium ($\geq 3 \leq 5$ mm diameter) or large (> 5 mm diameter). In (b) follicles are classified according to the O:T ratio as either oestrogen-inactive (OI; O:T ratio ≤ 1) or oestrogen-active (OA; O:T ratio > 1)

Treatment group	Follicle category	No. follicles/ewe	Oestradiol (pg/ μ l)	Testosterone (pg/ μ l)	O:T ratio	Inhibin A (ng/ μ l)	Activin A (ng/ μ l)	Follistatin (ng/ μ l)	Act:FS ratio
(a)	Medium Large	4.3 $\pm$ 0.9 ^a 8.0 $\pm$ 1.0 ^{***b}	26.5 $\pm$ 8.6 ^{***a} 87.5 $\pm$ 12.2 ^b	85.6 $\pm$ 15.8 [*] 58.5 $\pm$ 5.9	0.56 $\pm$ 0.25 ^a 2.35 $\pm$ 0.39 ^b	20.4 $\pm$ 2.81 ^{***} 18.7 $\pm$ 1.20 ^{***}	9.41 $\pm$ 2.06 ^{***} 5.76 $\pm$ 1.01 ^{***}	10.4 $\pm$ 1.49 ^a 6.05 $\pm$ 0.51 ^b	0.86 $\pm$ 0.10 ^{**} 0.91 $\pm$ 0.38 ^{***}
	Medium Large	1.9 $\pm$ 0.5 2.1 $\pm$ 0.3	3.4 $\pm$ 0.8 ^a 95.8 $\pm$ 30.9 ^b	44.7 $\pm$ 13.0 34.0 $\pm$ 6.3	0.28 $\pm$ 0.13 ^a 2.23 $\pm$ 0.57 ^b	4.64 $\pm$ 0.92 ^a 8.27 $\pm$ 0.69 ^b	1.59 $\pm$ 0.32 1.01 $\pm$ 0.15	7.82 $\pm$ 1.41 ^a 4.32 $\pm$ 0.98 ^b	0.33 $\pm$ 0.10 0.30 $\pm$ 0.04
(b)	OI OA	5.67 $\pm$ 0.84 ^{**} 5.33 $\pm$ 1.33 ^{**}	16.9 $\pm$ 3.42 ^{***a} 124.5 $\pm$ 13.6 ^b	83.4 $\pm$ 9.9 ^{**a} 48.9 $\pm$ 7.0 ^b	0.25 $\pm$ 0.04 ^a 3.47 $\pm$ 0.47 ^b	19.1 $\pm$ 1.86 ^{***} 19.2 $\pm$ 1.41 ^{***}	8.98 $\pm$ 1.40 ^{***a} 4.63 $\pm$ 0.98 ^{***b}	8.76 $\pm$ 1.06 ^a 5.93 $\pm$ 0.51 ^b	0.98 $\pm$ 0.12 ^{***} 0.81 $\pm$ 0.13 ^{***}
	OI OA	2.13 $\pm$ 0.52 1.50 $\pm$ 0.27	3.47 $\pm$ 0.53 ^a 119.5 $\pm$ 35.5 ^b	42.5 $\pm$ 10.1 35.8 $\pm$ 7.7	0.17 $\pm$ 0.04 ^a 2.88 $\pm$ 0.59 ^b	5.32 $\pm$ 0.76 ^a 8.71 $\pm$ 0.86 ^b	1.52 $\pm$ 0.26 0.92 $\pm$ 0.14	6.37 $\pm$ 1.18 5.31 $\pm$ 1.41	0.35 $\pm$ 0.08 0.26 $\pm$ 0.05

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the same follicle category in CON group. ^a and ^b denote a significant ($P < 0.05$) difference between follicle categories within a treatment group.

development (Li *et al.* 1995), increase granulosa cell expression of FSH receptors (Hasegawa *et al.* 1988, Xiao *et al.* 1992, Nakamura *et al.* 1993), aromatase activity (Miro *et al.* 1991) and enhance FSH-induced expression of LH receptors (Nakamura *et al.* 1994). Although comparable studies have not yet been reported in sheep, activin has been shown to inhibit premature luteinization/atresia

of cultured bovine granulosa cells consistent with a role in maintaining follicle health (Shukovski 1990, Shukovski *et al.* 1991). The second hypothesis is based on evidence that granulosa cells secrete free inhibin α subunit (Knight *et al.* 1989, Robertson *et al.* 1989, Sugino *et al.* 1989, 1992) and that full-length α inhibin precursor can compete with FSH for binding to its receptor (Schneyer *et al.* 1991). It proposes that antibodies against inhibin α block the binding of inhibin α precursor to FSH receptors, thereby increasing follicular responsiveness to FSH. Clearly further studies are now required to test these hypotheses. These should include experiments to determine the effect of long-term IMM on (i) expression of FSH and LH receptor mRNA/protein and (ii) steroidogenic responsiveness of cultured ovine granulosa cells to FSH and LH.

Follistatin binds to activin with high affinity and neutralizes its actions in most bioassay systems (Woodruff & Mather 1995), implying that the local activin:follistatin ratio is an important determinant of activin's ability to interact with its receptors. The chromatographic evidence we obtained suggests that nearly all of the activin A present in FF from IMM and CON animals is bound to follistatin, forming high molecular weight activin–follistatin complexes which presumably render the activin A biologically inactive. Despite this, it is evident from Table 1 that the levels of follistatin and activin A were similar in IMM ewes whereas there is a 3- to 4-fold excess of follistatin over activin A in CON ewes. The former ratio would be more favourable to dissociation of 'free' activin A for interaction with cellular receptors, consistent with our hypothesis that raised activin A in IMM ewes has a positive effect on granulosa cell function. In this study we did not enumerate or analyze follicles smaller than 3 mm in diameter. However, comparison of the inhibin, activin and follistatin contents of much smaller follicles would now be of great interest since activin-induced up-regulation of FSH receptors could be an important step by which preantral follicles become gonadotrophin-dependent (Findlay 1993, Nakamura *et al.* 1993, Findlay *et al.* 1996).

The mechanism(s) through which IMM promotes increased FF levels of inhibin A, activin A and, to a lesser extent, follistatin in these ewes remains to be established. While all three peptides are secreted from bovine granulosa cells in an FSH-dependent manner (Klein *et al.* 1991, D S Tannetta & P G Knight, unpublished observations), as discussed above, we have no evidence that FSH levels are raised in these long-term IMM ewes. Earlier studies have

Figure 5 Profiles of (a) inhibin A, (b) activin A and (c) follistatin immunoreactivity after chromatography of pools of FF collected during the normal follicular phase from IMM (●) and CON (○) ewes. The column used was a Superose-12 gel-permeation FPLC column calibrated using the following marker proteins: α_2 macroglobulin (750 kDa), apoferritin (450 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa) and recombinant human activin (25 kDa).

documented increased 'total' (i.e. antibody-bound+free) oestrogen and androgen levels in animals actively immunized against the respective steroid. These rises have been attributed to a compensatory increase in steroid production rate combined with a decrease in metabolic clearance rate (Nieschlag & Wickings 1978). Campbell *et al.* (1990) reported similar findings of raised ovarian androstenedione secretion rates in ewes actively immunized against androstenedione. In the same manner, since inhibin A is a direct target of the inhibin α antibodies generated, an increase in its total intrafollicular level is not surprising and could be attributed to (i) a compensatory increase in its production rate, or (ii) the formation of immune complexes which decrease its exit rate from the follicle and/or stabilize the dimer against proteolysis. Importantly, however, neither explanation could account for the marked increase in FF level of activin A we observed since the inhibin α subunit antibodies produced have no affinity for the $\beta\alpha\beta$ homodimer, as demonstrated by immunoblotting and antibody crossreactivity tests (D S Tannetta, N P Groome, P G Knight, unpublished observations). A more likely explanation, but one that will require further research to substantiate, is that immunoneutralization of inhibin (or monomeric α subunit) at a local intrafollicular level up-regulates granulosa cell $\beta\alpha$ subunit expression which, in turn, promotes the assembly of more $\beta\alpha\beta$ dimer. Therefore, an important objective of further studies will be to determine using an *in situ* hybridization approach, whether IMM affects the expression of mRNAs for the inhibin/activin subunits, follistatin and gonadotrophin receptors within the granulosa layer of individual follicles.

Acknowledgements

We thank Dr J P Mather (Genentech, San Francisco, CA, USA) for providing rh inhibins and activins, Dr P Smith (NIDDK, Baltimore, MD, USA) for rh follistatin and sheep FSH RIA reagents and Dr G S Pope (Department of Agriculture, University of Reading, UK) for steroid antisera. This work was supported by BBSRC and MAFF.

References

- Bleach ECL, Muttukrishna S, Cunningham FJ, Knight PG & Glencross RG 1996 Effect of inhibin immunization using different synthetic peptide fragments of the bovine α_C -subunit on plasma anti-inhibin titres, plasma FSH concentrations and the incidence of multiple ovulations in heifers. *Animal Reproduction Science* **41** 1–12.
- Cahill LP, Clarke IJ, Cummins JT, Driancourt MA, Carson RS & Findlay JK 1985a An inhibitory action at the ovarian level of ovine follicular fluid on PMSG-induced folliculogenesis in hypophysectomized ewes. In *Proceedings of the 5th Ovarian Workshop*, pp 35–38. Eds DE Toft & JR Ryan. New York: Plenum Press.
- Cahill LP, Driancourt MA, Chalmley WA & Findlay JK 1985b Role of intrafollicular regulators and FSH in growth and development of large antral follicles in sheep. *Journal of Reproduction and Fertility* **75** 599–607.
- Campbell BK & Scaramuzzi RJ 1995 Effects of acute immunoneutralization of inhibin in ewes during the late luteal phase of the oestrous cycle on ovarian hormone secretion and follicular development during the subsequent follicular phase. *Journal of Reproduction and Fertility* **104** 337–345.
- Campbell BK, Scaramuzzi RJ, Downing JA & Evans G 1990 Steroid secretion rates and plasma binding activity in androstenedione-immune ewes with an autotransplanted ovary. *Journal of Reproduction and Fertility* **89** 485–496.
- Campbell BK, Gordon BM, Tsonis CG & Scaramuzzi RJ 1995 The effect of acute immunoneutralization of inhibin in ewes during the early luteal phase of the oestrous cycle on ovarian hormone secretion and follicular development. *Journal of Endocrinology* **145** 479–490.
- England BG, Dahmer MK & Webb R 1981 Relationships between follicular size and antral fluid steroid concentrations at three stages of the estrous cycle in the ewe. *Biology of Reproduction* **24** 1068–1075.
- Findlay JK 1993 An update on the roles of inhibin, activin and follistatin as local regulators of folliculogenesis. *Biology of Reproduction* **48** 15–23.
- Findlay JK, Doughton BW, Tsonis CG, Brown RW, Hungerford JW, Greenwood PE & Forage RG 1993 Inhibin as a fecundity vaccine. *Animal Reproduction Science* **33** 325–343.
- Findlay JK, Drummond AE & Fry RC 1996 Intraovarian regulation of follicular development and ovulation. *Animal Reproduction Science* **42** 321–331.
- Fortune JE 1994 Ovarian follicular growth and development in mammals. *Biology of Reproduction* **50** 225–232.
- Fray MD, Wrathall JHM & Knight PG 1994 Active immunization against inhibin promotes a recurrent increase in litter size in sheep. *Veterinary Record* **134** 19–20.
- Fray MD, Lamming GE & Haresign W 1996 Inter-breed variation in the postpartum ewe response to continuous low-dose infusion of GnRH. *Theriogenology* **45** 1047–1064.
- Groome N & Lawrence M 1991 Preparation of monoclonal antibodies to the $\beta\alpha$ subunit of ovarian inhibin using a synthetic peptide immunogen. *Hybridoma* **10** 309–316.
- Hasegawa Y, Miyamoto K, Abe Y, Nakamura T, Sugino H, Eto Y, Shibai H & Igarashi M 1988 Induction of follicle-stimulating hormone receptor by erythroid differentiation factor on rat granulosa cells. *Biochemical and Biophysical Research Communications* **156** 668–674.
- Howles CM, Webster GM & Haynes NB 1980 The effect of rearing under a long or short photoperiod on testis growth, plasma testosterone and prolactin concentrations and the development of sexual behaviour in rams. *Journal of Reproduction and Fertility* **60** 437–447.
- Klein R, Robertson DM, Shukovski L, Findlay JK & de Kretser DM 1991 The radioimmunoassay of follicle-stimulating hormone (FSH)-suppressing protein (FSP): stimulation of bovine granulosa cell FSP secretion by FSH. *Endocrinology* **128** 1048–1056.
- Knight PG 1996 Roles of inhibins, activins and follistatin in the female reproductive system. *Frontiers in Neuroendocrinology* **17** 476–509.
- Knight PG & Muttukrishna S 1994 Measurement of dimeric inhibin using a modified two-site immunoradiometric assay specific for oxidized (Met O) inhibin. *Journal of Endocrinology* **141** 417–425.
- Knight PG, Beard AJ, Wrathall JHM & Castillo RJ 1989 Evidence that the bovine ovary secretes large amounts of monomeric inhibin α -subunit and its isolation from bovine follicular fluid. *Journal of Molecular Endocrinology* **2** 189–200.
- Knight PG, Groome N & Beard AJ. 1991a Development of a two-site immunoradiometric assay for dimeric inhibin using antibodies against chemically synthesized fragments of the α - and β -subunit. *Journal of Endocrinology* **129** R9–R12.

- Knight PG, Wrathall JHM, Glencross RG & McLeod BJ 1991b Effects of bovine follicular fluid on the secretion of LH and FSH in inhibin-immunized seasonally anoestrous ewes. *Journal of Endocrinology* **128** 403–410.
- Knight PG, Muttukrishna S & Groome NP 1996 Development and application of a two-site enzyme immunoassay for the determination of 'total' activin-A concentrations in serum and follicular fluid. *Journal of Endocrinology* **148** 267–279.
- Li R, Phillips DM & Mather JP 1995 Activin promotes ovarian follicle development *in vitro*. *Journal of Endocrinology* **136** 849–856.
- McNeilly AS, Crow W & Campbell BK 1991 Effect of follicular fluid and inhibin immunoneutralization on FSH-induced preovulatory growth in the ewe. *Journal of Endocrinology* **131** 401–409.
- Mann GE, Campbell BK, McNeilly AS & Baird DT 1989 Passively immunizing ewes against inhibin during the luteal phase of the oestrous cycle raises the plasma concentration of FSH. *Journal of Endocrinology* **123** 181–188.
- Mann GE, Campbell BK, McNeilly AS & Baird DT 1993 Follicle development and ovarian hormone secretion following passive immunization of ewes against inhibin or oestradiol. *Journal of Endocrinology* **136** 225–233.
- Miro F, Smyth CD & Hillier SG 1991 Development-related effects of recombinant activin on steroid synthesis in rat granulosa cells. *Endocrinology* **129** 3388–3394.
- Nakamura M, Minegishi T, Hasegawa Y, Nakamura K, Igarashi S, Ito I, Shinozaki H, Miyamoto K, Eto Y & Ibuki Y 1993 Effect of an activin A on follicle stimulating hormone (FSH) receptor messenger ribonucleic acid levels and FSH receptor expression in cultured rat granulosa cells. *Endocrinology* **133** 538–544.
- Nakamura K, Nakamura M, Igarashi S, Miyamoto K, Eto Y, Ibuki Y & Takashi M 1994 Effect of activin on luteinizing hormone-human chorionic gonadotropin receptor messenger ribonucleic acid in granulosa cells. *Endocrinology* **134** 2329–2335.
- Nieschlag E & Wickings EJ 1978 Biological effects of antibodies to gonadal steroids. *Vitamins and Hormones* **30** 165–202.
- Robertson DM, Giacometti M, Foulds LM, Lahnstein J, Goss NH, Hearn MTW & de Kretser DM 1989 Isolation of inhibin α -subunit precursor proteins from bovine follicular fluid. *Endocrinology* **125** 2141–2149.
- Schneyer AL, Sluss PM, Whitcomb RW, Martin KA, Sprengel R & Crowley WF 1991 Precursors of α -inhibin modulate follicle-stimulating hormone receptor binding and biological activity. *Endocrinology* **129** 1987–1999.
- Shukovski L & Findlay JK 1990 Activin A inhibits oxytocin and progesterone production by preovulatory bovine granulosa cells *in vitro*. *Endocrinology* **126** 2222–2224.
- Shukovski L, Findlay JK & Robertson D 1991 The effect of follicle-stimulating hormone-suppressing protein or follistatin on luteinizing bovine granulosa cells *in vitro* and its antagonistic effect on the action of activin. *Endocrinology* **129** 3395–3402.
- Sugino K, Nakamura T, Takio K, Titani K, Miyamoto K, Hasegawa Y, Igarashi M & Sugino H 1989 Inhibin α -subunit monomer is present in bovine follicular fluid. *Biochemical and Biophysical Research Communications* **159** 1323–1329.
- Sugino K, Nakamura T, Takio K, Miyamoto K, Hasegawa Y, Igarashi M, Titani K & Sugino H 1992 Purification and characterization of high molecular weight forms of inhibin from bovine follicular fluid. *Endocrinology* **130** 789–796.
- Terqui M, Wrathall JHM, Driancourt MA & Knight PG 1995 Modulation of ovarian function by steroid and inhibin immunization. *Livestock Production Science* **42** 181–192.
- Ulloa-Aguirre A, Rees-Midgley A, Beitins IZ & Padmanabhan V 1995 Follicle-stimulating hormone isoforms: characterization and physiological relevance. *Endocrine Reviews* **16** 765–787.
- Woodruff TK & Mather JP 1995 Inhibin, activin and the female reproductive axis. *Annual Review of Physiology* **57** 219–244.
- Wrathall JHM & Knight PG 1993 Production of immunoactive inhibin by bovine granulosa cells in serum-free culture: effects of exogenous steroids and FSH. *Domestic Animal Endocrinology* **10** 289–304.
- Wrathall JHM, McLeod BJ, Glencross RG, Beard AJ & Knight PG 1990 Inhibin immunoneutralization by antibodies raised against synthetic peptide sequences of inhibin α -subunit: effects on gonadotrophin concentrations and ovulation rate in sheep. *Journal of Endocrinology* **124** 167–176.
- Wrathall JHM, McLeod BJ, Glencross RG & Knight PG 1992 Effects of active immunization against a synthetic peptide sequence of the inhibin α -subunit on plasma gonadotrophin concentrations, ovulation rate and lambing rate in ewes. *Journal of Reproduction and Fertility* **95** 175–182.
- Xiao S, Robertson DM & Findlay JK 1992 Effects of activin and follicle-stimulating hormone (FSH)-suppressing protein/follistatin on FSH receptors and differentiation of cultured rat granulosa cells. *Endocrinology* **131** 1009–1016.

Received 24 March 1997

Revised manuscript received 29 July 1997

Accepted 13 November 1997